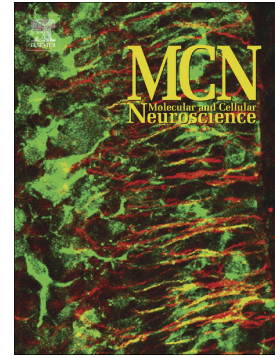# Journal Pre-proof

IRS1 expression in hippocampus is age-dependent and is required for mature spine maintenance and neuritogenesis

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IRS1 expression in hippocampus is age-dependent and is required for mature spine maintenance and neuritogenesis.

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Abstract: Insulin and insulin-like growth factor type I (IGF-1) play prominent roles in brain activity throughout the lifespan. Insulin/IGF1 signaling starts with the activation of the intracellular insulin receptor substrates (IRS). In this work, we performed a comparative study of IRS1 and IRS2, together with the IGF1 (IGF1R) and insulin (IR) receptor expression in the hippocampus and prefrontal cortex during development. We found that IRS1 and IRS2 expression is prominent during development and declines in the aged hippocampus, contrary to IR, which increases in adulthood and aging. In contrast, IGF1R expression is unaffected by age. Expression patterns are similar in the prefrontal cortex. Neurite development occurs postnatally in the rodent hippocampus and cortex, and it declines in the mature and aged brain and is influenced by trophic factors. In our previous work, we demonstrated that knockdown of IRS1 by shRNA impairs learning and reduces synaptic plasticity in a rat model, as measured by synaptophsyin puncta in axons. In this study, we report that shIRS1 alters spine maturation in adult hilar hippcampal neurons. Lastly, to understand the role of IRS1 in neuronal neurite tree, we transfect shIRS1 into primary neuronal cultures and observed that shIRS1 reduced neurite branching and neurite length.

Our results demonstrate that IRS1/2 and insulin/IGF1 receptors display different age-dependent expression profiles and that IRS1 is required for spine maturation, demonstrating a novel role for IRS1 in synaptic plasticity.

Keywords: shRNA, neurite branching, spine morphology, Sholl Analysis, IGF-1, insulin, IRS expression, hippocampus, prefrontal cortex.

1. Introduction

Insulin has both metabolic and non-metabolic functions in the central nervous system. The main metabolic function of insulin is the control of body energy homeostasis. After feeding, insulin plasma levels are elevated and act as an anorexigenic peptide directly on the arcuate nucleus (ARC) of the hypothalamus, contributing to decreased appetite and enhanced energy expenditure [1,2].

The non-metabolic functions of insulin overlap with Insulin-like growth factor-1 (IGF1) and include neuronal survival, neuronal proliferation and differentiation, and neuroprotective effects against toxic insults (for review, see [3]). Consequently, insulin/IGF1 signaling has been proven essential for learning, cognition, and memory [4,5], likely through the regulation of LTP by modulating the trafficking of NMDAR, AMPAR, and GABA receptors, release of neurotransmitters, and uptake of glucose (for review see [6,7]).

Dendrites are the main sites of information input into neurons, and the major neurite on neurons, and dendrite branching patterns are characteristic of neuronal phenotype. Proper branching is critical for the accuracy of neural transmission. Dendrites are dynamic and

undergo substantial modifications in response to the afferent transmission, trophic stimulation, or pathological processes (for review, see [8,9]). In a given neuron, the output signal is highly dependent on the dendritic tree pattern since it represents the integration of the individual synaptic signals [10,11]. Furthermore, defective neuronal arborization results in severe alterations in synaptic transmission, both in neurodegenerative [12] and neurodevelopmental diseases [13].

Trophic factors, such as neurotrophins BDNF, NGF, or NT3 and NT4, promote dendrite growth and complexity [14,15]. Likewise, insulin promotes functional circuits after axonal injury [16], promoting dendrite [17–19] and axonal sprouting [20,21]. Signaling pathways downstream of neurotrophic factors include the Ras cascade, phosphoinositide-3' kinase (PI3K), and mammalian target of rapamycin [22]. All of these signaling cascades are tightly regulated by the Insulin receptor substrates, the IRS, which are main mediators of Insulin and IGF-1, and other neurotrophic factor signaling cascades. There are four known IRS isoforms. IRS1 and IRS2 are highly expressed in the brain [23], and although their actions are generally overlapping, there may be important differences depending on the cell type in which they are expressed [24,25]. Insulin signaling is required for optimal synaptic transmission and long-term potentiation (LTP) [26]. Moreover, the role of insulin and IGF1 during development is well-recognized [27]. In addition, there is extensive support for a role for insulin and IGF1 signaling (IIS) in brain function, in cognitive impairment in neurodegenerative disease [28], and obesity models [29]. In addition, in neurodegenerative diseases (ie Alzheimer's disease) there is a strong brain insulin resistant component [30].

