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Abscisic Acid Supplementation Rescues High Fat Diet-Induced Alterations in Hippocampal Inflammation and IRSs Expression

Alberto Ribes-Navarro[1] · Mariam Atef[1] · Sandra Sánchez-Sarasúa[1] · María Teresa Beltrán-Bretones[1] · Francisco Olucha-Bordonau[1] · Ana María Sánchez-Pérez[1]

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Abstract

Accumulated evidence indicates that neuroinflammation induces insulin resistance in the brain. Moreover, both processes are intimately linked to neurodegenerative disorders, including Alzheimer's disease. Potential mechanisms underlying insulin resistance include serine phosphorylation of the insulin receptor substrate (IRS) or insulin receptor (IR) misallocation. However, only a few studies have focused on IRS expression in the brain and its modulation in neuroinflammatory processes. This study used the high-fat diet (HFD) model of neuroinflammation to study the alterations of IR, an insulin-like growth factor receptor (IGF1R) and IRS expressions in the hippocampus. We observed that HFD effectively reduced mRNA and protein IRS2 expression. In contrast, a HFD induced the upregulation of the IRS1 mRNA levels, but did not alter an IR and IGF1R expression. As expected, we observed that a HFD increased hippocampal tumor necrosis factor alpha (TNFα) and amyloid precursor protein (APP) levels while reducing brain-derived neurotrophic factor (BDNF) expression and neurogenesis. Interestingly, we found that TNFα correlated positively with IRS1 and negatively with IRS2, whereas APP levels correlated positively only with IRS1 but not IRS2. These results indicate that IRS1 and IRS2 hippocampal expression can be affected differently by HFD-induced neuroinflammation. In addition, we aimed to establish whether abscisic acid (ABA) can rescue hippocampal IRS1 and IRS2 expression, as we had previously shown that ABA supplementation prevents memory impairments and improves neuroinflammation induced by a HFD. In this study, ABA restored HFD-induced hippocampal alterations, including IRS1 and IRS2 expression, TNFα, APP, and BDNF levels and neurogenesis. In conclusion, this study highlights different regulations of hippocampal IRS1 and IRS2 expression using a HFD, indicating the important differences of these scaffolding proteins, and strongly supports ABA therapeutic effects.

Keywords Insulin resistance · Hippocampus · APP · BDNF · Neurogenesis · Neuroinflammation

Abbreviations

ABA	Abscisic acid
ANOVA	Analysis of variance
APP	Amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
ERK	Extracellular regulated kinases
HFD	High-fat diet
IGF1	Insulin-like growth factor
IGF1R	Insulin-like growth factor receptor
IR	Insulin receptor
IRS	Insulin receptor substrate
PFA	Paraformaldehyde
PPAR-γ	Peroxisome proliferator-activated receptor gamma
RTqPCR	Real-time quantitative polymerase chain reaction
SEM	Standard error of mean
SD	Standard diet
SGZ	Subgranular zone
TBS	Tris-buffered saline
TDZ	Thiazolidinediones
TNFα	Tumor necrosis factor alpha

✉ Ana María Sánchez-Pérez
sanchean@uji.es

[1] Department of Medicine, University of Jaume I, Castellón de la Plana, Spain

Introduction

Adult neurogenesis is a sensitive process that is very susceptible to different toxic insults, such as the presence of beta

amyloid plaques (characteristic in Alzheimer's disease) [1], insulin resistance [2], or inflammation [3]. Impairment of neurogenesis is associated with alterations of hippocampal-dependent functions, including spatial awareness, long-term memory, emotionality, and mood [4, 5].

For more than 20 years, neuroinflammation has been considered to underlie the onset of neurodegenerative disorders [6], including Alzheimer's disease [7–10]. Importantly, neuroinflammatory processes resulting from obesity and stress can induce insulin resistance [11]. These processes cause a considerable increase of reactive microglia, which secrete a variety of cytokines (e.g., TNFα), which activate specific intracellular cascades, including IKKβ and JNK [12]. Aside from this, a large body of evidence has accumulated correlating insulin resistance induced by chronic inflammatory processes and the development of the disease [13]. In contrast, numerous preclinical and clinical studies have shown how insulin sensitization improved cognitive damage, cytokine levels, and mitochondrial function after a fat enriched-diet period [14, 15].

Interestingly, insulin/IGF1 signaling has been shown to protect against these toxic insults and improves neurogenesis [16]. In line with this, physical exercise improves insulin sensitivity and increases hippocampal neurogenesis [17, 18]. The mechanisms of the exercise-induced improvement of neurogenesis may be a result of several factors, importantly the augmented blood flow to the brain [19] with the consequent affluence of growth factors, in particular IGF1 [20]. Highlighting the importance of IGF1, pilocarpine-induced epilepsy has been shown to increase the IGF1 expression via CREB stimulation in the SGZ-surrounding microglia, improving neurogenesis [21]. Another important factor in neurogenesis is the brain-derived neuronal factor (BDNF) [22]. In fact, there is evidence that hippocampal BDNF expression is also augmented by exercise [23]. Furthermore, a crosstalk between BDNF and IGF1 has been reported to play an important role in exercise-induced neurogenesis [24].

