Ageing alters the lipid sensing process in the hypothalamus of Wistar rats. Effect of food restriction

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ABSTRACT

Introduction: Lipids regulate a wide range of biological processes. The mechanisms by which fatty acids (FA) and its metabolites influence the hypothalamic regulation of energy homeostasis have been highly studied. However, the effect of ageing and food restriction (FR) on this process is unknown.

Methods: Herein, we analyzed the gene expression, protein and phosphorylation levels of hypothalamic enzymes and transcription factors related to lipid metabolism. Experiments were performed in male Wistar rats of 3-, 8- and 24-month-old Wistar rats fed *ad libitum* (AL), as ageing model. Besides, 5- and 21-month-old rats were subjected to a moderate FR protocol (equivalent to ≈ 80% of normal food intake) for three months before the sacrifice.

Results: Aged Wistar rats showed a situation of chronic lipid excess as a result of an increase in *de novo* FA synthesis and FA levels that reach the brain**,** contributing likely to the development of central leptin and insulin resistance. We observe a hypothalamic downregulation of AMP-activated protein kinase (AMPK) and stearoyl-CoA desaturase (SCD1) and an increase of carnitine palmitoyltransferase-1c (CPT1c) expression.

Discussion: Our results suggest an impairment in the physiological lipid sensing system of aged Wistar rats, which would alter the balance of the intracellular mobilization and trafficking of lipids between the mitochondria and the Endoplasmic Reticulum (ER) in the hypothalamus, leading probably to the development of neurolipotoxicity in aged rats. Lastly, FR can only partially restore this imbalance.

KEYWORDS: Ageing, lipotoxicity, food-restriction, leptin-resistance, lipid sensing

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Introduction

It is well established that the central nervous system (CNS) regulates food intake and energy expenditure in response to the nutrient signals. Nutrient sensitive hypothalamic neurons respond to peripheral glucose and FA, modifying the neuropeptides synthesis and modulating energy balance [1]. Besides, lipids, which constitute 50% of brain dry weight [1], are key components of intracellular signalling pathways involved in the regulation of a wide range of biological processes, including immune function, neuronal signalling, axonal myelination and synaptic function [2]. Furthermore, the brain expresses at high level enzymes responsible for the transport, utilization, and storage of lipids, suggesting that lipid metabolism is important in this organ, although glucose is its major energy source [3].

Since the discovery of lipid sensing neurons [4], recent evidence points at FA and their intermediary metabolites as important modulators of neuron activity in the hypothalamus, as well as key regulators of whole body energy balance through the control of insulin secretion, hepatic glucose production, adipose storage and food intake [3, 5, 6]. This phenomenon is known as “lipid sensing”. The mechanisms by which FA and their metabolites affect the CNS, particularly the hypothalamic regulation of energy homeostasis, have become an area of intense investigation. In this sense, intermediary fuel metabolism in the hypothalamus seems to play an important role in the regulation of energy homeostasis [2,3].

Cerebral lipids are derived from lipid uptake from the bloodstream or from local synthesis. Circulating FA enter the brain by passive diffusion [7] through the blood-brain barrier (BBB). Once in the brain, neurons and astrocytes take up FAs by translocation via carrier proteins such as CD36 and FA transport

proteins, as Albp/ap2 (also known as FABP4) [8]. Once inside glial cells and neurons, long chain FA (LCFA) are esterified to LCFA-CoA by acyl-CoA synthetase. These LCFA-CoA molecules have several fates, including mitochondrial uptake via CPT1a and subsequent FA oxidation (which is low in the brain), or ER uptake via a brain-specific CPT1 isoform (CPT1c) [9] and the conversion into structural and signalling lipid molecules. Regarding the local synthesis of FA, key enzymes involved in intracellular FA metabolism, such as acetyl-CoA carboxylase (ACCα), fatty acid synthase (FAS), malonyl-CoA decarboxylase (MCD), SCD1 and CPT1 are expressed at high levels in the most relevant hypothalamic areas that modulate energy homeostasis as arcuate (ARC), paraventricular (PVH), dorsomedial (DMH) and ventromedial (VMH) nuclei and lateral hypothalamic area (LHA) [10]. The synthesis of FA is modulated by AMPK, the cellular energy sensor [11], as well as by transcription factors. In fact, phosphorylation of ACCα by AMPK suppresses ACCα activity decreasing malonyl-CoA levels downregulating the FA biosynthetic pathway. This situation stimulates CPT1a and CPT1c, promoting LCFA-CoA uptake and metabolism by mitochondrial and/or ER, respectively [9]. Further, SCD1, a membrane-bound protein of the ER, catalyzes the desaturation of saturated LCFA-CoA substrates at the 9-delta position and its activity is associated with the myelin production and neuronal regeneration [12].

The activation of the lipid sensing system is driven by transcription factors, that regulate lipid metabolism. In fact, they are expressed abundantly in the hypothalamus. Among others, the lipogenic factor sterol regulatory-element binding protein-1c (SREBP-1c) enhances the transcription of genes associated with *de novo* FA synthesis such as ACC and FAS [13]. Moreover, the

carbohydrate response element-binding protein (ChREBP) mediates gene expression of multiple enzymes responsible for converting excess of carbohydrate to FA [14]. The forkhead transcriptional factors, FoxO1 and Foxa2 subfamilies, are important mediators of insulin and leptin signalling [15]. Accordingly, activation of FoxO1 and Foxa2 enhance transcriptional programs of nutritional reserves mobilization [16] and FA oxidation and ketogenesis [17], respectively.

FoxO1 decreases energy expenditure and stimulate food intake by modulating gene expression within the hypothalamic melanocortin system. Thus, in ARC, FoxO1 promotes the expression of agouti-related peptide (AgRP) and suppresses the expression of proopiomelanocortin (POMC) directly or antagonizing the signal transducer and activator of transcription 3 (STAT3) [18]. On the other hand, food intake can also be modulated by the mesolimbic reward system. In this sense, LHA orexin neurons, sensitive to peripheral signals of energy state, modulate reward-based feeding by activating ventral tegmental area (VTA) dopamine (DA) neurons [18-20]. Likewise, DA neurons in the VTA have receptors for peripheral hormones such as insulin and leptin, having a critical role in the regulation of food intake and energetic homeostasis [21]. Indeed, FoxO1 in dopaminergic neurons directs the coordinated control of energy balance, thermogenesis and glucose homoeostasis by regulating DA synthesis [22], while Foxa2 can act as a metabolic sensor in neurons of the lateral hypothalamic area to integrate metabolic signals, adaptive behavior and physiological responses [23].

It has been described that moderate FR confers protection to multiple age-related diseases, including neurodegenerative disease development during ageing [24].

Further, several results suggested that central lipid sensing could be regulated by FR and ageing through to the control of AMPK activity [25]. In fact, FR stimulates hypothalamic AMPK and improves AMPK pathways in the hippocampus associated with neuroprotective effects in mice [25]. Hence, activating AMPK is sufficient to extend lifespan in model organisms and mimics FR effects in AL fed animals [26], linking energy status, FR and lifespan [24].

It has been recently established that hypothalamic FA metabolism integrates peripheral hormonal and nutrient/metabolic molecules that affect the hypothalamic cytoplasmic pool of malonyl-CoA and LCFA-CoA, which brings about changes in the expression of neuropeptides controlling food intake [5, 27]. In this sense, it has been demonstrated that a 3 days intracerebroventricular infusion of oleic acid reduced neuropeptide Y (NPY) and AgRP mRNA expression in ARC nucleus decreasing food intake, as well as hepatic glucose production [5]. Moreover, it has been proposed that malonyl-CoA mediates leptin hypothalamic feeding control [28]. Thereby, leptin decreases phosphorylation and inhibits hypothalamic AMPK, and therefore, increase malonyl-CoA and LCFA-CoA levels promoting a fall in food intake [29]. In agreement with that, treatments with FAS inhibitors, such as cerulenin and C75 [30], as well as the specific genetic ablation of hypothalamic FAS [31], induce weight loss and an anorectic effect due to the accumulation of malonyl-CoA in the hypothalamus. This anorectic action is associated to decreased expression of orexigenic neuropeptides, AgRP and NPY, and elevated expression of anorexigenic ones, cocaine and amphetamine-regulated transcript (CART) and POMC, in the ARC nucleus [30, 31]. Related to this, it has been described that the anorexigenic response of corticotropin releasing hormone (CRH) and the

thyrotropin releasing hormone (TRH) neurons in PVH nucleus is induced by POMC neurons located in ARC [32].

It is now well established that alterations of hypothalamic function occur in both, diet-induced [33] and ageing associated [34] obesity models. In this sense, old Wistar rats are characterized by decreased hypothalamic expression of the long leptin receptor Ob-Rb and increased SOCS-3 expression [35], as well as by hiperleptinemia and increased adiposity [36]. Besides, aged Wistar rats present central alterations of the insulin signal transduction pathway, and therefore, develop hypothalamic insulin resistance during ageing [37]. Central leptin and insulin resistance are attenuated in this model by moderate long-term FR only in 8-month-old animals, but not in older animals, suggesting that the increase of adiposity associated with ageing plays a key role in the development of central leptin and insulin resistance [34-37]. Therefore, the aim of this study was to explore the molecular changes involved in the hypothalamic lipid sensing process in Wistar rats upon ageing. To this end, we analyzed the expression and protein levels of enzymes and transcription factors related to lipid metabolism in the hypothalamus of 3-, 8- and 24-month-old Wistar rats fed AL. The effects of moderate FR have also been analyzed.

