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Master's Thesis

A comparative study of the protein levels of Insulin and IGF-1 receptors and receptor substrates in a rat model of development and aging in different brain areas

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ABSTRACT

Alzheimer's disease (AD) is one of the most common causes of dementia. AD is a progressive, degenerative, and irreversible neurological disorder that cause early, cognitive impairment, memory loss, and behaviour changes. AD shares many age-related pathophysiological features of type 2 diabetes (T2D), importantly, insulin resistance defined as reduced cellular responsiveness to insulin. Insulin resistance is considered an etiological factor in AD. Insulin participates in synaptic plasticity through the activation of the insulin receptor (IR) and the PI3K signalling pathway.

In this study, western blots were used to analyze the protein levels of insulin and IGF-1 receptors and substrates during the development and aging in different brain areas (prefrontal area, dorsal hippocampus, amygdala, and cerebellum) in rats. We found receptors and receptors substrate levels may vary depending on age and area analysed. The implications of these results are discussed.

Key words: insulin resistance, AD, Insulin Receptor, IGF1 receptor, Insulin receptor substrates 1 y 2.

1. INTRODUCTION

Insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF1-R), are transmembrane receptors and tetrameric glycoproteins that belong to the receptor tyrosine (Tyr) kinase superfamily, they are composed of two α and two β subunits [1-4]. These receptors have the same structure and function and they are activated by insulin or insulin like growth factor-1 (IGF-1) ligands, however they exhibit higher affinity to own receptors. IRs are abundantly expressed in neurons and less abundantly in glia [5]. The IGF1 receptor is expressed in both neural stem cells and all neural cells throughout the lifespan [6, 7]. IGF1 receptors regulate dendritic sprouting, neuronal stem cell activation, cell growth, repair, synaptic maintenance and neuroprotection [8-12].

Peripheral glucose metabolism is regulated mainly by insulin signalling [13], but in brain insulin role is not directly involved in neuronal glucose metabolism [14]. The brain was considered to be an insulin insensitive tissue over the last several decades, while growing evidence from in vivo and in vitro studies confirm that insulin, as important neuroactive, neurotrophic peptide and its receptor, are present in different regions of the central nervous system [15]. IGF1 are important hormones for cell growth and survival. IGF-1expression is high in perinatal stage and decreases throughout the lifespan [16-18]. IGF1 stimulates normal brain development, and increases neuron number (likely by inhibiting apoptosis) [19].

Insulin, IGF-1 and IGF-2 are actively transported across the blood-brain-barrier (BBB) [20, 21]. Under normal physiological conditions (***Fig. 1A***), insulin or IGF-1 ligand bind to the α-subunit of the receptor which induces a conformational change in the β-subunit, which has tyrosine kinase activity. The receptor is autophosphorylated and subsequently phosphorylates the cytosolic protein named insulin receptor substrate 1 or 2 (IRS-1 or IRS-2). This results in the activation of several intracellular pathways, including PI3K and Akt, ERK, Src Fyn (intracellular tyrosine kinases) downstream. All these intracellular responses will facilitate neuronal growth, neuronal survival, synaptic plasticity, learning, and memory.

In pathological situations that result in insulin resistance (as in AD), this cascade does not function properly (***Fig. 1B***). In AD, accumulation of amyloid-β (Aβ) oligomers leads to increased tumor necrosis factor-alpha (TNF-α) levels and activation of stress kinases such as c-Jun N-terminal kinase (JNK) resulting in inhibitory serine phosphorylation of IRS-1 [22]. Aβ oligomers cause removal of IRs from the cell surface mediated by Casein

Kinase 2 (CK2) and Ca^{2+}/Calmodulin-Dependent Kinase II (CaMKII) [23, 24]. Insulin resistance lowers the expression of insulin degrading enzyme (IDE)[1] [25]. These enzyme also degrades AB, therefore lowered IDE expression further results in accumulations of Aβ [25-27]. Brain insulin signalling inhibits Glicogen Synthase Kinase 3 beta (GSK-3β) activity, a kinase that phosphorylates Tau. Thus, reduction in insulin signalling will increase abnormal tau phosphorylation [28, 29]. Deficient insulin signalling leads to impairment in nerve growth, synaptic plasticity, learning, and memory [18].

1.1. Insulin and Alzheimer's disease

Alzheimer's disease (AD) is one of the most common cause of dementia. About 35.6 million people worldwide are now suffering from AD, and disease prevalence is expected to affect 115 million by 2050 [30, 31]. This neurological disorder is characterized by a progressive, degenerative, and irreversible deterioration of memory, judgement, and reasoning. Alzheimer patients exhibit cognitive impairment, memory loss, and behavioural changes [32]. AD is characterized by neuronal loss and synaptic injury [34] [35]. The main pathological hallmarks of AD are extracellular insoluble beta amyloid (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) [36].

