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# Cross Talk Between Impaired Brain Glucose Metabolism and Tau hyperphosphorylation

*An Implication for Alzheimer Disease*

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# Disclosure

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*To My Family*

*(Alla mia famiglia)*

*Nothing in Life is to be feared*

*It is only to be understood*

*Now is the time to understand more*

*So that we may fear less*

- Marie Curie

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**GLOSSARY**

| | |
|---|---|
| APOE | Apolipoprotein |
| APP | Amyloid precursor protein |
| ARE | Antioxidant-responsive elements |
| Aβ | Beta Amyloid |
| ACH | Amyloid cascade hypothesis |
| AD | Alzheimer Disease |
| BR | Bilirubin |
| BVR | Biliverdin reductase |
| BV | Biliverdin |
| CAT | Catalase |
| DMEM | Delbecco's modified Eagle's medium |
| EAD | Early-onset AD |
| FAD | Familial Alzheimer disease |
| GSK-3β | Glycogen Synthase 3β |
| HBP | Hexose biosynthesis pathway |
| HBSP | Hexosamine biosynthetic pathway |
| HEK | Human embryonic kidney |
| HNE | 4-hydroxynonenal |
| HO | Heme-oxygenase |
| $H_2O_2$ | Hydrogen peroxide |
| IGF | Insulin-like growth factor |
| IRK | Insulin receptor kinase |
| iNOS | Inducible nitric oxide synthase |
| IPL | Inferior parietal lobule |
| LAD | Late-onset AD |
| MARK | Microtubule-affinity-regulating kinase |
| MARE | Maf recognition element |
| MCI | Mild Cognitive impairment |
| MLSP | Maximum lifespan potential |
| MMSE | Mini Mental State Examination |
| MS | Mass Spectrometry |
| NOX | NAPDH oxidase |
| Nrf2 | NF-E2-related factor 2 |
| OS | Oxidative stress |
| OGA | O-GlcNAcase |
| OGT | O-GlcNAc transferase |
| PBS | Phosphate buffer saline |

| | |
|---|---|
| PC | Protein carbonyl |
| PCAD | Preclinical AD |
| PD | Parkinson disease |
| PET | Positron emission tomography |
| PHF | Pre-helical filaments |
| PP2A | Protein phosphatase 2A |
| PS-1 | Presenilin |
| PTM | Post-translation modifications |
| PUFA | Polyunsaturated fatty acid |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SiRNA | short interfering RNA |
| SOD | Superoxide dismutase |
| SREs | Stress responsive element |
| T2DM | Type 2 diabetes mellitus |
| WT | Wild Type |
| 3-NT | 3-nitrotyrosine |
| 3×Tg-AD | Triple transgenic |

# Chapter 1
## *Introduction*

### 1.1 Alzheimer: What do we know?

*"Time is the hallmark of aging, that fades away your memories with the greatest challenge"*

The word Alzheimer, first time coined by a Bavarian psychiatrist and neuropathologist "Alois Alzheimer" in 1907 when his 51 years old female patient diagnosed with unfamiliar psychiatric symptoms and given a description of this clinico-pathological syndrome. Eventually this clinico-pathological symptom defines later a new neuropathological phenotype of the brain. He described it as a "peculiar disease of cortex" and found the strange alteration of the "neurofibrils" and "Foci (Focal deposits)" which are build up by peculiar substance and spread over the entire cortex along with the aging. (Alzheimer 1907).

In 1980, ninety years later structural evidence of those microscopic lesions confirmed as a "senile (Amyloid) plaques" and "neurofibrillary tangles (NFTs) "by Dr. Alzheimer. In the area of biochemical pathology, George Glenner first isolated and partially characterized the amyloid β-protein from the brains of patients who had died with Alzheimer disease or Down syndrome in 1984 (Glenner, G. G., 1984a; Masters, C. L., 1985). Two years later, several laboratories had identified the microtubule-associated protein Tau, as the principal constituent of the neurofibrillary tangles (Brion, J. P., 1985; Grundke-Iqbal, I., 1986; KoSIK, K. S., 1986; Nukina, N., 1986; Wood, J. G., 1986).