Methods

Experimental Animals

Experiments were performed in male Wistar rats of 3, 8 and 24 month-old from our in-house colony (Centre of Molecular Biology, Madrid, Spain). Animals were individually housed in and a 12h light/dark cycle at a controlled temperature (20-25 ºC) and humidity (50%) and fed AL standard laboratory chow and water. All animal

experiments were conducted according to the European Union laws (2010/63/EU) and following the Spanish regulations (RD 53/2013) for the use of laboratory animals. The experimental protocols were approved by the institutional committee of bioethics. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Food restriction protocol

Five- and 21-month-old rats were fed once daily randomly selected to undergo a moderate FR protocol as previously described [36, 38] with a standard diet (2014 Teklad Global 14% Protein Rodent Maintenance Diet). They were fed daily 18 and 21 g of chow, respectively (equivalent to ≈ 80% of normal food intake). After 2 months of nutritional restriction, the rats showed a body weight equivalent to ≈ 85% of AL fed aged mates. They were weighed weekly and the amount of food provided was adjusted individually in order to maintain their body weight for one additional month. FR rats were used at the age of 8 and 24 months, respectively. This FR protocol neither compromised the nutritional status of the animals and, confirming our previous published data [36-38], did not cause fasting hypoglycemia (Table 1). All the animals used in this study, fed AL or FR, were housed individually to avoid differences in their food intake, body weights and serum levels of lipids.

Before being sacrificed, all the animals were fasted overnight. Food was removed the day before sacrifice at 9 pm. All animals were decapitated the next day between 9-10 am, after the overnight fast to avoid variability between different animals. The hypothalamic regions were carefully dissected following the boundaries described by [39]. The block of hypothalamic tissue was 2 mm deep and was taken using the optic chiasm and the anterior margin of the mammillary bodies as caudal reference. After that, whole hypothalamic regions

were frozen in liquid nitrogen and stored at −80 °C until used. Also, blood samples were centrifuged, and plasma was frozen until use.

Serum metabolites and hormones analysis

Serum determinations were measured as previously described [40]. Triacylglycerides (TG) were determined by enzymatic kits from Biosystem (Barcelona, Spain). Non-esterified fatty acid (NEFA) levels were measured with an enzymatic assay from Wako Chemicals (Neuss, Germany) and glucose levels were determined using AmplexRed glucose/Glucose Oxidase Assay Kit (Molecular Probes, Inc., Eugene, OR). Serum insulin and leptin levels were measured by ELISA using rat specific kits from SPI-Bio (Montigny le Bretonneux, France). Serum resistin was assessed using a rat resistin ELISA kit (BioVendor, Brno, Czech Republic), following the manufacturer's instructions. Lee index was calculated as described previously [36].

RNA and protein isolation

Total RNA from whole hypothalamus was obtained using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of DNase-treated RNA [35]. Protein pellets obtained using QIAzol™ isolation reagent (Qiagen, Hilden, Germany) were resuspended in 4% SDS and 8 M urea in 40mM Tris–HCl (method adapted from [41]). The total recovery and integrity of these fractions were determined as described by [42].

Real time q-PCR analysis

Real time quantitative PCR (q-PCR) was performed by using ABI PRISM 7500 Fast Sequence Detection System instrument and software (Applied Biosystem, Foster City, CA). Relative quantification of target cDNA in each sample was performed from 10 ng

of cDNA in TaqMan One-Step real time PCR Master Mix, using Pre-Developed TaqMan Assay Reagents (PE Applied Biosystem) for *Crh, Ampk, Albp/ap2, FoxO1a, Foxa2, Chrebp* with FAM and 18S rRNA with VIC as real time reporter was used as control to normalize gene expression (Supplementary Table S1), whereas for *Npy, Pomc, Trh, Accα, Srbpc-1c, Cpt1a, Cpt1c, Scd1 and rRNA* as housekeeper to normalize gene expression, we used SYBR-Green One-Step real time PCR Master Mix with the primers supplied by Bonsai Technologies (Supplementary Table S1).

The ΔΔCT method was used to calculate the relative differences between experimental conditions and control groups as fold change in gene expression [43].

Western Blot analysis

Equal amounts of loaded proteins were fractionated on a SDS-PAGE gel and blotted for 2h at 90 V. Western Blot analyses was performed with the corresponding primary antibody followed by incubation with secondary antibody conjugated with horseradish peroxidase (Goat anti-rabbit IgG-HRP and rabbit anti-Mouse IgG-HRP; Biorad) according to standard protocols (Supplementary Table S2 for additional information). ECL (Amersham) reagent was used for developing. Bands were quantified by scanning densitometry with a G-Box densitometer with exposure in the linear range using Gene Tools software (Synergy, Cambridge, UK). Relative levels of phosphorylated proteins were normalized to the corresponding amount of total protein mass and β-actin levels in the same sample.

Statistical analysis

Data are expressed as mean ±SEM. Statistical analysis was performed using one-way ANOVA (GraphPad Prism 5.03 software, GraphPad Software, Inc., San Diego, CA). For statistical information about the effect of ageing or ageing and diet, values of the

Fisher criterion are presented (see Supplementary information to data in Figures 1-5 and Table 1 for further details). When the main effect was significant, the Bonferroni post hoc test was applied to determine individual differences between means. Animals under FR were compared with their AL age mates using non-paired Student's t-test. A *p*-value < 0.05 was considered significant.

Results

Effect of ageing and food restriction on body weight and systemic metabolism

As expected, body weight increased progressively with ageing while FR decreased it by ~ 15% in both 8- and 24-month-old rats [35, 36]. No significant effect of age was observed on fasting serum glucose and insulin levels, but they were only decreased in 8-month-old FR rats. Aged Wistar rats also showed higher levels of serum TG but lower levels of NEFA with respect to young animals. Besides, FR caused a significant decrease in adiposity and in Lee index in both, 8- and 24-month-old rats. Serum leptin concentration increased progressively with ageing reaching values six-fold higher in 24- than in 3-month-old rats (27.6±4 versus 4.6±0.5, respectively). In middle-age 8-month-old rats, FR markedly decreased serum leptin levels up to values below than those observed in younger rats. In 24-month-old rats, a further moderate decrease in leptin level was observed under FR. Also, serum resistin levels increased significantly with ageing, but only in mature 8-month-old animals FR significantly decreased circulating resistin. [*Table 1 near here*]

Ageing modified the hypothalamic gene expression of neuropeptides that control food intake and energy expenditure in Wistar rats. In fact, *Npy* gene expression was significantly increased in 24-month-old rats fed AL (Figure 1A). In parallel, we observed a significant decline in the mRNA level of *Pomc* at this age (Figure

1B). According to that, our results indicated that both, *Crh* and *Trh* gene expression, decreased in 24-month-old animals (Figure 1C, D). Further, a greater fall was observed in *Pomc* and *Trh* expression under FR conditions only in aged rats (Figure 1B, D). [*Figure 1 near here*]

Effect of ageing and nutritional status on the hypothalamic gene expression and protein level and activity of the transcription factors involved in lipid metabolism

To determine the effect of ageing and FR on central lipid metabolism, we first analyzed the gene expression and protein level of transcription factors involved in lipid synthesis (SREBP-1c and ChREBP), lipolysis (FoxO1) and FA oxidation (Foxa2) (Figures 2 and 3) in the hypothalamus of our animal model.

As shown in Figure 2A, the hypothalamic mRNA level of the lipogenic transcription factor *Srebp-1c* increased significantly in old but not in middle-aged Wistar rats. No significant differences were found when the animals were subjected to FR in any case. Besides, the protein levels of native and the mature form of SREBP-1c (mSREBP-1c) were not altered either with ageing or with FR. However, the ratio mSREBP-1c/native SREBP-1c was higher only in middle aged rats with FR (Figure 2B). [*Figure 2 near here*]

On the other hand, *Chrebp* gene expression was upregulated in both 8- and 24-month-old animals fed AL, and FR reverted that at both ages (Figure 2A). However, no change was observed at the protein level (Figure 2C). The same expression pattern was observed when the mRNA levels of *FoxO1a* were analyzed (Figure 3A). Next, we look at the phosphorylation levels to evaluate its activity. As shown in Figure 3B, a significant decrease in the phosphorylation degree was observed in 24-month-old rats. Only old animals under FR showed

an increase in the ratio p-FoxO1a/FoxO1a (Figure 3B), without significant differences in the total levels of FoxO1a and pFoxO1a. [*Figure 3 near here*]

Likewise, our results indicate that in the hypothalamus of 8-month-old FR rats, the gene expression of the transcription factor gene *Foxa2* was significantly increased. However, the mRNA level of this transcription factor significantly diminished in 24-month-old animals regardless the nutritional status (Figure 3A). Nevertheless, no change was observed at protein level (Figure 3C).