Although we know enough about AD, its etiology remain largely unidentified. Recent human and preclinical studies have provided convincing evidence that AD is a degenerative metabolic disease, which is mediated by impairments in brain insulin responsiveness, glucose utilization, and energy metabolism leading to increased oxidative stress, inflammation, and worsening of insulin resistance [37-50]. This symptoms are common to type 2 diabetes (T2D), in fact both pathologies share many age-related pathophysiological features [47], such as insulin resistance, disrupted glucose metabolism in non-neural tissues [51, 52], oxidative and inflammatory stress, amyloid aggregation, neural atrophy and/or degeneration, and cognitive decline [53, 54].

Brain imaging studies have demonstrated deficits in glucose utilization in AD [55, 56], thereby this hypometabolism of glucose may precede the onset of cognitive deficits [57-59]. Similar reduction was detected in the brain of several AD mouse models [60-62].

This reduced glucose utilization occur early in the course of disease, suggest a role for impairment insulin signalling in pathogenesis of neurodegenerative diseases [63-65].

[1] Insulin Degrading Enzyme, is secreted at high levels from microglial cells and degrades *Aβ extracellularly.*

Insulin participates in synaptic plasticity through the activation of the insulin receptor and phosphoinositide 3-kinase (PI3K) signalling pathway, and these impairments in brain insulin coupled with insulin-like growth factor (IGF) signalling is associated with increased accumulation of Aβ, phosphorylated tau, reactive oxygen/nitrogen species, pro-inflammatory and pro-apoptosis molecules [50, 66, 67]. The relationship between insulin impairment and accumulation of amyloid-β peptide with changes in glucose utilization is unknow [63].

Aβ oligomer induced JNK activation leading to phosphorylation and degradation of the protein IRS-1. IRS deficiency contributes to insulin resistance in diabetes and in AD brain. For this reason, AD has come to consider as type 3 diabetes due to insulin resistance [40, 68].

1.2. Insulin Resistance

Insulin resistance is a hallmark of metabolic syndrome and T2D, it is defined as the reduced capacity of the body to respond to insulin. Some studies indicate that a major cause of insulin resistance in T2D is a chronic upregulation of a normally adaptive feedback/feed-forward mechanism that attenuates insulin signalling via phosphorylation of IRS-1 at S312, S616, and/or S636 (equivalent to S307, S612, and S632, respectively, in rodents). These phosphorylation residues are therefore candidate biomarkers of brain insulin resistance [69-72].

Thus, increased insulin resistance in the brain may contribute to pathological processes that lead to acceleration of AD. For that reason, restoring insulin signalling to normal levels in the brain by insulin treatment may provide therapeutics benefits in AD subjects. Some studies find that intranasal administration of insulin in AD patients improve performance on hippocampus-dependent memory tasks [73]. This therapy has been selected by the National Institute of Health (NIH) as one of the two therapeutic strategies as part of the National Alzheimer's Plan in the US [74].

1.3. Protein expression in development and aging

Given the importance of Insulin IGF1 receptors and their substrates in human development and aging and pathological aging, there are few comparative studies regarding the expression of proteins involved in the insulin/IGF-1 signalling during the development and aging. A decrease of the expression levels of IGF-1 in human plasma [75], decrease levels of mRNA and protein IGF-1 in plasma of rats [76], decrease levels

of IGF1-R/Akt/GSK3 signalling pathway and decrease levels of IGF-1 in brain but high levels of IGF1-R in mice hippocampus [77] with aging.

IGF-1 has important roles as an autocrine, proliferative and prosurvival factor in early brain development [78]. Thus, IGF-1 has been demonstrated to have a crucial role in the differentiation of Mesenchymal Stem Cells (MSCs) into cells of neuronal lineage through its pro-proliferative and anti-apoptotic effects. IGF-1 appears to have important effects on the proliferation of Neural Stem Cells towards either neuronal or glial cell types [79, 80]. In the developing brain, IGF-1 overexpression results in an increased number of total neurons in the cerebral cortex [78]. IGF-1 overexpression stimulates an increase in synapse number and it is also an important anti-apoptotic signal in different neurons [81, 82]. The level of IGF1-R expression decreases to adult levels soon after birth but remains relatively high in the choroid plexus, meninges, and vascular sheaths [83]. Insulin and IGF-1 regulate brain development [84], in IGF-1 or IGF1-R knockout mice studies the brain development was retarded compared with their wild-type counterparts [85, 86].

Other studies report maternal insulin and glucose can affect fetal brain development [87], in fact, the development of hyperglycemia or hyperinsulinism in diabetic mothers leads serious outcomes such as macrosomia, retarded development [88, 89] and malformations in the fetal brain [90]. Diabetic mothers could produce offspring with abnormal insulin secretion and obesity [91, 92]. In mice mode, diabetic mothers have embryos with increased apoptosis on the neural crest during organogenesis [93].

