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Kelly Meneyrol, Ánxela Estévez-Salguero, Ismael González-García, Jeanne Guitton, Mohammed Taouis, et al.. Ovarian insufficiency impairs glucose-stimulated insulin secretion through activation of hypothalamic de novo ceramide synthesis. *Metabolism*, 2021, 123, pp.154846. 10.1016/j.metabol.2021.154846 . hal-03407983

HAL Id: hal-03407983

<https://hal.science/hal-03407983v1>

Submitted on 22 Aug 2023

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Ovarian insufficiency impairs glucose-stimulated insulin secretion through activation of hypothalamic de novo ceramide synthesis

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Abstract

Oestrogens regulate body weight through their action on hypothalamus to modulate food intake and energy expenditure. Hypothalamic *de novo* ceramide synthesis play a central role on obesity induced by oestrogen deficiency. Depletion in oestrogens is also known to be associated with glucose intolerance, which favours type 2 diabetes (T2D). However, the implication of hypothalamic ceramide in the regulation of glucose homeostasis by oestrogen is unknown. Here, we studied glucose homeostasis and insulin secretion in ovariectomized (OVX) female rats. OVX induces body weight gain associated with a hypothalamic inflammation and impaired glucose homeostasis. Genetic blockade of ceramide synthesis in the ventromedial nucleus of the hypothalamus (VMH) reverses hypothalamic inflammation and partly restored glucose tolerance induced by OVX. Furthermore, glucose-stimulated insulin secretion (GSIS) is increased in OVX rats due to a raise of insulin secretion second phase, a characteristic of early stage of T2D. In contrast, GSIS from isolated islets of OVX rats is totally blunted. Inhibition of ceramide synthesis in the VMH restores GSIS from isolated OVX islets and represses the second phase of insulin secretion. [Stimulation of oestrogen receptor \$\alpha\$ \(ER \$\alpha\$ \) by oestradiol down-regulates ceramide synthesis in hypothalamic neuronal GT1-7 cells but no in microglial SIM-A9 cells. In contrast, genetic inactivation of ER \$\alpha\$ in VMH upregulates ceramide synthesis.](#) These results indicate that hypothalamic neuronal *de novo* ceramide synthesis triggers the OVX-dependent impairment of glucose homeostasis which is partly mediated by a dysregulation of GSIS.

1. Introduction

Ovarian oestrogens are known to exert important anti-obesity effects in humans and animal models. Indeed, lower levels of oestrogens in postmenopausal women are associated with obesity (1, 2). In men, total oestrogen deficiency due to the inactivating mutations of the CYP19 gene, which codes for aromatase (an enzyme responsible for a key step in the biosynthesis of oestrogens), produces metabolic dysregulation (3). Interestingly, oestradiol replacement reverses the progression of obesity and metabolic dysfunctions in postmenopausal women (4).

Due to their lipophilic nature, oestrogens readily diffuse through cellular membranes and operate via interaction with intracellular receptors. Two classical nuclear receptors for oestrogens (ER) have been described: ER α and ER β (5, 6). Both ER α and ER β operate as ligand-dependent transcription factors that, upon ligand binding, form dimers to interact with canonical ER response elements (ERE) in the promoter regions of oestrogen-regulated genes (5, 6). This classical mode of action, however, accounts for just a fraction of the effects of oestrogens, whose capacity to modulate gene expression and cellular functions are far more pleiotropic. Numerous actions of oestrogens, while conducted via ER and ER β , do not involve classical direct interactions with EREs, but rather the interplay with other transcriptional mediators, which operate at non-ERE regions (7). In addition, distinct membrane receptors for oestrogens have been described, whose nature is not fully elucidated yet. These are likely to include both ion channels, which can be modulated by oestrogens, as well as bona fide surface receptors, such as the G-protein coupled receptor, GPR30, also named GPER1, which seem to conduct, even in an overlapping fashion, some of the rapid effects of oestrogens on different cellular systems (8). Conflicting results on the actual cellular location (membrane vs. intracellular) and pharmacology of GPR30/GPER1 have been reported, and considerable controversy persists on the actual physiological roles of some of these non-classical receptors in mediating oestrogen actions (9, 10). Associated with the higher risk to develop obesity, oestrogen deficiency also predisposes males and females to impaired glucose homeostasis (3, 11, 12). Indeed, male and female mice with global knockout of oestrogen receptor α (ER α) develop not only obesity but also glucose intolerance (13, 14). The loss of ER α in pancreatic islets also predisposes male and female mice to β cell dysfunction and failure in response to metabolic stress (15, 16). Oestrogen replacement has been shown to prevent metabolic complications, such as type 2 diabetes (T2D). Recent evidence reported that, besides their direct action on peripheral insulin sensitive tissues, oestrogen also acts centrally to modulate energy balance (17-19).

Obesity is associated with a hypothalamic lipotoxicity (20, 21), which promotes central insulin resistance and body weight gain. Among the molecular mechanisms involved in hypothalamic lipotoxicity, endoplasmic reticulum (ER) stress and inflammation have been proposed (22, 23). In peripheral tissues, these processes occur through the accumulation of reactive lipid species, such as diacylglycerol and ceramides (24, 25). More recently, *de novo* synthesis of ceramide has been shown to induce ER stress in the hypothalamus leading to brown adipose tissue thermogenesis (19, 26). Accumulation of ceramide in the hypothalamus is not only responsible for the installation of central insulin resistance but also obesity-induced dysregulation of glucose homeostasis (27, 28). Hypothalamic ceramide inhibit insulin secretion by regulating parasympathetic tone and intrinsic β pancreatic insulin secretion (27). Compelling evidence suggests a mechanistic link between ceramides and the central effect of ovarian steroids on energy balance. Recent data from our groups have shown that central E2 decreases hypothalamic ceramide levels and endoplasmic reticulum ER stress, leading to increased sympathetic tone and brown fat activation (29). Moreover, we have recently shown that central ceramide synthesis mediates obesity-induced precocious puberty (30). Although lower levels of oestrogens have been associated with energy balance deregulation with a central role of hypothalamic ceramides (29), the role of these lipids in the deregulation of insulin secretion and higher risk of T2D induced by oestrogen deficiency is not known. In the present study, we aimed to determine the importance of *de novo* ceramide synthesis in the hypothalamus as a major player on the central effects of oestrogens on glucose homeostasis and especially insulin secretion.