Alzheimer is the most common form of the neurodegenerative disease that affects the vast area of cerebral cortex and hippocampus leads to dementia within the aging (Masters, C. L., 2015).

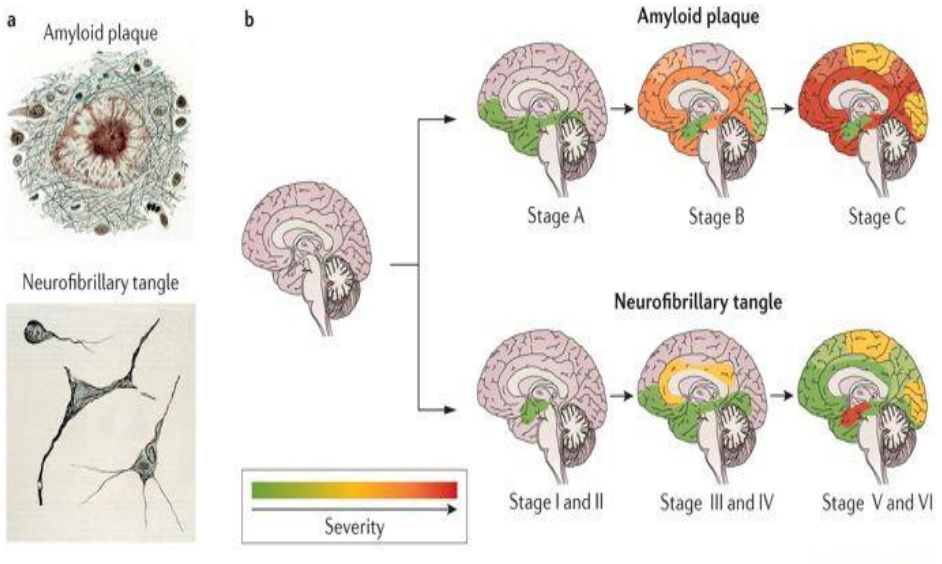



**Figure 1**: Progression of AD at Aβ deposition (Stage A-C) and neurofibrillary tangles (Stage I-VI, adopted from Braak and Braak)

Surprisingly AD affects 5.3 million Americans and is the 6$^{th}$ leading cause of death in the US. Alzheimer has been projected as a socially and economically burden on the society, and it reduces the quality of life for the patients who are suffering from this neurodegenerative disease. Prevalence of AD is 3 % for age 65-74 and 18.7% for 75-84 years and 47.2 % for those over 85 years old (Evans, D. A., 1989). On the broad spectrum, this disease has been classified into two categories, based on their occurrence in the

Population; 1) familial AD (FAD) affects the people younger than 65 ages and 2) Sporadic affects the people at the age of 65 and older than this. In less than 1% of cases of AD population are the FAD and caused by a specific point mutation in all three proteins; i) APP ii) Presenilin 1(PS-1) and iii) Presenilin 2 (PS-2) (Campion, D., 1999) known as a membrane protein. Some of these mutations inherit to next generations that leads to Aβ and Tau pathology by altering signal transduction probably for protein phosphatase 2A (PP2A) and glycogen synthase-3β (GSk-3β) (Iqbal, K.,2005) but not always this alteration shows significant changes or instead decrease Aβ generations, which have been observed in longitudinal studies (Blanchard, J., 2010). However, for instance, some individuals from FAD/sporadic AD cases are characterized by APOE polymorphism at the genetic level with three alternative allele expressions (ε2, ε3, ε4). Apolipoprotein E (APOE) is lipolipoprotein controls synaptic repair in case of tissue injury and maintains the synaptic structure at the tissue level (Poirier, J., 1995, Soininen, H., 1995) but also considered as a risk factor (other than age) for AD development (Strittmatter, W. J., 1993).