Up to now, comparative studies of the expression of IIS proteins during development and into adulthood have not been reported. Thus, the goal of the present investigation was to determine changes in IRS1, IRS2, IR, and GSK protein expression during development and into adulthood. We focused on the hippocampus and prefrontal cortex due to their involvement in cognitive function and our previous report that knockdown of IRS1 in GABAergic neurons in rat hippocampus *in vivo* impairs spatial memory in rats [31]. Here, we suggest completely new function for IRS1 in glutamatergic neurons, specifically, neuritogenesis.

2. Materials and Methods

Animals

Male Wistar rats were used in this study for neuronal culture and Western blot studies. All protocols were approved by the Animal Ethics Committees of the Universitat Jaume I (code 2017/USC/PEA/00091). All procedures were in line with directive 86/609/EEC of the European Community on the protection of animals used for experimental and other scientific purposes.

Hippocampal cultures and transfections

Neuronal cultures were prepared from hippocampi of rat embryos at 18 days gestation as described [32]. The hippocampi were dissociated, and cells were plated on poly-D-lysine-treated glass coverslips (12 mm in diameter) at a density of 1800 cells/mm^2. Cultures were plated and maintained in Neurobasal medium supplemented with B27, penicillin, streptomycin, and L-glutamine. Hippocampal cultures (DIV 7) were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Hippocampal neurons cultured in these conditions begin active dendritic branching at approximately DIV 6 until approximately DIV 12 [33–35]. Neurons were fixed at DIV 12 with 4% paraformaldehyde

plus 4% sucrose in PBS. Coverslips were mounted onto frosted glass microscope slides using Fluoromount G (Southern Biotechnology). Labeled cells were visualized by immunofluorescence using an Olympus BX61 microscope with a Leica DFC550 digital camera (Leica, Germany). Images acquired with software Leica Application Suite v4.11.0 (Leica, Germany) were contrasted with Photoshop (Adobe). Images were transformed to black and white color , level of birghtness and contrast was identical for all images analyzed, and the experimenter blid to the condition. Neurite branching was examined using Sholl analysis [36] and expressed as intersections versus distance from the soma. Neurite length was determined with the software ImageJ plugin NeuronJ.

Construction of vectors pSUPER IRS1 shRNA

The shRNA sequences against IRS1, fully characterized showing silencing ability and specificity in [31]; and against IRS2 (5 ′GCAACACACCTGACTCCATT 3′) were used for subcloning into the pSUPER-GFP HindIII and BglII hanging er ds were added for cloning into the pSUPER-GFP vector (Oligoengine) according to manufacturer's instructions. Oligonucleotides were synthesized and purchased from Thermofisher.

Antibodies

Anti-IRS1 rabbit polyclonal antibody against IRS1 (1:1000, 06-248) was purchased from Merck Millipore (Billerica, MA, USA); IRS2 rabbit polyclonal antibody (1:1000, #4502) was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA); IR rabbit monoclonal antibody (1:5000, ab5500) was obtained from Abcam (Cambridge, UK); IGF1 rabbit monoclonal antibody (1:1000, #3018) was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA); tubulin mouse monoclonal antibody, #sc-8035) was obtained from Santa Cruz Biotech (CA, USA); and mouse monoclonal anti-ß-actin (1:2000, #A2228) was from Sigma Aldrich (MO, USA). Secondary antibodies linked to horseradish peroxidize (anti-mouse #122011 and anti-rabbit #122825) were purchased from Jackson Immuno Research Laboratories, Inc. (Baltimore, PA).

Western blot

Tissue was lysed in RIPA buffer containing protease and phosphatase inhibitors (Halt protease and phosphatase inhibitor, Thermo Scientific, Waltham, MA, USA). Protein (30-40µg) was resolved by polyacrylamide gel (8%) electrophoresis, then transferred to PDVF membranes, and incubated in blocking solution (5% bovine serum albumin TBS-Tween 0.05% in PBS) for 1h. The membranes were incubated with primary antibodies overnight at 4C. On the following day, the membranes were incubated with secondary antibody for 1h at room temperature (RT) After several washes, blots were developed with Enhanced chemiluminescence ECL (BioRad, Hercules CA, USA). Bands were visualized using an imager developer (IMAGEQUANT LASc 4000, GE Healthcare Little Chalfont, UK). Images were quantified with ImageJ blots toolkit software (National Institutes of Health, Baltimore, MD, USA).

Statistical analysis

One-way ANOVA followed by Tukey post hoc test was used to analyze protein expression differences throughout development. Two-way ANOVA followed by Bonferroni, was used to determine statistically significant differences in Sholl analysis. Student t-test was applied for analysis of neurite length.

