# Role of brain JNK2 in the control of the insulin receptor and its relationship with cognitive performance in a high-fat diet preclinical model

Oriol Busquets [1, 2, 3, 4], Àuria Eritja[1], Blanca M López[1], Miren Ettcheto [1, 2, 3, 4], Patricia R. Manzine [1, 5], Rubén D. Castro-Torres [1, 6, 7], Ester Verdaguer [3, 4, 6], Jordi Olloquequi [8], Manuel Vázquez-Carrera [1,9,10], Carme Auladell [3, 4, 6], Jaume Folch [$, 2, 3], and Antoni Camins [$, 1, 3, 4]

[1] Departament de Farmacologia, Toxicologia i Química Terapèutica, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain.

[2] Departament de Bioquímica i Biotecnologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain.

[3] Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, Madrid, Spain.

[4] Institut de Neurociències, Universitat de Barcelona, Barcelona, Spain.

[5] Department of Gerontology, Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil.

[6] Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain.

[7] Departamento de Biología Celular y Molecular, C.U.C.B.A., Universidad de Guadalajara y División de Neurociencias, Sierra Mojada 800, Col. Independencia, Guadalajara, Jalisco, 44340, Mexico.

[8] Instituto de Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca, Chile.

[9] Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, Barcelona, Spain.

[10] Institut de Recerca Sant Joan de Déu (IR-SJD), Esplugues de Llobregat, Barcelona, Spain.

Correspondence and reprint requests to Antoni Camins PhD, Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Barcelona, Spain. Av. Joan XXIII 27/31, E-08028 Barcelona, Spain. Tel: +34 93 4024531, Fax: +34 934035982, Mail: camins@ub.edu

## Abbreviations.

78-KDa Glucose Regulated Protein (BIP), Alzheimer's disease (AD), Activation Transcription Factor 3 (ATF3), Activation Transcription Factor 4 (ATF4),Amyloid Precursor Protein (APP), cAMP Response Element Binding (CREB), C/EBP Homologous Protein (CHOP), Cluster of Differentiation 86 (Cd86), Cluster of Differentiation 206 (Cd206) c-Jun N-terminal Kinase (JNK), Control Diet (CT), Discrimination Ratio (DI), Endoplasmic Reticulum (ER), Eukaryotic Initiation Factor 2α (EIF2α), Glial Fibrillary Acidic Protein (GFAP), Glycogen Synthase Kinase 3β (GSK3β), Glucose Tolerance Test (GTT), High-Fat Diet (HFD), Insulin Receptor (IR), Insulin Tolerance Test (ITT), Immunofluorescence (IF), Ionized Calcium-Binding Adapter Molecule 1 (IBA1), JNK2 Knock-out animals (*Jnk2*$^{-/-}$), Mitogen Activated Protein Kinases (MAPK), Novel Object Recognition Test (NORT), Nuclear Factor Kappa-Light-Chain of Activated B Cells (NFκB), Paraformaldehyde (PFA), Phospho-Activation Transcription Factor 2 (P-ATF2), Phospho-cAMP Response Element Binding (P-CREB), Phospho-Eukaryotic Initiation Factor 2α (P-EIF2α), Phospho-Glycogen Synthase Kinase 3β (P-GSK3β), Phospho-Insulin Receptor (P-IR), Phospho-Protein Kinase B (P-AKT), Phospho-Protein Kinase R-like Endoplasmic Reticulum Kinases (P-PERK), Protein Kinase R-like Endoplasmic Reticulum Kinases (PERK) Protein Kinase B (AKT), Protein Tyrosine Phosphatase 1B (PTP1B), Post-Synaptic Density Protein 95 (PSD95), Real Time – Polymerase Chain Reaction (RT-PCR), Research Resource Identifiers (RRID), Type 2 Diabetes Mellitus (T2DM), Type 3 Diabetes (T3D), X-Box Protein 1 (XBP1),Wild-type (WT).


## Abstract

Insulin resistance has negative consequences on the physiological functioning of the nervous system. The appearance of Type 3 Diabetes in the brain leads to the development of the sporadic form of Alzheimer's disease. The c-Jun N-terminal Kinases (JNK), a subfamily of the Mitogen Activated Protein Kinases, are enzymes composed by three different isoforms with differential modulatory activity against the insulin receptor (IR) and its substrate.

This research focused on understanding the regulatory role of JNK2 on the IR, as well as study the effect of a High-Fat Diet (HFD) in the brain. Our observations determined how JNK2 ablation did not induce compensatory responses in the expression of the other isoforms but, led to an increase in JNKs total activity. HFD-fed animals also showed an increased activity profile of the JNKs. These animals also displayed endoplasmic reticulum (ER) stress

and upregulation of the Protein Tyrosine Phosphatase 1B (PTP1B) and the Suppressor of Cytokine Signalling 3 protein (SOCS3). Consequently, a reduction in insulin sensitivity was detected and it correlated with a decrease on the signalling of the IR. Moreover, cognitive impairment was observed in all groups but, only wild-type genotype animals fed with HFD showed neuroinflammatory responses.

In conclusion, HFD and JNK2 absence cause alterations in normal cognitive activity by altering the signalling of the IR. These affectations are related to the appearance of ER stress and an increase in the levels of inhibitory proteins like PTP1B and SOCS3.

## 1. Introduction.

In the last few decades, the prevalence of obesity has maintained a growing trend all over the world (Abarca-Gómez *et al*., 2017). The dramatic increase in the number of individuals suffering this condition is the consequence of an easy availability to calorie-dense foods, sedentary lifestyles, and a combination of genetic predisposition and learnt behaviour. In addition, inappropriate dietary upbringing and unhealthy childcare of infants is causing for early-on appearance of obesity which, over the years, will have further complications as they age. Multiple data has demonstrated that obesity constitutes an important risk factor for the appearance of a number of chronic conditions including hypertension, heart disease, stroke, certain cancers, and other pathologies like Type 2 Diabetes Mellitus (T2DM) (Bray *et al*., 2004).