Relative to AD, strong insulin immunoreactivity in pyramidal neurons and increased IR density in frontal, temporal, parietal, and occipital cortex of AD human patients compared to age-matched controls has been found [5]. In addition, low levels of IR, tyrosyl-phosphorylated IR, IRS-1, IRS-2, and IGF1-R, and insulin in hippocampus, hypothalamus, and cortex, have been observed in AD patients [11]. Moreover, insulin sensitivity is reduced whereas greater in responses to IGF-1 (IGF1-R→IRS-2→PI3K signalling pathway in cortex and hippocampus of AD patients compared to controls. Moreover, in this study they report a positive correlation between IRS-1 pS616 and IRS-1 pS636/639 with Aβ plaques and a negative correlation with episodic memory [47], suggesting that serine phosphorylation in IRS-1 (that leads to IRS-1 degradation and insulin resistance) is a major factor in AD formation and memory impairment.

In this study, we analysed the IR, IGF1-R, IRS-1 and IRS-2 protein levels in prefrontal cortex, hippocampus, amygdala and cerebellum during the development and aging in

rats. As described in the introduction, many studies have been carried out regarding the expression of some of these proteins in different brain areas and conditions, especially concerning aging and AD, however, no comparative study of the receptors and substrates in different areas during development and aging has been carried out.

This work shows that under the same conditions, certain brain areas show a decrease in IRS1 and IRS2 protein levels with aging, indicating a possible link to insulin resistance. Their high levels during normal development suggest a pivotal role of these proteins for optimal brain growth.

2. MATERIALS AND METHODS

2.1. Animals

Females and males Wistar rats were used in this study for Western blot studies. The ages of rats were 18 to 19 days in embryonic stage (E18/E19), 3 days post-natal (P3), 10 days post-natal (P10), 20 days post-natal (P20), 1 month post-natal (1M), 9 months post-natal (9M), and 15 months post-natal (15M). All protocols were approved by the Animal Ethics Committees of the Universitat Jaume I (Spain). 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.

2.2. Antibodies

We use anti-IRS1 as rabbit polyclonal antibody against IRS1 (1:500, #06-248) from rat, mice and human species that was obtained from Merk Millipore (Billerica, MA, USA); anti-IRS2 as rabbit polyclonal antibody against IRS2 (1:1000, #4502) was obtained from Cell Signalling Technology, Inc. (Danvers, MA, USA); anti-IR as rabbit monoclonal antibody against IR (1:5000, ab5500) from rat and human, was obtained from Abcam plc. (Cambridge, UK); anti-IGF1 as rabbit monoclonal antibody against IGF1 (1:1000, #3018) from rat, mice, monkey and human, from Cell Signalling Technology, Inc. (Danvers, MA, USA); anti-tubulin as mouse monoclonal antibody use as control (1:1000, #sc-8035) from mouse, rat, human and porcine obtained from Santa Cruz Biotech (CA, USA); and monoclonal anti-ß-actin antibody produced in mouse (1:2000, #A2228) also use as control from Sigma Aldrich (MO, USA). Secondary antibodies against mice and rabbit (1:5000, #122011 and #122825, respectively) linked to horseradish peroxidise were purchased from Jackson Immuno Research Laboratories, Inc. (Baltimmore, PA).

2.3. Immunoblotting

Tissue was lysed in RIPA buffer containing protease and phosphatase inhibitors (Halt protease and phosphatase inhibitor, Thermo Scientific, Waltham, MA, USA). 30-40µg of protein were resolved in Polyacryamide geles (8%), then transferred to PDFV membranes, and incubated in blocking solution (5% bovine serum albumin TBS-Tween 0.05%) for 1h. The membranes were exposed to primary antibodies overnight. Following day, secondary antibody was incubated for 1h. After several washes blots were developed with 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).

2.4. Statistical analysis

All data are expressed as mean ± SEM. Statistical significance was calculated using one way-ANOVA, followed by a Bonferroni post-test to analyze differences between conditions.

3. RESULTS

3.1. Insulin/IGF1 receptors and substrates protein levels in rat prefrontal cortex.

The levels of insulin and IGF-1 signalling pathway proteins under study were the Insulin Receptor (IR), Insulin Receptor Substrate 1 and 2 (IRS-1, IRS-2), and the Insulin-like growth factor 1 receptor (IGF1-R).

We have observed that in the rat prefrontal cortex the expression of IR increases during development (***Fig. 2A***). Quantification of immunoblot bands indicate that at P20 and 1M the protein levels have increased significantly from early postnatal and embryonic and at 9 and 15M age increased significantly from early embryonic and postnatal.

On the other hand, IRS-1 do not change significantly during development but we observed a tendency to decrease (no significant) at 15M (***Fig. 2B***). The expression of IRS-1 and IRS-2 increases at P20, but decrease with age to almost embryonic levels again (***Fig. 2C***). In contrast, the expression of IGF1-R is homogeneous during the development and aging in the prefrontal area of rat (***Fig. 2D***).

3.2. Dorsal Hippocampus

The expression of IR increases with age in the dorsal hippocampus of rats similarly to what we found in the prefrontal area (***Fig. 3AFig. 3*****).** However, the expression of IRS-1 indicate it decreases with age in the dorsal hippocampus of rats ***(Fig. 3B)***.