3. Results

Expression of insulin and IGF signaling proteins (IRS1, IRS2, and receptors IR and IGF1R)

during hippocampal development (postnatal 10 and 20 days); adult (1 and 9 months), and aged subjects (15 months) was evaluated by Western blot analysis (Fig 1A). We found that the expression of IRS1 and IRS2 reaches a maximal level at early ages and decreases to minimal levels in 15 month old rats. Quantification of IRS1 expression was at 6.89 ±1.36 fold at P10, 7.5 ± 1.37 fold at P20 , and 6.05 ± 1.6 fold at 1month with respect to 15 months. Expression declines at 9 months (1.8 ± 0.6 fold; Fig 1B and Fig S1). In contrast, IRS2 levels decreased at earlier time points: P10 (6.1 ± 0.8 fold), P20 (3.6± 0.8 fold), 1 month (1.5± 1.19; Fig 1C and Fig S1). Interestingly, the IR and IGF1R protein demonstrates a completely different expression pattern; IR protein levels were lower at early ages P10 (0.43 ± 0.04 fold); P20 (0.55 ± 0.12 fold), 1 month (0.84± 0.07 fold) and 9 months (0.99 ± 0.1 fold) with respect to 15 months of age (Fig 1D and Fig S1). Finally, IGF1R expression did not change during development and aging (Fig 1E and Fig S1).
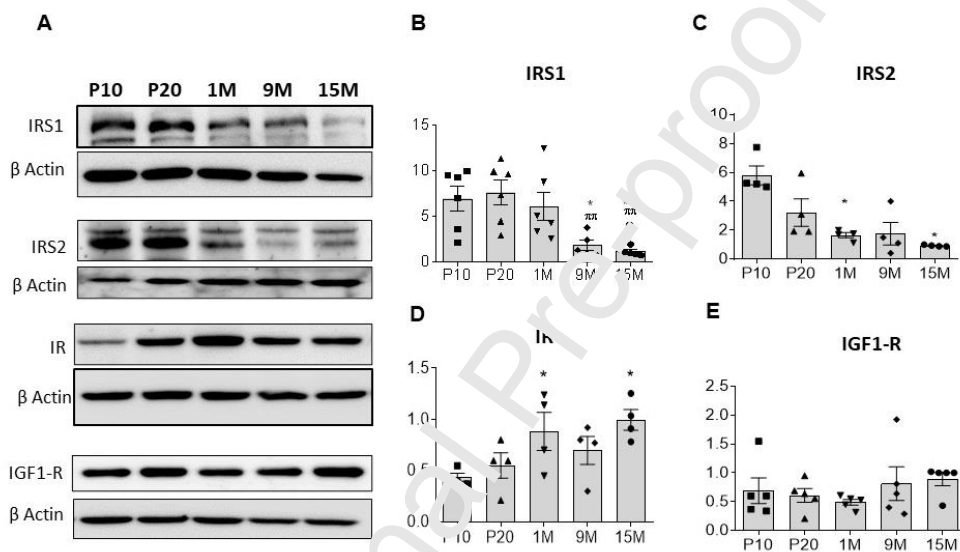

Figure 1. Expression of IR, IRS1, IRS2, and IGF1R in the hippocampus during development and aging. (A) Representative Western blots. (B-E) Quantification of protein bands for IRS1 (B), IRS2 (C), IR (D), and IGF1R (E) normalized to β-actin and relative to the value at 15 months at the indicated ages. Data are presented as mean +SEM (n 4-6 rats) and analyzed by one way ANOVA followed by Tukey post hoc test (*$p < 0.05$ compared to p10, π $p<0.05$ compared to P20, and ω $p<0.05$ compared to 1 month). P= Postnatal days;   M=months.