2. Materials and Methods

2.1. Animals

Adult female Sprague-Dawley rats (250–300 g; Animalario General USC, Santiago de Compostela, Spain) were used for the experiments. All animals were housed on a 12-hour light (8:00 to 20:00)/ 12 hour dark cycle in a temperature- and humidity-controlled room and they were allowed free access to standard laboratory chow 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 and 15012/2020/010)

2.2. Ovariectomy

Rats were bilaterally ovariectomized (OVX) or sham operated under ketamine/xylazine anesthesia (50 mg/kg, intraperitoneally), as described previously (19, 29, 31). Both flanks of the rat were shaved. First, in the right flank, an incision was made in the skin and muscle layers. Carefully, the ovary was pulled out. Then, the distal part of uterine horn was ligated with surgical suture and the ovary was removed after making a dissection between the suture and the ovary. Finally, the uterine horn was returned into the abdominal cavity and the wound was closed by suturing the muscle layer and applying surgical staples to the skin. The procedure was repeated in the left ovary. In sham rats, the ovary was exposed but not dissected. Central treatments on OVX rats were carried out 2 weeks after surgery to ensure a total wash out of endogenous ovarian hormones.

2.3. Stereotaxic Microinjection of Adenoviral or Lentiviral Expression Vectors

Rats were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) under ketamine-xylazine anesthesia (50 mg/kg, intraperitoneally). The VMH was targeted bilaterally using a 25-gauge needle (Hamilton, Reno, NV). Two injections were performed in each VMH and were directed to the following stereotaxic coordinates for the VMH: 2.4/3.2 mm posterior to the bregma, ± 0.6 mm lateral to midline and 10.1 mm ventral, as previously reported (19, 20, 32-39). Adenoviral (Ad) vectors (Viraquest, North Liberty, IA) containing green fluorescence protein (GFP, used as control) or short hairpin serine palmitoyltransferase long chain subunit 1 (shSPTLC1), at 1.1×10^{12} pts/mL, were delivered at a rate of 200 nL/min for 5 min (1 μ L/injection site), as previously reported (19, 20, 32-39). After the Ad treatment, animals were monitored for 7 days. In some experiments, lentiviral (GFP, ER α shRNA; Santa Cruz Biotechnology; Santa Cruz) were stereotaxically delivered in sham rats as previously reported (19).

2.4. Glucose and Insulin Tolerance Tests

Insulin tolerance test (ITT) was performed after 7 days of Ad treatment and oral glucose tolerance test (OGTT) after 10 days of Ad treatment. Glycemia was measured at 0, 15, 30, 45, 60, 90 and 120 min with an Accu-Check glucometer (Roche, Barcelona, Spain) after an intraperitoneal injection of 0.5 UI/kg insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) for ITT or after 2 mg/g D-glucose (Sigma-Aldrich) administered orally via gavage for OGTT. Animals were fasted 5h or overnight for the ITT and OGTT, respectively.

2.5. Plasma insulin levels

During the OGTT, tail blood samples at 0, 15, 30 and 60 min were collected in microtubes with EDTA (Microvette 100 K3E, Sarstedt, Germany). Then, microtubes were centrifuged at 2000 rpm 15 min at 4°C. Plasma was collected and stored at -80°C until assayed for insulin by ELISA (Rat Ultrasensitive Insulin Elisa, ALPCO, Eurobio, Courtaboeuf, France).

2.6. In vitro insulin secretion from isolated islets

Islets of Langerhans were isolated after collagenase digestion of the pancreas as previously described (27, 40). In vitro insulin release was assayed from islets under static conditions after overnight culture. Floating Islets were pre-incubated in KRBH-0.05% BSA with 2.8 mM of glucose for 30 min, followed by 60 min incubation in KRBH-0.05% BSA with 2.8 or 16.7 mM glucose to measure glucose-induced insulin secretion. The supernatant was stored at -20°C until assayed for insulin by ELISA (Rat Ultrasensitive Insulin Elisa, ALPCO, Eurobio, Courtaboeuf, France). Islets were homogenized in protein extraction buffer and stored until insulin determination.

2.7. Sample processing

Rats were killed by cervical dislocation. From each animal, VMH (dissected from the whole hypothalamus) were harvested and immediately homogenized on ice to preserve phosphorylated protein levels. VMH was dissected by micropunches under the microscope, as previously reported (19, 20, 33, 37). Samples were stored at -80°C until further processing.

2.8. Western blot

Protein lysates from VMH tissue were homogenized in lysis buffer (consisting of a mix of 0.05M Tris-HCl, 0.01M EGTA, 0.001M EDTA, 0.016M Triton X-100, 0.001 M sodium orthovanadate, 0.05M sodium fluoride, 0.01 M sodium pyrophosphate and 0.25 M sucrose, made up with distilled water and adjusted to 7.5 pH; all of them from Sigma-Aldrich; St. Louis, MO, USA) and freshly added protease inhibitor cocktail tablets (Roche Diagnostics; Indianapolis, IN, USA). Bradford Method (Protein assay dye concentrate, Bio-Rad Laboratories; Hercules, CA, USA) was used to determine the protein concentration and the total protein content of the tissues was calculated.

The protein lysates were subjected to SDS-PAGE, electrotransferred to polyvinylidene difluoride membranes (PVDF; Bio-Rad Laboratories; Hercules, CA, USA) with a semidry blotter and probed with antibodies against IKK, pIKK, SPTLC1, SPTLC2 (Abcam; Cambridge, UK), TNF- α (Cell Signaling; Danvers; MA, USA) and β -actin (Sigma; St. Louis, MO, USA).

