Pregnancy induces resistance to the anorectic effect of hypothalamic malonyl-CoA and the thermogenic effect of hypothalamic AMPK inhibition in female rats

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ABSTRACT

During gestation, hyperphagia is necessary to cope with the metabolic demands of embryonic development. There were three main aims of this study: Firstly, to investigate the impact of pregnancy on hypothalamic fatty acid metabolism, a key pathway for the regulation of energy balance. Secondly, to study whether pregnancy induces resistance to the anorectic effect of fatty acid synthase (FAS) inhibition and accumulation of malonyl-CoA in the hypothalamus, and, thirdly, whether changes in hypothalamic AMPK signaling are associated to BAT thermogenesis during pregnancy. Our data indicate that in pregnant rats, the hypothalamic fatty acid pathway shows an overall state that should lead to anorexia and elevated brown adipose tissue (BAT) thermogenesis: decreased activities of AMP-activated protein kinase (AMPK), FAS and carnitine palmitoyltransferase 1 (CPT1), coupled to increased acetyl-CoA carboxylase (ACC) function with subsequent elevation of malonyl-CoA levels. This profile seems dependent of estradiol levels, but not prolactin or progesterone. Despite the apparent anorexic and thermogenic signaling in the hypothalamus, pregnant rats remain hyperphagic and display reduced temperature and BAT function. Actually, pregnant rats develop resistance to the anorectic effects of central FAS inhibition, which is associated with a reduction of proopiomelanocortin (POMC) expression and its transcription factors phospho-signal transducer and activator of transcription 3 (pSTAT3) and phospho-forkhead box O1 (pFoxO1). This evidence demonstrates that pregnancy induces a state of resistance to the anorectic and thermogenic actions of hypothalamic cellular signals of energy surplus, which, in parallel to the already known refractoriness to leptin effects, likely contributes to gestational hyperphagia and adiposity.

INTRODUCTION

Pregnancy is a hypermetabolic state with a major increase in maternal body weight and fat mass, associated with several neuroendocrine changes. During gestation, the energy balance becomes positive, primarily because of an increase in feeding, required to cope with the metabolic demands of the growing fetus and also to be ready for the subsequent lactation requirements (1-3). This energy-conserving mechanism needs to be extremely accurate to ensure a correct embryonic development, and failure to increase fat mass during pregnancy causes considerable neonatal and maternal morbidity (1-3). In fact, either maternal under-nutrition or excessive over-nutrition can lead to long-term negative effects on the offspring, such as increased risk of obesity and type 2 diabetes (1;3).

The molecular mechanisms leading to maternal hyperphagia during gestation are poorly understood, but increased in food intake is commonly mediated by central resistance to anorectic signals. Therefore, during pregnancy, the hypothalamus, a key brain area modulating energy balance (4-7), loses sensitivity to anorectic signals, such as the hormones leptin and cholecystokinin (CCK) (1;8-12), as well as the neuropeptide α-melanocyte-stimulating hormone (α-MSH) (13). Those impaired responses are associated with decreased proopiomelanocortin (POMC; the precursor of α-MSH) and increased agouti-related protein (AgRP) and neuropeptide Y (NPY) expression in the arcuate nucleus of the hypothalamus (ARC) of pregnant rats (9;11). Despite this evidence, little is known about the hypothalamic molecular mechanism leading to gestation-induced hyperphagia.

Data gleaned during the last decade, have demonstrated that besides the above neuropeptide systems, key cellular metabolic pathways in the central nervous system play a major role in the regulation of whole body energy homeostasis. Much attention has been on lipid metabolism, with physiological, genetic and pharmacological evidence demonstrating that inhibition of key enzymes within this metabolic pathway, such as AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), carnitine palmitoyltransferase 1 (CPT1) and lipoprotein lipase (LPL), impacts feeding {Wolfgang, 2006 33034 /id;Lage, 2008 33322 /id;Yue, 2012 16137 /id;Cansell, 2014 16445 /id;Pimentel, 2013 16453 /id} and brown adipose tissue (BAT) thermogenesis {López, 2010 33344 /id;Whittle, 2012 33372 /id;Martínez de Morentin, 2012 33357 /id;Tanida, 2013

16253 /id;López, 2013 33383 /id;Seoane-Collazo, 2014 16447 /id;Martínez de Morentin, 2014 33390 /id;Beiroa, 2014 16446 /id;Contreras, 2014 33392 /id}.