To provide the preventive attention and care , It is important to define the risk factors for AD and they are mostly, aging (Evans, D. A., 1989), presence of APOE4 alleles (Strittmatter, W. J., 1993), a family history for AD dementia (Henderson, A. S., 1986), head injury ( Mortimer, J. A., 1985), low educational attainment (Zhang, M.,1990) and low linguistic ability early in life (Snowdon, D. A., 1996). The very first manifestation of a dementing disorder is behavioral symptoms those appear before the cognitive declines (Rubin, E. H., 1989) and later during the course of illness (Rubin, E. H., 1989; Petry, S., 1988). Early detection of these symptoms is extremely important as it does cause the impairment in daily living activities (Lyketsos, C. G., 1997; Mok, W. Y., 2004), accelerate cognitive decline (Stern, Y.,1987)

and worsen patient's quality of life (González-Salvador, T., 2000). In the symptomatic assessment, episodic memory lost very early because at the earliest of AD usually neurofibrillary changes occur in medial temporal lobe structures e.g., hippocampus, entorhinal cortex (Braak, H., & Braak, E., 1991) which is critical for episodic memories and thus a deficit in the ability to learn and remember new information (i.e., anterograde amnesia) which is something not surprising but a clinical hallmark of earliest AD (Warren, J. D., 2012). However, the short-term or working memory is maintained until very late in the course of MCI or AD development (Perry, R. J., 1999; Chen, P., 2001).

Based on diagnosis stages, Alzheimer disease has categorized into preclinical AD (PCAD), amnestic mild cognitive impairment (MCI), early-onset AD (EAD), and a late-stage AD (LAD) respectively. MCI is often referred to as the first stage or transition stage of PCAD to AD, and it is the stage in which the patient and those around them begin first to notice signs of memory loss (Jicha, G. A., 2006). Aβ load and NFT density increases in the number and the concentration through the progression of MCI to LAD. LAD presenting the highest amounts and subsequently the lowest neuron density and highest cognitive impairment and ultimately death. However, PCAD is an unusual stage in which the patients have significant levels of Aβ and NFT yet perform normally in cognitive testing and activities of daily living (Bradley, M. A., 2010).

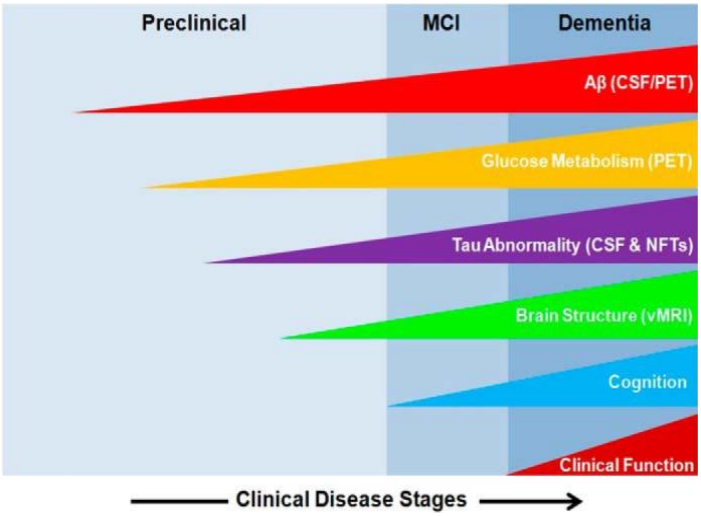



**Figure 2:** Different stages of AD progression with different biomarkers

Because of limited availability of diagnostic tools, a definitive diagnosis cannot be made without neuropathological observation so only post -mortem analysis can reveal the condition of patient's death, whether it was the AD or some other neurodegenerative disorders.