Each membrane was then incubated with the corresponding secondary antibody: anti-mouse or anti-rabbit (all of them from DAKO; Glostrup, Denmark). The membranes were exposed to an X-ray film (Fujifilm; Tokyo, Japan) and developed using developer (Developer G150; AGFA HealthCare: Mortselsel, Belgium) and fixator (Manual Fixing G354; AGFA Health-Care: Mortselsel, Belgium). Autoradiographic films were scanned and the bands signal was quantified by densitometry using ImageJ-1.33 software (NIH; Bethesda, MD, USA). Values were expressed in relation to β -actin. Representative images for all proteins are shown. In all the Figures showing images of gels, all the bands for each picture come always from the same gel, although they may be spliced for clarity.

2.9. Cell culture and Real-time PCR

GT1-7 murine hypothalamic cells (kindly provided by the Dr. P. Mellon, UCSD, USA) were cultured in DMEM medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin (27). SIM-A9 murine microglial cells were cultured in 50% DMEM 4.5g/L de glucose, and 50% HamF12 supplemented with 15% FBS and 100 U/mL penicillin/streptomycin. Oestradiol (E2) and the ER α agonist 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) was dissolved in DMSO. Both cell lines were treated with various concentrations of E2 or PPT for 24h or 48h. 2.9 Real-time PCR. RNA isolation, cDNA synthesis, and mRNA quantitation were carried out as described (27). A list of the primer sequences is shown in Supplementary Table 1.

2.4. Statistical analysis

Data are expressed as mean \pm SEM. Protein data were expressed in relation to control Sham GFP rats. Data analysis was performed using GraphPad Prism statistical software. Statistical significance was determined by multiple t-test, unpaired t-test, ordinary one-way ANOVA and two-way ANOVA. $P < 0.05$ was considered significant.

3. Results and Discussion

3.1. Ovariectomy increases body weight of female rats

Ovariectomy (OVX) in female rats significantly increases body weight gain as soon as 5 days after surgery (after 14 days after surgery: sham: 19.9 +/- 1.99 g; OVX: 43.85 g +/- 2.16; $p < 0.001$) (Fig.1A). This raise is associated as expected with an increase of serine palmitoyl-transferase long chain (SPTLC) 1 and 2 protein levels in the hypothalamus of OVX female rats (Fig.1B and C) comforting a role of hypothalamic *de novo* ceramide synthesis in dysregulation of energy balance (29). Stereotaxic microinjection of adenoviral vectors encoding for a short hairpin SPTLC1 (Ad-shSPTLC1) in the VMH was used to down-regulate SPTLC1 and decrease ceramide accumulation induced by OVX as previously shown (29). Selective infection in the VMH was assessed by visualization of GFP expression (Fig.1D) following injection of a control adenoviruses expressing green fluorescence protein (GFP) alone as previously shown (29). Down-regulation of hypothalamic SPTLC1 does not alter the increased body weight gain (Fig.1E), nor daily food intake (Fig.1F) in OVX females over a period of 7 days post-injection.

3.2. Silencing of SPTLC1 specifically in the VMH inhibits hypothalamic inflammation in OVX rats

Over-nutrition is known to induce hypothalamic inflammation, which favours obesity and related diseases (41). The master inflammatory NF- κ B pathway plays a central role in hypothalamic inflammation and energy balance deregulation (42, 43). OVX has been shown to potentiate hypothalamic inflammation in response to obesity (44, 45). Therefore, we explored whether OVX regulates the NF- κ B pathway in the hypothalamus through a *de novo* ceramide synthesis-dependent mechanism. OVX increases the protein levels of IKK and its phosphorylation status in the hypothalamus (Fig.2A and B). Activated IKK induces the phosphorylation of its substrate I κ B α and its proteasomal degradation. This promotes the release of NF- κ B and its translocation into the nucleus where it induces the transcription of inflammatory cytokine genes (46). Down-regulation of SPTLC1 by Ad-shSPTLC1 does not alter hypothalamic IKK overexpression but blunted its increased phosphorylation state in OVX female rats (Fig.2A and B). These data suggest that depletion of oestrogen induces hypothalamic inflammation in a *de novo* ceramide synthesis-dependent manner. In agreement, OVX increases the hypothalamic TNF α protein expression, which is reversed by deletion of SPTLC1 in VMH (Fig.2C).

3.3. Oestradiol inhibits the expression of *de novo* ceramide synthesis genes in neuronal but not in microglial cell lines

Hypothalamic inflammation can occur in neurons (42) but also in other neural cells (43, 47). Therefore, we determined whether oestrogen targets *de novo* ceramide synthesis in neuronal or microglial cells in order to inhibit inflammation. Oestradiol (E2) at low dose (1nM) slightly increases the mRNA expression of SPTLC2 subunit but drastically reduces the mRNA expression of SPTLC1 and of several ceramide synthases (CerS) in the GT1-7 neuronal cell line (Fig.3A and C). E2 has no effect on the mRNA expression of the dihydro-ceramide desaturase 1 (DES1) (Fig.3A). At the same concentration, E2 is unable to alter DES1, SPT or CerS mRNA expression in the SIMA-9 microglial cell line (Fig.3B and D). We and others have previously shown that VMH administration of a lentivirus encoding a shRNA that silences ER α to sham rats induced weight gain without changes in feeding behaviour (19, 48). Interestingly, a specific ER α agonist, PPT, also decreases the mRNA expression of SPTLC1 and CerS1 in GT1-7 cells (Fig.3E). Moreover, VMH-specific delivery of adeno-associated viral vectors silencing ER α in sham rats is associated with an increase of SPTLC2 protein levels in the hypothalamus (Fig.3F). These results support a role of ER α in the negative regulation of *de novo* ceramide synthesis in hypothalamic neurons. Among the CerS expressed in GT1-7 cells, E2 (1 nM) and PPT decreases CerS1 mRNA levels by almost 70% (Fig.3C and E). Recently, it has been shown that CerS1 global knockout mice exhibit a higher energy expenditure protecting them from diet-induced obesity (49). It will be therefore tempting to suggest that in vivo oestrogen depletion could raise ceramide levels by increasing CerS1 expression in hypothalamic neurons to induce inflammation and later on dysregulate energy balance and favour obesity.