T2DM is associated with alterations in glucose homeostasis due to insulin resistance (Kahn, 1978). The insulin receptor (IR) is a transmembrane receptor that is found ubiquitously in peripheral and central tissues and has major roles in cellular function (De Meyts, 2016). Several years ago, the Rotterdam Study reported evidence on a relationship between T2DM and the risk of developing dementia (Ott *et al*., 1996). Specifically, preclinical research data has revealed associations between IR activity and neuroplasticity, as well as modulation of learning and memory processes (Cheng *et al*., 2010; Liu *et al*., 2015). This link has been studied by different research groups and, the concept of a brain-specific insulin resistance has been hypothesised (Hennenberg *et al*., 1995; de la Monte, 2012). This condition has been labelled as Type 3 Diabetes (T3D) and, it is believed to be the progression towards the sporadic form of Alzheimer's disease (AD) (de la Monte *et al*., 2008; Mittal *et al*., 2016).

High-fat diet (HFD) preclinical models have been established as an approach to reproduce the consequences of obesity on the organism while affecting both peripheral and central systems (Kothari *et al*., 2017). It has been reported that HFD reduces the activity of the IR, increases endoplasmic reticulum (ER) stress, as well as up-regulates neuroinflammatory responses and, impairs cognitive function leading to the development of AD-like pathologies (Correia *et al*., 2012; De Felice *et al*., 2014). Published evidence from our research group and others have demonstrated how HFD leads to increases in gene expression and protein levels of the amyloid precursor protein (APP) and its product both on its soluble and insoluble deposited states (Nuzzo *et al*., 2015, Ettcheto *et al*., 2016; Busquets *et al*., 2017).

The c-Jun N-terminal Kinases (JNK), a subfamily of the Mitogen Activated Protein Kinases (MAPK) are modulators of many cellular pathways (Johnson *et al*., 2007; Solinas *et al*., 2017). These proteins are expressed from 3 genes (*Mapk8*, *Mapk9* and *Mapk10*) that turn up into 10 variant protein products, which are grouped within three different isoforms (JNK1, JNK2 and JNK3). Interestingly, these isoforms have been described to be heterogeneously distributed throughout the body and tissues, as well as characterized for their different functions (Haeusgen *et al*., 2009; Pal *et al*., 2016). Several studies have reported their presence in the brain (Carboni *et al*., 1998; Brecht *et al*., 2005).

The JNKs have been described to be activated in obesity situations, promoting the inhibition of the IR and favouring inflammatory responses (Hirosumi *et al*., 2002). Interestingly though, it seems that the isoforms have divergent effects. When studying different isoform-specific knockout mice it was seen that *Jnk1*$^{-/-}$ mice were leaner than the control and, when they were fed with HFD, the ablation protected them from developing impaired glucose tolerance and insulin resistance (Hirosumi *et al*., 2002). *Jnk2*$^{-/-}$ animals are the least studied models and thus, very little information can be provided. Finally, *Jnk3*$^{-/-}$ mice showed severe increases in body weight when fed with HFD. Body weight values were well over those observed in wild-type (WT) mouse (Vernia *et al*., 2016).

In addition to this data, the JNKs are also regulators of cellular homeostasis and controllers of intracellular stresses from the ER, which is also affected by exposition to a HFD. Lastly, these kinases control some essential elements for proper cognitive performance like the cytoskeleton (Sánchez *et al*., 2000; Bjorkblom *et al*., 2005), playing important roles in the definition of dendritic and neurite architecture and morphogenesis. They also play specific roles in synaptic plasticity and neurogenesis in the brain (Zdrojewska *et al*., 2014).

Consequently, the main purpose of the present study was to evaluate the role of the JNK2 isoform and HFD on the development of altered conditions in the brain. We aimed to understand better how it affects IR signalling and ER stress while observing its consequences on cognitive performance and neuroinflammation.

## 2. Materials and Methods.

### *2.1 Animals and Diet.*

Male C57BL/6J wild-type (WT) (RRID: MGI:5657312) and knockout transgenic mice for the *Mapk9* gene, that codifies for the JNK2 protein, (*Jnk2* $^{-/-}$) were used. Transgenic animals were obtained and characterized following the method described by Dong *et al.*, 1998. In all cases, animals were obtained from established breeding couples in the animal facility (Animal facility from the Pharmacy and Food Sciences Faculty from the University of Barcelona; approval number C-0032). Study was not pre-registered.

Right after the weaning and throughout their growth, animals were fed control (CT) diet or palmitic acid-enriched HFD (45% fat content. Research Diets, Inc.; product D12451; **Supplementary Material 1**). Animals were allocated by using consecutive litters. Specifically, each litter was fully assigned to an experimental group until enough animals had been obtained for the study: WT CT (n=15; after excluding 2 animals during the NORT (see later)), WT HFD (n=12; after excluding 4 animals during the NORT), *Jnk2*$^{-/-}$ CT (n=15; after excluding 3 animals during the NORT), *Jnk2*$^{-/-}$ HFD (n=17; after excluding 1 animal during the NORT). The number of mice per group differed because in each litter there was a different numbers of animals born and only males were used for the study. Litters were born one after the other in the different breeding pairs of WT and *Jnk2*$^{-/-}$ animal genetic lines. Animals were number 1 to 69 and identified by using ear markings. Animals were housed in boxes of no less than 2 animals per cage and no more than 4. No previous sample size calculations were performed. Although unlikely, it cannot be discarded that littermate pooling might create differences in the general data sets due to the purity of the homozygous background of the breeding pairs. Animals were grown until 9 months of age and underwent monthly weight controls.

The animals were kept under stable conditions of temperature and humidity, 12h light/dark cycles and food and water *ad libitum* (Animal facility from the Pharmacy and Food Sciences Faculty from the University of Barcelona). Animals were treated at all times under the ethic directions defined by the European Committee (European Communities Council Directive 2010/63/EU) and the manipulation protocols were previously approved by the ethic committee from the University of Barcelona. It was made sure that animal numbers, their stress and pain were kept under a necessary minimum following the appropriate animal manipulation ethic methodologies. An experimental design flow-chart has been depicted in **Figure 1**.