On the basis of the above evidence, in this study we investigated the role of hypothalamic fatty acid metabolism on pregnancy-induced feeding and decreased BAT thermogenesis (28;29). Despite enhanced feeding in pregnant rats, we found evidence of enhanced satiety signaling, including decreased AMPK activation and FAS expression and increased levels of the anorectic signal, malonyl-CoA. This discrepancy probably represents a state of resistance to the molecular satiety signals induced by the energy surplus. On the other hand, we observed alterations of POMC and its transcriptional regulators compatible with gestational-induced hyperphagia, suggesting a role for this pathway in overruling the anorectic signals of malonyl-CoA. Our data are the first to conclusively demonstrate that during gestation there is a deregulation of key elements of lipid metabolism in the hypothalamus.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (non-pregnant 250–300 g and pregnant at day 16-17 of gestation; *Animalario General USC*; Santiago de Compostela, Spain) were used for the experiments. Non-pregnant rats in proestrus were used as controls. All animals were housed on a 12 h light (8:00 to 20:00), 12 h dark cycle, in a temperature and humidity controlled room. The animals were allowed free access to standard laboratory pellets of rat chow and tap water. The experiments were performed in agreement with the *International Law on Animal Experimentation* and were approved by the *USC Local Ethical Committee* and the *Ministry of Science and Innovation* of Spain (Project ID 15010/14/006).

Surgical procedure, validation of ovariectomy and peripheral treatments

Sprague-Dawley rats were bilaterally ovariectomized (OVX) or sham-operated as described previously (25;30;31). Absence of gonadal function was confirmed by increased luteinizing hormone (LH) serum levels measured using a double-antibody method and radioimmunoassay kits (supplied by *Dr. AF Parlow; National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program;* Torrance, CA) as reported (25;30;31). Peripheral treatments (estradiol, progesterone or prolactin) and central treatments (estradiol, estrogen receptors agonists or cerulenin) were carried out two weeks after surgery to ensure a total washout of endogenous ovarian hormones.

For the experiments of estradiol replacement, ovariectomized rats received a daily injection of estradiol (estradiol benzoate; 2 μg dissolved in 100 μL of sesame oil; both from *Sigma;* St Louis, MO, USA) or vehicle (100 μL of sesame oil; control rats) during 7 days (25;30;31). For the experiments of progesterone replacement, animals were subcutaneously implanted with *Silastic* brand silicon tubing (*Dow Corning*, Midland, MI, USA) elastomers (20 mm length; inner diameter, 0.062 cm; exterior diameter, 0.125 cm) containing progesterone (50mg/capsule; *Sigma,* St Louis, MO, USA) (32) and exposed to the sex steroid for 7 days, as previously shown (30;32). Rats implanted with empty

capsules served as controls. Finally, for the experiments of prolactin replacement, rats were subcutaneously treated with prolactin (4 mg dissolved in 100 μL of phosphate buffered saline, PBS; supplied by *Dr. AF Parlow; National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program;,* Torrance, CA) or vehicle (100 μL of PBS) during 4 days, every 8 hours, as previously reported (33). Food intake and body weight recordings were made daily along every treatment schedule; a daily average of food intake (g/24h) and body weight change since the beginning of each treatment was calculated.

Implantation of intracerebroventricular cannulae and central treatments

Chronic intracerebroventricular (ICV) cannulae were implanted under ketamine-xylacine anesthesia (50 mg/kg, intraperitoneal, IP), as described previously, with correct positioning in the lateral ventricle was confirm by postmortem histological examination (19;20;25;34-37). The animals were caged individually and used for experiments four days later. During this post-operative recovery period the rats became accustomed to the handling procedure under non-stressful conditions. For the estradiol and estrogen receptor agonists experiments, OVX rats received one daily bolus of either estradiol (17 β-Estradiol; 5 nmol dissolved in 5 μL of DMSO; *Sigma;* St Louis, MO, USA), the selective estrogen receptor alpha (ERα) agonist 4,4',4"-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, 5 nmol dissolved in 5 μL of DMSO, *TOCRIS Bioscience*; Bristol, UK), the selective estrogen receptor beta (ERβ) agonist 2,3-bis(4-hyroxyphenyl)-propionitrile (DNP; 5 nmol dissolved in 5 μL of DMSO, *TOCRIS Bioscience*; Bristol, UK) or vehicle (5 μL of DMSO; control rats) during 7 days (25). For the experiments with cerulenin, pregnant rats were ICV cannulated as described above at gestational day 14, with subsequent recovery for four days before centrally treated with cerulenin (10 μg dissolved in 5 μL of DMSO; *Sigma;* St Louis, MO, USA) or vehicle (5 μL of DMSO), each 12 hours during 2 days (19).