Ageing decreases and FR restores hypothalamic Ampk gene expression and protein phosphorylation levels

We have analyzed the mRNA and the protein phosphorylation levels of AMPK as a potentiating protein of the ATP-generating oxidative processes. As showed, *Ampk* gene expression significantly decreased in 24-month-old Wistar rats fed AL (Figure 4A). According to that, we observed a fall in the phosphorylation protein levels (Figure 4B), however, no changes were observed in the total protein levels of AMPK and pAMPK. FR decreased *Ampk* mRNA levels only in 8-month-old animals (Figure 4A), however, restored the AMPK activity, as suggested by the increase in its phosphorylation levels (Figure 4B), as well as by the increase in the phosphorylation levels of ACCα in aged Wistar rats (Figure 4C). The analysis of *Accα* mRNA and protein indicates that neither ageing nor FR influenced gene expression pattern or protein levels in the hypothalamus of Wistar rats (Figure 4A and B). [*Figure 4 near here*]

Effect of ageing and food restriction on the expression of genes involved in lipid transport and metabolism in the hypothalamus of Wistar rats

The study of *Cpt1a* and *Albp/ap2* hypothalamic gene expression in our model showed that mRNA levels of both genes decreased significantly in aged rats (Figure 5A and B). On the other hand, the analysis of *Cpt1c* gene expression indicates that this isoform is

significantly increased in 24-month-old rats (Figure 5C). Likewise, FR reduces its gene expression only in old animals (Figure 5C). In addition, our results suggest that the unsaturation degree of the FA in the hypothalamus of Wistar rats may be decreased with ageing, since the levels of *Scd1* mRNA fell significantly from 8 months. FR restores *Scd1* mRNA levels only in mature 8-month-old Wistar rats (Figure 5D). [*Figure 5 near here*]

Discussion

Different studies have highlighted the importance of brain lipid metabolism. The CNS is sensitive to the nutritional status of the body, responding to fasting and nutrients, such as glucose or FA, by releasing signals from the hypothalamic nuclei that modulate metabolic homeostasis [4, 44, 45]. In this regard, both glucose and fatty acids in a different way could modulate the membrane potential, firing rate, neurotransmitter and neuropeptide release, as well as gene expression in neurons involved in nutrient sensing mainly in VMH [1, 46].

In this way, it has been demonstrated that neuronal networks in the hypothalamus could detect variations of circulating and endogenous LCFA to regulate food intake and energy expenditure [47]. Regarding to fasting, the hypothalamus responds activating orexigenic pathways whereas anorexigenic response is inhibited which result in an increment of food intake and the downregulation of energy expenditure with activation of anabolic pathways [48]. In the present work, our results support previous data about a clear deterioration in the central signalling pathway of the anorectic hormones, leptin and insulin with ageing [34, 37]. Thus, in 24-month-old rats an increment of *Npy* gene expression is observed concomitant with a decreased of *Pomc* mRNA level,

although serum leptin concentration is increased. In this sense, the decrease in the gene expression of *Crh* and *Trh* observed in these animals suggest that the anorexigenic effects of POMC peptide might be also suppressed. Furthermore, only in 8-month-old rats upon FR decreased serum leptin and insulin levels, suggesting an improvement in leptin and insulin response with FR in middle-aged but not in old animals, as it has previously been described [36, 37]. On the basis that alterations in lipid sensing in the hypothalamus may underlie the origin of this central insulin and leptin resistance during ageing, we evaluate herein the gene expression, protein content and protein phosphorylation levels of several key enzymes and transcription factors involved in the hypothalamic lipid metabolism with ageing. Besides, the consequences of moderate FR have been also studied.

Activation of FoxO1 in the hypothalamus increase food intake and body weight by stimulating the gene expression of *Npy* in the ARC nucleus by interacting with FoxO1-binding sites in *Npy* promotor region. Besides, FoxO1 decrease the anorexigenic *Pomc* neuropeptide mRNA levels by antagonizing the activity of STAT3, which is a downstream transcription factor activated by the leptin signalling [49]. According to that, a central insulin and leptin resistance state would possibly favor an increase in the gene expression of this factor and a downregulation in the phosphorylation degree, like we observed in 24-month-old rats. In this situation, FoxO1 would be active being responsible, at least in part, of the increase in *Npy* and the decrease in *Pomc* mRNA levels, observed during ageing. Additionally, FR, which can partially restore leptin and insulin signal [36, 37], decreased *FoxO1* gene expression and enhanced its phosphorylation in old food restricted rats without modification of *Npy* and

Pomc mRNA levels, confirming that FR in old rats is not enough to totally restore leptin sensitivity.

On the other hand, Foxa2 favors fasting adaptation in the hypothalamus interacting with promoter regions of melanin concentrating hormone (*Mch*), increasing its expression and activating the orexigenic response [23]. Our results seem to agree with this possibility, since we appreciated a significant increase in the gene expression of this factor in the hypothalamus of 8-month-old food restricted rats. However, the same effect was not observed in rats of 24 months under FR, suggesting that the adaptation to fasting in old Wistar rat may be impaired. Likewise, Foxa2 is implicated in protection processes against neuronal degeneration in the hypothalamus [50]. This could be one of the causes that the gene expression of this factor is significantly diminished in old Wistar rats. Besides, the low mRNA levels of *Foxa2* observed in old rats agreed with a downregulation of the lipid oxidative process. Indeed, our results indicate that the hypothalamus of old Wistar rats achieves less oxidation coinciding with the data obtained by other authors [51]. Thus, although FA are not mostly oxidized in the brain and CPT1a mRNA levels are usually very low in this tissue [52], the decrease observed in the hypothalamic gene expression of *Cpt1a* and *Albp/ap2* could indicate a lower mobilization and trafficking of intracellular FA to the mitochondria and an impairment of the hypothalamic ketogenic routes in 24-month-old rats which could attenuate the neuroprotective effects of ketone bodies [53].

Next, it has been described that in the brain from old rats, *Srebp-1c* mRNA levels are increased without changes in the gene expression of *Acc*α or *Fas* [51]. In agreement with that, we showed an upregulation of mRNA levels of *Srebp-1c*

in 24-month-old rats and in *Chrebp* since 8-month-old of age, which would indicate an increment of the hypothalamic lipogenic routes with ageing. However, no changes were observed in the mSREBP-1c (65kDa)/SREBP-1c (130 kDa) ratio. According to that, we found no changes in the gene expression of the SREBP-1c targets genes *Accα,* and *Fas* (data not shown) with ageing. Despite these results, it is known that ACCα can be inactivated by phosphorylation via AMPK [54]. Hence, it is quite possible that the low gene expression levels and protein activation of AMPK observed in old rats, would contribute to the synthesis of malonyl-CoA and therefore to keep *de novo* FA synthesis with ageing. FR restores AMPK protein activity in 24-month-old rats according with an increment in ACCα phosphorylation promoting its inactivation.

However, besides the intracellular synthesis, astrocytes and neurons can uptake FA from the bloodstream. In this sense, recent studies have highlighted the role of neuronal lipoprotein lipase (LPL) mediating the hydrolysis of TG and the brain FA uptake [6]. Further, it has been described that a twofold increase in plasma TG by infusing them directly into carotid arteries, the main route by which FAs reach to forebrain, was related to an impairment of hypothalamic insulin and leptin signalling [55] and hence, to a decline in the sympathetic activity in rats [56]. Accordingly, the gain of adiposity in aged Wistar rats leads to an increase of serum TG levels from the age of 8 months, reaching almost twofold higher levels in 24-month-old compared with 3-month-old animals. These data support that high FA levels could be reaching the brain during ageing contributing, together with *de novo* synthesis, to increase the intracellular LCFA/LCFA-CoA concentrations. In agreement with that, it has been described

that postprandial dyslipidemia progressively increased with ageing [38] and this could be associated with liver steatosis and hepatic ChREBP activity [57]. Moreover, moderate FR is required to preserve antisteatotic actions of central leptin in the liver of middle-aged Wistar rats [40]. Therefore, as it has been proposed by [58], an imbalance between lipid sensing mechanisms in the brain and liver may contribute to obesity associated to central leptin and insulin resistance.

Interestingly, the upregulation of *Cpt1c* gene expression observed in old Wistar rats seems to indicate that, regardless of the origin of the LCFA-CoA, they are preferably destined to the ER during ageing. Indeed, CPT1c has been described as belonging to the brain specific CPT family [59] and it is present in ER of neurons [52]. Besides, several studies involve CPT1c isoform with CNS control of food intake [9, 60]. Certainly, high levels of CPT1c in ARC nucleus of rats up-regulates *Npy* gene expression and increases food intake. Interestingly, CPT1c raise the ER *de novo* ceramide biosynthesis in the hypothalamus [61, 62]. Since leptin reduces ceramides levels in ARC, CPT1c has been postulated as a potential mediator in leptin´s hypothalamic anorectic actions [61]. Our results agree with this possibility since we observed an increase of *Npy* gene expression in leptin resistant 24-month-old Wistar rats concomitant with the increased gene expression of *Cpt1c*. Furthermore, FR in aged animals decreased adiposity, serum TG and leptin levels, together with *Cpt1c* gene expression. Importantly, in old rats FR reduced *Cpt1c* gene expression to values similar to those of young animals. Consequently, although we have not analyzed the hypothalamic ceramide content from old rats, according to the upregulation of the CPT1c gene expression and the increased lipid supply from serum to the hypothalamus with

ageing, as suggested by the circulating higher lipid content reported herein, it is tempting to speculate that the hypothalamic ceramide levels could be increased in old rats. In this sense, the increased ceramide content in the hypothalamus from old rats could contribute to the dysregulation of glucose homeostasis and insulin resistance observed in obese old rats leading to type 2 diabetes [6, 63]. In fact, it has been shown that ceramides could induce lipotoxicity, ER stress and insulin resistance in the hypothalamus [6, 64], being all these conditions present during ageing in Wistar rats. Nevertheless, additional studies must be done before a final conclusion may be drawn.