### *2.2 Glucose Tolerance Test (GTT) - Insulin Tolerance Test (ITT).*

Previous to the beginning of the test, animals were fasted for 6 hours. In both tests, animals were injected in the intraperitoneal cavity. In GTT mice were administered a glucose dosage of 1 g/kg, whereas in ITT, insulin was administered at a dosage of 0.75 ui/kg. Next, blood samples were analysed from the tail vein in consecutive time periods. For GTT, samples were extracted at 5, 15, 30, 60, 120 and 180 min after the administration of glucose, in ITT the measurements were made 15, 30, 45, 60 and 90 min after insulin injection. Animals were continuously observed and monitored and, in those cases in which blood glucose concentrations dropped under a concentration of 20 mg/dl, animals were administered a glucose dosage of 1 g/kg and kept under observation until glucose blood levels stabilized and normal behaviour was observed.

### *2.3 Novel Object Recognition Test (NORT).*

In the NORT test, animals were evaluated in a room with an open field box (50x50x20 cm) surrounded by black curtains and constant controlled illumination. The animals were placed in the open field area 3 consecutive days for 10 minutes in order to habituate them to the space. The amount of time spent in the central area of the field was registered. In the $4^{th}$ day, two identical objects were placed in the open field space. Exploration time for each of the objects was evaluated over a total of 10 minutes. Animals that showed significant preference for one of the objects over the other were excluded from posterior analysis. Significant preference was defined after statistical comparison of observation time for each

object. A total of 10 animals were excluded from all experimental groups. In the 5th day, one of the objects was replaced for a new one and the same observation and data acquiring was made.

All spaces and objects were properly cleaned and prepared between animals in order to eliminate odour cues. All objects were chosen randomly for the test in order to eliminate the variability associated to colours and shapes preference. Simple randomization, the objects were blindly chosen from a box and assigned for each test. All data was obtained from recordings using the program Smart 3.0 (Panlab). Quantifications of exploration time were determined through a ratio: Discrimination ratio (DI)= (Time spent exploring the new object – Time spent exploring the known object)/Total exploration time.

### *2.4 Golgi Stain.*

Golgi Stain procedures were done following the direction of the Kit purchased from FD Neurotechnologies, Inc. (FD Rapid GolgiStain™ Kit; Cat #PK401). Images were obtained from a BX61 Laboratory Microscope (Melville NY-Olympus America Inc.).

Dendritic spine numbers were quantified by selecting granular neurons in the dentate gyrus (DG) of the hippocampus. Measurement was done at least 50 μm from the soma along consecutive 10 μm fragments on secondary branches starting 10 μm after branching from the primary dendrite. Spine density was calculated by dividing the number of spines per segment and was expressed as the number of spines per 10 μm of dendrite. Three animals were chosen from each experimental group by taking animals from different litters. It allowed to correct for any biases developed due to factors like pregnancy conditions or number of siblings as it has been described to affect cognitive development. From each animal at least 5 neurons were checked. Neurons were chosen from those that showed clear staining. No neurons were included in the analyses if their dendritic arborisation crossed paths with those nearby. Spine density value for each neuron was the result of the mean of 5 consecutive measurements in the same secondary branch. Quantifications were performed by a blinded experimenter.

### *2.5 Immunoblot analysis.*

Fresh brains were extracted right after euthanasia (neck dislocation). Hippocampus area was dissected and kept frozen at -80°C until use. Samples were cryohomogenised using liquid nitrogen. Proteins were extracted using a lysis buffer (Tris HCl 1M pH 7.4, NaCl 5M,

EDTA 0.5M pH 8, Triton, distilled $H_20$) containing a protease (Complete Mini, EDTA-free; Protease Inhibitor cocktail tablets, 11836170001, Roche Diagnostics GmbH, Germany) and phosphatase inhibitor cocktail (Phosphatase Inhibitor Cocktail 3, P0044, Sigma-Aldrich, USA). Samples concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™) and 10 µg were used for each assay.

Immunoblot procedure was already described in Busquets *et al*., 2018. In brief, for electrophoresis acrylamide gels of 10-12% concentration were used and run at 100V. Transference was performed through standard wet transference at constant 200mA for 100 minutes or semi-dry at 2.5 A for 20 minutes. Primary and Secondary antibodies are described in **Supplementary Material 2**.

Results were obtained from chemoluminescence detection using the Pierce® ECL Western Blotting Substrate (#32106, Thermo Scientific, USA), a Bio-Rad Universal Hood II Molecular Imager and the Image Lab v5.2.1 software (Bio-Rad Laboratories). Measurements were expressed in arbitrary units and all results were normalised with the corresponding loading control (Glyceraldehyde-3-phosphate dehydrogenase; GAPDH).

### *2.6 Kinase activity assay.*

The P-JNK enzyme was immunoprecipitated from hippocampal protein samples. Assay preparation and procedure were previously described in Busquets *et al*., 2018.

### *2.7 Immunofluorescence (IF).*

Animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After making sure the animals were in the no-pain sleep phase they were intracardiacally perfused with 4% paraformaldehyde (PFA) diluted in 0.1 M phosphate buffer (PB). After perfusion, brains were removed and stored in PFA overnight at 4°C. The next day the solution was changed into PFA+30% sucrose. Coronal sections of 20 µm were obtained by a cryostat (Leica Microsystems), kept in a cryoprotectant solution and -20°C in the freezer.

IF protocol has already been described in previous publications (Busquets *et al*., 2017 and Busquets *et al*., 2018). Information regarding the antibodies used in IFs can be found in **Supplementary Material 3**.

Image acquisition was performed with an epifluorescence microscope (Olympus BX61 Laboratory Microscope, Melville, NY-Olympus America Inc.). Fluorescence intensity was quantified as Corrected Total Cell Fluorescence Value as described in Busquets *et al*., 2017.