In the prefrontal cortex, however, the changes in expression patterns are different from those observed in the hippocampus. First, changes to IRS1 and IRS2 expression were not significant, suggesting that these proteins remain expressed at the same levels throughout development and aging. The maximal change was at P20 (2.01 ± 0.5 fold) for IRS1 (Fig 2B and fig S2) and P20 (2.22 ± 0.7) for IRS2 (Fig 2C and Fig S2) compared to 15 months of age. Similar to the hippocampus, IR expression increased with maturity, with lowest expression at P10 (0.17± 0.08 fold) compared to 15 months. In addition, IGF1R levels in the prefrontal cortex, like in the hippocampus, do not change significantly throughout life.
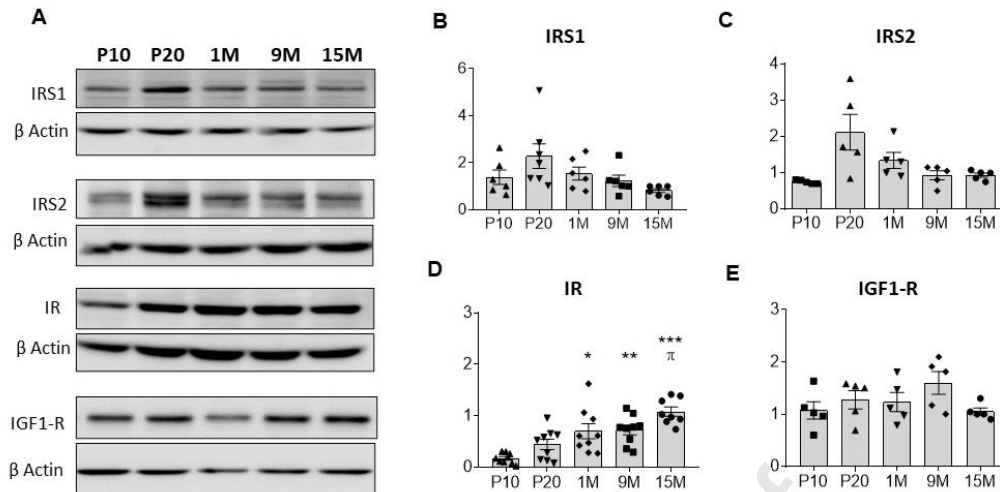
Figure 2. Expression of IR, IRS1, IRS2, and IGF1R in the prefrontal cortex. (A) Representative Western blots. (B-E) Quantification of protein bands for IRS1 (B), IRS2 (C), IR (D), and IGF1R (E) normalized to ß-actin and relative to the value at 15 months at the indicated ages. Data are presented as mean±SEM (n=4-6 rats) and analyzed by one way ANOVA followed by Tukey post hoc test (*p < 0.05, **< 0.01, ***<0.001 compared to p10; π p<0.05 compared to P20). P= Postnatal days;   M=months.

In our previous studies, we observed that knock down of IRS1 expression alters learning capabilities and impairs synaptic plasticity as measured by synaptophysin puncta labeling in axons [31]. shIRS1 was introduced by a viral particle that infects mostly GABA-ergic neurons. In these neurons we have now measured the dendritic spines morphology. We found that knock down of IRS1 reduces the spine number (6.98±0,49 in control versus 4.5 ± 0.92 in shIRS1 spines in 10μm length of dendrite, Fig 3C) and alters spine maturation, since the number of thin (1.47 ± 0.2) and mushroom (1.18 ± 0.14) type were significantly lower than control (thin 2.31 ± 0.36; mushroom 1.78 ± 0.26) (Fig 3E and G, respectively). Moreover, inmmature spines, were higher in the shIRS1 infected neurons (filopodia type in control 0.3 ± 0.13 and shIRS1 0.8 ± 0.23; Fig3D). Branched type of neurons are very scarce compared to the other types, and did not change in control and shIRS1 infected neurons.
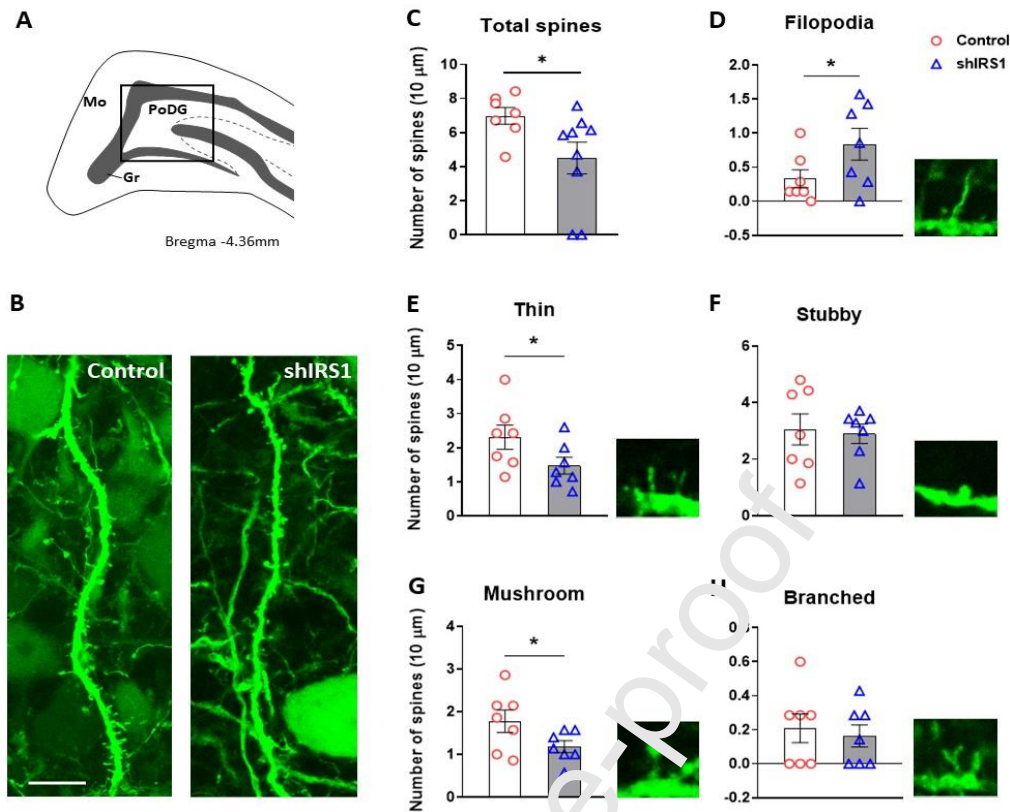
Figure 3. AAV shIRS1 alters dendritic spine maturation in hippocampal neurons. Schematic representation of the dentate gyrus of the dorsal hippocampus, area of injection of the viral particle **(A)**. Representative images of AAV control and AAV shIRS1 labeled dendrites analysed in the polimorphic area (PoDG) of the dentate gyrus **(B)**. Quantification of total dendritic spines **(C)** filopodia spines **(D)**; thin spines (E); stubby **(F),** mushroom **(G)** and branched (**H**) in 10 μm. Representative images at high-magnification (x63) of the dendritic spine shapes analysed. Data is represented as the mean ± SEM (n = 7). We analyzed 7 neurons (considered replicates in one animal) and analyezed 7 subjects. Data were analyzed using one-tailed Student t test *p < 0.05. Scale bar =10 μm.