It has been reported that the desaturation of FA in astrocytes is an important route in the oleic acid synthesis, which promotes neuronal differentiation [65]. As the critical committed step in oleic acid synthesis is the introduction of the cis-double bond at position Δ9, catalyzed by the ER enzyme SCD1 [66], our results suggest that ageing in Wistar rats is connected to a decrease in hypothalamic FA desaturation process, as suggested by the decrease of *Scd1* gene expression observed from the age of 8-months. On the other hand, unsaturated FA can act as repressors of *Srebp-1c* factor expression [67] and our results agree with this possibility. Indeed, in 24-month-old animals we observed a decrease in *Scd1* gene expression together with an increase in mRNA levels of *Srebp-1c*, supporting impairment in unsaturated FA synthesis during ageing. FR restores *Scd1* mRNA levels only in 8-month-old rats suggesting an improvement in this process.

It has been described that the plasma lipidomic profile [68] as well as plasma membrane lipid composition in white adipose tissue [69], are regulated by fasting and ageing respectively in rats. Further, our results showed alterations in

the central lipid sensing metabolism linked with ageing, involving the expression levels and protein activity of the neuropeptides, transcription factors and enzymes implicated in this process. Some of these dysregulations cannot be reversed by FR, and this could contribute to hypothalamic leptin and insulin resistance development during ageing, described in this rat model [36,37].

Conclusions

In conclusion, our results suggest that in the hypothalamus of aged Wistar rats, the physiological lipid sensing could be impaired due to a situation of chronic high circulating lipid excess, that may contribute to the development of central leptin and insulin resistance and, therefore, to the progress of a pathophysiological state. Thus, the increase of both, adiposity and serum TG levels, together with a probably increased *de novo* FA synthesis, as suggested by the gene expression data reported herein, would provide high lipid levels in the hypothalamus that might trigger lipotoxic mechanisms. Hence, we propose a hypothetical model in which under these conditions of lipid oversupply, as seen in aged rats, the ER could increase FA uptake together with a decrease in the unsaturation degree according to the changes in *Cpt1c* and *Scd1* gene expression reported herein. These events might likely contribute to the development of central leptin and insulin resistance, along with the increase of the neurodegeneration and demyelination process associated to age. Finally, although moderate FR seems to improve lipid sensing in middle aged rats it does not seem enough to totally restore this process in old animals.

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Disclosure statement

The authors declare no potential conflict of interests.

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Figure legends

Figure 1. Effect of ageing and FR on the gene expression of the hypothalamic neuropeptides and releasing hormones. mRNA levels of *Npy* (A), *Pomc* (B), *Crh* (C) and *Trh* (D) in hypothalamus of 3-, 8- and 24-month-old Wistar rats. Values are the means ± SEM (n=6-12) per group of animals, each sample made in duplicate. Values were normalized to 1 versus 3-month-old rats. The effect of ageing was evaluated by one-way ANOVA test, followed by a Bonferroni posthoc test. FR was compared with its AL age mate group using non-paired Student's t-test. *$p< 0.05$ compared to 3 months; **$p< 0.05$ compared to 8 months; $^{a}p< 0.05$ vs. same age fed AL.

Figure 2. **Effect of ageing and FR on the hypothalamic lipogenic transcription factors SREBP-1c and ChREBP.** (A) mRNA levels of *Srebp-1c* and *Chrebp*, (B) Representative western blot of the native, mature SREBP-1c and β-actin. Native/mature SREBP-1c, mature SREBP-1c/β-actin and native SREBP-1c/β-actin ratios were determined by optical density analysis. (C) Representative western blot of ChREBP and

β-actin. ChREBP/ β-actin ratio determined by optical density analysis. Each sample was loaded in duplicate. 40 µg of total extracts from hypothalamus were used for immunoblot analysis; Values are the means ± SEM (n=6-12) per group of animals, each sample made in duplicate. Values were normalized to 1 versus 3-month-old rats. The effect of ageing was evaluated by one-way ANOVA test, followed by a Bonferroni posthoc test. FR was compared with its AL age mate group using non-paired Student's t-test. *p< 0.05 compared to 3 months; **p< 0.05 compared to 8 months; [a]p< 0.05 vs. same age fed AL.

Figure 3. Effect of ageing and FR on hypothalamic FoxO1 and Foxa2 transcription factors. (A) mRNA levels of *FoxO1a* and *Foxa2*. (B) Representative western blot of p-FoxO1a, total FoxO1a and β-actin. pFoxO1a/Total FoxO1a, pFoxO1a/β-actin and total FoxO1a/β-actin ratios were determined by optical density analysis. (C) Representative western blot of Foxa2 and β-actin. Foxa2/β-actin ratio was determined by optical density analysis. Each sample was loaded in duplicate. 40 µg of total extracts from hypothalamus were used for immunoblot analysis. Values are the means ± SEM (n=6-12) per group of animals. each sample made in duplicate. Values were normalized to 1 versus 3-month-old rats. The effect of ageing was evaluated by one-way ANOVA test, followed by a Bonferroni posthoc test. FR was compared with its AL age mate group using non-paired Student's t-test. *p< 0.05 compared to 3 months; **p< 0.05 compared to 8 months; [a]p< 0.05 vs. same age fed AL.

Figure 4. **Effect of ageing and FR on the hypothalamic gene expression and phosphorylation levels of AMPK and ACC**α. (A) mRNA levels of *Ampk* and *Accα.* (B) Representative western blot of pAMPK, total AMPK and β-actin. pAMPK/Total AMPK, pAMPK/β-actin and total AMPK/β-actin ratios were determined by optical density analysis. (C) Representative western blot of pACCα, total ACCα and β-actin.

pACCα /Total ACCα, pACCα/β-actin and total ACCα/β-actin ratios were determined by optical density analysis. Each sample was loaded in duplicate. 40 µg of total extracts from hypothalamus were used for immunoblot analysis. Values are the means ± SEM (n=6-12) per group of animals, each sample made in duplicate. Values were normalized to 1 versus 3-month-old rats. The effect of ageing was evaluated by one-way ANOVA test, followed by a Bonferroni posthoc test. FR was compared with its AL age mate group using non-paired Student's t-test. *p< 0.05 compared to 3 months; **p< 0.05 compared to 8 months; ^{a}p< 0.05 vs. same age fed AL.

Figure 5. Effect of ageing and food restriction on the expression of genes involved in lipid transport and metabolism in the hypothalamus of Wistar rats. mRNA levels of *Cpt1a* (A), *Albp/ap2* (B), *Scd1* (C) and *Cpt1c* (D) in hypothalamus of 3, 8 and 24-month-old Wistar rats. Values are the means ± SEM (n=6-12) per group of animals, each sample made in duplicate. Values were normalized to 1 versus 3-month-old rats. The effect of ageing was evaluated by one-way ANOVA test, followed by a Bonferroni posthoc test. FR was compared with its AL age mate group using non-paired Student's t-test. *p< 0.05 compared to 3 months; **p< 0.05 compared to 8 months; ^{a}p< 0.05 vs. same age fed AL.

Graphical abstract. Schematic representation of the fate of LCFA-CoA in the hypothalamus of young and old rats. Blood circulating LCFAs in young Wistar rats reach the hypothalamus, where they are esterified to LCFA-CoA. Into glial cells or neurons, LCFA-CoA are driven to mitochondria (CPT1a) or ER (CPT1c) where could be desaturated by SDC1 and, thereby, converted into structural and signalling unsaturated lipids as oleic acid, related with neuronal myelinization and differentiation. However, the excess of LCFA that reach to the hypothalamus in old animals, could generate an increase in LCFA-CoA, which together with an increase in CPT1c levels,

could favor the capture of LCFA-CoA to the ER. The decrease in the levels of SCD1 in old rats would decrease FA unsaturation degree that could trigger lipotoxicity process and neurodegeneration, both related to the development of neurodegenerative diseases linked to age.

Tables

Table 1. General characteristics of the animals. Results are the mean ±SEM of 10-12 rats per group. The effect of ageing was evaluated by one-way ANOVA test, followed by a Bonferroni posthoc test. FR was compared with its AL age mate group using non-paired Student's t-test. Significant differences $^{*}p<0.05$ from 3-month-old animals, $^{**}p<0.001$ from 3-month-old animals, $^{\#}p<0.001$ from 8-month-old animals, $^{a}p<0.01$ from its AL age-mates, $^{b}p<0.001$ from its AL age-mates.