### *2.8 Real Time – Polymerase Chain Reaction (RT-PCR).*

For RNA extraction, samples were added TRIsure™ (BIO-38033; Bio line GmbH), homogenized using a vortex and left to rest for several minutes. They were centrifuged for 5 min at 12000*g* and 4 °C. Supernatant was transferred into new tubes and chloroform was added. After another centrifuge, phases were separated and the superior layer was collected into a new tube. Later, isopropanol was added and samples were left to rest in ice. After at least 10 min, they were centrifuged again for 10 min at 14000g and 4 °C. Next, 70% ethanol was added and samples were centrifuged for 5 min at 7500g and 4°C. Lastly, supernatant was removed, pellet was left to dry and it was dissolved in DEPC $H_2O$. RNA extraction products were kept at -80 °C until use. RNA concentration and integrity were assessed using a NanoDrop (Thermo Scientific).

For reverse transcription, 2 μg of RNA were used following the protocols described by the High Capacity cDNA Reverse Transcription Kit (4368813; Applied Biosystems). cDNA samples were used in equivalent quantities and each was analysed in triplicate for each gene. Primer sequences can be found in **Supplementary Material 4**. SyBr Green reagent was used coupled with ROX (Thermo Scientific Maxima SYBR Green qPCR Master Mix (2X); K0253; Thermo Scientific) on a Step One Plus Real Time-PCR system (Life Technologies). RT-PCR cycle parameters (Stage 1: 95 °C 10 min; Stage 2: 95 °C 10 sec, 60 °C 30 sec, 70 °C 1 min (40 cycles; Melting curve: 95 °C 15 sec, 60 °C 60 sec, 95 °C 15 sec).

All results were normalised with *Gapdh* as housekeeping gene. The WT CT experimental group was used as the calibrator to evaluate gene expression variations. Measurements were calculated through the Double delta Ct method and expressed in arbitrary units.

### *2.9 Xbp1 splicing detection.*

The qualitative evaluation of the spliced state of XBP1 was examined by gel electrophoresis after a PCR amplification (Forward Primer: TGAGAACCAGGAGTTAAGAACAC; Reverse Primer: TTCTGGGTAGACCTCTGGGAGTTCC). The methodology has been previously described by Guo *et al*., 2007. PCR cycle parameters (Stage 1: 94 °C 2 min; Stage 2: 94 °C 1 min, 62°C 1 min, 72 °C 1 min (34 cycles); Stage 3: 72 °C 5 min).

### *2.10 Statistical analysis.*

Data was presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25$^{th}$ to 75$^{th}$ percentile in the extremes. The maximum and minimum values were represented as whiskers. All four experimental groups were compared through two-way ANOVA. Tukey's was performed (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Researchers were blinded to treatment when data sets were analysed. All analyses and graph representations were performed in the program Graph Pad Prism for Windows version 6.01; Graph Pad Software, Inc. Data was previously checked for normality (D'Agostino-Pearson normality test) and outliers (Grubb's test). In all figures, only relevant significant comparisons were shown: WT CT *vs* WT HFD, WT CT *vs Jnk2 -/-* CT, WT CT vs Jnk2-/- HFD, *Jnk2 -/-* CT *vs Jnk2 -/-* HFD and WT HFD *vs Jnk2 -/-* HFD.

## 3. Results.

### *3.1 Mapk9 knockdown and HFD feeding increase activity rates of JNK in the hippocampus.*

Gene expression for *Mapk8*, *Mapk9* and *Mapk10* was quantified through RT-PCR (**Figure 2A**). WT HFD experimental group showed significant upregulation on the expression of all genes when compared to WT CT ($p<0.001$ for *Mapk8* and *Mapk9*; $p<0.01$ for *Mapk10*). *Jnk2-/-* experimental groups had no expression of the *Mapk9* gene ($p<0.001$).

Evaluation of the ratio between phosphorylated (Thr183/185) and total JNK protein levels determined significant increased values: $p<0.001$ WT CT *vs* *Jnk2*$^{-/-}$ CT, $p<0.001$ WT CT *vs* *Jnk2*$^{-/-}$ HFD and $p<0.05$ WT HFD *vs* *Jnk2*$^{-/-}$ HFD (**Figure 2B**). Similar results were observed

when performing an activity assay for this same enzyme (P-JNK Thr183/185): $p<0.05$ WT CT *vs* WT HFD, $p<0.05$ WT CT *vs* *Jnk2*$^{-/-}$ CT and $p<0.01$ WT CT *vs* *Jnk2*$^{-/-}$ HFD (**Figure 2C**).

### *3.2 Alteration in physiological metabolic parameters.*

Several measurements were performed on the animals during their growth (**Figure 3**). First, body weight was controlled monthly (**Figure 3A**). HFD groups showed higher body weight values at 9-months of age ($p<0.001$ WT CT *vs* WT HFD and $p<0.05$ *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD). Also, JNK2 ablation led to increased body weight: $p<0.05$ WT CT *vs* *Jnk2*$^{-/-}$ CT and $p<0.001$ WT CT *vs* *Jnk2*$^{-/-}$ HFD. Additional data was gathered with the GTT and ITT tests. Time-related variation of blood glucose concentration was determined by comparing AUC values between experimental groups extrapolated from the curves of response throughout the tests. In the GTT, all experimental groups showed significant increases when compared against the control: WT HFD ($p<0.05$), *Jnk2*$^{-/-}$ CT ($p<0.05$) and *Jnk2*$^{-/-}$ HFD ($p<0.001$) (**Figure 3B**). Similarly, ITT assays showed similar responses ($p<0.001$ WT CT *vs* WT HFD, $p<0.05$ WT CT *vs* *Jnk2*$^{-/-}$ CT and $p<0.001$ WT CT *vs* *Jnk2*$^{-/-}$ HFD) (**Figure 3C**).