The levels of expression of IRS-2 in hippocampus is very low, barely detectable (***Fig. 3C***) Quantifications of the blots suggest an early increase in IRS2 protein levels and stabilization with age. The expression of IGF1-R is uniform during the development and aging in the dorsal hippocampus similarly to what we observed in the prefrontal area ***(Fig. 3D)***.

3.3. Amygdala

The expression of IR decreases during the development but increases during the aging (***Fig. 4A***). IRS-1 expression increases during early postnatal development, it peaks at P3 and P10, but it decreases in the amygdala of aged rats (***Fig. 4B***). The expression of IRS-2 is barely detectable in amygdala, similarly to dorsal hippocampus (***Fig. 4C***). The expression of IGF1-R appears quite uniform through age (***Fig. 4D***).

3.4. Cerebellum

The expression of IR is difficult to state at this point in cerebellum, given the low signal obtained (***Fig. 5A***). The expression of IRS-1 and IRS-2 does not seem to change drastically in cerebellum thourgh life time (***Fig. 5B-C***). The expression of IGF1-R, seems quite stable during development and aging the cerebellum (***Fig. 5D***).

4. DISCUSSION

In this study, we studied whether the proteins levels of insulin and IGF-1 receptors together with their substrates change during development and aging. Similarly, whether this changes can be dependent on the brain area. We analysed prefrontal cortex, dorsal hippocampus, amygdala, and cerebellum in a rat model. According to our results, in the prefrontal cortex IRS1 and IRS2 levels have a tendency to decrease with aging, similarly a one study [96] report a decrease of IRS-2 with aging but in hypothalamus. While the IR increase with age and the IGF1R does not change.

The lower expression of IR during embryonic stages compared to adults in all brain areas studied may reflect a small contribution of this factor to development. However, as

mentioned above, IR density increases in frontal, temporal, parietal, and occipital cortex of AD humans patients compared to age-matched controls [67]. In contrast with other study that showed lower levels of IR in AD patient's hippocampus, hypothalamus, and cortex compared to controls [40].

On the other hand, we have found that the expression of IGF1-R in all brain areas studied remains stable through development and aging contrary to other studies that report a decreased in aged mice [90], and high levels of IGF1-R in mice hippocampus with aging [74]. Whether this difference is due to diverse animal model remains to be elucidated and may question animal model to study human processes.

Regarding the expression of IRS-1 and IRS-2, both showed a tendency to decrease with age in the prefrontal area and hippocampus, but the effect is more dramatic with IRS-1 in the hippocampus. We hypothesized that the decrease of IRS-1 protein levels would account for the insulin signalling impairment and hippocampal function alterations reported during aging [75, 76]. In other studies [40] reported low levels of IRS-1 and IRS-2 in AD patients. In contrast, amygdala nor cerebellum do not show such reduction in the levels of IRS-1 and IRS-2 during aging, Withers et al. reported that IRS-2-associated PI-3 kinase activity is markedly increased in IRS-$1^{-/-}$ mice, suggesting that IRS-2 may be compensating in certain areas for the lack of IRS1 [96]. It is possible that this effect is due to compensation during development and it may not reflect the real contribution of both substrates to different.

In our studies IGF-1R protein levels are quite constant throughout life, in contrast with other reports where IGF1-R levels decrease expressions in mice hippocampus with aging [77]. Interestingly, increase in IGF1-R levels were found in AD cases reported [73-75]. Other reports found few neurons containing IGF1-R receptors but increased in astrocytes in AD patients [76, 77].

We must be aware that in this study we do not have sufficient statistical power to conclude the findings in amygdala, cerebellum or hippocampus given the few samples we have analysed. Proof of this are the error bars of the graphs of quantification.
In summary, we have found that the expression of these proteins involved in the insulin and IGF1 signalling pathways changes with the age and it depends on the brain area. Thus the substrates IRS-1 and IRS-2 decreases with the aging due to the serine phosphorylation of IRS-1 [97, 98].

5. FINAL REMARKS

Our study has focused on the observation of the IR, IGF1-R, IRS-1, and IRS-2 protein levels during the development and aging in prefrontal area, hippocampus, amygdala, and cerebellum of rats. We use western blot to detect these proteins involved in insulin signalling. To complete the study, and being aware of the low samples used in certain brain areas, we will increase the samples to achieve statistical power. In addition we intend to analyze other areas as the ventral hippocampus, and other tissues such as muscle and liver. We also considered that given the differences between animal models and human in terms of proteins expression. It would be interesting to extend the studies to human tissues.