Finally, given the fact that the peak of IRS1 protein expression is highest postnatally in the hippocampus, we set out to determine whether IRS1 regulates neuritogenesis during neuronal development as this process occurs at the same time period. To that end, we transfected our shIRS1 sequences [31] into primary rat neuronal hippocampal cultures at DIV7 and analyzed neuritic branching at DIV12. Neurons transfected with pSUPER-EFGP control and pSUPER-scramble-RNA-EGFP showed no differences in neurite branching complexity, whereas knockdown of IRS1 expression resulted in neurons with a more simplified arborization pattern as measured by Sholl analysis (Fig 4A). Statistical analysis indicated significant differences between shRNA IRS1 and shRNA control at 40μm, between 50-70μm, and at 75-100μm distance from the soma. Moreover, reduction in IRS1 expression resulted in a significantly shorter neurite tree as measured by total (Fig 4B) neurite length. The neurons transfected with shRNA IRS1 have fewer primary branches and fewer ramifications of these primary neurites.
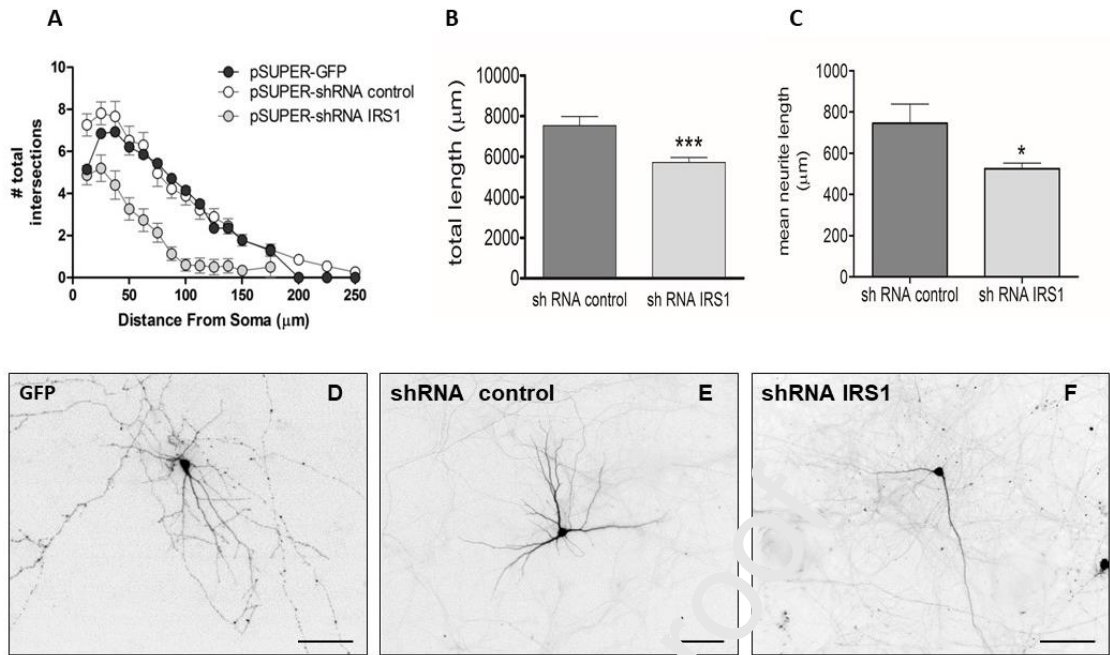
Figure 4. IRS1 knockdown in hippocampal neurons reduces dendritic arborization.
(A) Sholl analysis of transfected neurons shows that knockdown of IRS1 expression (grey circles) significantly reduces the number of intersections (neurites) at 40μm (*p<0.05); between 50-70μm (**p<0.01); and at 75-100μm (***p<0.001) distances from soma compared to control shRNA (white circles) and GFP alone (black circles). Neurons were analyzed from four independent cultures, and the numbers of neurons used for the study were GFP n=27, shRNA IRS1 n=20, and control n=35. Data on the graph were analyzed for statistical differences with two-way ANOVA followed by Bonferroni post-test. (B) Total neurite length was reduced in shRNA IRS1 and shRNA IRS2, compared to shRNA control transfected neurons. Data are represented as mean and SEM (C-F) Representative pictures of neurons transfected with pSUPER-GFP (D), pSUPER-control (E), pSUPER-IRS1shRNA (F) at DIV7 and fixed for analysis at DIV12. Scale bar, 100μm.