3.4. Specific down-regulation of SPTLC1 in the VMH ameliorates glucose tolerance of OVX rats

Obesity is known to induce glucose intolerance through hypothalamic inflammation (21) and *de novo* ceramide synthesis (27). OVX also induces glucose intolerance (50, 51). However, it is unknown whether *de novo* ceramide synthesis is involved. Therefore, we aimed to determine if the down-regulation of SPTLC1 in the VMH might restore glucose homeostasis in OVX female rats. Oral glucose tolerance test (OGTT) shows that OVX rats exhibit glucose intolerance (Fig.4A) as previously shown (50, 51). We noticed a significant increase of AUC-(15-30min) for the OVX group compared to the Sham group (Fig.4B). After 7 days-post injection, Ad-shSPTLC1 significantly reduced the peak of glycemia in OVX group compared

to Sham group, (OVX: 156.9 +/- 6.11 mg/dl; OVX +shSPTLC1: 140.9 +/- 6.76 mg/dl; $p < 0.001$) during the OGTT (Fig.4A). Notably, these results suggest an improvement of glucose homeostasis independent of a reduction of weight in OVX rats, since body weight was not altered during this period (Fig.1E). Insulin tolerance test (ITT) showed no difference of insulin sensitivity between OVX and Sham group (Fig.4C) whereas down-regulation of SPTLC1 seems to maintain the drop in glycaemia at later times in OVX female rats (at 120 min: OVX: 40.9 +/- 4.29 mg/dl; OVX + shSPTLC1: 24.44 +/- 4.85 mg/dl; $p < 0.002$). These results suggest that the reduction of the peak of hyperglycemia during OGTT induced by down-regulation of hypothalamic SPTLC1 in OVX is not related to decreased insulin sensitivity in agreement with previous studies (51) but would rather involves an effect on glucose-induced insulin secretion. Increased glycaemia at the end of ITT could be related to a counter-regulation of the induced hypoglycaemia (52). Knowing the role of VMH in the counter-regulation (53), future experiment will be required to determine how hypothalamic ceramides can regulate the counter-regulation of hypoglycaemia.

3.5. Hypothalamic SPTLC1 contributes to the dysregulation of insulin secretion in OVX rats

Glucose-induced insulin secretion, which is partly regulated by hypothalamic circuitry (54), is altered by inflammation (55). Hypothalamic *de novo* ceramide synthesis has also been associated with a deregulation of insulin secretion by islets of Langerhans from obese rats (27). Therefore, we explored insulin secretion during the OGTT in OVX female rats. OVX does not increase significantly the first phase of insulin secretion (at t15 min: sham: 0.70 +/- 0.12 ng/ml; OVX: 1 +/- 0.22; $p < 0.312$) whereas it maintains secretion at later times (at t30 min: sham: 0.35 +/- 0.03 ng/ml; OVX: 0.61 +/- 0.08; $p < 0.014$), which represents the second phase of insulin secretion (Fig.4D). **In agreement, the increment of insulin levels between 0 and 30 min in OVX females is significantly increased compared to sham rats whereas there is no difference between groups at 0 vs 15 min (Fig4E and F). This late increase of insulin was associated with a 2-fold increase of the AUC Insulin (0-60 min) of OVX compared to Sham female rats (Fig.4G).** The appearance of an exaggerated second phase of insulin secretion during OGTT is a hallmark of the early stages of T2D (56). Interestingly, this defect is also found in knock-out male and female mice for the nuclear ER α (57). Down-regulation of hypothalamic SPTLC1 partially decreases this increase of the second phase of insulin secretion during the OGTT (Fig.4D), with the AUC between the Sham + shSPTLC1 and OVX + shSPTLC1 that become statically not different (Fig.4G). **In agreement, the increment of insulin levels between 0 and 30 min in OVX + shSPTLC1 females is not significantly**

different from Sham + shSPTLC1 (Fig4F). These results suggest down-regulation of hypothalamic SPTLC1 markedly alters insulin secretion in OVX rats. In order to determine if the dysregulation of insulin secretion induced by hypothalamic ceramide in OVX female rats is islet cell autonomous, we explored glucose-stimulated insulin secretion from isolated islet of Langerhans from these rats. Sham islets respond to stimulatory concentration of glucose (16.7 mM) whereas glucose-induced insulin secretion from OVX islets is completely blunted (Fig.4H). Deletion of SPTLC1 in VMH totally reverses the inhibitory effect of OVX (Fig.4H). Moreover, insulin content is not altered in islets of OVX female treated or not with the Ad-shSPTLC1 (Fig.1 supp). These data suggest that oestrogen depletion induces a hypothalamic *de novo* ceramide synthesis-dependent dysregulation of insulin secretion.

4. Conclusion

Hypothalamic ceramide metabolism has been shown to play a major role on energy balance (20, 26), in particular regulating the effects of gonadal axis on metabolism (29, 30). Here, our data show that *de novo* ceramide synthesis, in addition to its role in energy balance impairment (26), also contributes to the dysregulation of glucose homeostasis induced by oestrogen deficiency. In agreement with a previous study (51), this defect is not related to the installation of an insulin resistant state, but rather comes from a deregulation of glucose-induced insulin secretion in OVX females. Indeed, hypothalamic *de novo* ceramide synthesis contributes to the in vitro defect of insulin secretion induced by glucose from isolated islets of OVX rats. Thus, hypothalamic ceramides could negatively modulate intracellular insulin secretion pathways in islets. OVX seems to have no effect on glucose metabolism in islets but down-regulates the expression of SNARE proteins such as syntaxin-1A (51). This latter mechanism reduces the number of secretory granules docked onto the plasma membrane (58) and could contribute to the alteration of glucose-induced insulin secretion in OVX females. We recently showed that hypothalamic ceramides decrease parasympathetic tone in genetic obese rats which result in a drastic alteration of glucose-induced insulin secretion from isolated islets (27). Whether inhibitory effect of hypothalamic ceramide on parasympathetic tone is responsible for this effect in islets of OVX females will require further studies.