We used 7-10 rats per group and the experiments were repeated at least twice; animals were treated at 9:00 AM (one hour after the light cycle had commenced) in the fed state. Rats were sacrificed by cervical dislocation. From each animal, the hypothalamus, the VMH or the ARC (for

western blotting, enzymatic activities of malonyl-CoA assays) was immediately homogenized on ice to preserve phosphorylated protein levels. In a separate experiment, the whole brains (for *in situ* hybridization) were dissected, and stored at -80ºC until further processing. Dissection of the VMH and the ARC was performed by micropunches under the microscope, as previously shown (19;25;37-39).

Enzymatic and malonyl-CoA assays

The CPT1 and FAS activities, as well as the malonyl-CoA assays were performed as previously described (19;21;25;34;35;40).

Temperature measurements

Body temperature was recorded twice at day 16-17 with a rectal probe connected to digital thermometer (*BAT-12 Microprobe-Thermometer; Physitemp*; NJ, US). Skin temperature surrounding BAT was recorded with an infrared camera (*B335: Compact-Infrared-Thermal-Imaging-Camera; FLIR;* West Malling, Kent, UK) and analyzed with a specific software package (*FLIR-Tools-Software; FLIR;* West Malling, Kent, UK) (20;21;24;25;37).

Western blotting

Protein lysates from the whole hypothalamus, VMH or ARC were subjected to SDS-PAGE, electrotransferred on a PVDF membrane and probed with the following antibodies **(Antibody Table)**: ACCα, AMPKα1, AMPKα2 (*Millipore*; Darmstadt, Germany), ACCα/β, (*Upstate;* Temecula, CA, USA); FAS (*BD*, Franklin Lakes, NJ, USA), pACC-Ser^{79}, pAMPK-Thr^{172}, STAT3, FoxO1, pFoxO1-Ser^{256} (*Cell Signaling;* Danvers; MA, USA); CPT1c (*Proteintech*; Chicago, IL, USA); β3-adrenergic receptor (β3-AR), pSTAT3-Tyr^{705}, UCP1 (*Abcam, Cambridge, UK*); CaMKKα, CaMKKβ, POMC (*Santa Cruz*; Santa Cruz, CA, USA); β-actin, α-tubulin (*Sigma;* St. Louis, MO, USA) as previously described {López, 2006 27789 /id;López, 2008 33316 /id;Roa, 2009 33347 /id;Lage, 2010 33340 /id;López, 2010 33344 /id;Martínez de Morentin, 2012 33357 /id;Varela, 2012 33358 /id;Whittle, 2012 33372 /id;Imbernon, 2013 33380 /id;Ramírez, 2013 33382 /id;Martínez de Morentin, 2014 33390 /id;Beiroa, 2014 16446 /id;Contreras, 2014 33391 /id}.

In situ hybridization

Coronal brain sections (16 μm) were probed with a specific oligonucleotide for FAS (*GenBank Accession Number*: NM_017332; 5'-GGG TCC ATT GTG TGT GCC TGC TTG GGG TG-3') as previously published (19-21;25;34;35;38-41).

Immunohistochemistry

Double labeling was performed as described (19;35;36;38), using a mouse anti-FAS (1:150; *Abcam,* Cambridge, UK*)*) and/or rabbit anti-estrogen receptor alpha (ERα) (1:100, *Santa Cruz Biotechnology, Inc;* Santa Cruz CA, USA). We used 4 female rats. The specificity of anti-ERα antibody was shown by preadsorption with control peptide (10 nmol/ml; data not shown). The specificity of anti-FAS antibody was previously validated (35).

Statistical analysis

Data are expressed as mean ± SEM. mRNA and protein data were expressed in relation (%) to control (either non-pregnant or vehicle-treated) rats. We used 6-19 animals per experimental group, depending on the experiments, as specified in each figure legend. Statistical significance was determined by Student's t-test when two groups were compared or ANOVA and *post-hoc* two-tailed Bonferroni test when more than two groups were compared. $P<0.05$ was considered significant.