Insulin and IGF1 signaling cascades are very similar: both peptides exert their actions through tyrosine kinase receptors via the subsequent activation of the IRSs [25–27]. Of the four known receptor substrate isoforms, the IRS1 and IRS2 isoforms are highly expressed in the brain [28, 29]. Insulin and IGF1 receptors are expressed in dentate gyrus in rat and mouse brains [30–33], but the specific expression of the IRS in these areas is largely unknown. Neuroinflammation may induce insulin resistance through IRS inactivation by specific serine phosphorylation. In humans, IRS1 P-Ser 307 may determine insulin resistance in type 2 diabetes [34, 35]. One of the consequences of P-Ser in IRS1 is to increase the degradation of the protein using ubiquitination [36]. However, this process does not appear to occur for IRS2 degradation [37], which suggests differential regulation of both proteins, at least in certain cell types.

In addition to exercise, other factors, such as phytohormones have been proposed as neuroprotectors, precisely due to their insulin sensitizer activity and anti-inflammatory properties [38]. For instance, ABA a PPARPγ agonist [39] can improve glucose tolerance in obesity models, reduce neuroinflammation, and restore HFD-induced neurological alterations [40–42]. The mechanism underling ABA action involves the activation of lanthionine synthetase C-like 2 (LANCL2) [43, 44].

In this study, we evaluate the effects of HFD-induced neuroinflammation and ABA treatment in the expression levels of several members of the insulin/IGF1 pathway, along with inflammation markers, in the hippocampus. One of the well-known effects of HFD exposure is the reduction of adult hippocampal neurogenesis [43]; therefore, we evaluated whether ABA treatment could rescue the HFD-induced reduction in hippocampal neurogenesis via its anti-inflammatory and insulin sensitizing properties.

Materials and Methods

Animals and Diet

Eight-week-old male Wistar rats (Janvier Labs, Saint-Berthevin, France) were kept at the animal facility of the University Jaume I. The procedures followed directive 86/609/EEC of the European Community on the protection of animals used for experimental and other scientific purposes. The experiments were approved by the Ethics Committee of the University Jaume I (approval number 2014/VSC/PEA00209). The animals were maintained on a 12 h:12 h light–dark cycle and housed in pairs to reduce stress due to social isolation. Rats were divided randomly into four experimental groups: SD, control animals fed the standard rodent diet (Ssniff, Soest, Germany); SD-ABA, animals fed standard diet supplemented with ABA (Fernandez-Rapado, Spain) in their drinking water (20 mg/L); HFD, animals fed an HFD (5736 kcal/kg, Ssniff), and HFD-ABA, animals fed an HFD and that had ABA in their drinking water (20 mg/L). We have used ABA concentration as described [40]. The four groups were fed ad libitum for 12 weeks.

Immunoblotting

Rats were lightly anesthetized (Dolethal, 200 mg/Kg Vetoquinol S.A., Madrid, Spain) and then killed by decapitation. Brains were rapidly removed and frozen in cold isopentane (Sigma-Aldrich, St Louis, MO, USA). Hippocampi were dissected at − 15 °C (using a cryostat) to preserve the protein phosphorylation. Tissue was lysed in a RIPA buffer containing protease and phosphatase inhibitors (SERVA Electrophoresis, Heidelberg, Germany). Mechanical tissue lysis was achieved using a sonicator (Hielsher Ultrasound Technology, Teltow, Germany). Thirty micrograms of the total protein were subjected to SDS-

PAGE, transferred to Immobilon-P membranes (MERCK Millipore, Darmstadt Germany), which were blocked for non-specific binding and incubated with primary antibodies: anti-phospho IKKαβ (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500); anti-phospho TAU (Abcam, Cambridge, UK; 1:1000); anti-SOD (Abcam, Cambridge, UK; 1:5000); anti-IRS1 (EMD Millipore, Temecula, CA, USA; 1:500); anti-IRS2 (Cell Signaling, Danvers, MA, USA 1:1000), and anti-β-Actin (Sigma-Aldrich, St Louis, MO, USA; 1:2000) overnight at 4 °C. After several washes with a washing buffer containing 0.3% Triton X-100, the membranes were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse, Jackson Immunoresearch, Suffolk, UK). Staining was developed using ECL (BioRad, Hercules CA, USA), and digital images were captured with a charge-coupled device imager (IMAGEQUANT LAS 4000, GE Healthcare Little Chalfont, UK). Immunoreactive bands were quantified with Image J blots toolkit software (National Institutes of Health, Baltimore, MD, USA), which was normalized to the β-Actin signal in each sample. Data were expressed as a percentage of the control (standard diet) normalized signal as the mean plus and minus the standard error of mean (SEM).