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APPENDICES

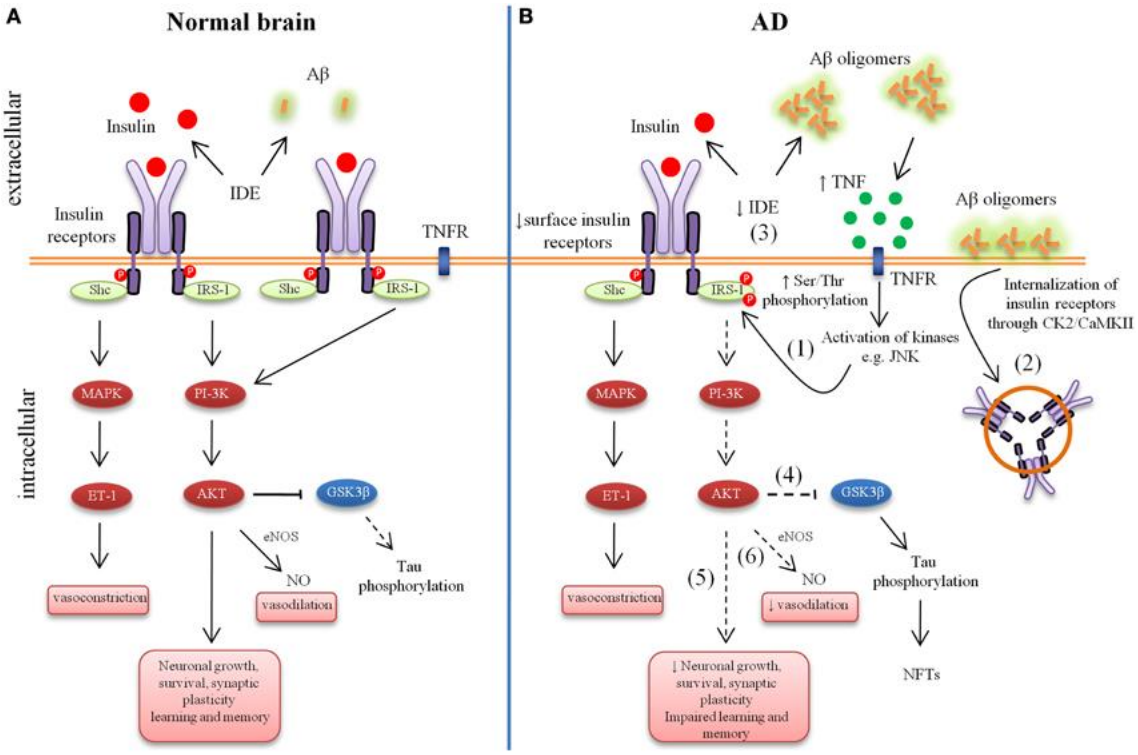

Fig. 1 *Aberrant brain insulin signalling in Alzheimer's disease (AD). Schematic outline of neuronal insulin signalling in the normal brain (A) and AD brain (B)*