RESULTS

Pregnancy inhibits AMPK and differentially modulates the expression and activity of enzymes required for fatty acid synthesis in the hypothalamus

Pregnant rats developed a marked hyperphagia at day 16-17 of gestation, represented as a daily average of those consecutive days **(Figure 1A)**. To analyze the central mechanisms that could mediate the effects of pregnancy on feeding, we studied the hypothalamic levels of the serine/threonine protein kinase AMPK, an important regulator of fatty acid biosynthesis and food intake (15;42;43). AMPK is activated by phosphorylation of Thr172 in the α subunit (pAMPKα) by several upstream kinases, such as calcium/calmodulin-dependent protein kinase kinase alpha and beta (CaMKKα and CaMKKβ). Once activated, pAMPK phosphorylates and thus inactivates ACC, resulting in decreased production of malonyl-CoA and, normally, a subsequent increase in food intake (15;42;43). It was therefore interesting that pregnant rats showed a marked decrease in phosphorylated hypothalamic CaMKKβ, AMPKα (pAMPKα) and ACCα (pACCα, which is then inactive) compared to non-pregnant female rats **(Figures 1B and 1C)**. In addition, the concentration of AMPKα2, but not AMPKα1, was decreased **(Figures 1B and 1C)**. This was also the case for hypothalamic FAS, an important enzyme for fatty acid biosynthesis with a direct role in regulating feeding (34;35;44;45). Our data showed a marked decrease in both FAS protein **(Figures 1B and 1C)** and activity **(Figure 1D)** in the hypothalamus of pregnant rats. Given the simultaneous activation of ACC and inactivation of FAS, we also measured malonyl-CoA levels in the hypothalamus of pregnant rats. Our results showed that gestation increased the concentration of malonyl-CoA in the rat hypothalamus **(Figure 1E)**, associated with a reduction in CPT1c protein levels **(Figures 1B and 1C)** and activity **(Figure 1F)**. Of note, the detected levels of malonyl-CoA in the hypothalamus of pregnant rats were the highest we have ever detected in a non-pharmacological or genetic setting (19;34;35;40;41;45) **(Supplementary Table 1).**

Pregnancy inhibits FAS expression specifically in the ventromedial nucleus of the hypothalamus

Having shown that pregnancy inhibits the expression of hypothalamic FAS, we aimed to investigate whether this effect was restricted to certain hypothalamic areas. Our data showed that pregnancy decreased FAS mRNA expression specifically in the ventromedial nucleus of the hypothalamus (VMH) and not in other hypothalamic nuclei, such as the ARC and the paraventricular nucleus (PVH) **(Figures 2A and 2B)**. Hypothalamic FAS expression is normally tightly regulated by nutritional status, being downregulated by either fasting or food restriction and increased by refeeding (34;35;45;46). We therefore investigated whether the effect of nutritional manipulations was preserved in the hypothalamus of pregnant rats. Our data showed that, neither fasting nor refeeding, did impact FAS expression in the VMH of pregnant rats, with FAS levels consistently decreased when compared with non-pregnant female rats **(Supplementary Figures 1A and 1B)**. Next, we investigated the effect of pregnancy on the protein levels of the fatty acid synthesis pathway. Western blot analysis also showed that pregnancy decreases the levels of pAMPKα and its downstream target pACCα, as well as AMPKα1 and FAS in the VMH **(Figures 2C-D)**. Of note, no effect was detected either in pAMPKα, pACC, AMPKα1 or FAS in the neighboring ARC **(Supplementary Figures 1C-D).**

FAS expression in the VMH is specifically inhibited by peripheral estradiol but not by other pregnancy hormones, such as progesterone and prolactin

Pregnancy is associated with several neuroendocrine changes, among them a marked increase in the circulating levels of progesterone, prolactin, placental lactogens and estrogens at the end of gestation (1-3;9). Recent data from our group have also shown that estradiol modulates AMPK activity in the hypothalamus (25). Thus, we aimed to investigate whether any of those hormones may mediate the effect of pregnancy on FAS mRNA expression. In order to avoid the possible interference of the endogenous ovarian steroids milieu, we analyzed the effects in OVX females (25;30;31). OVX rats displayed elevated body weight relative to sham-operated rats (data not shown) as well as the expected increase in LH serum levels 2 weeks after the ovaries were removed (Sham-operated: 0.5 ±

0.19 ng/mL *vs.* OVX: 11.7 ± 0.74 ng/mL, P<0.001). OVX rats subcutaneously treated with estradiol showed decreased body weight **(Figure 3A)** and food intake (daily average of the 7 days of treatment; **Figure 3B).** The loss in body weight was due to the effect of the estrogen and not related to differences in the body mass of the groups at the beginning of the treatment (vehicle: 281.91 ± 5.75 g *vs.* estradiol: 280.23 ± 3.65 g). FAS mRNA expression was specifically reduced in the VMH but not in the ARC and the PVH of estradiol-treated rats **(Figures 3C and 3D)**. On the contrary, neither progesterone nor prolactin, when given peripherally, affected body weight **(Figures 3E and 3H)**, food intake (daily average of the 7 or 4 days of treatment, respectively; **Figures 3F and 3I)** or FAS mRNA expression in any of the analyzed nuclei **(Figures 3G and 3J)**. Overall, these data suggest that among the pregnancy hormones analyzed, only estradiol affects FAS mRNA levels, and is thus the hormone likely to mediate the pregnancy-induced decrease of FAS expression.