### 1.2 Molecular Basis of AD Pathology

At neuropathological level, neurofibrillary tangle (NFT) formation begins in the entorhinal cortex (universal over the age of 65 and considered as a part of healthy aging) and spread to the neocortical region which is the hallmark of AD development or may coincide with another tauopathy. (Bouras, C., 1994; Braak H and Braak E 1995). On the other hand, Amyloid deposition begins in neocortical regions, and while it is a hallmark feature of AD at least

40% of individuals dying after age 65 years without signs of dementia but have neocortical amyloid plaques at autopsy suggesting it may be a part of either healthy or "pathological" aging (Bouras, C., 1994; Braak H, Braak E 1997; Price, J. L.,1999). For decades it has been noticed that many nondemented patients have diagnosed with AD-like neuropathology (Dayan, A. D. 1970; Katzman, R., 1988) but recently this incidental pathology has been correlated with the presence of APOE genotype. Some individuals carrying e4 copies and dying at 50's had some neocortical amyloid depositions, and some of them had neurofibrillary tangles, but no mental status was reported as they died young (Kok, E., 2009). Thus, no clear explanation defines the pathogenesis and occurrence of AD-like characteristics in nondemented patients, and the reasons remain unknown.

NFTs and Aβ depositions are region specific and catalyzed by many up-streams signaling cascades. Lately, the molecular mechanism of APP process and NFT formation has become the spotlight in the field of Alzheimer.

### 1.2.1 Amyloid Protein Precursor (APP) and Tau in Physiology and Pathology

Plaques are extracellular deposits mainly composed of Aβ1-40 and Aβ1-42 peptide those generated by proteolytic cleavage process of the amyloid precursor protein (APP) by β-secretase cleavage and known as the amyloidogenesis (Glenner, G. G., 1984b; Kang, J., 1987). The APP is a transmembrane protein, and the primary function is synaptic formation and repair (Priller, C., 2006) thus it has a critical role in the maintenance of membrane and its level is upregulated during neuronal differentiation (Zheng, H., 2006). APP expression is probably also increased in response to specific

genetic, biological, chemical and other environmental insults, all resulting in increased metabolism and production of Aβ. Somehow Aβ is catabolized by neprilysin and insulin-degrading enzyme (Miners, J. S., 2011). Aβ1-42 have the tendency of fibrillization and insolubility than Aβ1-40, and therefore it has higher abundance in the plaques within AD pathology. Interestingly Aβ depositions are supposed to begin 20 years before the development of clinical symptoms (Villemagne, V. L., 2013). Aβ depositions occur in the region of the hippocampus, microglia, (Thal, D. R., 2002; Braak, H., 2006) where they found as extracellularly diffuse or focal deposits (Hyman, B. T., 2012; Montine, T. J., 2012). Aβ (1-42) a significant component of plaques shows more toxicity than Aβ (1-40) (Mohmmad Abdul 2004; Boyd-Kimball, D., 2005; Boyd-Kimball, D., 2005b; Boyd-Kimball, D., 2005c; Mohmmad Abdul, H., 2006).

According to the Amyloid cascade hypothesis (ACH), Aβ deposition is an initial and central trigger to the disease and which subsequently leads to NFTs (neurofibrillary tangles) formations, other secondary events, neuronal cell death, and dementia. Although the mechanism of Aβ neurotoxicity is not fully known and many studies are focused on understanding the basics of Aβ mediated neurotoxicity (Hardy, J. A., 1992; Selkoe, D. J.1991). However genetic studies have identified the mutation in APP (Goate, A., 1991), presenilin-1 (PSEN-1) and presenilin-2 (PSEN-2) genes in the families in early-onset AD (FAD, <60 years) and this could lead to Aβ deposition (Levy-Lahad, E., 1995; Sherrington, R., 1995).This observation resulted in the formulation of “Amyloid cascade hypothesis” in which it has believed that early onset phenotype in FAD shares similarity with late-onset AD and thus these amyloid depositions could explain pathogenesis of all types AD (Reitz, C., 2012).Thus according to the “Amyloid cascade hypothesis”, Aβ is

a central to the pathogenesis of AD and has capacity to induce protein oxidation, inhibit the activity of antioxidant enzyme and Aβ toxicity. Later this Aβ toxicity confirms the elevated oxidative damage in the brain. (Butterfield, D.A., 1997; Hardy, J., 2002).

Other than senile plaques, second major neuropathogenesis includes the formation of Tau containing neurofibrillary tangles (NFTs). Moreover, in AD-brain all the isoform of Tau has been identified hyperphosphorylated and accumulated as PHF (pre-helical filaments) (Grundke-Iqbal, I.,1986a; Grundke-Iqbal, I., 1986b; Iqbal, K., 1989).