Next, hippocampal proteins of the IR signalling pathway were detected. The calculation of the ratio between total and phosphorylated protein, determined significant reductions due to the lack of JNK2, as well as for the effects of HFD ($p<0.05$ WT CT *vs* *Jnk2*$^{-/-}$ CT). Additional response elements to the signalling of the IR were examined. In all cases there was a reduction on their phosphorylated ratios: P-AKT (Ser473)/AKT ($p<0.05$ WT CT *vs* WT HFD and WT CT *vs* *Jnk2*$^{-/-}$ HFD), P-CREB (Ser133)/CREB ($p<0.01$ WT CT *vs* WT HFD and $p<0.05$ WT HFD *vs* *Jnk2*$^{-/-}$ HFD), P-GSK3B (Ser9)/GSK3B ($p<0.01$ WT CT *vs* WT HFD and WT CT *vs* *Jnk2*$^{-/-}$ HFD) (**Figure 4A**). Also, PTP1B presented noticeable increases in its protein levels in all experimental groups versus the control ($p<0.01$ WT CT *vs* WT HFD, $p<0.05$ WT CT *vs* *Jnk2*$^{-/-}$ CT and $p<0.001$ WT CT *vs* *Jnk2*$^{-/-}$ HFD).

Finally, quantification of gene expression variations was performed. *InsR* showed decreased values in all experimental groups versus the control ($p<0.01$ WT CT *vs* WT HFD, WT CT *vs* *Jnk2*$^{-/-}$ CT and WT HFD and *Jnk2*$^{-/-}$ HFD; $p<0.01$ WT CT *vs* *Jnk2*$^{-/-}$ HFD) while *Ptpn1* and *Socs3* showed increases (**Figure 4B**): *Ptpn1* ($p<0.05$ WT CT vs WT HFD and *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD; $p<0.01$ WT CT *vs* *Jnk2*$^{-/-}$ HFD) and *Socs3* ($p<0.001$ WT CT *vs* WT HFD, $p<0.05$ WT CT *vs* *Jnk2*$^{-/-}$ CT and *Jnk2-/-* HFD).

### *3.3 Evaluation of HFD-induced ER stress.*

Due to the importance of proper ER function in the memory process, levels of several proteins of the ER were assessed (**Figure 5A**): BIP ($p<0.05$ WT CT *vs* WT HFD, $p<0.01$ WT CT *vs* *Jnk2*$^{-/-}$ CT and $p<0.001$ WT CT *vs* *Jnk2*$^{-/-}$ HFD), P-EIF2a(Ser51)/EIF2a ($p<0.01$ WT CT *vs* *Jnk2*$^{-/-}$ HFD and $p<0.05$ WT HFD *vs* *Jnk2*$^{-/-}$ HFD), ATF4 ($p<0.01$ WT CT *vs* WT HFD, *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD and WT CT *vs* *Jnk2*$^{-/-}$ HFD), CHOP ($p<0.01$ WT CT *vs* WT HFD and *Jnk2*$^{-/-}$ CT vs *Jnk2*$^{-/-}$ HFD; $p<0.001$ WT CT *vs* *Jnk2*$^{-/-}$ HFD and ATF6 ($p<0.05$ WT CT *vs* WT HFD and *Jnk2*$^{-/-}$ CT).

Complementarily, a qualitative evaluation of the splicing of the XBP1 mRNA showed how all the experimental groups but the WT CT showed a spliced product of lower molecular weight (in the gel it can be observed as a faint band at the lower part of the image (arrow) (**Figure 5B**).

### *3.4 Determination of cognitive decline through NORT, quantification of dendritic spines and involved biomarkers.*

When the animals reached the 8 months of age, they were tested for memory and learning capabilities through the NORT (**Figure 6**). No differences were observed between groups in the open field exploration time in the habituation period. Results showed reductions in the discrimination ratio of all groups when compared to WT CT (**Figure 6B**): $p<0.05$ for WT CT *vs* *Jnk2*$^{-/-}$ CT and $p<0.01$ for WT CT *vs* WT HFD and *Jnk2*$^{-/-}$ HFD

Some samples were used for Golgi Stain in order to determine the state and number of dendritic spines of pyramidal neurons in the DG of the hippocampus (**Figure 6C-F**). Experimental groups that had been exposed to HFD or lacked JNK2 presented smaller and shorter dendritic spines (qualitative evaluation). Quantification of dendritic spines number determined significant differences in all experimental groups when compared with the WT CT: $p<0.001$ WT CT *vs* WT HFD and *Jnk2*$^{-/-}$ HFD, $p<0.01$ WT CT *vs* *Jnk2*$^{-/-}$ CT and *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD (**Figure 6G**).

Protein levels for spinophilin, a protein highly present in dendritic spines, and the post-synaptic density protein 95 (PSD95) were determined through immunoblot **(Figure 6H)**. Spinophilin showed significant reductions between WT CT and WT HFD ($p<0.05$), WT CT

and *Jnk2*$^{-/-}$ HFD ($p<0.001$) and *Jnk2*$^{-/-}$ CT and *Jnk2*$^{-/-}$ HFD ($p<0.05$). Also, statistical analysis for PSD95 showed significant decrease between WT CT and both *Jnk2*$^{-/-}$ transgenic groups ($p<0.05$).

### *3.5 Analysis of changes in neuroinflammatory-related cells and other biomarkers.*

Visualization of astroglia and microglia was performed through the detection of GFAP and IBA1 proteins. Representative images of all four experimental groups can be found in **Figure 7 A-D** for GFAP and **E-H** for IBA1. Quantification of the CTCF determined significant increases in the relative intensity fluorescence value in the WT HFD experimental group for both experiments: GFAP (**Figure 7I**; $p<0.001$ WT CT *vs* WT HFD and WT HFD *vs Jnk2-/-* HFD) and IBA1 (**Figure 7J**; $p<0.01$ WT CT *vs* WT HFD and $p<0.001$ WT HFD *vs* *Jnk2*$^{-/-}$ HFD).