FAS expression in the VMH is specifically inhibited by central estradiol through the estrogen receptor alpha

Next, we wanted to investigate whether the effect of estradiol on FAS expression in the VMH was centrally mediated or if it was a secondary effect, mediated by changes in peripheral hormones as a result of subcutaneous estradiol administration. In addition, we aimed to investigate which estrogen receptor (ER) isoform (alpha or beta) could mediate that action. OVX rats were ICV-treated with estradiol or the specific ERα or ERβ agonists, PPT and DPN, respectively (25). The efficiency of the three treatments was controlled by analyzing serum LH levels: OVX-vehicle: 8.97 ± 1.33 ng/mL; OVX-estradiol: 2.27 ± 0.49 ng/mL, P<0.001 *vs.* OVX-vehicle; OVX-PPT: 1.97 ± 0.39 ng/mL, P<0.001 *vs.* OVX-vehicle; OVX-DPN: 8.56 ± 1.11 ng/mL, P<0.001 *vs.* OVX-estradiol and OVX-PPT. Our data showed that central injection of estradiol and PPT, but not DPN, reduced body weight **(Figure 4A)**, food intake **(Figure 4B).** The loss in body weight was due to the effect of the drugs and not related to differences in the body mass of the groups at the beginning of the treatment (vehicle: 278.9 ± 7.14 g; estradiol: 275.91 ± 6.81 g; PPT: 271.65 ± 5.66 g; DPN: 272.53 ± 6.81 g). FAS mRNA expression in the VMH was reduced after estradiol and PPT but not DPN ICV administration **(Figures**

4C and 4D). Such ERα-mediated effect of estradiol on FAS expression in the VMH was further supported by the fact that double-labeling studies in female rats demonstrated high levels of colocalization of FAS and ERα in the VMH **(Figure 4E)**. Overall these data suggest that estradiol modulates FAS expression in the VMH through the ERα. In keeping with this observation, FAS mRNA expression **(Supplementary Figure 2A)** and activity **(Supplementary Figure 2B)** were decreased in the VMH of normal female rats in proestrus, the stage of the estrous cycle characterized by the higher circulating levels of estradiol. Similarly, FAS expression in the VMH of proestrus females was significantly decreased when compared to male rats **(Supplementary Figure 2C).**

Pregnant rats do not respond to the anorectic effect of central inhibition of FAS and accumulation of malonyl-CoA

Our data showed that pregnant rats were hyperphagic despite having higher levels of hypothalamic malonyl-CoA than non-pregnant female rats. This indicates that, in the gestational state, the mechanisms controlling food intake do not respond to the anorectic effect of hypothalamic malonyl-CoA. We therefore tested whether further central inhibition of FAS (19;34;44;45) would reverse hyperphagia in pregnant animals. To evaluate this, pregnant rats were treated with the FAS inhibitor cerulenin for 2 days (ICV administration) (19). In non-pregnant female rats, cerulenin increased malonyl-CoA (**Figure 5A)**, accompanied by marked anorexia and reduction in body weight **(Figures 5B and 5D)**. This increase in hypothalamic malonyl-CoA was not statistically significant in pregnant rats, probably due to the already extremely elevated concentration in the gestational state **(Figure 5A and Supplementary Table 1)**. Accordingly, cerulenin failed to change both food intake **(Figure 5C)** and body weight **(Figure 5E)** in pregnant rats. To further characterize the mechanism underlying resistance to the anorectic effects of malonyl-CoA accumulation in the hypothalamus of pregnant rats, we analyzed the expression of POMC, the key effector of cerulenin's anorectic effect (19). Our data showed that cerulenin induced a marked increase in POMC protein levels in the ARC of non-pregnant female rats **(Figures 5F and 5G)** but not in pregnant rats **(Figures 5H and 5I)**. This

increased POMC expression in non-pregnant female rats was accompanied by parallel changes in pFoxO1 and pSTAT3 **(Figures 5F and 5G)**, both of which regulate transcription of POMC (19;47;48). None of these effects were present in the ARC of pregnant rats. Overall, these data suggest that the normal anorectic response to cerulenin is blunted in pregnant rats, likely as a consequence of an impaired regulation of POMC and its putative regulators, the transcription factors pFoXO1 and pSTAT3.