4. Discussion

There is overwhelming evidence that both Insulin and IGF1 are important neurotrophic agents in the central nervous system. Amongst other roles, insulin sensitivity has been proposed as a good predictive player in cognitive function [37]. Low IGF-1 serum levels have been associated with decline in cognitive function due to aging [38]. In our study, we observed that the expression of IRS1 and IRS2, key mediators of Insulin and IGF1 signaling, vary through the lifespan, whereas this pattern is different from expression of the IR and IGF1R receptors. To our knowledge, this is the first report comparing the levels of IR, IGF1R, and the IRS1/2 in the hippocampus and cortex during development and aging. A systematic analysis of the expression profile of the proteins in the IIS in the whole brain, comparing different areas and ages, will be useful in the field but it is beyond the scope of this study.

The use of rodent models for human pathophysiologic processes, even with limitations, is widely used as it provides useful information. Amongst them Wistar rat is a widely used rodent model. Lifespan in rodents is much shorter than human. 15 months of age in a rat is considered equivalent to 45-50 years in human [39].

In the hippocampus, IRS1 and IRS2 expression is highest postnatally (P10 and P20) and decreases in aged rats. Because IRS-1 and IRS2 can mediate not only insulin and IGF1 action but also action of other trophic factors, such as BDNF [40] or NGF [41], we postulate that the

observed downregulation of the IRS expression would have a deleterious impact on brain activity since several trophic factor signaling cascades converge to IRS1/2, which would be impaired. We found that this reduction is more prominent in the hippocampus, whereas in the prefrontal cortex changes in IRS1/2 expression during lifespan are not significant, and we observed only a slight peak of expression, which was not statistically significant, at P10. Aging mediates several alterations to neurons, including decreased expression of synaptic structures, augmented oxidative stress, and reduced glucose metabolism in the hippocampus [42]. Based on our results, we hypothesize that some of these effects could be due to the resistance of insulin and/IGF1 and other trophic factors, mostly in the hippocampus, due to downregulation of IRS1/2 expression.

In contrast to the IRS1/2 expression pattern, we found that the IR expression increases with maturity. This result is contrary to early reports in humans, where radioligand binding suggested a reduced IR density in the aged temporal cortex compared to young subjects [43]. However, radioligand binding is not a direct measure of protein expression, but instead, measures functionality. In addition to protein levels, other alterations could attenuate ligand binding, and increased expression could be the result of a compensatory mechanism. Importantly, Alzheimer's patients showed increased insulin binding, consistent with an increased expression [43]. Thus, the optimal expression of IR in physiological and pathophysiological aging remains elusive and requires further studies. Given the characteristics of IR promoters, IR has initially been considered a housekeeping gene [44]. However, it is now accepted that IR expression is not only tissue-dependent but also regulated by external and internal factors (for review, see [45]). Whether expression levels correlate with function is not always clear, and for that reason, our study can only point out the different patterns of expression throughout the life span, which may suggest different roles of these proteins during different life stages e.g. that IR may not be crucial for hippocampal and prefrontal cortical development as IRS1 and IRS2 are important. Thus, our data indicate that during development, these scaffolding proteins may mediate the action of IGF1, with a role in development and aging that is increasingly acknowledged [46]. In our model, the expression of IGF1R is stable throughout life in both the hippocampus and prefrontal cortex, and this is consistent with previous reports [47], where the receptor levels do not change during life span in contrast to IGF1 serum levels. The role of IGF1 signaling in aging is also still controversial (for review, see [48]). Moreover, the studies addressing a specific role for IGF1 in different brain areas are not conclusive. Thus, it is yet unknown what are the optimal levels of IGF1 signaling for proper brain function. In addition, in most studies, only the circulating IGF1 levels have been measured, but the receptor sensitivity, and importantly, the downstream mediators, such as IRS1 and IRS2, have not been evaluated. Thus, our report is the first comparative study in the hippocampus and cortex that evaluates the expression of the Insulin/IGF1 signaling.