In vivo, the intrinsic defect of glucose-induced insulin secretion by islets of OVX rats is associated with an increase of the second phase of insulin secretion during OGTT, a characteristic of pre-diabetes (56). Importantly, this latter adaptation is also under the control of hypothalamic *de novo* ceramides synthesis. OVX female had higher levels of insulin-degrading enzyme in liver and this is correlated with higher insulin clearance (51) suggesting that the raise of the second phase of insulin secretion is not due to a defect in elimination of insulin. These data suggest that despite alteration of glucose-induced insulin secretion by hypothalamic ceramide, additional mechanisms which raise insulin secretion are still functional in OVX females. These mechanisms remain to be elucidated but could involve inhibition of the sympathetic tone, known to decrease insulin secretion (59). Higher sensitization of islets to incretins such as glucagon-like-peptide 1 (GLP-1) would be not be possible since OVX induces a partial decrease in GLP-1 secretion from pancreatic α cells and intestinal L cells (60). In contrast, an organ-organ communication with the endocrine pancreas involving liver and adipose could be relevant to regulate the second phase of insulin secretion. Indeed, these organs are known to regulate islet function (61, 62). However, due to intrinsic

defect of islets [observed in OVX rats](#), it will be expected at longer time that the [increase of second phase of insulin secretion](#) will be lost and favour the onset of T2D in these rats.

This early deregulation of glucose-induced insulin secretion seemed to be initiated independently from the regulation of body weight gain by ceramide. [Moreover](#), our data suggest that the molecular link between hypothalamic ceramides and the alteration of insulin secretion could be the induction of hypothalamic inflammation (23) known as a factor involved in inhibition of insulin secretion (55). It will remain to determine the hypothalamic neural cell subtype responsible for the regulation of the ceramide/inflammation axis by oestrogens and their role on the deregulation of glucose homeostasis by OVX. Our data suggest a selective inhibitory effect of oestrogen [through the ER \$\alpha\$ receptor](#) on *de novo* ceramide synthesis [pathway](#) in neuronal cells. ER α is expressed in steroidogenic factor 1 (SF1) derived neurons of the VMH, which are known to control, [besides BAT thermogenesis](#), insulin sensitivity and glucose tolerance [but not directly link to the regulation of insulin secretion](#) (39). This could originate from the existence of several sub-populations of SF1 neurons, as it happens for example for POMC neurons (63). Indeed, it has been shown that some SF1 neurons seem to be specialized in the regulation of blood glucose levels, whereas others are responsible for insulin sensitivity in the periphery (64). In addition to the identification of SF1 neurons sub-populations, the communication of SF1 neurons with other neurons outside the VMH to trigger metabolic functions could be relevant. Proopiomelanocortin (POMC) neurons are key regulator of insulin secretion (65). Indeed, chronic POMC neuronal inhibition has been shown to be associated with a decrease in a defect in glucose-induced insulin secretion (66). Interestingly, POMC neurons could receive excitatory inputs from the VMH (67) and high fat diet controls SF1 neuronal activity, which leads to the attenuation of POMC neurons activation, resulting in obesity and diabetes (68). Whether *de novo* ceramides synthesis in the VMH regulate the communication between SF1 and POMC neurons in order to regulate insulin secretion remain to be determined. Alternatively, whether different SF1 populations could also modulate different autonomic populations and/or endocrine axes (i.e. adrenal function) involved in counter-regulatory responses would need to be addressed. Aging has been shown to be associated with an increase in circulating ceramide levels, which in post-menopausal women is at least partially linked to lower oestrogen levels (69). Whether hypothalamic ceramide levels are increased in post-menopausal women remains unknown. Nevertheless, these recent data with ours suggest that a hypothalamic *de novo* synthesis of ceramide can be central in the [deregulation of glucose homeostasis and will favour the development](#) of T2D induced by oestrogen depletion.

Further studies on this topic will be necessary to understand the physiological link between central ceramides, oestrogen and the regulation of whole-body metabolism.

Acknowledgement

The research leading to these results has received funding from the Xunta de Galicia (ML: 2016-PG068); Ministerio de Economía y Competitividad (MINECO) co-funded by the FEDER Program of EU (ML: RTI2018-101840-B-I00); Atresmedia Corporación (ML); “la Caixa” Foundation (ID 100010434), under the agreement LCF/PR/HR19/52160022 (ML); Centre National de la Recherche Scientifique (CM and HLS); the Innovative Medicines Initiative Joint Undertaking under grant agreement n° 155005-IMIDIA (CM); the Agence Nationale de la Recherche (CM: ANR PRCI-15-CE14-0027-01 BetaDiamark); Société Francophone du Diabète (HLS). IG-G is recipient of a fellowship from the European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie actions (842080 - H2020-MSCA-IF-2018). J. Guitton received a doctoral fellowship from the Université Paris Saclay. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

HLS, ML and CM contributed to the study concept and design. HLS, ML, CM, KM, and YB contributed to the analysis and interpretation of the data. KM, AES, IGG, JG, YB and HLS contributed to the acquisition of the data. HLS and ML wrote/edited the manuscript with contributions from CM, KM, AES, IGG and YB. HLS and ML are responsible for its content. All authors revised the article and approved the final version.

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Legend to figures

Figure 1: Ovariectomy induces body weight gain in association with higher expression of hypothalamic SPTLC1 in female rats.

Female rats underwent surgery with (OVX group: empty symbols) or without (Sham group: plain symbols) ovariectomy. **A)** Animals were weighed for 14 days post-surgery (day 0 correspond to day of OVX surgery). **B and C)** After the end of experiments, rats were killed and hypothalamus were sampled in order to quantify protein levels of SPTLC 1 and 2. **D)** 2 weeks post OVX-surgery, stereotaxic microinjection of adenoviral vectors encoding for a short hairpin SPTLC1 (Ad-shSPTLC1) in ventromedial hypothalamus (VMH) was used to down-regulate SPTLC1. **Direct fluorescence of GFP in the VMH of OVX rats.** **E)** Rats were weighed from day 0 (day of stereotaxic surgery) up to day 7. **F)** Daily food intake was also assessed during this latter period. Data represent means \pm SEM. * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$, Sham vs OVX groups and Sham GFP vs OVX GFP groups and **** $p < 0,001$, Sham shSPTLC1 vs OVX shSPTLC1 groups (n= 18-20 rats per group for A and n= 9-10 for B,C,E,F).