Immunohistochemistry

Rats were anesthetized with pentobarbital (120 mg/kg Eutanax, Fatro, Barcelona, Spain) and transcardially perfused with saline (0.9%), followed by a 4% paraformaldehyde (PFA) fixative in 0.1 M phosphate buffer, pH 7.4. After perfusion, the brains were removed and post-fixed overnight at 4 °C in PFA, followed by 48 h in a 30% sucrose solution for cryoprotection. Sliding Microtome Leica SM2010R (Leica Microsystems, Heidelberg, Germany) was used to obtain 40-μm thick coronal frozen sections. The brains were cut in rostrocaudal direction; six series of slices were collected from each brain and stored at − 20 °C. Sections were rinsed twice in 0.05 M Tris-buffered saline (TBS), pH 8.0, containing 0.2% Triton X-100 at room temperature. Slices were incubated in blocking solution (containing 2% donkey serum and 4% serum albumin bovine for 1 h); anti-Doublecortin (DCX) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000) was used. After several washes, the slides were incubated with the biotinylated secondary antibody (Jackson 1: 200) in TBS with 0.2% Triton X-100. Then sections were rinsed 3× for 10 min and transferred to a 1:50 avidin–biotin–horseradish peroxidase complex solution for 90 min (VECTASTAIN® Elite® ABC-HRP Kit; Peroxidase, Standard, USA). Then, sections were rinsed 2× for 10 min in TBS and 2× for 10 min in 0.05 M 7.6 Ph Tris Buffer (TB). Color reaction was achieved by incubation with DAB (Sigma-Aldrich, St Louis, MO, USA) and 2 μl of H_2O_2 in TB for 15–20 min. The reaction was stopped by several rinses in phosphate buffered saline solution. Finally, the slices were mounted on gelatinized slides and air dried overnight. Then, sections were rehydrated, alcohol-dehydrated, xylene-cleared and cover-slipped with a DPX mounting medium.

Image Analysis

Images were taken by a Nikon optical microscope (Nikon, Tokyo, Japan) attached to a Leica camera (Leica Microsystems), which was connected to the Leica software (Leica Microsystems), to acquire the images. To quantify the DCX positive neurons, six images were taken for each anatomical subdivision of the hippocampus in the four treatments (HFD, HFD-ABA, SD, and SD-ABA). The DCX-positive neurons were counted with Image J (National Institute of Health, Baltimore, MD, USA) at levels rostral (Bregma − 3.72 mm) and caudal (Bregma − 6.00 mm) levels (Paxinos and Watson [45]). Data were expressed as the mean and the SEM of the DCX positive neurons ($n = 6$). The researcher performing the quantification of DCX-positive neurons was blind to the experimental condition.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from the rat hippocampus ($n = 5$–11) and homogenized in 500 μL of lysis buffer according to the Norgen Fatty Tissue RNA Purification Kit (Norgen Biotek Corp, product no. 36200, ON, Canada). Genomic DNA was removed using a spin-column process during the RNA extraction. In addition, DNAse I treatment (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) was performed to ensure the complete removal of genomic DNA. RNA samples were eluted in 50 μL of nuclease-free water and reverse transcribed to cDNA using a PrimeScript™ RT reagent kit (Takara, Shiga, Japan) following the manufacturer's instructions. Primers were designed using the Primer3 software tool (http://primer3.ut.ee/) (Table 1). RT-qPCR reactions were carried out using SYBR PREMIX Ex Taq (Tli RNase H Plus) (TAKARA Bio Inc., Shiga, Japan) in an Applied Biosystems StepOne Plus™ Real-Time PCR System (Foster City, CA, USA). At the end of each PCR reaction, a melting curve stage was performed to confirm that only one PCR product was amplified in these reactions. The relative gene expression to SD was calculated by using the $2^{-\Delta\Delta Ct}$ method for each reaction and by using the housekeeping gene GAPDH as internal control.

Statistics

Data were expressed as mean ± SEM and subjected to a Kolmogorov-Smirnov normality test, and only when the test was positive for normality ($\alpha = 0.05$) were the data analyzed using a parametric one-way analysis of variance (ANOVA) followed by a post-hoc Newman-Keuls test. To evaluate the

Table 1 Primers sequences obtained from Primer3 software tool

Gene	Accession number	Forward primer (5′- > 3′)	Reverse primer (5′- > 3′)
GAPDH	NM_017008.4	TGCCCCCATGTTTGTGATG	TGGTGGTGCAGGATGCATT
INSR	M29014.1	TCCTAAAGATCCGTCGCTCC	AAGAGCTTGCCCTGAGTGAT
IGF-1R	NM_052807.2	TGTCCTCTCGGCATCAAACT	TATCACCACCGCACACTTCT
IRS-1	NM_012969.1	ATTGGAGGTGGGTCTTGCAG	TGGGGATCTTCTGGGCCATA
IRS-2	NM_001168633.1	TCACCACAGGACACAGATGC	GCATGAAGTGTGGCAAACGT
APP	NM_019288.2	CCCCAAGATCCGGTTAAACT	TACTTGTCGACTGCGTCAGG
BDNF	NM_001270638.1	GAGACAAGAACACAGGAGGAAA	CCCAAGAGGTAAAGTGTAGAAGG
TNF α	NM_012675.3	GACCCTCACACTCAGATCATCTTCT	TGCTACGAGGTGGGCTACG

associations between variables, we used a two-tailed Pearson correlation test. The correlation coefficient r values (± 0.1 to ± 0.3) reveal a weak association, are a (± 0.3 to ± 0.5) medium, and are higher than ± 0.5 strong correlation [46–48]. Positive or negative r values mean positive or negative correlations, respectively [49].