Pregnant rats do not respond to the thermogenic effect of AMPK inhibition in the VMH

Recent data from our group have demonstrated that inhibition of AMPK in the VMH induces an activation of brown adipose tissue (BAT) thermogenesis through the sympathetic nervous system (SNS) {López, 2010 33344 /id;Whittle, 2012 33372 /id;Martínez de Morentin, 2012 33357 /id;López, 2013 33383 /id;Seoane-Collazo, 2014 16447 /id;Martínez de Morentin, 2014 33390 /id;Beiroa, 2014 16446 /id}. Having shown that pregnancy induced a marked decrease in the pAMPK levels specifically in that nucleus **(Figures 2C-D)**, we investigated BAT thermogenesis in pregnant rats (16-17 day of gestation). Notably, as previously demonstrated (28;29), our results showed decreased UCP1 and β3-AR protein concentration in the BAT of pregnant rats **(Figures 6A-B),** lower body temperature **(Figure 6C)** and BAT area temperature **(Figures 6D-E).** Overall, this evidence indicates that central control of thermogenesis by AMPK is altered in pregnant rats.

DISCUSSION

Pregnancy is associated with considerable metabolic demands that are met by a number of homeostatic mechanisms. There is a remarkable increase in body fat mass, body weight and feeding in the mother during gestation. The mechanisms involved in the regulation of hyperphagia in this state are still largely unknown but it is likely that pregnancy-induced hyperphagia is driven by altered response to hormonal signals, including the development of leptin resistance and impairment in the hypothalamic responses to leptin (1;8-11). The molecular mechanism underlying that effect is double: a reduction in the transport of leptin through the blood-brain-barrier (BBB) and an alteration in the hypothalamic intracellular leptin signaling pathway, such as STAT3 and phosphoinositide-3-kinase (PI3K) (1;8-11;49). In the current study, we demonstrate for the first time that during pregnancy, inhibition of hypothalamic AMPK, FAS and CPT1 activities, increased ACC function and accumulation of malonyl-CoA do not induce the expected anorectic response. These observations suggest that, besides refractoriness to the effects of leptin and its effectors (e.g., α-MSH), a state of resistance to the effects of cellular signals of energy surplus develops during gestation in order to cope with the high caloric demand for embryonic development and to prepare for the subsequent demands of lactation (1-3).

Evidence over the last decade has demonstrated that modulation of hypothalamic lipid metabolism plays a major role in the regulation of energy balance, affecting both feeding and energy expenditure (14-16;18;25;50). Anatomical data showed that central enzymes involved in lipid metabolism, namely AMPK, ACC, FAS and CPT1, are expressed at particularly high levels in key hypothalamic nuclei modulating energy homeostasis, such as the ARC, PVH and VMH in rodents and humans (14-16). Recent data from our group has demonstrated that central effects of estradiol on energy balance are mediated by modulation of VMH AMPK trough ERα (25). Interestingly, treatments with FAS inhibitors, such as cerulenin and C75 (44;51), drugs that down-regulate FAS expression, such as tamoxifen (TMX) (34), and genetic downregulation of FAS in the VMH (45) induce a remarkable weight loss and anorexia. One common feature to these manipulations is that all of them require hypothalamic malonyl-CoA accumulation for the hypophagic effect to take place.

Thus, malonyl-CoA has been proposed to act as a critical (anorectic) signal of nutrient abundance in the hypothalamus (44;51).

Although it is clear that fatty acid metabolism is a *bona fide* component of the hypothalamic networks that modulate energy homeostasis, it is not known whether sensing of cellular signals, such as lipids and/or derivates, could mediate the hyperphagic and overall anabolic state that characterizes pregnancy. Of note, our data show an overall state of hypothalamic fatty acid metabolism that should normally lead to anorexia, namely decreased pAMPK, FAS and CPT1 activities, as well as increased ACC function with concomitant elevated malonyl-CoA concentration. The fact that the pregnant rats remain hyperphagic suggests that they are resistant to the accumulation of malonyl-CoA in the hypothalamus. Of note, this effect seems to resemble the mechanism leading to leptin resistance, because the impaired response to the FAS inhibitor cerulenin is associated by alteration on STAT3 phosphorylation, which has been linked with altered leptin's responsiveness (11;49;52;53). Thus, we conclude that the hyperphagic state induced by pregnancy involves resistance to the usual effect of malonyl-CoA on food intake. Although the mechanisms by which this occurs are unclear, we speculate that accumulation of specific lipid species in the hypothalamus of pregnant rats might have a role. Moreover, it might not be unexpected that under conditions of elevated energy demand, specific allostatic responses may overrule usual inhibition of energy intake by malonyl-CoA. In support of this, recent evidence from our group has shown that in the hyperthyroid state, which also requires high energy intake to overcome the overall catabolic situation, hypothalamic resistance to malonyl-CoA also occurs (19). Another possibility is that changes in lipid species other than malonyl-CoA might interfere with the usual mechanisms controlling feeding, an idea that would require further investigation.