Figure 2: Silencing of SPTLC1 specifically in the VMH inhibits hypothalamic neuronal inflammation in OVX rats

Female rats underwent 2 sets of surgery with (OVX groups: shaded colors) or without (Sham groups: plain colors) ovariectomy followed by stereotaxic microinjection of Ad-shSPTLC1 (in grey) or Ad-GFP (in black) in VMH. After the end of experiments, rats were killed and hypothalamus were sampled in order to quantify protein levels of **A)** IKK, **B)** pIKK and **C)** TNF α by western blot (standardization on β -actin and relative density calculated in reference to the mean of Sham GFP group). Data represent means \pm SEM. * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$ (n= 6-7 rats per group).

Figure 3: Oestradiol decreases SPTLC1 and ceramide synthases mRNA expression in neuronal cells but not in microglial cells.

Neuronal cells (GT1-7) and microglial cells (SIMA-9) were treated with various doses (1 nm, 10 nm and 100 nm) of oestradiol for 48h. mRNA levels of several genes involved in the *de novo* ceramide biosynthesis (CerS1, CerS2, CerS3, CerS4, CerS6, DES1, SPT1 and SPT2) were determined using RT-qPCR. Each sample was processed twice and every condition was made in triplicate. mRNA levels were calculated using $2^{(-\Delta\Delta CT)}$ method with RPL 19 and β -

actin as housekeeping genes and the first control group as calibrator. **A)** and **C)** mRNA levels in GT1-7 neuronal cells on the left and **B)** and **D)** in SIM-A9 microglial cells on the right. Data represent floating bars (min to max) with line at mean \pm SEM. * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$ versus control. **E)** GT1-7 cells were treated with 100 nM of PPT or E2 for 24h. mRNA levels of SPTLC1 and CerS1 were determined using RT-qPCR.. Data represent means \pm SEM. ** $p < 0,01$, *** $p < 0,001$ versus control. **F)** SPTLC2 protein levels in the VMH of rats stereotaxically treated within the VMH with lentivirus encoding short hairpin RNA of the ER α . Error bars represent SEM; n = 6 animals per experimental group. ** $p < 0.01$ versus GFP.

Figure 4: Specific down-regulation of SPTLC1 in the VMH ameliorates glucose tolerance and impact insulin secretion of OVX rats

Female rats underwent 2 sets of surgery with (OVX groups: shaded colors) or without (Sham groups: plain colors) ovariectomy followed by stereotaxic microinjection of Ad-shSPTLC1 (in grey) or Ad-GFP (in black) in VMH. **A)** An oral glucose tolerance test (OGTT) was performed with glycaemia measurements 10 days post-stereotaxic procedure (fasting overnight, 2 g/kg). **B)** AUC-1(15-30 min) based on those values allowed a better assessment of glucose tolerance at the pic area. **C)** Insulin sensitivity was evaluated with an insulin tolerance test (ITT) with glycaemia measurements 7 days post-stereotaxic procedure (5h of fasting, 0.5 UI/kg). Data represent means \pm SEM. * $p < 0,05$, *** $p < 0,001$ T15 min, T30 min versus T0 min. n= 6-10 rats per group for A,B and D and n=3-6 rats per group for C. **D)** During the OGTT was performed, four blood samples on tail at 0, 15, 30 and 60 min were made. Plasma insulin levels were obtained after ELISA tests. **E and F)** Increment of insulin secretion was determined between T0 vs T15 min as representative of the first phase of insulin secretion (**E**) and T0 vs T30 min as representative of the second phase of insulin secretion (**F**). Data represent the percent of T0 \pm SEM. * $p < 0,05$ Sham-GFP vs OVX-GFP and Sham-shSPTLC1 vs OVX-shSPTLC1. **G)** AUC based on those values allowed a better assessment of glucose induced insulin secretion variations as a whole. **H)** Glucose induced insulin secretion test on isolated islets from Sham and OVX rats subjected to Ad-shSPTLC1. The amount of insulin secreted after stimulation with low (basal) or high glucose concentration was estimated in ng per islet (each condition was made in triplicate for each rat). Data represent as a percent of stimulated insulin secretion over basal in Sham \pm SEM. * $p < 0,05$, ** $p < 0.01$, *** $p < 0,001$.

Figure 1 :