	3 months	**8 months**	**8 months-FR**	**24 months**	**24 months-FR**
Body weight (g)	438 ± 17	562 ± 21**	422 ± 9^{b}	778 ± 22$^{**,\#}$	607 ± 13^{b}
Glucose (mg/dl)	123 ± 4	131 ± 5	109 ± 4^{a}	126 ± 4	125 ± 4
Leptin (ng/ml)	4.6 ± 0.5	7.5 ± 0.9*	2.0 ± 0.1^{b}	27.6 ±4.0$^{**,\#}$	7.1 ± 1.2^{b}
Insulin (ng/ml)	1.0 ± 0.1	1.0 ± 0.2	0.67 ± 0.03^{b}	1.27 ± 0.28	1.09 ± 0.03
Resistin (ng/ml)	16.2 ± 2.0	34.2 ± 2.3**	16.7 ± 1.0^{b}	21.9 ± 1.8$^{**,\#}$	22.8 ± 1.6
TG (mg/dl)	90 ± 4	156 ± 9**	98 ± 14^{b}	171 ± 8$^{**,\#}$	103 ± 4^{b}
NEFA (mM)	0.46 ± 0.13	0.41 ± 0.1	0.48 ± 0.12	0.44 ± 0.13	0.25 ± 0.14
Lee Index	311 ± 3	315 ± 4	297 ± 3^{a}	319 ± 3	302 ± 4^{a}
Adiposity (%)	3.1 ± 0.1	5.3 ± 0.3**	1.4 ± 0.06^{b}	5.2 ± 0.4$^{\#}$	1.8 ± 0.1^{b}

Ageing alters the lipid sensing process in the hypothalamus of Wistar rats. Effect of food restriction.

María Rodríguez, Cristina Pintado, Rodrigo Torrillas-de la Cal, Eduardo Moltó, Nilda Gallardo, Antonio Andrés and Carmen Arribas

INVENTORY OF SUPPLEMENTARY INFORMATION

Supplementary Figure Legend
Supplementary Table S1
Supplementary Table S2
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Supplementary Figure Legends

Supplementary Table S1 Primers and Probes used for real time PCR

Supplementary Table S2 Primary Antibodies used for Western Blot analysis

Supplementary Table S3: ***Supplementary information to data in table 1.*** Summary of the effects of ageing obtained by one-way ANOVA followed by Bonferroni´s post hoc test on body weight, levels of glucose, leptin, insulin, resistin, TG, NEFA, Lee Index and % of adiposity. Statistical significance was set at $p<0.05$.

Supplementary Table S4: ***Supplementary information to data in Figures 1, 2, 3, 4 and 5.*** Summary of the effects of ageing obtained by one-way ANOVA followed by Bonferroni´s post hoc test on the mRNA level of *Npy, Pomc, Crh, Trh, Srebp-1c, Chrebp, Foxo1a, Foxa2, Ampk, Accα, Cpt1a, Albp/ap2, Cpt1c* and *Scd-1*; protein level of mSREBP, native SREBP, ChREBP, Foxa2, FoxO1a, pFoxO1a, AMPK, pAMPK, ACCα and pACCα and mSREBP-1c/ SREBP-1c ratio, pFoxO1a/FoxO1a ratio, pAMPK/AMPK ratio and pACCα/ACCα ratio. Statistical significance was set at $p<0.05$.

Supplementary Table S5: ***Supplementary information to data in table 1.*** Summary of the effects of ageing and diet obtained by one-way ANOVA followed by Bonferroni´s post hoc test on body weight, levels of glucose, leptin, insulin, resistin, TG, NEFA, Lee Index and % of adiposity. Statistical significance was set at $p<0.05$.

Supplementary Table S6: ***Supplementary information to data in Figures 1, 2, 3, 4 and 5.*** Summary of the effects of ageing and diet obtained by one-way ANOVA followed by Bonferroni´s post hoc test on the mRNA level of *Npy, Pomc, Crh, Trh, Srebp-1c, Chrebp, Foxo1a, Foxa2, Ampk, Accα, Cpt1a, Albp/ap2, Cpt1c* and *Scd-1*; protein level of mSREBP, native SREBP, ChREBP, Foxa2, FoxO1a, pFoxO1a, AMPK, pAMPK, ACCα and pACCα and mSREBP-1c/ SREBP-1c ratio, pFoxO1a/FoxO1a ratio, pAMPK/AMPK ratio and pACCα/ACCα ratio. Statistical significance was set at $p<0.05$.

Supplementary Figure S1: Uncropped western blot images corresponding to Figure 2B. The membrane was cut into the corresponding size of the proteins of interest. Native and mature form of SREBP-1c was detected at the same membrane. The membrane was re-incubated with β-actin antibody.

Supplementary Figure S2: Uncropped western blot images corresponding to Figure 2C. The membrane was cut into the corresponding size of the proteins of interest.

Supplementary Figure S3: Uncropped western blot images corresponding to Figure 3B. The membrane was cut into the corresponding size of the proteins of interest. Each protein (phosphorylated and total form) was detected in the same membrane. The membrane was re-incubated with β-actin antibody.

Supplementary Figure S4: Uncropped western blot images corresponding to Figure 3C. The membrane was cut into the corresponding size of the proteins of interest. The membrane was re-incubated with β-actin antibody.

Supplementary Figure S5: Uncropped western blot images corresponding to Figure 4B. The membrane was cut into the corresponding size of the proteins of interest. Each protein (phosphorylated and total form) was detected in the same membrane.

Supplementary Figure S6: Uncropped western blot images corresponding to Figure 4C. The membrane was cut into the corresponding size of the proteins of interest. Each protein (phosphorylated and total form) was detected in the same membrane.

SUPPLEMENTARY INFORMATION

Supplementary Table S1

Gene	**ABI assay ID**
Crh	Rn01462137_m1
Ampk	Rn00576935_m1
Albp/ap2	Rn00670361_m1
FoxO1a	Rn01494868_m1
Foxa2	Rn00562517_m1
Chrebp	Rn00591943_m1
18S rRNA	4319413E
	Primers
Npy	sense primer 5´-CCGCCATGATGCTAGGTAAC-3´ antisense primer 5´- CACCACATGGAAGGGTCTTC-3´
Pomc	sense primer5´-AGCAACCTGCTGGCTTGCAT-3´ antisense primer 5´-CCAGCACTGCTGCTGTTTCT-3´
Trh	sense primer 5´-AGCTCAGCATCTTGGAAAGC-3´ antisense primer 5´-CCAGCAGCAACCAAGGTC-3´
Accα	sense primer 5´-TGGGCGGGATGGTCTCTTT-3´ antisense primer 5´-GCCATCAGTCTTGATAGCCA-3´
Srbp-1c	sense primer 5´-CTCATCAACAACCAAGACAGT-3´ antisense primer 5´-GTGCAGGTGTCACCTTGGGT-3´

Cpt1a	sense primer 5´- CATGTCAAGCCAGACGAAGA-3´ antisense primer 5´-CCATAGCCATCCAGATCTT-3´
Cpt1c	sense primer 5´-TGGAGCACTCATGGGCTGA-3´ antisense primer 5´- TGCCAGACAAGGTCTTGGCT-3´
Scd1	sense primer 5´- TGCCAGAGGGAATAGGGAAA-3´ antisense primer 5´-ATGGACTGGAAGGAGTCAT-3´
rRNA 18S	sense primer 5´-CGGCTACCACATCCAAGGAA-3´ antisense primer 5´-GCTGGAATTACCGCGGCT-3´

Supplementary Table S2

Primary Antibody	**Dilution**
β-actin (abcam)	1:1000
SREBP-1c (Santa Cruz)	1:250
p-S-309-FoxO1a (abcam)	1:500
FoxO1a (abcam)	1:500
Foxa2 (abcam)	1:500
ChREBP (abcam	1:500
p-T-172-AMPK (abcam)	1:500
AMPK (abcam)	1:1000
p-S-79-ACCα (abcam)	1:500
ACCα (abcam)	1:1000

Supplementary Table S3

F STADISTIC

	AGE
Body weight (g)	F(2,25)= 70,57 p<0,05
Glucose (mg/dl)	F(2,25)=0,7454 p= 0,4848 ; ns
Leptin (ng/ml)	F(2,25)= 18,53 p<0,05
Insulin (ng/ml)	F(2,25)= 0,4752 p= 0,6273; ns
Resistin (ng/ml)	F(2,25)= 18,07 p<0,05
TG (mg/dl)	F(2,25)= 29,92 p<0,05
NEFA (mM)	F(2,25)= 0,03402 p= 0,9666; ns
Lee Index	F(2,25)= 1,501 p= 0,2422; ns
Adiposity (%)	F(2,25)= 12,26 p<0,05