Results

A HFD Alters Hippocampal IRS1 and IRS2 mRNA Expression. ABA Treatment Can Prevent These Changes

We found that TNFα levels were increased significantly by HFD treatment, and ABA administration, along with a HFD, prevented this significant increase ($F_{3,39} = 9.363$). Similarly, we observed that the BDNF mRNA level in the hippocampus was significantly reduced in HFD-treated animals compared to SD and that the treatment with ABA in the drinking water prevented this reduction ($F_{3,16} = 5.898$). Interestingly, the APP mRNA expression levels also showed a significant increase with HFD exposure ($F_{3,19} = 4.524$).

Next, we wanted to evaluate the effects of a HFD on the proteins' expression of the insulin-signaling pathway. Even though it is widely accepted that a HFD and neuroinflammation induces insulin resistance, whether a HFD affects mRNA levels of IR, IRS1, and IRS2 is largely unknown. In our model, we observed a significant reduction of IRS2 mRNA levels in the hippocampus of rats fed a HFD with respect to SD (Table 2). Most interestingly, ABA treatment prevented this significant reduction in the hippocampal IRS2 levels in rats fed a HFD ($F_{3,37} = 5.911$). Moreover, there was a significant increase in the mRNA levels of IRS1 in those animals fed a HFD, which can also be rescued by ABA administration ($F_{3,22} = 4.301$) (Table 2). Hence, these results suggest that the modulation of IRS1 and IRS2 gene expression may be different, depending on the environmental conditions, which suggests that these proteins may be involved in different cellular needs, in insulin signaling, and in neuroinflammation. IR did not change with diet or ABA treatment; however, the IGF1R did show a change in those control samples that were treated with ABA ($F_{3,16} = 3.025$) (Table 2).

ABA Can Effectively Restore HFD-Induced Reduction in Hippocampal IRS2 Protein

To evaluate the effect of a HFD on protein expression in the hippocampus, we measured the levels of both IRS1 and IRS2 and the inflammatory markers phosphrorylated IKK (pIKK) [50] and Tau (pTau) [51] using a western blot (Fig. 1a). We observed that IRS2 protein levels were significantly lower in the HFD group (0.61 ± 0.05; $n = 11$) compared to SD group (0.99 ± 0.02; $n = 9$), HFD supplemented with ABA group (1.00 ± 0.16; $n = 12$), and the SD-ABA group (1.29 ± 0.17; $n = 7$), ANOVA ($F_{3,35} = 4.897$; $p = 0.006$) (Fig, 1b). In contrast, hippocampal IRS1 protein levels from matched samples were not different from each other [HFD (0.97 ± 0.12; $n = 5$), HFD-ABA (1.05 ± 0.12; $n = 5$), SD (0.96 ± 0.02; $n = 5$), SD-ABA (1.31 ± 0.25; $n = 5$)] (Fig. 1c). On the other hand, pIKK did not show any significant differences between groups [HFD (1.38 ± 0.32; $n = 6$), HFD-ABA (1.28 ± 0.20, $n = 6$), SD (0.98 ± 0.01, $n = 7$), SD-ABA (1.15 ± 0.07, $n = 7$)] (Fig. 1d). We found that ABA increased pTau expression in SD-ABA animals (1.37 ± 0.19; $n = 7$) with respect to the SD group (0.99 ± 0.02; $n = 9$), that the HFD-treated animals had lower levels, although not significantly lower (0.68 ± 0.12, $n = 11$), and ABA added to HFD group (0.88 ± 0.07; $n = 11$) did not significantly alter the pTau levels, ANOVA ($F_{3,34} = 6.012$; $p = 0.002$) (Fig. 1e).