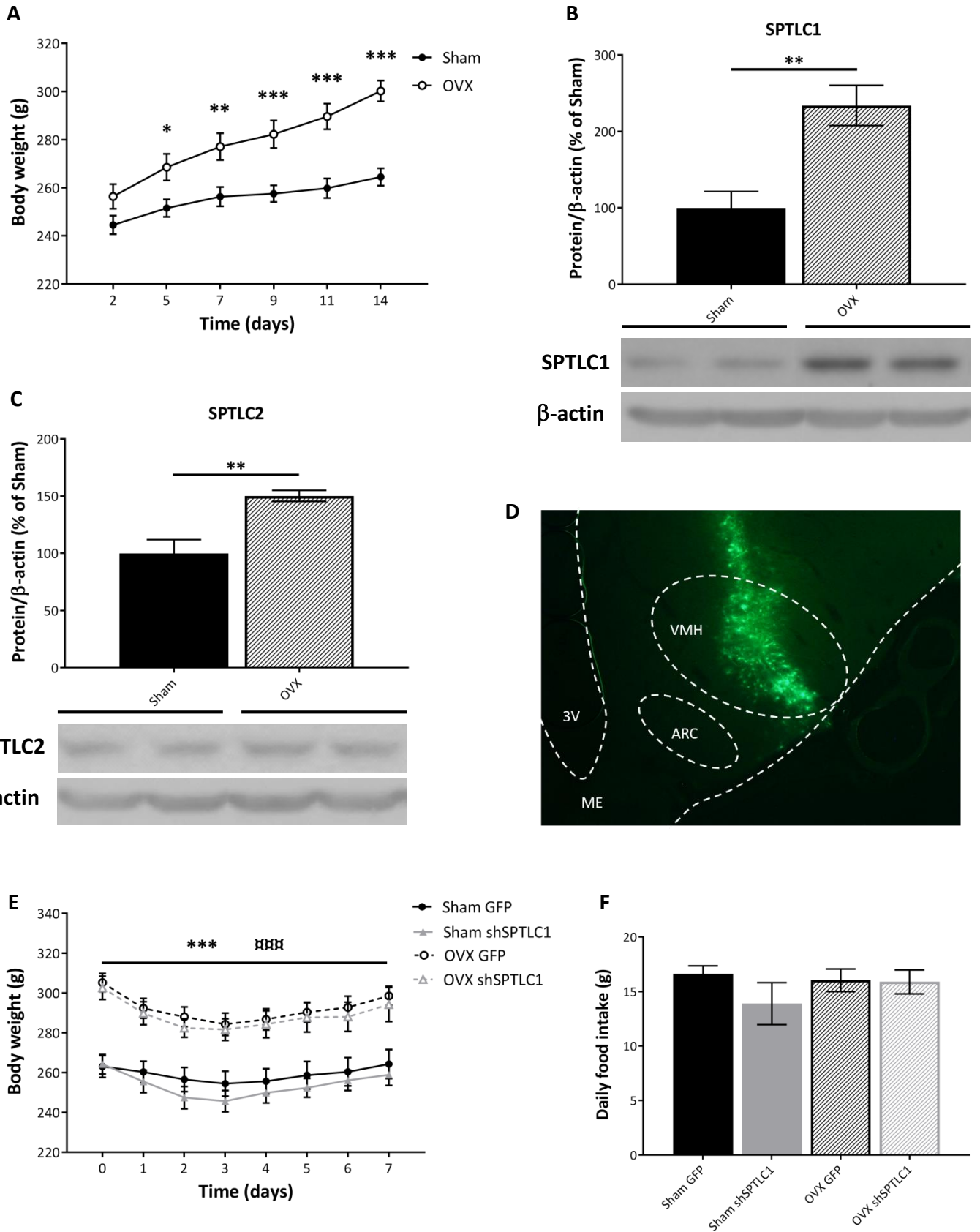


Figure 2 :

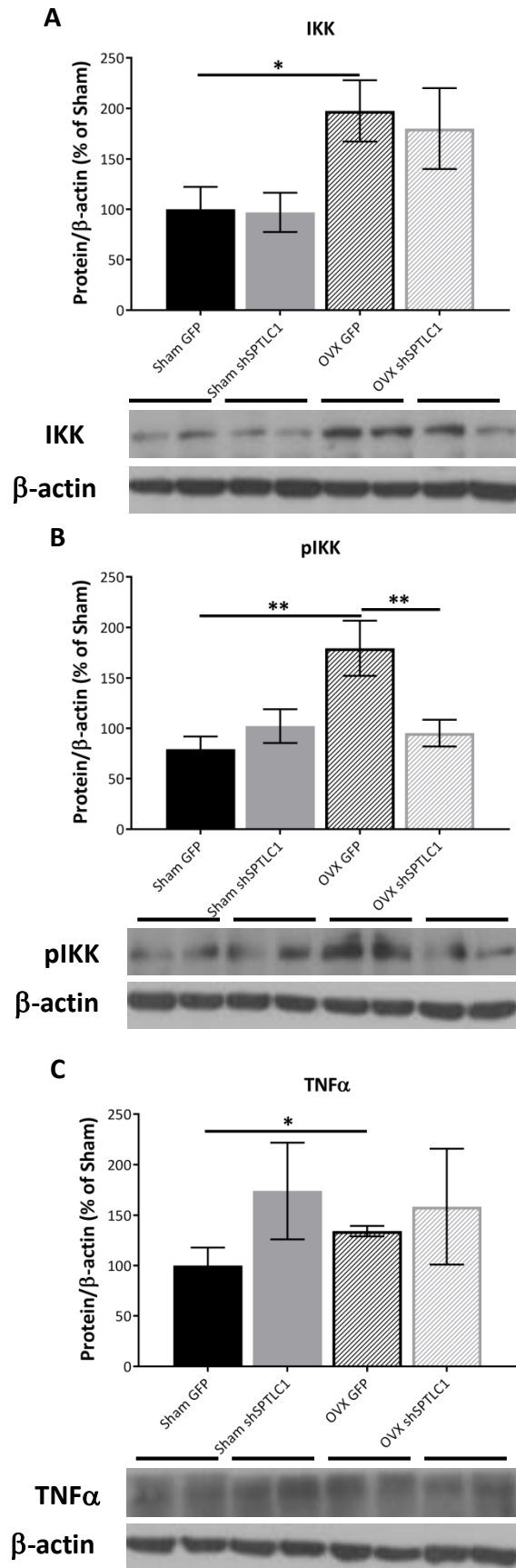


Figure 3 :