Supplementary Table S4

F STADISTIC

Gene	**AGE**	**Protein**	**AGE**
Npy	F(2,14)= 8,549 *p*<0,05	mSREBP-1c/SREBP-1c	F(2,15)=0,1642 *p*= 0,8501; ns
Pomc	F(2,17)= 3,007 *p*<0,05	mSREBP-1c	F(2,15)= 1,335 *p*= 0,3689; ns
Crh	F(2,13)= 13,36 *p*<0,05	SREBP-1c	F(2,15)= 1,920 *p*= 0,1726; ns
Trh	F(2,14)= 9,582 *p*<0,05	ChREBP	F(2,8)= 1,205 *p*= 0,3487 ; ns
Srebp-1c	F(2,14)= 22,77 *p*<0,05	pFoxO1a/FoxO1a ratio	F(2,13)= 4,762 *p*<0,05
Chrebp	F(2,8)= 11,41 *p*<0,05	pFoxO1a	F(2,13)= ,5895 *p*= 0,5688;ns
Foxo1a	F(2,15)= 11,25 *p*<0,05	FoxO1a	F(2,13)= 0,3743 *p*= 0,6945; ns
Foxa2	F(2,15)= 15,01 *p*<0,05	Foxa2	F(2,8)= 0,6040 *p*= 0,5698; ns
Ampk	F(2,12)= 12,04 *p*<0,05	pAMPK/AMPK ratio	F(2,10)= 22,69 *p*<0,05
Accα	F(2,13)=0,3820 *p*= 0,6899; ns	pAMPK	F(2,10)= ,3181 *p*= 0,7412; ns
Cpt1a	F(2,16)= 18,57 *p*<0,05	AMPK	F(2,10)= ,2909 *p*= 0,7594; ns
Albp/ap2	F(2,13)= 4,653 *p*<0,05	pACCα/ACCα ratio	F(2,12)= ,4808 *p*= 0,6297 ; ns
Cpt1c	F(2,15)= 9,691 *p*<0,05	pACCα	F(2,12)= ,8121 *p*= 0,4712; ns
Scd-1	F(2,17)= 25,08 *p*<0,05	ACCα	F(2,12)= ,4135 *p*= 0,6733; ns

Supplementary Table S5

F STADISTIC

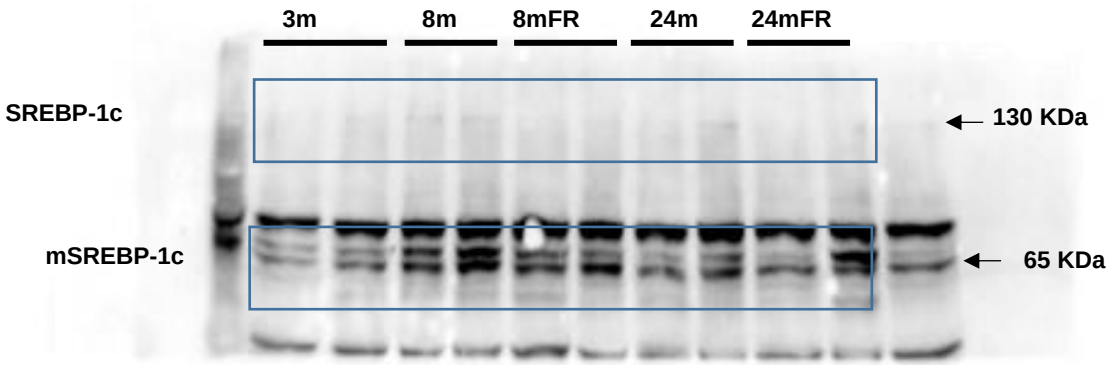
	AGE & DIET
Body weight (g)	F(4,37)= 67,81 *p*<0,05
Glucose (mg/dl)	F(4,37)=3,538 *p*<0,05
Leptin (ng/ml)	F(4,37)= 18,61 *p*<0,05
Insulin (ng/ml)	F(4,37)= 0,3135 *p*= 0,3125; ns
Resistin (ng/ml)	F(4,37)= 14,42 *p*<0,05
TG (mg/dl)	F(4,37)= 18,59 *p*<0,05
NEFA (mM)	F(4,37)= 0,3975 *p*= 0,8091; ns
Lee Index	F(4,37)= 7.550 *p*<0,05
Adiposity (%)	F(4,37)= 38,74 *p*<0,05

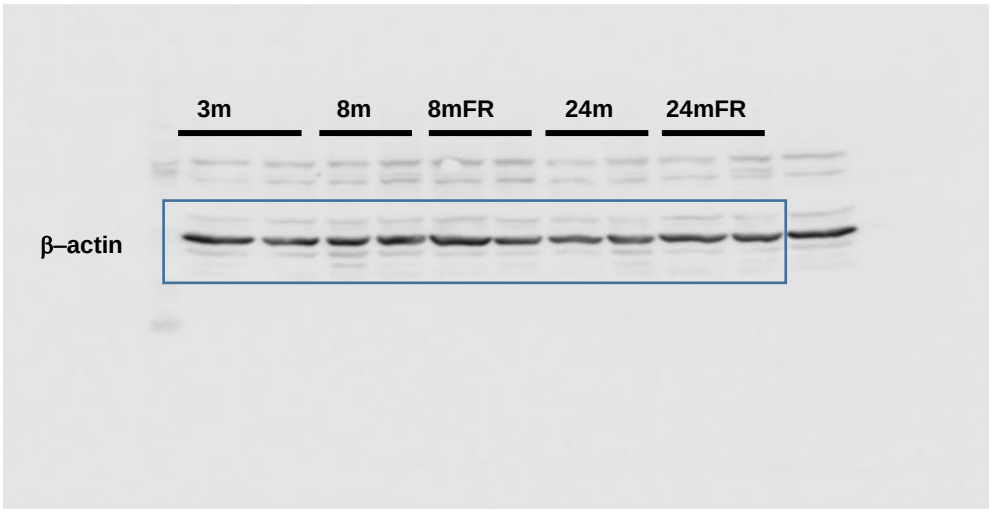
Supplementary Table S6

F STADISTIC

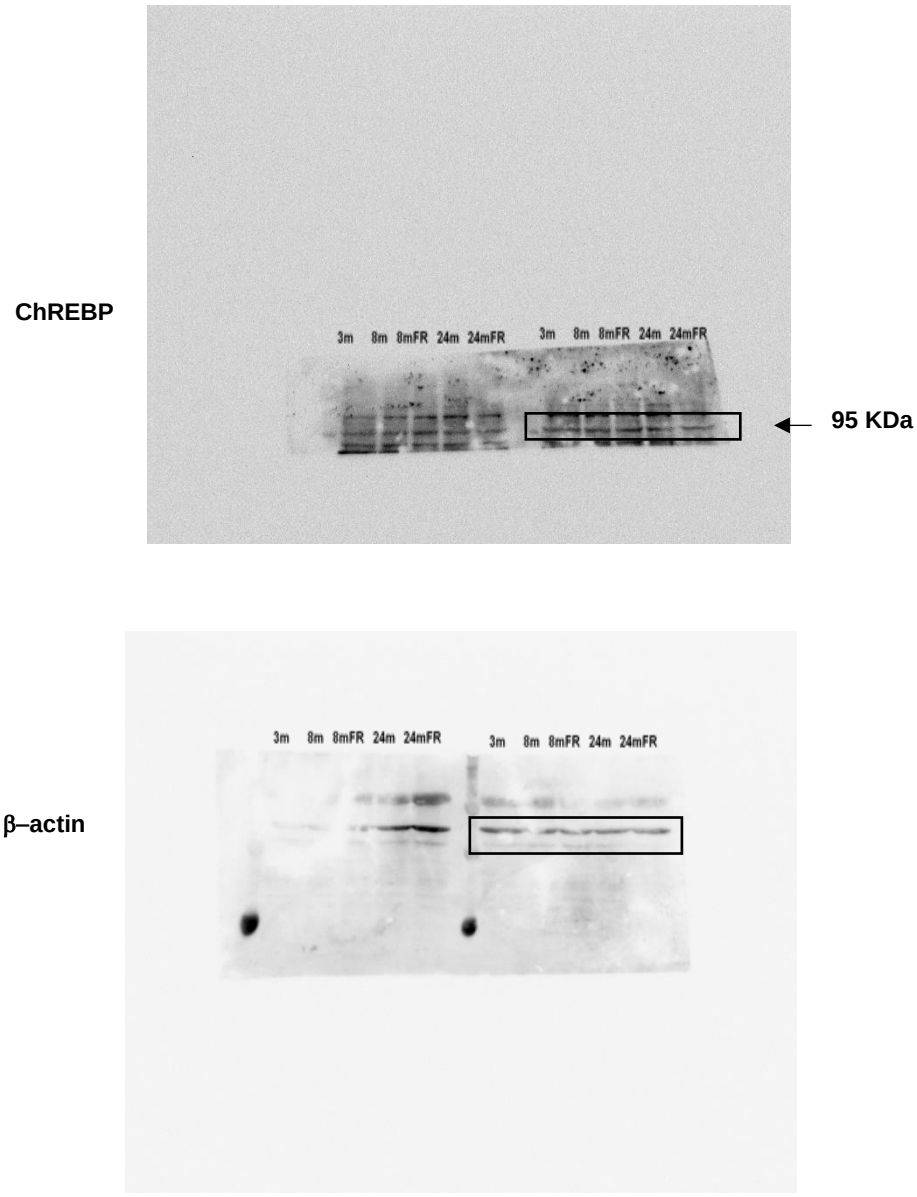
Gene	**AGE & DIET**	**Protein**	**AGE & DIET**
Npy	F(4,28)= 2,563 *p*=0,602; ns	mSREBP-1c/SREBP-1c	F(4,24)=3,190 *p*<0,05
Pomc	F(4,30)= 2,078 *p*=0,1085; ns	mSREBP-1c	F(4,28)= 1,817 *p*= 0,1536; ns
Crh	F(4,27)= 4,752 *p*<0,05	SREBP-1c	F(4,32)= 1,299 *p*= 0,2913; ns
Trh	F(4,27)= 10,05 *p*<0,05	ChREBP	F(4,16)= 0,5619 *p*= 0,6936; ns
Srebp-1c	F(4,23)= 3,470 *p*<0,05	pFoxO1a/FoxO1a ratio	F(4,23)= 4,419 *p*<0,05
Chrebp	F(4,14)= 18,28 *p*<0,05	pFoxO1a	F(4,23)=0,5166 *p*= 0,7243; ns
Foxo1a	F(4,23)= 19,42 *p*<0,05	FoxO1a	F(4,23)= 0,2372 *p*=0,9147 ; ns
Foxa2	F(4,25)= 33,23 *p*<0,05	Foxa2	F(4,13)= 1,308 *p*= 0,3179; ns
Ampk	F(4,19)= 8,302 *p*<0,05	pAMPK/AMPK ratio	F(4,17)= 15,52 *p*<0,05
Accα	F(4,21)= 1,550 *p*= 0,2242; ns	pAMPK	F(4,17)=0,8039 *p*= 0,5597; ns
Cpt1a	F(4,23)= 12,34 *p*<0,05	AMPK	F(4,17)= 1,196 *p*= 0,3829; ns
Albp/ap2	F(4,19)= 4,232 *p*<0,05	pACCα/ACCα ratio	F(4,20)= 1,404 *p*= 0,2689; ns
Cpt1c	F(4,24)= 11,60 *p*<0,05	pACCα	F(4,20)= 0,8821 *p*= 0,4995; ns
Scd-1	F(4,25)= 22,08 *p*<0,05	ACCα	F(4,20)= 0,1673 *p*= 0,95193; ns