Further gene expression variation analyses allowed for the characterization of the state of M1 (proinflammatory) and M2 (immunosuppressant) microglia states (**Figure 7K**): *Cd86* ($p<0.01$ WT CT *vs* WT HFD and $p<0.05$ WT HFD *vs* *Jnk2*$^{-/-}$ HFD), *Ccl3* ($p<0.01$ WT CT *vs* WT HFD), *Tlr4* ($p<0.01$ WT CT *vs* WT HFD and $p<0.05$ WT CT *vs* *Jnk2*$^{-/-}$ CT and *Jnk2*$^{-/-}$ HFD), *Tnfa* ($p<0.001$ WT CT vs WT HFD and WT HFD *vs* *Jnk2*$^{-/-}$ HFD), *Arg1* ($p<0.05$ WT CT vs WT HFD, WT CT *vs* *Jnk2*$^{-/-}$ HFD and *Jnk2*$^{-/-}$ CT vs *Jnk2*$^{-/-}$ HFD) and *Cd206* ($p<0.05$ WT CT vs WT HFD).

Complementarily, inflammation response-related proteins were examined: TLR4 ($p<0.05$ WT CT *vs* *Jnk2*$^{-/-}$ HFD) and ATF3 ($p<0.05$ WT CT *vs* WT HFD, *Jnk2*$^{-/-}$ CT and *Jnk2-/-* HFD; $p<0.05$ WT HFD *vs* *Jnk2*$^{-/-}$ HFD) (**Figure 7L**).

## 4. Discussion.

Previous publications have described how obesity increases the activity of the JNKs (Ip *et al*., 1998; Hirosumi *et al*., 2002). This increase causes proinflammatory and proapoptotic responses, as well as reductions of insulin sensitivity (Nguyen *et al*., 2005; Solinas *et al*., 2006). However, in most cases, this activity is evaluated as a whole and not in an isoform-specific manner. Also, their role in the hippocampus has never been studied attentively. Considering their prominent control on synaptic plasticity (Sánchez *et al*., 2000; Zdrojewska *et al*., 2014), our research group considers them key targets for the treatment of

neurodegenerative pathologies in the brain. In the present study, their regulation of the IR and its consequences on cognition were evaluated in a model of JNK2 ablation and HFD feeding.

Many authors have confirmed the presence of all JNK isoforms in the brain, including in the hippocampus (Carboni *et al*., 1998; Brecht *et al*., 2005, Coffey *et al*., 2014)). Reinecke *et al.* reported that the lack of one of the isoforms caused for compensatory changes in the expression of the others (Reinecke *et al*., 2013). However, no differences were observed in the *Jnk2*$^{-/-}$ experimental groups when compared to the control. Interestingly, when evaluating the ratio between the phosphorylated state of P-JNK and total protein, a significant increase was detected. Similar results were observed when running a kinase activity assay. Thus, compensatory mechanisms seem to exist between the isoforms; independent of gene expression but linked to their activity.

Published data has described the effects of specific knock-outs of JNK1 and JNK3 on the control of body weight (Hirosumi *et al*., 2002; Vernia *et al*., 2016) but, there is controversy on the effect of JNK2. In our case, analysis of body weight values detected increases in those animals that had been knocked out for the *Mapk9* gene. Also, long-term feeding of HFD caused for body weight gain. The increased activity of the JNKs in all experimental groups might be the reason for this variation; favouring IR inhibition and higher formation of adipose tissue reserves (Morton *et al*., 2006). This hypothesis would be supported by our results demonstrating a reduced sensitivity to insulin through the GTT and ITT tests. Furthermore, alterations on IR functioning would have consequences on cognitive performance. Grillo and co-workers reported how when administering lentiviral virus that contained antisense sequences for the brain IR, cognitive capabilities were affected (Grillo et al., 2015). Analysis of the PTP1B, SOCS3 and other IR signalling downstream proteins would back up these conclusions.

PTP1B has been postulated to be a possible target for the treatment of pathologies like T2DM (Ganou *et al*., 2018) since it is a direct inhibitor of the signalling of the IR through its phosphatase activity (Vieira *et al*., 2017). Higher levels of PTP1B would result in higher blood glucose concentration, increased food ingestion and body weight. Similarly, research on SOCS3 has shown similar effects. (Mori H et al., 2004; Torisu T et al., 2007). Furthermore, it is also believed that these proteins regulate other pathways like ER homeostasis, synaptic plasticity and neuroinflammation (Panzhinskiy *et al*., 2013). Finally, the JNKs are inductors of these two proteins as it has been demonstrated in models of HFD-

induced obesity (Mohammad-Taghvaei *et al*., 2012; Szegezdi *et al*., 2006) or by using JNK inhibitors like in the research by Gao and colleagues (Gao S et al, 2017)

In our results, all experimental groups showed increased ER stress when compared against the control group. This data was in accordance with that reported by Raciti and colleagues (Raciti *et al*., 2012), which described how JNK2 activation was required in order to maintain pro-survival mechanisms in the cell. Increased ER stress would also favour the activation of the JNKs by increasing the activation of ATF6 and the posterior splicing of the XBP1 mRNA (Salvadó *et al*., 2014). Moreover, multiple research groups have linked alterations of the ER and the appearance of alterations in synaptic plasticity and cognitive dysfunction (Özcan *et al*., 2003; Liang *et al*., 2015). It has also been demonstrated how phosphorylation of the protein EIF2α has profound effects on synaptic function and memory impairment in several preclinical models of neurological diseases (Tao *et al*., 2014; Trinh *et al*., 2014; Lourenco *et al*., 2015).

Hence, the evidence suggests that all these mechanisms would be the cause for the subsequent cognitive decline. In our study, we evaluated the cognitive response of the animals using the NORT behavioural test. The results showed clear impairment in the animals for memory formation due to the HFD and the transgenesis of the *Mapk9* gene. In addition, the use of a GolgiStain in brain slices and the immunoblot of synaptic proteins like Spinophilin and PSD95, demonstrated significant decline in their values versus the control. Some researchers have linked these alterations to the reduction of proteins like the brain derived neurotrophic factor (Molteni *et al*., 2002; Kanoski *et al*., 2008). These results would support the evidence on the relationship between the development of insulin resistance and the appearance of reduction in cognitive performance.