ABA Rescues the HFD-Induced Reduction in Hippocampal Neurogenesis

To evaluate hippocampal neurogenesis, we quantified DCX, which was expressed in differentiating and migrating neurons during embryonic and postnatal development

Table 2 Hippocampal mRNA expression of neuroinflammation markers and Insulin/IGF-1 pathway genes

GENES	HFD	HFD-ABA	SD	SD-ABA
TNFα (n=7-15)	1.67 ± 0.16	1.19 ± 0.14 *	1.01 ± 0.04***	0.99 ± 0.04 **
BDNF (n=5)	0.59 ± 0.04	0.85 ± 0.10	1.00 ± 0.02**	0.87 ± 0.09
APP (n=5-6)	1.8 ± 0.29	1.41 ± 0.22	1.00 ± 0.01**	0.95 ± 0.13 *
IRS1 (n=7)	1.28 ± 0.08	1.06 ± 0.04	1.01 ± 0.02*	1.06 ± 0.08
IR (n=5-7)	0.97 ± 0.07	0.95 ± 0.14	1.01 ± 0.01	0.99 ± 0.04
IGF1-R (n=5)	1.24 ± 0.14	1.11 ± 0.10	1.00 ± 0.01	0.80 ± 0.13*
IRS2 (n=7-14)	0.59 ± 0.06	1.02 ± 0.09 *	1.00 ± 0.06**	0.83 ± 0.15

The expression of all the genes was quantified using GAPDH as a housekeeping gene and normalized to SD levels. Data passed KS normality test and was analyzed by One Way ANOVA followed by post-hoc Tukey's test. *p<0.05, ** p <0.01 *** p <0.001 compared to HFD

[52] using immunohistochemistry in the rostral (Bregma − 3.72 mm) and caudal hippocampus (Bregma − 6.00 mm) (Fig. 2a), following the rat brain atlas [45]. Data were expressed as the mean of DCX positive neurons ± SEM. In the rostral hippocampus, we found that the number of DCX-positive neurons decreased significantly in HFD-fed rats (131.9 ± 41.4; n = 6) compared to SD-fed controls (275 ± 34.15; n = 6). The ABA treatment rescued the DCX expression in HFD-ABA animals (339.1 ± 43.99; n = 6) and did not alter the DCX in SD animals (257.2 ± 34.15; n = 6). ANOVA ($F_{3,20}$ = 6.150; p = 0.0039)] (Fig. 2b). There were representative images of DCX positive neurons in SD (Fig. 2c), a HFD (Fig. 2d), SD + ABA, (Fig. 2e), and HFD + ABA (Fig. 2f). An identical pattern was followed in the caudal hippocampus (data not shown).

IRS1 and IR21 mRNA Levels Correlate Differently with TNFα, BDNF and APP

We have observed that diet and ABA treatment can differently affect the expression of IRS1 and IRS2. To further understand the relationships of IRS expression and other biomarkers of inflammation, we aimed to ascertain if there was a relationship between the levels of IRS1 and IRS2 gene expression with TNFα, APP, and BDNF. We observed that IRS1 correlates positively with TNFα (Pearson r = 0.574; p = 0.02) (Fig. 3a), whereas IRS2 correlates negatively with TNFα (Pearson r = − 0.498; p = 0.05) (Fig. 3b). Interestingly, IRS1 showed a positive correlation with APP (Pearson r = 0.672; p = 0.003) (Fig. 3c). In contrast, IRS2 did not correlate with APP either way (Fig. 3d). None of the substrates showed any correlation with BDNF levels (Fig. 3e, f, respectively).

The IRS1 Expression Correlates with Weight Gain but IRS2 Does Not

Due to the different patterns of expression showed by both isoforms of IRS, we aimed to elucidate if there was a relationship between the percentage of weight gain and the IRS expression. Interestingly, IRS1 showed a positive correlation with the percentage of weight gain (Pearson r = 0.498; p = 0.019) (Fig. 4a). However, the IRS2 expression did not show any significant correlation when plotted versus the percentage of weight gain (Fig. 4b).