UNCROPPED WESTERN BLOT IMAGES



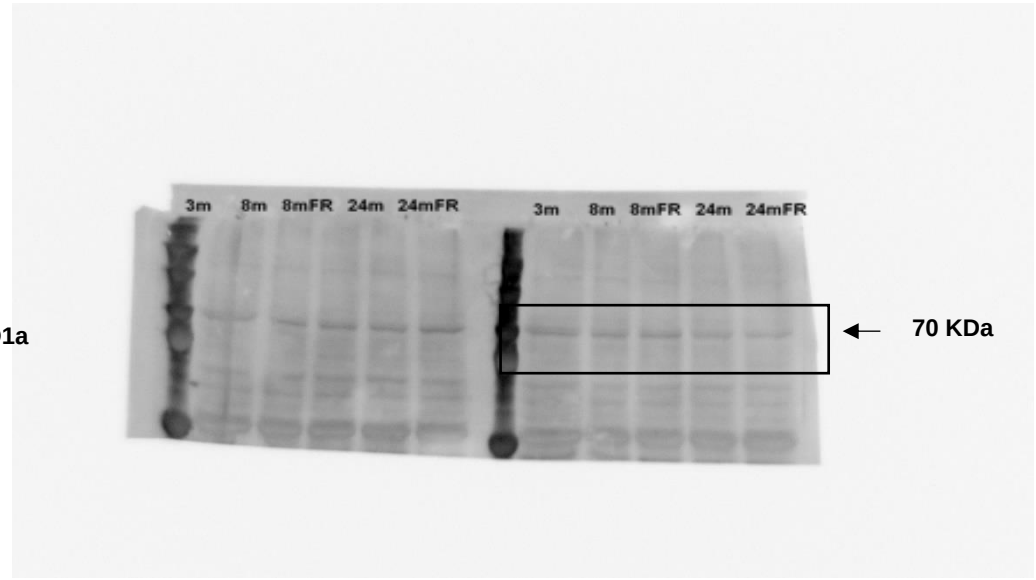


Supplementary Figure S1

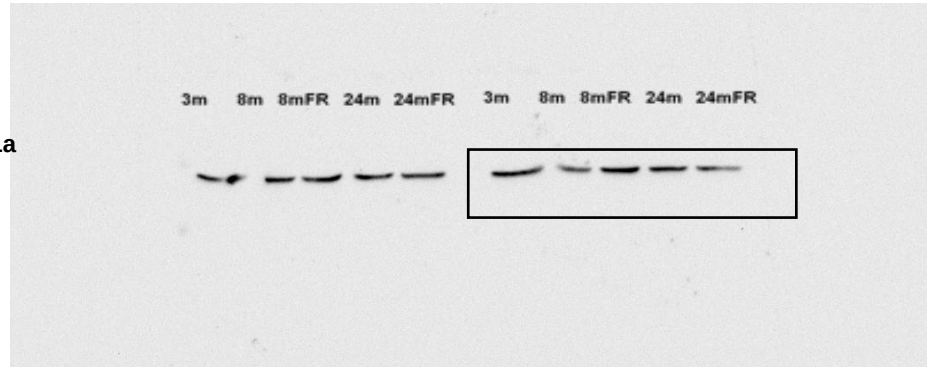


Supplementary Figure S2

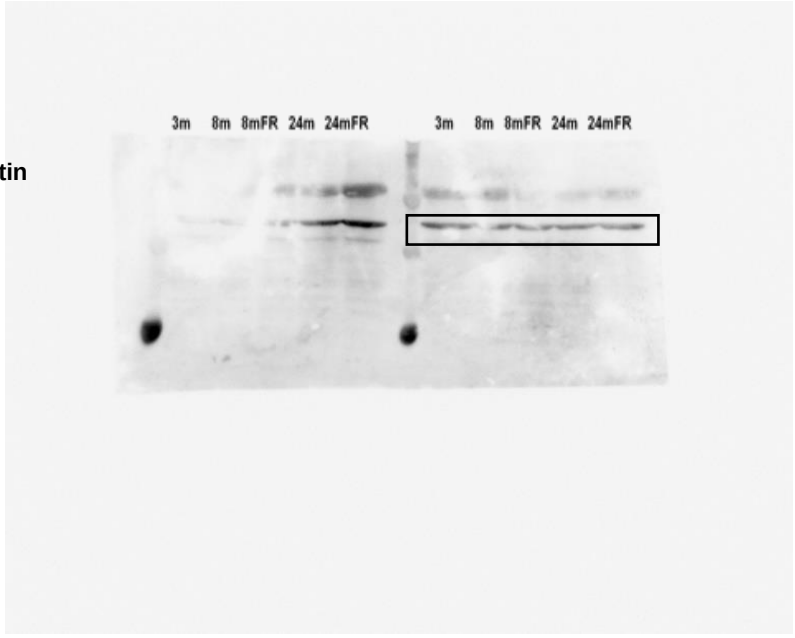
p-FoxO1a



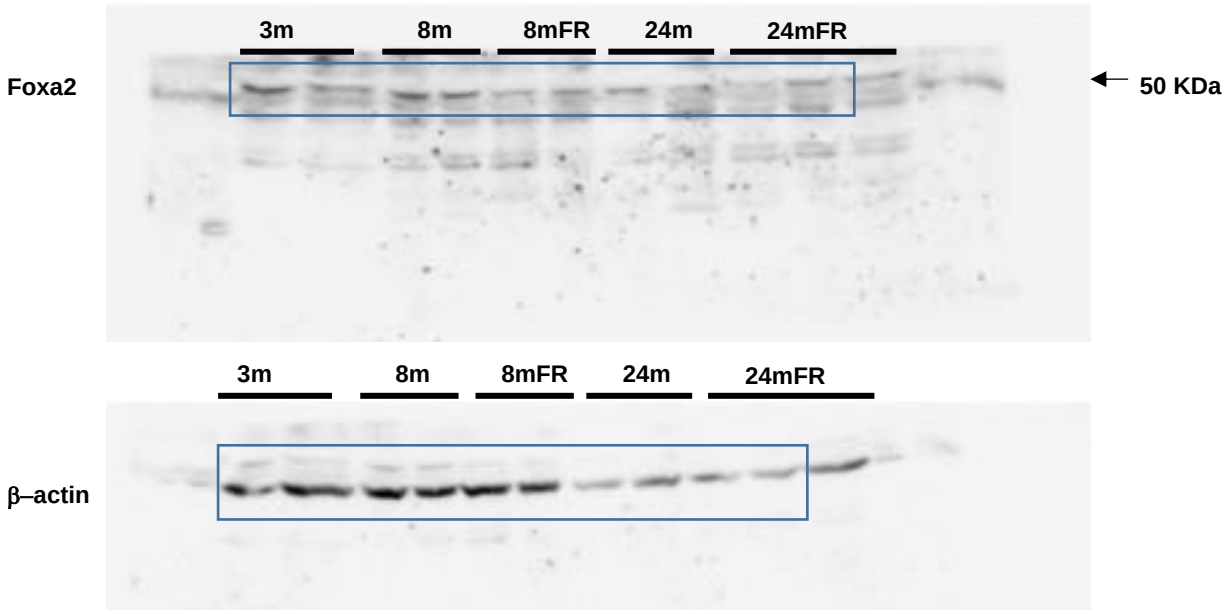
FoxO1a



β–actin