Conformational changed (Jicha, G. A., 1997; Jicha, G. A., 1999a; Jicha, G. A., 1999b) and truncated Tau (Cotman, C. W., 2005; Gamblin, T. C., 2003; Novak, M., 1991) followed after its hyperphosphorylation were reported and reasonably represents the Tauopathy in AD-brain (Delobel, P., 2008). Tau is a one of the phosphoproteins, and physiologically phosphorylated Tau plays a varied role in microtubule stability, axonal transport, (Cuchillo-Ibanez, I., 2008) neuronal intracellular trafficking and neurite growth (Johnson, G. V., 2004; Götz, J.,2010; Wang, Y.,2015). Normally Tau contains 2-3 mole phosphate per protein moiety at its functional level but in AD brain this phosphorylation increases by 3-4fold (Köpke, E., 1993) and thus hyperphosphorylated Tau inhibits its protein binding affinity and microtubule assembly promoting activity in AD-brain (Grundke-Iqbal, I., 1986a, Lindwall, G., 1984). However basal level of these tangles is the sign of universal aging but abruptly presence of hyperphosphorylated Tau tangles gradually increase the neuron instability, promote the loosening of neuron network and neuron loss (Gómez-Isla, T., 1997) in some area of hippocampus where processes involved in prior to store experiences into the permanent memories, thus this characteristic

memory deficits result in interference for learning and making new memories.

Tau contains mainly serine, threonine and tyrosine sites for phosphorylation but although can be phosphorylated on histidine, lysine, and arginine which determines unfolded native Tau to folded or aggregated functional Tau moiety. Parallel to phosphorylation, many other post-translation modifications (PTM) are subjected to the Tau, i.e. glycosylation (O-linked or N-linked), glycation, deamidation, isomerization, nitration, methylation, ubiquitylation, sumoylation, and truncation, etc but majority of mentioned all PTMs are prominently found in AD brain than normal brain (Wang, Y., 2015). Among the all, phosphorylation depends on a balanced interplay between kinases and phosphatases but has not found persistent under pathological conditions. On the other hand, Phosphorylation can also be affected by O-glycosylation since some serine and threonine residues can either be O-glycosylated or phosphorylated on Tau (Götz, J., 2010).

To date, PHF-Tau has been identified at least for 29 phosphorylation sites (Liu, S. J.,2004). Tau is target for several kinases including, GSK-3β; Cdk5; MAP kinases (JNK, ERK, and p38); microtubule-affinity-regulating kinase (MARK); CK2; DYRK1A; TTBK1; and P70S6 kinase, known as "Tau kinases" (Götz, J., 2010; Dolan, P. J., 2010). Although, thus far only few of them are considered to be good candidates for bona fide in vivo Tau kinases and among them "GSK-3β" is pathologically evident for Tau hyperphosphorylation. GSK-3β is expressed at high levels in the brain (Woodgett JR. 1990), where it localizes to neurons (Leroy, K., 1999), and thus it is in an appropriate compartment to access Tau. GSK-3β also

associates with microtubules (Ishiguro, K., 1993) and, when this kinase is overexpressed in cells, the phosphorylation state of Tau dramatically increases at numerous sites (Cho, J. H., 2003; Lovestone, S.,1996; Wagner, U., 1996). However, all the protein kinases that contribute to the pathological phosphorylation of Tau in the AD and other neurodegenerative diseases remain elusive. Amongst, GSK-3β is a potential upstream trigger in Tau pathology in AD-brain (Jope, R. S., 2004) and also facilitates APP proteolysis to generate Aβ (Su, Y., 2004).