Finally, inflammation has been reported to have a close relationship with the appearance of cognitive dysfunctions (Bocarsly *et al*., 2015; de Felice *et al*., 2015). As it has been previously described, HFD caused an induction of reactive astrocytes and microglia (Busquets *et al*., 2017) but, animals that have had their JNK2 ablated showed no changes in the cellular reactivity neither when being fed CT or HFD diet. Multiple biomarkers of the M1 and M2 microglia profiles were evaluated, as well as proteins like TLR4 and ATF3. The detection of increased gene expression for *Ccl3*, *Arg1*, *Cd86* and *Tnfa* in the WT HFD experimental group would indicate an increased presence of M1 (proinflammatory) microglia (Zhang *et al*., 2017) but, in those animals that lacked JNK2 no such increase appeared. Possibly, the lack of proinflammatory response in these animals would be derived on the

direct control of the JNKs on the activation of glial cells or due to other compensatory mechanisms. Increased levels of the antiinflammatory ATF3 could be one of the reasons for the observed results (Gilchrist *et al*., 2006; Hunt *et al*., 2012).

In conclusion, the importance of the JNK2 isoform in the proper cellular function of the brain tissue has been demonstrated, just like the negative effects of the HFD. Also, the relationship between metabolic alterations like insulin resistance, ER stress and neuroinflammation for proper cognitive performance has been confirmed; specially by biomarkers like PTP1B and SOCS3 proteins on the modulation of the IR. Further study of the isoforms and the effects of insulin-related biomarkers will favour the design of new drugs for the treatment of AD-like sporadic neurodegenerative pathologies (**Figure 8**).

## 5. Conflict of interest.

No authors have any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations. All authors have reviewed the contents of the manuscript being submitted, approve of its contents and validate the accuracy of the data.

6. Involves human subjects:
7. If yes: Informed consent & ethics approval achieved:
8. => if yes, please ensure that the info "Informed consent was achieved for all subjects, and the experiments were approved by the local ethics committee." is included in the Methods.

9. ARRIVE guidelines have been followed:
10. Yes
11. => if it is a Review or Editorial, skip complete sentence => if No, include a statement: "ARRIVE guidelines were not followed for the following reason:
12. "

## 13.

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## Figure Footnotes.

**Figure 1.** Flow-chart schematic describing the experimental design of the study. n = number of animals. aft. ex. = after excluding during the NORT.

**Figure 2.** *Analysing the JNKs:* A) RT-PCR quantification for the *Mapk8*, *Mapk9* and *Mapk10* genes expression. Multiple comparison: *Mapk8* (*** WT CT *vs* WT HFD.), *Mapk9* (*** WT CT *vs* WT HFD, *** WT CT *vs* *Jnk2*$^{-/-}$ CT, *** WT HFD *vs* *Jnk2*$^{-/-}$ HFD.) and *Mapk10* (** WT CT *vs* WT HFD and ** WT HFD *vs* *Jnk2*$^{-/-}$ HFD). B) Immunoblot evaluation of hippocampal total and phosphorylated JNK ratio (Thr183/185). Multiple comparison: *** WT CT *vs* *Jnk2*$^{-/-}$ CT, *** WT CT *vs* *Jnk2*$^{-/-}$ HFD and * WT HFD *vs* *Jnk2*$^{-/-}$ HFD. C) Quantification of relative kinase activity value for the P-ATF2 (Thr71) protein. Multiple comparison: * WT CT *vs* WT HFD, * WT CT *vs* *Jnk2*$^{-/-}$ CT and ** WT CT *vs* *Jnk2*$^{-/-}$ HFD. $n \geq 4$ (n = number of animals). Data was presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Two-way ANOVA and Tukey's were used for statistical analysis (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

**Figure 3.** *Evaluating metabolic parameters*: A) Analysis and representation of the increase of animal body weight in the 9-month period of the study. Multiple comparison: *** WT CT *vs* WT HFD, * WT CT *vs* *Jnk2*$^{-/-}$ CT, *** WT CT *vs* *Jnk2*$^{-/-}$ HFD and * *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD. B) Extrapolation of GTT results by determination of AUC values and posterior normalization to control group. Multiple comparison: * WT CT *vs* WT HFD, * WT CT *vs* *Jnk2*$^{-/-}$ CT and *** WT CT *vs* *Jnk2*$^{-/-}$ HFD. C) Extrapolation of ITT results by determination of AUC values and posterior normalization to control group. Multiple comparison: *** WT CT *vs* WT HFD, * WT CT *vs* *Jnk2*$^{-/-}$ CT and *** WT CT *vs* *Jnk2*$^{-/-}$ HFD. $n \geq 12$ (n = number of animals). Data was presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Two-way ANOVA and Tukey's were used for statistical analysis (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

**Figure 4.** *Study of the IR signalling pathway:* A) Immunoblot detection of IR and related signalling proteins: P-IR (Thr1150/1151)/IR ratio (* WT CT *vs* *Jnk2*$^{-/-}$ CT, *** WT CT *vs* *Jnk2*$^{-/-}$ HFD and *** *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD), PTP1B (** WT CT *vs* WT HFD, * WT CT *vs* *Jnk2*$^{-/-}$ CT and *** WT CT *vs* *Jnk2*$^{-/-}$ HFD), P-AKT (Ser473)/AKT ratio (* WT CT *vs* WT

HFD and * WT CT *vs* *Jnk2*$^{-/-}$ HFD), P-CREB (Ser133)/CREB ratio (** WT CT *vs* *Jnk2*$^{-/-}$ HFD and * WT HFD *vs* *Jnk2*$^{-/-}$ HFD), P-GSK3B(Ser9)/GSK3B ratio (** WT CT *vs* WT HFD and ** WT CT *vs* *Jnk2*$^{-/-}$ HFD). B) Quantification of gene expression variations: *InsR* (** WT CT *vs* WT HFD, ** WT CT *vs* *Jnk2*$^{-/-}$ CT, *** WT CT *vs* *Jnk2*$^{-/-}$ HFD and ** *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD), *Ptpn1* (* WT CT vs WT HFD, ** WT CT *vs* *Jnk2*$^{-/-}$ HFD and * *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD) and *Socs3* (*** WT CT *vs* WT HFD, * WT CT *vs* *Jnk2*$^{-/-}$ CT and * WT CT *vs* *Jnk2*$^{-/-}$ HFD). $n \geq 4$ (n = number of animals). Data was presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Two-way ANOVA and Tukey's were used for statistical analysis (* $p<0.05$).