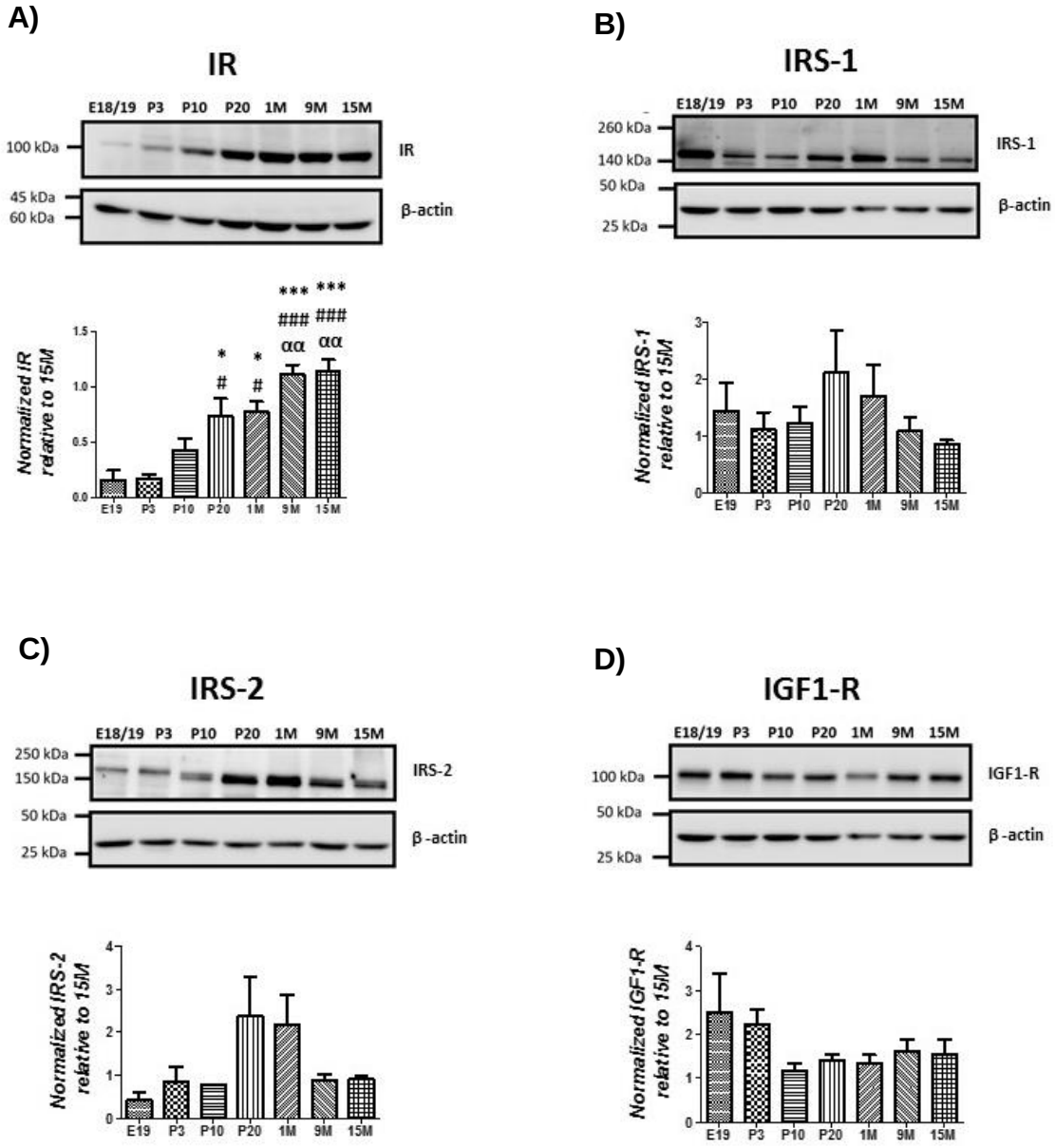

Fig. 2. *Insulin and IGF-1 receptors and substrates expression level in the rat prefrontal area during development and aging. Representative blots of IR (A) (n=8); IRS-1 (B) (n=7); IRS-2 (C) (n=4), IGF1-R (D) (n=4) at the indicated ages. Bottom panel shows the quantification of protein bands normalized to ß-actin, and relative to the value at 15M. Data are expressed as mean ± SEM (n=4-8) and analysed by One way ANOVA, followed by Bonferroni post hoc test (*p < 0.05). *P<0.01, **P<0.001, ***P<0.0001 vs E19; #P<0.01, ##P<0.001, ###P<0.0001 vs P3; and αP<0.01, ααP<0.001, αααP<0.0001 vs P10.*