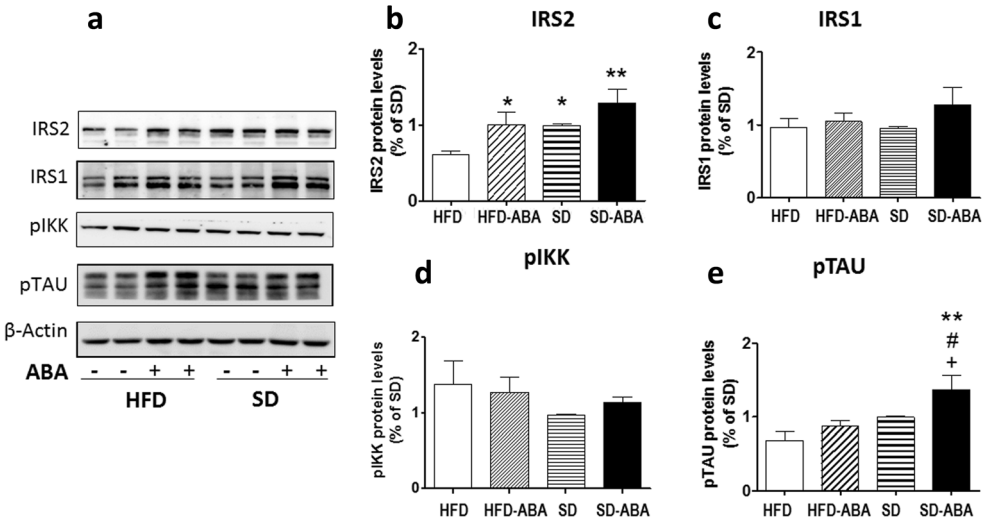

Fig. 1 A western blot analysis was carried out to evaluate the expression of the proteins in the hippocampus. Representative images of IRS2, IRS1, pIKK, pTAU, and loading control β-Actin (**a**). Quantifications of IRS2 protein levels (**b**), IRS1 (**c**), pIKK (**d**), and pTAU (**e**). HFD (white columns); HFD-ABA (diagonal lines); SD (horizontal lines), and SD-ABA (black columns). Data are represented as the mean ± SEM (n = 6). The ANOVA was followed by a post-hoc Newman-Keuls test (n = 5–11). *p < 0.05; **p < 0.01;*vs. HFD; #vs. HFD-ABA; +vs. SD

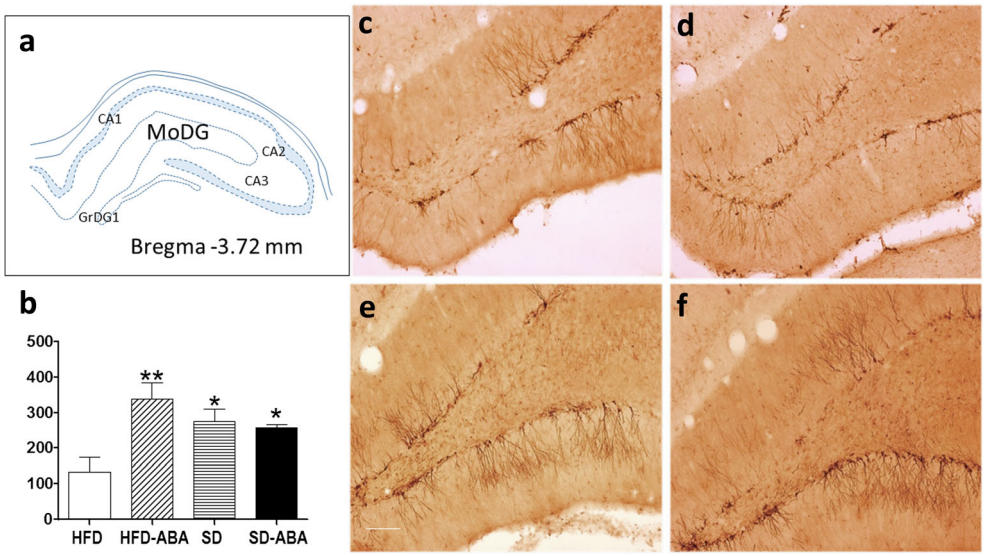

Fig. 2 ABA rescues the HFD-induced reduction in the number of DCX positive neurons in the hippocampus. A Patxinos drawing of a rostral hippocampus (Bregma − 3.72 mm) (**a**). The quantification of DCX positive neurons (**b**). Data are represented as the mean ± SEM ($n = 6$) and analyzed using a one-way ANOVA followed by post-hoc Newman-Keuls test. *$p < 0.05$; **$p < 0.01$, with respect to HFD. The representative images are of DCX imunolabeling in SD (**c**), HFD (**d**), SD + ABA (**e**), and HFD + ABA (**f**). Calibration bar, 100 μm

Discussion

Excessive caloric intake can induce neuroinflammation and insulin resistance. Both processes are considered major risk factors that lead to cognitive alterations [53–55].

In this study, we have made use of a well-established model of neuroinflammation—feeding rats a HFD for 3 months—to study its effects on the insulin-signaling pathway. In addition, we have evaluated the effects of the phytohormone ABA in hippocampal insulin signaling. ABA targets peroxisome proliferator-activated receptor gamma (PPAR-γ) in a manner similar to the Thiazolidinediones class of anti-diabetic drugs [56, 57] and curcumin, a well-known potent anti-inflammatory molecule [58] and also PPAR-γ agonist [59]. In fact, given their capability to reduce inflammation, this family of PPAR-γ agonist molecules has been proposed as a new class of molecules with which to treat central nervous system disorders [60].