In addition to survival and lifespan, IGF1 plays an important role in neurite outgrowth and differentiation [19], mainly through the activation of the PI3 kinase pathway. This effect of IGF1 has been shown in cell lines [49], in *Drosophila* [50], and in hippocampal cultures [51]. However, whether this effect is mediated through IRS1 or any other IRS isoform had not yet been

addressed. The PI3 kinase pathway is the main regulator of GSK3β, which has been demonstrated to be a key factor in dendritogenesis [52]. Our study demonstrates that IRS1 is required for proper neurite outgrowth, both in arbor complexity and neurite length. Other studies have suggested that IRS1 mediates the effects of IGF1 in brain growth, but not in glia or oligodendrocytes, thus suggesting that IRS1 effects may be specific for neurons [53].

Highlighting the importance of correct neurite arborization during brain development is the fact that significant alterations in neurites are found in several mental pathologies of early-onset, like autism [54,55], Rett syndrome [56], or Down syndrome [57], and mental retardation [58]. Moreover, deficient neurite complexity has also been reported in neurodegenerative disorders, such as Alzheimer´s disease (AD) [59]. Interestingly, a negative correlation between neuronal arborization and neurofibrillary tangles, but not with Aβ plaque densities, was observed in the subiculum of AD patients, compared to controls subjects, further confirming the relationship of Tau microtubule function and neurite tree [60].

We found a decreased expression of IRS1 and IRS2 in the aged brain, mostly manifesting in the hippocampus compared to the prefrontal cortex, providing another mechanistic explanation for increased insulin resistance in aging (and AD). Our results on IRS1 expression levels agree with the data obtained in the SAMP8 mice mouse model, where IRS1 expression is reduced in mice suffering from accelerated aging, compared to age matched controls[61]. In this study, we show that IRS1 knockdown in primary neuronal cultures reduces neurite branching in primary cultures of hippocampal cultures. Our shIRS1 construct is specficic for IRS1 and does not affect IRS2 expression, as we shown in our previous paper [31]. Given that IRS1 is required for dendritic branching and IRS1 levels are down-regulated in physiological aging, it is tempting to hypothesize that IRS1 reduction/dysfunction may underlie or strongly contribute to the reduced dendritic arbor observed in senescent models [62]. This result is in agreement with a recent study showing that obesity conditions cause dendrite arbor and LTP deficits, which are rescued by improving IRS1/Akt pathway by exendin-4 treatment [63].

Moreover, we found that reduction of IRS1 expression in adult hippocampus, GABAergic neurons reduced the number of total spines (not counting filpoida inmature spines) and reduced the number of mature spines (thin and mushroom), this is consitent with the metabolic induced insulitn resistance effects found in [63]. Interestingly, we found that the number of inmature (filpodia-like) spines are increased in shIRS1 infected neurons, suggesting that IRS1 is specficially required for maturation. Inmature filpoida like spines are considered not fully functional given the absence of posynaptic density [64]. Our data strongly suggests that IRS1 role is required for mature spine formation and maintenance.

We have analyzed shRNA against IRS2 and a low viability of shIRS2 transfected neurons. This result   agrees with other studies showing that IRS2 is neuroprotective [65]. We observed that the surviving shIRS2 transfected neurons showed alterations in the neurite arbor, and neurite length compared to control neurons. In addition, IRS2 knockout mice are viable, but their brains smaller than those of wildtype mice suggesting a pivotal role for IRS2 in neuronal growth and survival [66].