Current evidence has also linked hypothalamic AMPK specifically located in the VMH with BAT thermogenesis (27). Central signals that modulate BAT, such as thyroid hormone, bone morphogenetic protein 8b (BMP8b), leptin, glucagon-like peptide 1 (GLP-1) and estradiol decrease the activity of AMPK in the VMH, leading to increased sympathetic tone, elevated thermogenesis and weight loss {López, 2010 33344 /id;Whittle, 2012 33372 /id;Martínez de Morentin, 2012 33357

/id;Tanida, 2013 16253 /id;López, 2013 33383 /id;Seoane-Collazo, 2014 16447 /id;Martínez de Morentin, 2014 33390 /id;Beiroa, 2014 16446 /id}. In fact, the *VMH AMPK-SNS-BAT axis* seems to act as a canonical central mechanism, integrating central and peripheral regulation of energy homeostasis (27). Our data showed that hypothalamic AMPK signaling is specifically inhibited in the VMH of pregnant rats, despite the fact that BAT thermogenesis is markedly diminished in the same animals (28;29). Overall, this evidence indicates the development during pregnancy of an AMPK-resistant state which leads to lower thermogenesis and consequently the highest possible anabolic state.

The eventual role of maternal gestational hormones in the alterations of hypothalamic fatty acid metabolism in pregnant rats was also investigated in the current study. The hormonal changes in rat pregnancy start with the mating-induced surges of prolactin, followed by elevation of progesterone from the *corpus luteum* and the loss of the cyclical elevations of estrogens. Estradiol increases slowly in the second half of pregnancy, achieving high levels at the end of the gestation. The development of the placenta and the appearance of the placental hormones around mid-gestation, especially placental lactogens, introduces new factors regulating the energy homeostasis of the mother (2). As a first approach, we evaluated the possible involvement of the progesterone, prolactin and estrogens on the mRNA levels of FAS in the VMH. For that, we administered those hormones to OVX rats. Although no significant effects were found after treatment with progesterone or prolactin, our data showed that treatment with estradiol (peripherally and centrally), or the ERα specific agonist, PPT (centrally), recapitulate the pregnancy-induced decrease in FAS expression and activity in the VMH. Recent data from our group also indicate that pAMPK, pACC and CPT1 activities, as well as malonyl-CoA levels, are regulated in a similar manner in OVX rats treated with estradiol and PPT (25). Of note, neither prolactin nor progesterone induced any effect on the hypothalamic levels of FAS, suggesting that estradiol is the main regulator of FAS function. These results are also consistent with our previous report showing that TMX, a selective estrogen receptor modulator (SERM), which mimics as estrogen's actions in the VMH, inhibits FAS expression in that hypothalamic site (34). Whether prolactin and progesterone modulate AMPK signaling in the VMH will deserve further investigation.

However, considering that recent data have demonstrated that estradiol inhibits AMPK through ERα in the VMH of female rats (25), it is likely that increased estrogenic tone contributes to the reduced pAMPK levels in the VMH also during pregnancy.

In summary, our study shows that pregnant rats display resistance to the anorectic effects of hypothalamic inhibition of AMPK, FAS and CPT1, as well as accumulation of malonyl-CoA. Also our data show that pregnant rats do not respond to the thermogenic effect of AMPK inhibition in the VMH. Since estradiol induces a state of negative energy balance (54-57), and very recent evidence indicates that this effect is mediated by the hypothalamic AMPK pathway (25), our data demonstrate that during pregnancy there is a dissociation between such fatty acid pathway profile and the expected anorectic and thermogenic effects, through a state of resistance linked to altered hypothalamic POMC expression (and its transcriptional regulators, pSTAT and pFoxO1), as well as impaired UCP1 and β3-AR expression in the BAT. Therefore pregnancy constitutes a situation of increased food intake and fat deposit in the face not only of rising levels of leptin, but also rising levels of estradiol at the end of the gestational period. This evidence adds new proof to the fact that during pregnancy there exists an overall *"turning off"* of the *"anorectic and thermogenic switches"*, in order to get the optimal hyperphagic and anabolic status that guarantees a healthy state for the mother and, especially, ensure embryonic development and post-partum lactation demands. Furthermore, given that hypothalamic estradiol regulates glucose metabolism (58), it will be important to investigate whether the changes in glucose homeostasis that characterize pregnancy may be mediated by hypothalamic fatty acid pathway and AMPK, which is a major regulator of glycaemia (59;60). Understanding those events at the molecular level might be relevant not only for a better understanding of the physiology of pregnancy but also in the context of pathological alterations of energy balance, as obesity, where resistance to anorectic signals, such as leptin and insulin, together with hypothalamic AMPK alterations (6;61-64), have been also described.