### 1.2.2 Glycogen Synthase Kinase 3-β (GSK-3β): A hallmark kinase in AD

Neuronal cell survival and synaptic plasticity by promoting Tau phosphorylation is one of the activated GSK-3β dependent event and has widely reported in many studies (Mandelkow, E. M., 1992; Hong, J. G., 2012). GSK-3β is ubiquitous serine/threonine kinase under physiological condition and inactivated by phosphorylation through upstream kinases. Activated GSK-3β negatively regulates many cells signaling pathways (i.e., Wnt signaling and insulin signaling pathway) and also promotes cell death (Grimes, C. A., 2001). GSK-3β requires phosphorylation at Tyr216 in the activation loop for its activity (Hughes, K., 1993; Lochhead, P. A., 2006) although this Tyr phosphorylation is thought to be autophosphorylation (Cole, A., 2004), however, phosphorylation by Src family tyrosine kinases has also been reported (Goc, A., 2014). Inhibition of GSK-3β activity regulates by phosphorylation at the N-terminal Ser9 by several protein kinases, such as PKA, PKB, p90RSK and Akt (V 2001; Doble, B. W., 2003; Jope, R. S., 2004, Sutherland, C., 1993; Jensen, J., 2007). Phosphorylated Ser9 binds to a pocket for priming phosphorylation in the substrate-binding

region and to reduce the binding affinity for substrates (Dajani, R., 2001; Ter Haar, E., 2001). However, regulation at the molecular level is not yet fully understood, for example, it isnot known what amount of GSK-3β remains inactive without Tyr216 phosphorylation. Therefore, it is unclear whether all GSK-3β molecules are automatically activated by autophosphorylation after de novo synthesis or if they require any signal for activation. For instance, insulin and other growth factors activate Akt, which in turn phosphorylates GSK-3β at Ser9, rendering the kinase inactive and resulting in decreased phosphorylation of downstream substrates, such as glycogen synthase, Tau, β-catenin, etc. (Liu, S. J.,2004; Zhang, Y., 2018).

In general GSK-3β is able to phosphorylate Tau at multiple sites although the most prominent phosphorylation sites are, Ser-198, Ser-199, and/or Ser-202 (Tau-1 site) and Ser-396 and/or Ser-404 (PHF-1 site) (Godemann, R., 1999; Agarwal-Mawal, A., 2003; Lovestone, S., 1996; Sun, W., et al 2002). It has been seen that phosphorylation at Ser396 and Ser404 seems to precede phosphorylation at the other sites (Godemann, R., 1999). GSK-3β preferentially phosphorylates many of its substrates after they have been pre-phosphorylated by other kinases, whereas the stretch of amino acids in Tau that includes the phosphorylatable residues, Ser396, Ser400, and Ser404, can be directly phosphorylated by GSK-3β without the prior activity of other kinases (Leroy, A., 2010; Hanger, D. P., 2011) and thus, these sites apparently have become the potential target to be studied in detail in the AD brain. However, phosphorylation at Ser404 is critical to this process, and substitution of this residue by alanine ablates phosphorylation of both Ser396 and Ser400. It appears, therefore, the primary phosphorylation of Ser404 by GSK-3β can itself serve as a primed residue for the subsequent sequential

phosphorylation of Tau at Ser400 and Ser396 by GSK-3β (Leroy, A., 2010; Hanger, D. P., 2011).Nonetheless, a recent study strongly supports and confirms that phosphorylation at Tau Ser 396-404 (PHF-1) is one of the earliest events in the AD and thus this study highlights the chronological order of tau phosphorylation sites along with the AD progression levels (Mondragón- Rodríguez, S., 2014). Aiming to identify early events and modification at phosphorylation level, many studies are currently focused on understanding the role of GSK-3β for Tau phosphorylation in early-onset AD-brain.