**Figure 5.** *ER stress status evaluation:* A) Detection of ER stress-related proteins: BIP (* WT CT *vs* WT HFD, ** WT CT *vs* *Jnk2*$^{-/-}$ CT and *** WT CT *vs* *Jnk2*$^{-/-}$ HFD), P-EIF2a (Ser51)/EIF2a ratio (** WT CT *vs* *Jnk2*$^{-/-}$ HFD and * WT HFD *vs* *Jnk2*$^{-/-}$ HFD), ATF4 (** WT CT *vs* WT HFD, ** WT CT *vs* *Jnk2*$^{-/-}$ HFD and ** *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD), CHOP (** WT CT *vs* WT HFD, *** WT CT *vs* *Jnk2*$^{-/-}$ HFD and ** *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD) and ATF6 (* WT CT *vs* WT HFD and * WT CT *vs* *Jnk2*$^{-/-}$ HFD). B) Qualitative evaluation of ER stress through XBP1 mRNA splicing. In WT HFD, *Jnk2*$^{-/-}$ CT and *Jnk2*$^{-/-}$ HFD experimental groups, the presence of a smaller band in the lower part of the gel, would indicate the presence of a spliced version of XBP1 mRNA, one of the mechanisms of signalling of stress in the ER. $n \geq 4$ (n = number of animals). Data was presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Two-way ANOVA and Tukey's were used for statistical analysis (* $p<0.05$, ** $p<0.01$).

**Figure 6.** *Behavioural assessment of cognitive performance and related biomarkers*: A) Novel Object Recognition Test (NORT) open field habituation. Time spent in inner quadrant (seconds; sec). B) Results for the quantification of the discrimination ratio (DI). Multiple comparison: ** WT CT *vs* WT HFD, * WT CT *vs* *Jnk2*$^{-/-}$ CT and ** WT CT *vs* *Jnk2*$^{-/-}$ HFD. $n \geq 12$ (n = number of animals). C-F) Optical microscope images of brain GolgiStain. Scale bar: 10 µm. G) Quantification of dendritic spines for each 10 µm. Multiple comparison: *** WT CT *vs* WT HFD, ** WT CT *vs* *Jnk2*$^{-/-}$ CT, *** WT CT *vs* *Jnk2*$^{-/-}$ HFD and * *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD. n = 3 (n=number of animals). From each animal at least 5 neurons were checked. Spine density value for each neuron was the result of the mean of 5 consecutive measurements in the same secondary branch. H) Immunoblot detection of Spinophilin (* WT

CT *vs* WT HFD, *** WT CT *vs* *Jnk2*$^{-/-}$ HFD and *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD) and PSD95 (* WT CT *vs* *Jnk2*$^{-/-}$ CT and * WT CT *vs* *Jnk2*$^{-/-}$ HFD). $n \geq 4$ (n = number of animals). Data was presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Two-way ANOVA and Tukey's were used for statistical analysis (* $p<0.05$, ** $p<0.01$).

**Figure 7.** *Evaluation of inflammatory responses*. Representative images for the detection of astrocytes (A-D) and microglia (E-H) in the DG of the hippocampus All samples are co-stained with Hoechst for the detection of cellular nucleus (blue). Scale bar: 200 µm. Quantification of relative fluorescence was calculated as Corrected Total Cell Fluorescence for each experimental group: GFAP (I; *** WT CT *vs* WT HFD and *** WT HFD *vs* *Jnk2*$^{-/-}$ HFD) and IBA1 (J; ** WT CT *vs* WT HFD and *** WT HFD *vs* *Jnk2*$^{-/-}$ HFD). $n \geq 15$ (n=number of biological replicates) K) Quantification of expression variation for multiple microglia profile characterization-related genes: *Cd86* (** WT CT *vs* WT HFD and * WT CT *vs* *Jnk2*$^{-/-}$ HFD), *Ccl3* (** WT CT *vs* WT HFD), *Tlr4* (** WT CT *vs* WT HFD, * WT CT *vs* *Jnk2*$^{-/-}$ CT and * WT CT *vs* *Jnk2*$^{-/-}$ HFD), *Tnfa* (*** WT CT *vs* WT HFD and *** WT HFD *vs* *Jnk2*$^{-/-}$ HFD), *Arg1* (* WT CT *vs* WT HFD, * WT CT *vs* *Jnk2*$^{-/-}$ HFD and *Jnk2*$^{-/-}$ CT *vs* *Jnk2*$^{-/-}$ HFD) and *Cd206* (* WT CT *vs* WT HFD). L) Semi-quantification of protein levels for TLR4 (* WT CT vs Jnk2-/- HFD) and ATF3 (* WT CT *vs* WT HFD, * WT CT *vs* *Jnk2*$^{-/-}$ CT, * WT CT *vs* *Jnk2*$^{-/-}$ HFD and * WT HFD *vs* *Jnk2*$^{-/-}$ HFD). $n \geq 4$ (n = number of animals). Data was presented as interleaved boxes and whiskers. The box represents the median in the middle and the 25th to 75th percentile in the extremes. The maximum and minimum values were represented as whiskers. Two-way ANOVA and Tukey's were used for statistical analysis (* $p<0.05$).

**Figure 8.** *Final schematic for the mechanisms involved in the present study.*

**Supplementary Material 1.** Description of nutritional content for control (CT) and High-Fat Diet (HFD).

**Supplementary material 2.** List of primary and secondary antibodies used for immunoblot analysis.

**Supplementary material 3.** List of primary and secondary antibodies used for immunofluorescence (IF) assays.

**Supplementary material 4.** Sequence specification for primers used in Real Time – Polymerase Chain Reaction (RT-PCR).