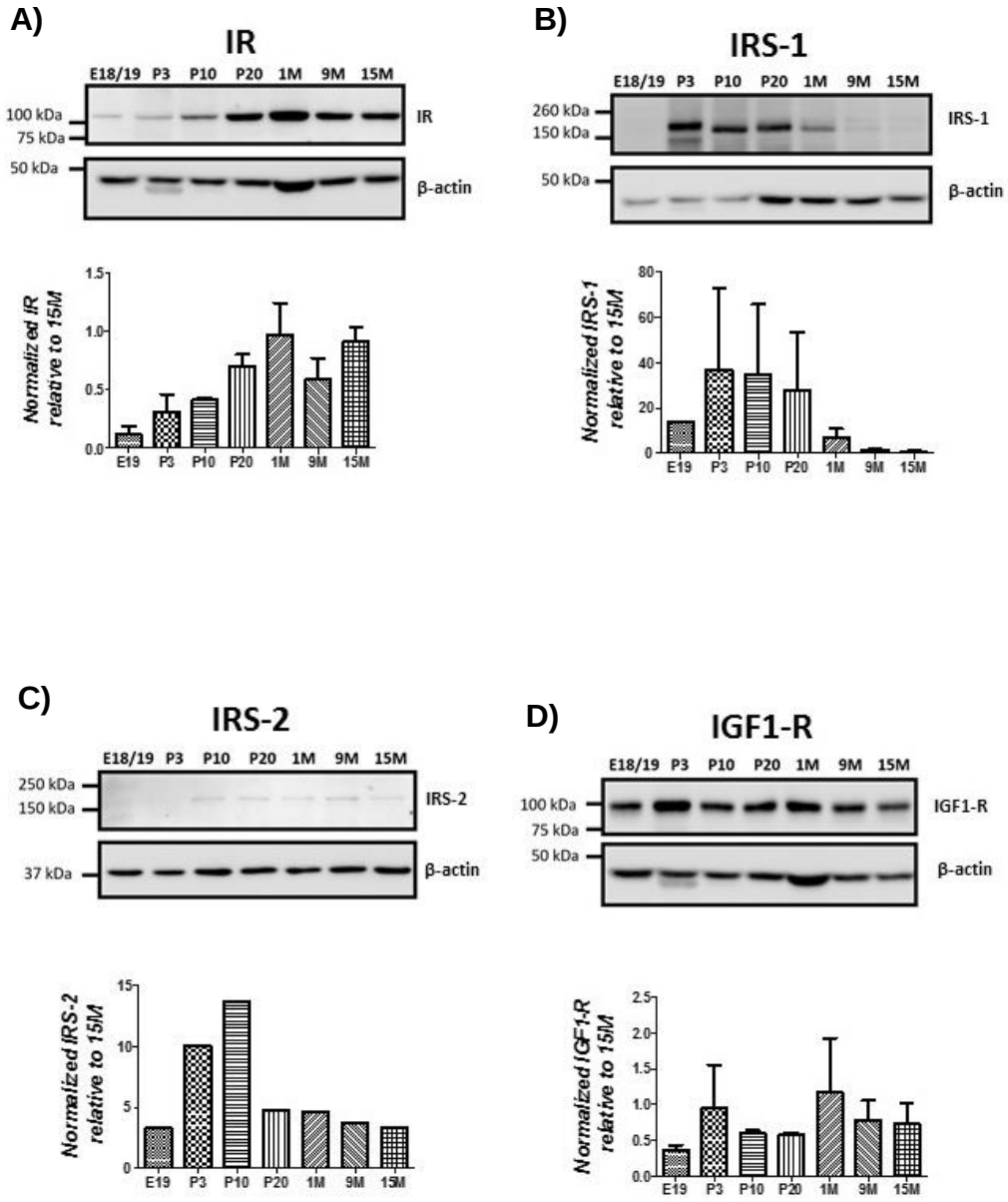

Fig. 3. *Insulin and IGF-1 receptors and substrates expression level in the dorsal hippocampal during development and aging. Representative blots of IR (A) (n=2); IRS-1 (B) (n=3); IRS-2 (C) (n=1), IGF1-R (D) (n=1) at the indicated ages. Bottom panel shows the quantification of protein bands normalized to ß-actin, and relative to the value at 15M. Data are expressed as mean ± SEM (n=1-3) and analysed by One way ANOVA, followed by Bonferroni post hoc test (*p < 0.05).*

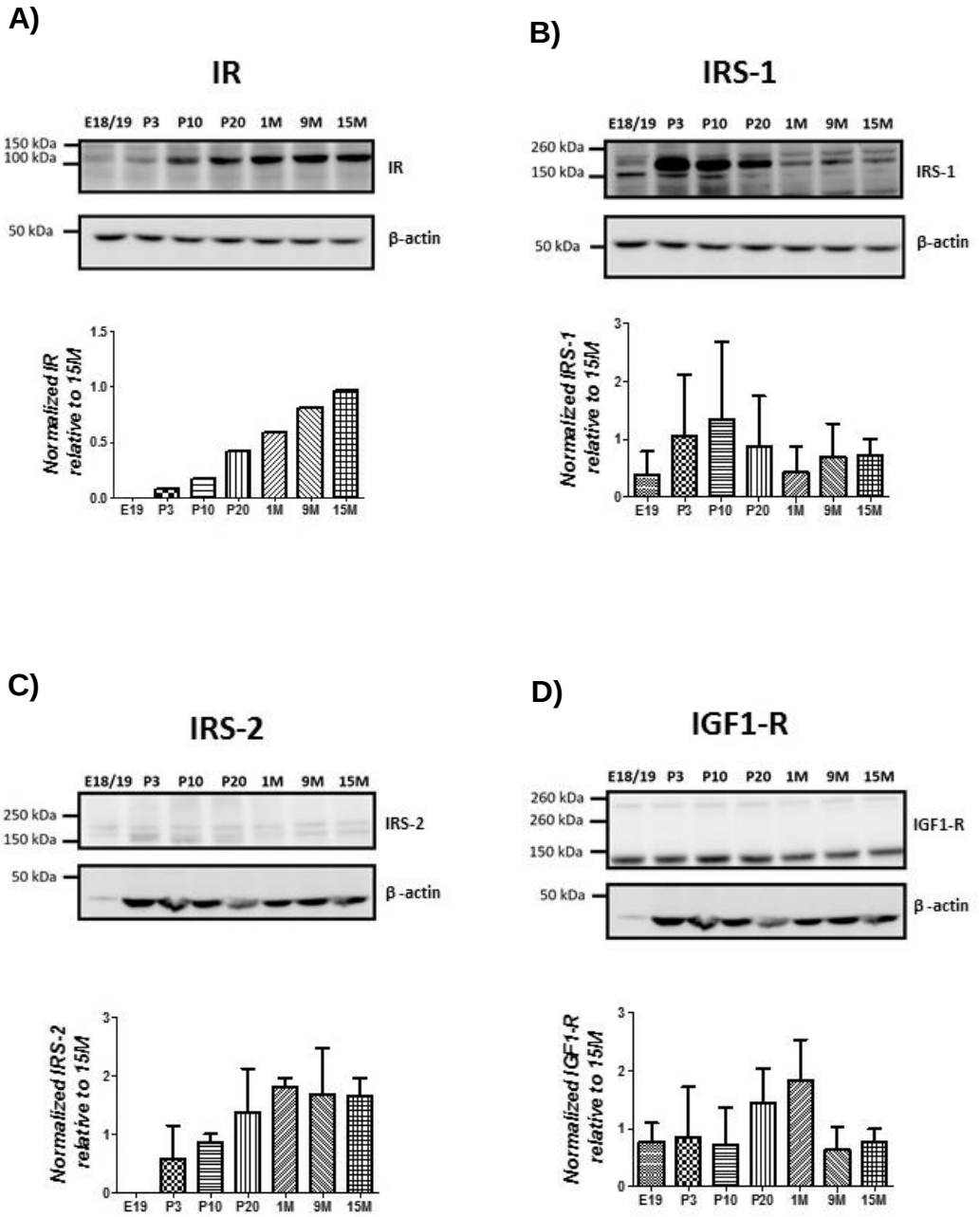

Fig. 4. *Insulin and IGF-1 receptors and substrates expression level in the amygdala area during development and aging. Representative blots of IR (A) (n=2); IRS-1 (B) (n=2); IRS-2 (C) (n=2), IGF1-R (D) (n=2) at the indicated ages. Bottom panel shows the quantification of protein bands normalized to ß-actin, and relative to the value at 15M. Data are expressed as mean ± SEM (n=2) and analysed by One way ANOVA, followed by Bonferroni post hoc test (*p < 0.05).*