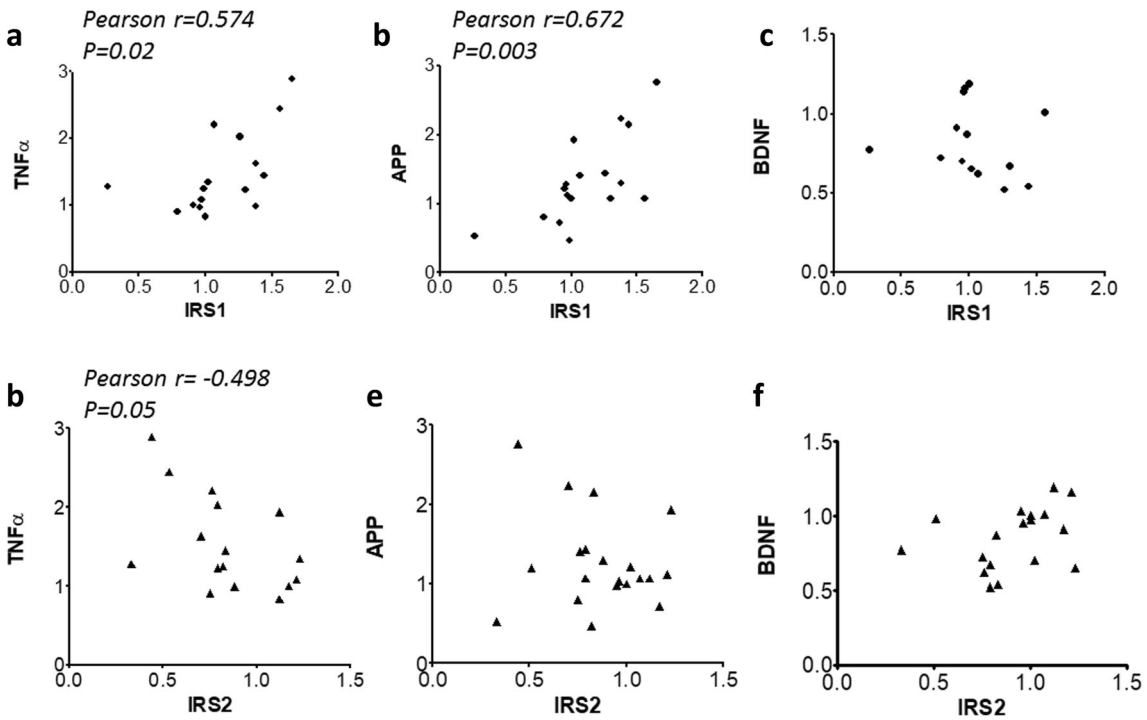

Fig. 3 The correlation of IRS1 and IRS2 with TNFα, APP, and BDNF. The IRS1 expression correlates positively with the TNFα (**a**) and APP (**b**) expression. IRS1 does not correlate with the BDNF expression (**c**). The IRS2 gene expression correlates negatively with TNFα and does not correlate with the APP (**e**) or BDNF (**f**) expression

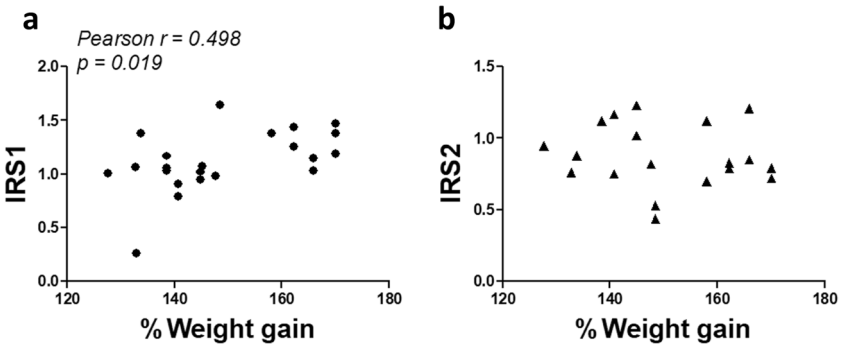

Fig. 4 The correlation of IRS1 and IRS2 versus the percentage of weight gain. The IRS1 expression correlates positively with the percentage of weight gain (**a**). There is no significant correlation between IRS2 and the percentage of weight gain (**b**)

We have analyzed the mRNA of IR and IGF1R and their substrates, IRS1 and IRS2. We found no changes in hippocampal IR or IGF1R in the hippocampus of HFD-fed animals compared to the controls. However, our study shows for the first time that a HFD can reduce IRS2 expression in hippocampal tissue, both at the messenger RNA and protein levels, therefore providing an additional plausible mechanism of hippocampal insulin resistance in obesity and metabolic syndrome. These results are also in line with previous reports showing that IRS2 is reduced in dorsal root ganglia from diabetic (insulin resistance) rodent models [61]. Moreover, in the gastrocnemius muscle, IRS2 expression was found to be significantly reduced in diabetic animals compared to the controls [62]. On the contrary, we observed that HFD increased IRS1 levels. These results may be paradoxical; however, they are in line with earlier reports indicating that IRS1 mRNA is increased in the hippocampus of APP/PS1 transgenic mice compared to aged-matched controls [63].

Interestingly, we have observed that treatment with ABA restored IRS1 and IRS2 expression, which could account for beneficial effects improving the cognitive performance of HFD-fed animals [40]. These results are supported by studies showing that PPAR-γ agonists can restore pathological IRS levels, that curcumin reduced the IRS1 levels in double APP/PS1 transgenic mice [63], and that pioglitazone increased the pancreatic IRS2 expression in a model of diabetic transgenic mice [64].