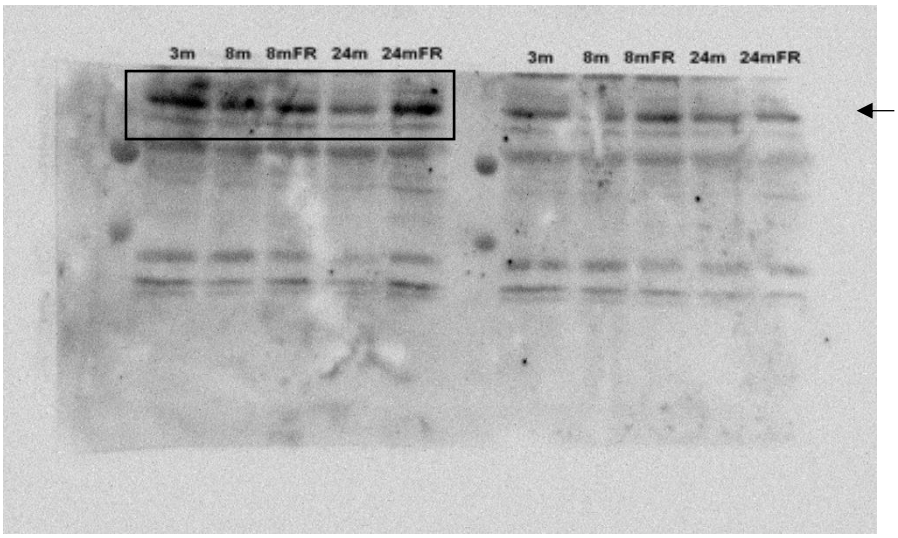


Supplementary Figure S3



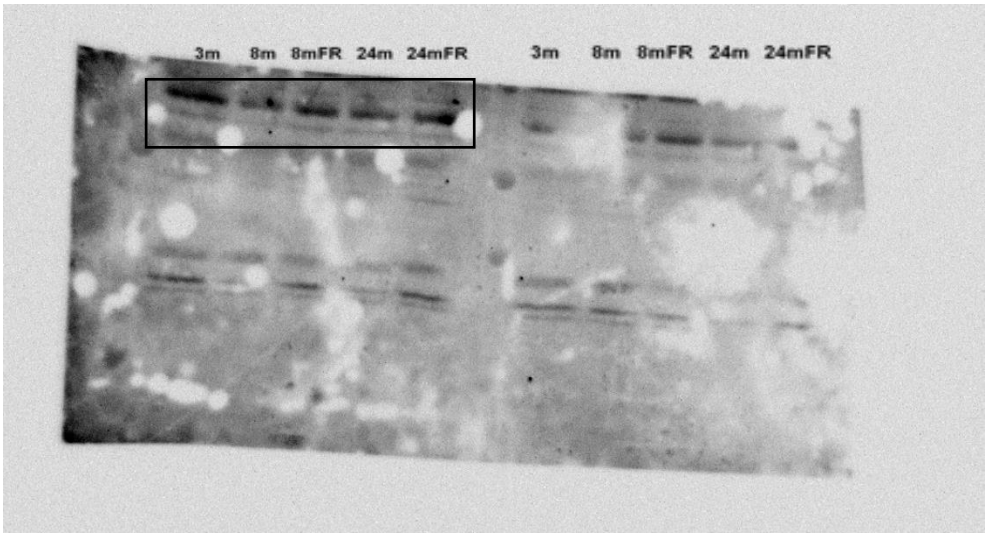
Supplementary Figure S4

p-AMPK

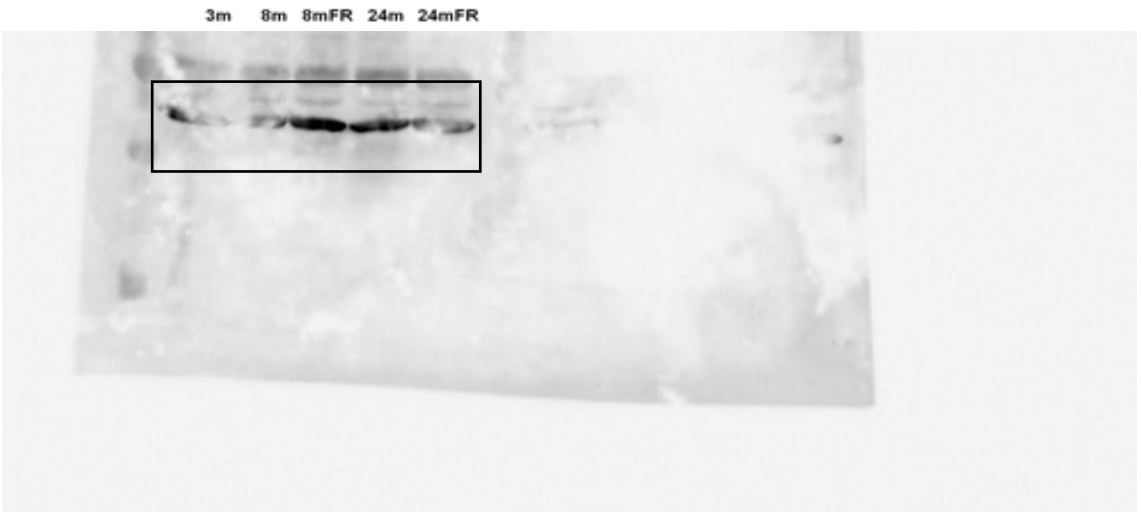


62 KDa

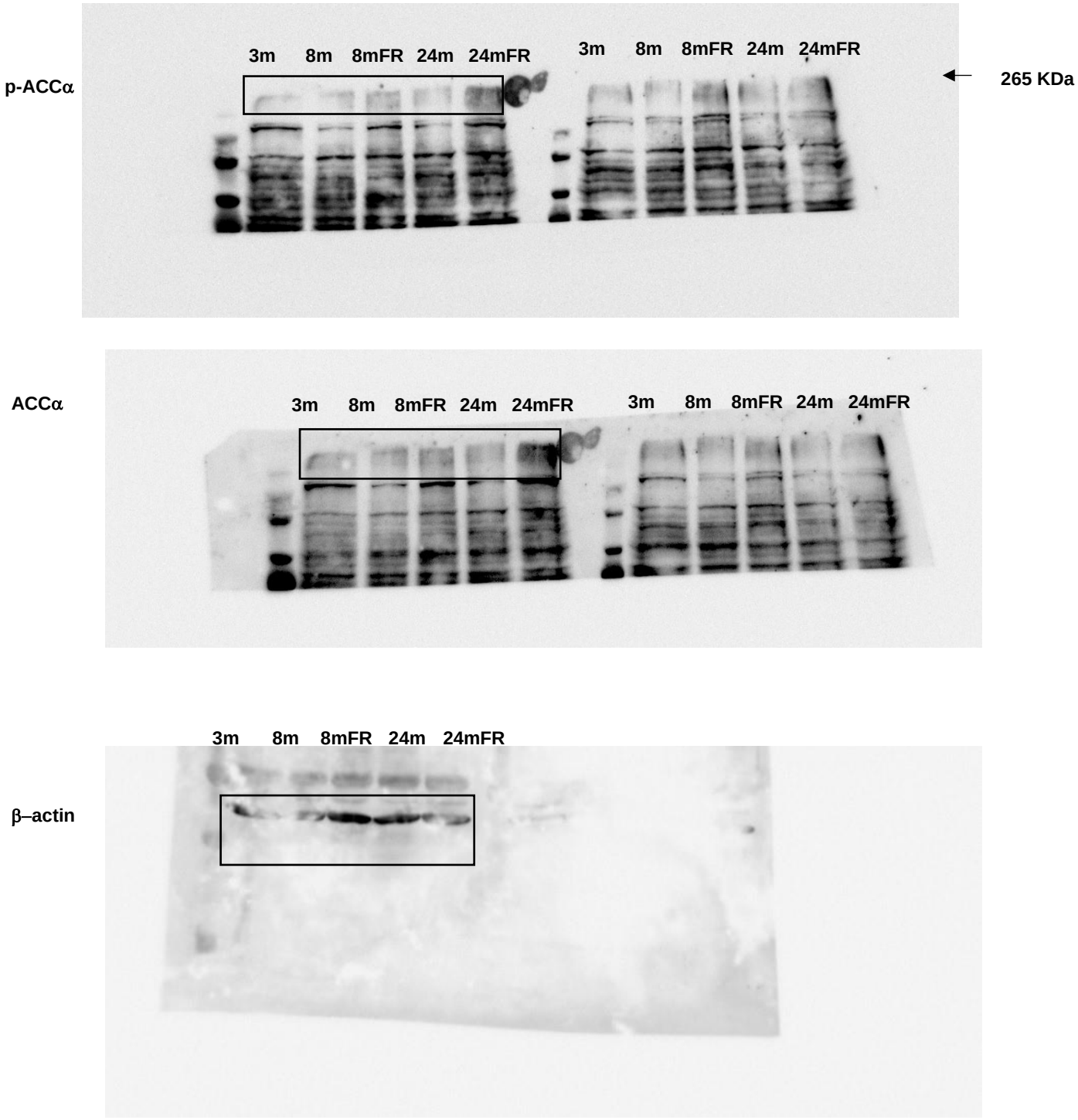
AMPK



β–actin



Supplementary Figure S5



Supplementary Figure S6