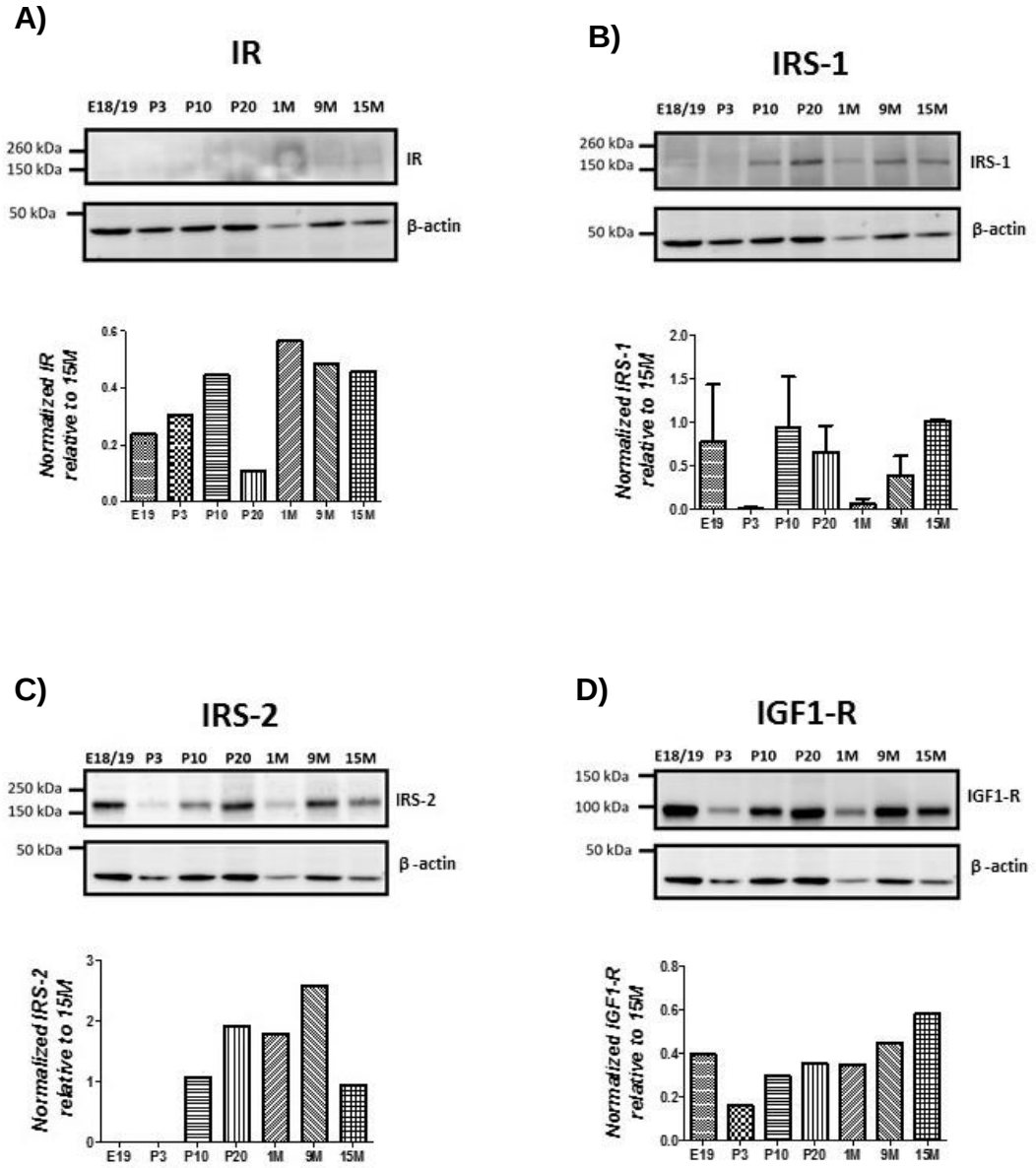

Fig. 5. *Insulin and IGF-1 receptors and substrates expression level in the cerebellum area during development and aging. Representative blots of IR (A) (n=2); IRS-1 (B) (n=2); IRS-2 (C) (n=1), IGF1-R (D) (n=1) at the indicated ages. Bottom panel shows the quantification of protein bands normalized to ß-actin, and relative to the value at 15M. Data are expressed as mean ± SEM (n=1-2) and analysed by One way ANOVA, followed by Bonferroni post hoc test (*p < 0.05).*

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