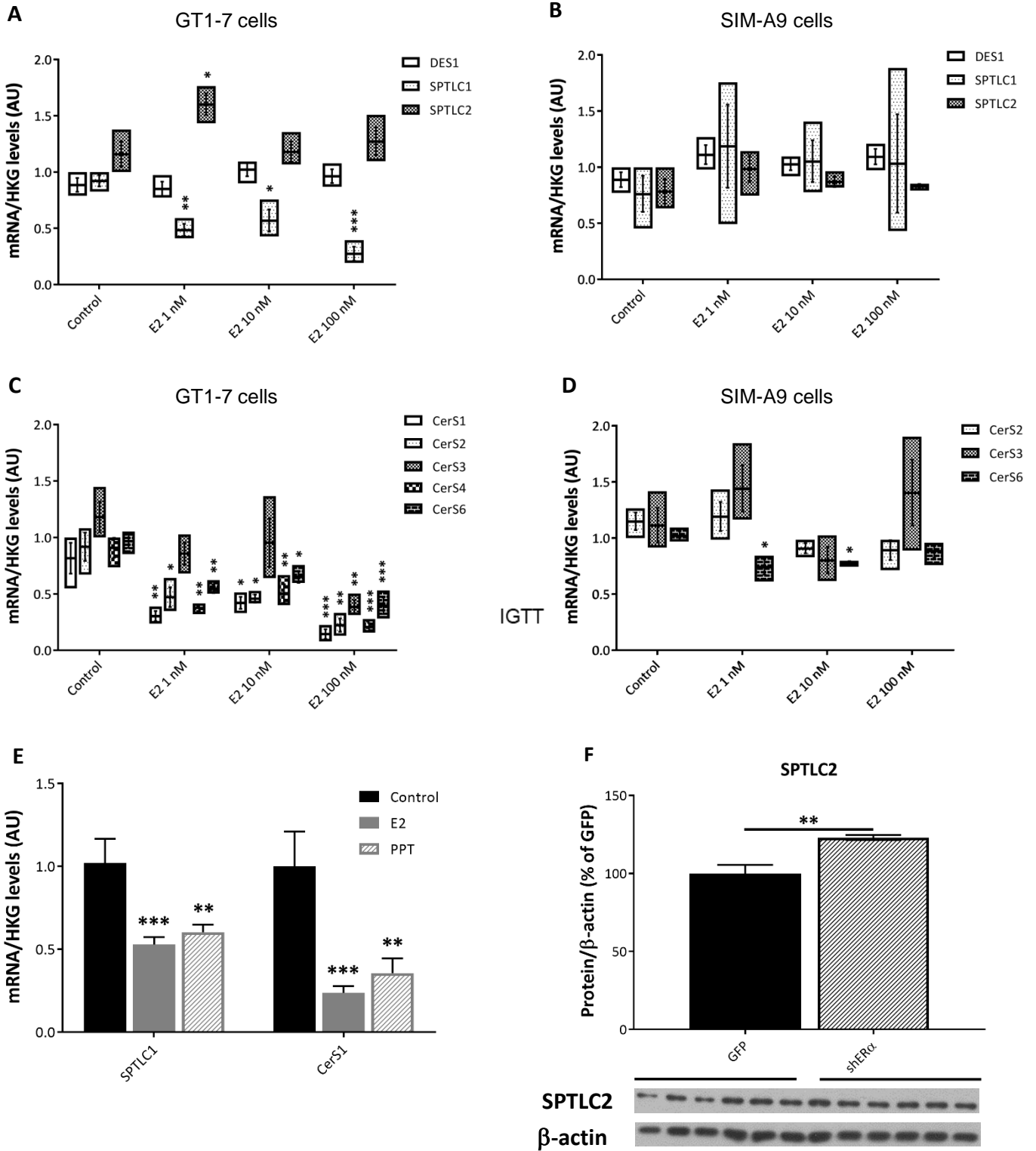
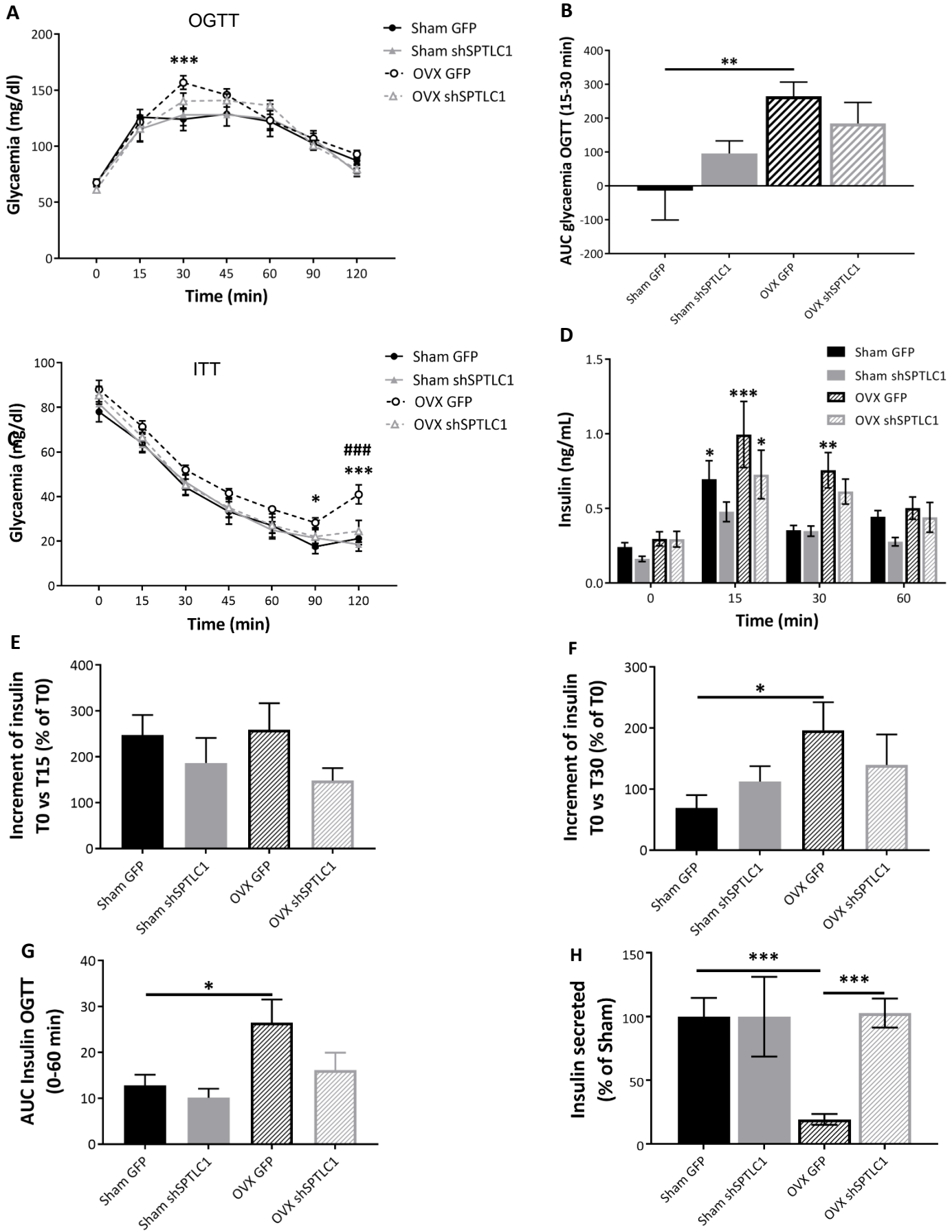


Figure 4 :



Supplementary data :

Figure S1 :