To our knowledge, our study reports for the first time that HFD alters hippocampal IRS1 and IRS2 expression and that supplementation with ABA is capable of restoring these alterations.

Protein expression was studied using immunodetection and confirmed the alterations observed in the IRS2 mRNA levels, but not IRS1 mRNA. This discrepancy in messenger and protein expression could be due to the fact that the IRS1 protein levels may be regulated by degradation, and, therefore, the increase in mRNA is not being detected in the protein. Paradoxically, pIKK and pTau were not found to be increased in a HFD as expected given the fact that inflammation augments the phosphorylation of IKK [65, 66] and that insulin resistance results in tau hyperphosphorylation [67]. Nevertheless, supporting our results, pIKK levels are not altered in HFD-treated rats [68], indicating that pIKK may not be a universal and reliable marker for neuroinflammation. However, further studies should be carried out to elucidate the role of pIKK in hippocampal HFD-induced inflammation. Similarly, future research will help to understand the conditions required for accumulation of pTau derived from an insulin-resistance situation. It is plausible that time is an important factor for observing such accumulation.

Furthermore, we have observed that ABA treatment is capable of restoring a HFD-induced reduction in neurogenesis, as evaluated by DCX positive neurons in the subgranular layer of the dentate gyrus. It is well accepted that a HFD reduces neurogenesis and that exercise and learning can improve it [69]. Consistent with our data, many insulin sensitizer molecules used against type 2 diabetes have been proven effective in restoring altered neurogenesis [70]. In addition, BDNF signaling is closely related to neurogenesis modulation, synaptic plasticity, and the formation and recall of hippocampal-dependent memories [71]. BDNF reduction has also been proposed as one of the earliest events in HFD-induced cognitive impairment [72, 73]. In this study, in addition to showing impaired neurogenesis, we observed that the BDNF mRNA levels were reduced in the hippocampus with HFD and that ABA treatment could efficiently prevent this reduction. This is in line with previous studies reporting that neurogenesis and BDNF levels can be rescued using insulin and BDNF-sensitizing nutritional compounds [74] and anti-inflammatory supplementation [75]. BDNF may be able to induce IRS1 and IRS2 tyrosine phosphorylation [76] but, to date, it has not been reported to regulate IRS1 or IRS2 expression. In fact, we found no correlation between IRS1 and IRS2 with BDNF levels.

On the other hand, TNFα, a well-accepted marker of neuroinflammation, insulin resistance, and cognitive impairment [77], increases in the brain of HFD-treated animals [78]. As

expected, we found that TNFα levels were increased in the hippocampus of HFD-treated rats and that ABA treatment significantly could prevent it. This result confirms that ABA is an effective anti-neuroinflammatory molecule. Furthermore, since TNFα levels are also closely linked to impaired neurogenesis and reduction in BDNF [79], our study confirms the close relationship of these processes with IRS expression.

APP transgenic mice have been extensively used as a model for Alzheimer's disease in an attempt to over-express a mutated protein that promotes oligomer aggregation [80]. We have found that endogenous APP levels are increased in the hippocampus of HFD-fed rats. It is plausible that this increment can favor Aβ accumulation [81] and mediate some of the deleterious effects of a HFD in cognition. We found that ABA treatment could effectively counteract these effects, preventing the overexpression of APP. Other studies have also shown that supplementary nutrients can effectively downregulate APP endogenous expression [82]. Therefore, the anti-inflammatory and insulin-sensitizing actions also regulate APP expression, preventing further complications of Aβ deposition.

Finally, to understand the relationship between IRSs, the biomarkers used in this study and obesity, we analyzed the normalized levels of IRS1 and IRS2 with TNFα, APP, BDNF rat hippocampal tissue, and the percentage of weight gain. Interestingly, we observed the levels of TNFα correlate inversely with the expression of IRS2 and positively with IRS1. This fact strongly suggests that IRS1 and IRS2 can be differentially affected by neuroinflammatory processes and therefore modulates distinct aspects of insulin resistance. Further confirming this hypothesis, we found that APP correlates positively with IRS1 but does not correlate at all with IRS2; thus, it is plausible that the signaling pathway that affects APP can target only IRS1 but not IRS2. Moreover, the percentage of weight gain correlates positively with IRS1 but not with IRS2 expression.

Considering all these data, we conclude that both IRS1 and IRS2 expression regulation, although modulating insulin and IGF1 signaling, are totally independent of each other, suggesting that their implication in neuroinflammation and brain insulin resistance may also be different. Further studies should be carried out to establish the mechanism underlying these differences, such as location, protein interactions, and promoter sensitivity. These findings will be crucial for opening different methods of brain insulin resistance treatment.

Moreover, we confirm that ABA can effectively restore neuroinflammation and neurogenesis, supporting the beneficial effects on memory that we have reported previously [40]. In addition, we show that ABA can also influence IRS1 and IRS2 expression levels, providing a mechanistic link to how ABA can restore insulin signaling.

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