Furthermore, because reduced the dendritic arbor has been linked to reduced hippocampal volume in stress-related disorders [67] it is likely that reduction of IRS1 in aged subjects, may

contribute to the observed reduction of hippocampal volume in the elderly [68]. Moreover, the involvement of IGF1 signaling in dendritic complexity is well known, and thus, we could hypothesize that an increment in IGF1 resistance (due to IRS1 downregulation) in the brain could also account for the impairment in neurite, and hence, dendrite maintenance (Fig 4).
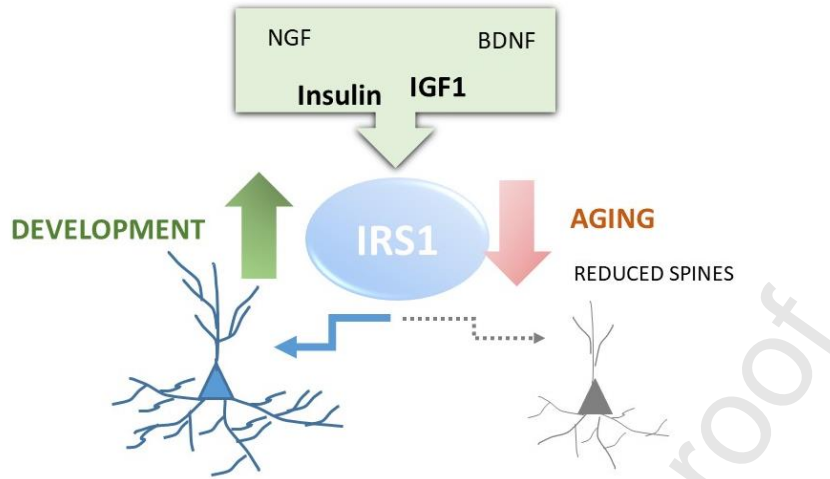

Figure 5 Model. Our data show changes in IRS1 and IRS2 proteins levels, mostly in the hippocampus and to a lesser extent also in the prefrontal cortex, during development and aging. The expression profile of these scaffolding proteins is distinct from that of IR and IGF1R. We also demonstrate the requirement of IRS1 expresión for neurite branching, and thus, our data suggest that IRS1/2, as a scaffolding proteins mediating trophic factor mediate neuritic arbour development and maintenance of mature spines.

In our previous paper [31] we reported that AAV serotype DJ8 infects mostly somatostatin (SOM), parvalbumin (PV) interneurons and long-range SOM neurons in the hippocampal region, where the virus was injected. In that previous report, we focused on the long-range SOM neurons because the greatest number of AAV labelled fibers were observed in the inner layer of the medial septum, where these neurons had been shown to project. This hippocampal-septum connection had already been proved essential in spatial memory processes. However, GABAergic neurons account for up to 15% in hippocampal formation [69], confirmed by the estimation of inhibitory/excitatory low ratio [70]. This is recapitulated in hippocampal primary cultures, since they have been shown to be 10% inhibitory neurons [33]. For that reason, the observed decrease in expression during aging in the hippocampal lysate is probably due to a general decrease in both GABAergic and glutamatergic neurons, with a large component of this change in glutamatergic neurons.

In addition, in the Allen atlas mouse brain, IRS1/2 expression is strongly expressed in hippocampal principal neurons, in the pyramidal layer of CA1 and CA3 and granule layer of dentate gyrus. This observation further supports the idea that IRS1/2 downregulation in glutamatergic neurons occurs during aging. Finally, we cannot rule out the abundant presence of IRS1 in glia, where it has been shown to play a role mediating IGF1 signaling [71]. The study of changes to specific neuron types is beyond the scope of the current work and is a future study.

Thus, IRS1 dysfunction could be at the crossroad of developmental and degenerative processes. This has been proposed for IGF1 signaling, independent of IGF1 serum levels [46], further indicating resistance to neurotrophic factors. Our data support that IRS1 can mediate IGF1 actions in development and aging since changes in IRS1 expression correlate better with IGF1 actions than does the expression of the IGF1 receptor itself. Finally, both BDNF and NGF are important trophic factors required for proper neuronal synaptic plasticity, and both activate tyrosine kinase receptors. In some reports, IRS1/2 scaffolding proteins have been shown to mediate trophic factor action [40,41,72], although weaker than insulin/IGF1 signaling in cortical neurons [73]. Taken together, it is likely that IRS1 downregulation in aging would attenuate the effects of trophic factors on dendrite maintenance.

How these mechanisms are regulated requires further research, especially for specific and targeted therapies. Our studies provide a further understanding of the signaling molecules involved in the regulation of mature spine maintenance, neurite branching and outgrowth, and thus helping to identify the mosaic of pathways involved in the complex regulation of neuronal processes during development and aging.

Author Contributions: SSS performed spine morphology quantification and Western blot analysis and contributed to manuscript writing, MMM quantified spine morphology; CG and MTB performed Western blots analysis; NEM carried out the Sholl analysis. AMSP made the primary cultures, designed the study, and wrote the manuscript.

Institutional Review Board Statement: All protocols were approved by the Animal Ethics Committees of the Universitat Jaume I (code 2017/ JSC/ EA/00091). All procedures were in line with directive 86/609/EEC of the European Community on the protection of animals used for experimental and other scientific purposes.

Conflicts of Interest: The authors declare no conflict of interest.

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