Since GSK-3β phosphorylates Tau at many sites seen in PHFs, it is suggested that activation of GSK-3β strongly promotes NFTs in the AD-brain but many studies which are focused on understanding the biochemical regulation of GSK-3β in AD brain are in contradiction, and many of them have demonstrated no up-regulation of this kinase in AD-brain. Thus, one of the possibilities is that abnormal hyperphosphorylation might be because some other modifications occur on Tau which makes it a better substrate for phosphorylation (Liu, F., 2002). While another study from Pei and Jin-Jing et al. has investigated the regional and intracellular distributions of active and inactive forms of GSK-3β in brain staged for neurofibrillary changes and demonstrated increased active GSK-3β, but no changes in inactive GSK-3β status were found in AD-brain (Pei, J. J., 1999; Leroy, K., 2002). Further to broaden our understandings, one of the recent epigenetic-based studies suggested the hyperactivity of GSK-3β along with NFT formation could be just targeted in the early stage of AD brain (Braak stage I-II) whereas turn off in a later stage, i.e. braak stage IV-VI (Nicolia, V., 2017). Therefore, the exact mechanism of GSK-3β activation/inhibition in Tau hyperphosphorylation is a misconception and not fully known.

Although further work is needed to clarify these controversial findings.

### 1.2.3 GSK-3β and oxidative stress

Biochemical analysis of different regions (i.e., pre-cortex, cortex, hippocampus, inferior parietal lobule) in postmortem AD-brain at various stages (i.e. pre-clinical AD (PCAD), mid impaired cognition (MCI) and AD) demonstrated that AD brain is highly sensitive to increased oxidative stress (Butterfield, D. A., 2006; Butterfield, D. A.,2007; Sultana, R., 2007 Butterfield DA 2008). Despite of mild OS, increased OS is being addressed in many mediating regulatory pathways, i.e. Akt activation, down-stream inhibited Gsk-3β (Grimes, C. A., 2001) and insulin resistance (Yu, T., 2006). Although contrary to this finding, one noticeable effect of oxidative stress has demonstrated in a recent study mentioning GSK-3β activation and so Tau phosphorylation at the specific site could be mediated by oxidative stress. This study suggested a novel mechanism of GSK-3β regulation in which N-terminal cleavage of GSK-3β is catalyzed by calpain; a calcium-dependent non-lysosomal cysteine protease expressed ubiquitously in mammals. This cleavage results in loss of the inhibitory domain and producing two fragments with kinase activity.

Moreover, it is very well known that oxidative stress usually induces calcium overloads through mitochondria ROS overproductions and results in cellular calcium sparks (Hamilton, D. J., 2013) and calpain activation (Feng, Y.,2013). Based on such studies, activation of GSK-3β is not only restricted to upstream kinases in insulin cascade, i.e. Akt, but also can be achieved through other parallel mechanisms, i.e., inhibited protein phosphatase 2A (PP2A) and activated calpain proteases. These studies widely open the

possibilities to further enquire the complexity of GSK-3β activation/inhibition-mediated signaling in AD brain in various aspects.