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FIGURE LEGENDS

FIGURE 1. Effect of pregnancy on the enzymes required for fatty acid synthesis in the hypothalamus

(A) Daily food intake, **(B)** representative western blot images, **(C)** hypothalamic levels of proteins of fatty acid metabolism pathway, **(D)** FAS activity, **(E)** malonyl-CoA concentration and **(F)** CPT1 activity in the hypothalamus of non-pregnant female and pregnant rats. Dividing lines show spliced bands from the same gel. Data are expressed as mean±SEM; n=7-10 animals per experimental group; *, ** and ***P<0.05, 0.01 and 0.001 *vs.* non-pregnant.

FIGURE 2. Effect of pregnancy on fatty acid metabolism pathway in the hypothalamus

(A) Representative *in situ* hybridization autoradiographic images and **(B)** FAS mRNA levels in the hypothalamic arcuate (ARC), paraventricular (PVH) and ventromedial (VMH) nuclei of non-pregnant female and pregnant rats. **(C)** Representative western blot images and **(D)** levels of proteins of fatty acid metabolism pathway in the VMH of non-pregnant female and pregnant rats. Dividing lines show spliced bands from the same gel. 3V: third ventricle. Data are expressed as mean±SEM; n=7-10 animals per experimental group; *, ** and ***P<0.05, 0.01 and 0.001 *vs.* non-pregnant.

FIGURE 3. Effects of peripheral administration of estradiol, prolactin and progesterone on FAS expression in the hypothalamus of OVX rats

(A) Body weight, **(B)** daily food intake **(C)** representative *in situ* hybridization autoradiographic images and **(D)** FAS mRNA levels in the hypothalamic arcuate (ARC), paraventricular (PVH) and ventromedial (VMH) nuclei of OVX rats subcutaneously treated with vehicle or estradiol. **(E and H)** Body weight change, **(F and I)** daily food intake and **(G and J)** FAS mRNA levels in the ARC, PVH and VMH of OVX rats subcutaneously treated with vehicle, or progesterone **(E, F and G)** and vehicle or prolactin **(H, I and J)**. 3V: third ventricle. Data are expressed as mean±SEM; n=8-11animals per experimental group; *, ** and ***P<0.05, 0.01 and 0.001 *vs.* vehicle.

FIGURE 4. Effects of central administration of estradiol and estrogen receptor agonists on FAS expression in the hypothalamus of OVX rats

(A) Body weight change, **(B)** daily food intake **(C)** representative *in situ* hybridization autoradiographic images and **(D)** FAS mRNA levels in the ventromedial nucleus of the hypothalamus (VMH) of OVX rats centrally treated with vehicle, estradiol, PPT or DPN. **(E)** Photomicrographs showing double immunohistochemistry of FAS and ERα coexpression in the VMH (upper scale bar: 50 μm, lower scale bar: 25 μm). 3V: third ventricle. Data are expressed as mean±SEM; n=7-19 animals per experimental group or n=4 (immunohistochemistry); *, ** and ***P<0.05, 0.01 and 0.001 *vs.* vehicle.

FIGURE 5. Effects of central administration of cerulenin on energy balance in pregnant rats

(A) Hypothalamic malonyl-CoA levels, **(B and C)** daily food intake and **(D and E)** body weight change, **(F and H)** representative western blot images and **(G and I)** hypothalamic levels of proteins involved in POMC pathway in the arcuate nucleus of the hypothalamus (ARC) of non-pregnant female and pregnant rats centrally treated with vehicle or cerulenin. Data are expressed as mean±SEM; n=7-19 animals per experimental group; *, ** and ***P<0.05, 0.01 and 0.001 *vs.* vehicle.

FIGURE 6. Effects of pregnancy on BAT thermogenesis

(A) Representative western blot images, **(B)** of BAT UCP1 and β3-AR protein levels, **(C)** body temperature, **(D)** infrared thermal images and **(E)** quantification of temperature of the skin surrounding interscapular BAT of non-pregnant female and pregnant rats. Data are expressed as mean±SEM; n=6-8 animals per experimental group; *** P<0.001 *vs.* non pregnant.