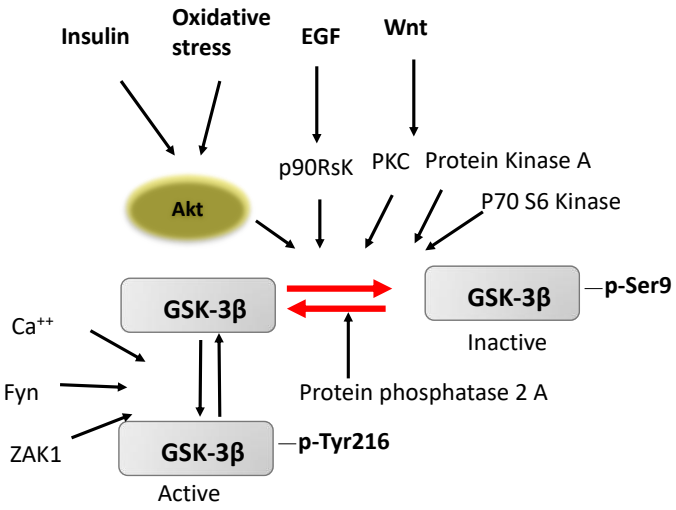



**Figure** 3: Multiple mechanism for GSK- 3β activation/inhibition regulation through various upstream signaling.

### 1.2.4 GSK-3β and Insulin Resistance (IR): A double sided player in AD and IR

Lately, it has been attributed in few studies that impaired cognition in the AD-brain can be recovered by insulin/glucose administration (Barone, E., 2018). Such evidence directly indicates a crucial link between AD-brain and insulin resistance (impaired glucose utilization). Glucose utilization regulated by insulin and insulin-like growth factor (IGF) but reduced insulin signaling (clinically known as insulin resistance) resulted in inhibited downstream insulin cascade signaling; PI3K/Akt kinase and ultimately led to increased GSK3-β activity (Talbot, K., 2012; Rivera, E. J., 2005; Steen, E.,2005) Tau phosphorylation. (van der Harg, J. M., 2017; 56 Bomfim, T. R., 2012, Zhang, X., 2016). In addition, autopsies were performed on patients with DM and/or

AD, which resulted in reducing levels and activity of PI3K/Akt signaling pathway in their frontal cortex, with a large amount of phosphorylated GSK-3β and abnormal tau hyperphosphorylated proteins (Steen, E.,2005; Nicolia, V., 2017; Liu, Y., 2011) . Interestingly, the defects of this signaling pathway are more severe in individuals who suffer both DM and AD (T2DM-AD). Excessive activation of GSK-3β through inactivated Akt is closely related to Tau hyperphosphorylation. However, this is not the case always followed in AD progression as other studies reported increased GSK-3 β activation without decreased Akt activation levels in the postmortem AD brain (Griffin, R. J., 2005; Yarchoan, M., 2014; Pei, J. J., 2003; Tramutola, A., 2015). So, it is remained to reconcile all the evidence that can provide a precise mechanism at the specific time point of aging (i.e., early onset to late onset).

Although besides human data, new insights in AD and insulin resistance signaling have been intervened by Eugenio et al., using 3xTg-AD mice model This study demonstrated increased insulin resistance mainly in old age of mice (12 months) which further led to reduced Akt activation and increased GSK-3 β, however intranasal insulin treatment recovered the Akt activation (Barone, E., 2018; Barone, E., 2016). From all the observations, one convincible explanation could be that insulin resistance mediated GSK-3β activation is one of the early events in AD-brain which no longer activated along with the aging. In the agreement of this hypothesis, one another study explained that the mechanism responsible for the increase in Tau phosphorylation in response to insulin deficiency in the late-onset AD is unlikely to be an elevated GSK-3β activity (in fact, a decrease in the active form of the kinase was observed); instead, the specific inactivation of protein phosphatase 2A (PP2A) may be responsible (Liu, C., 2010)

## 1.3 Brain and Oxidative Stress: A Staircase to AD Development

### 1.3.1. Oxidative Stress

"Reactive oxygen species" are one of the major byproducts of any aerobic metabolism known as free radicals. Principally, a free radical is any chemical species that contains one or more free unpaired electrons in the outer orbital of their atomic structure which makes them highly reactive species that reacts with any other species to achieve the stability (Halliwell, B., 1995). Endogenously ROS production governed by multiple cellular metabolisms: i) NADPH oxidase (NOX) complex in cell membranes, mitochondria, peroxisomes, and endoplasmic reticulum (Muller, F., 2000; Derick, H. A. N., 2001) ii) oxidative phosphorylation of electron chain reaction in mitochondria, iii) in inflammatory responses through neutrophils and macrophages. Exogenously ROS can be generated by several pollutants, smoke, UV radiation, Drugs, hormone, X-Rays, Xenobiotics, and gamma rays through direct or indirect methods (Valko, M., 2006).

The cellular system can tolerate modest and mild oxidative stress and potentially respond to the cellular damage by upregulating stress-responsive gene (e.g., HO-1/2) expression, upregulating antioxidant defense enzyme (e.g., Superoxide dismutase: SOD) and some other protective protein system (Chaperone). Nevertheless, severe OS may be responsible for reversible or non-reversible damage to the DNA, Proteins, and lipids that causes the structural and functional alterations (Nakamura, H., 1997) an imbalance between this two-counteract activity consequences the causes of oxidative stress (OS) (Sies, H., 1989).

OS participates in the biological system at various levels. At low concentration, ROS could be responsible for increased free intracellular Ca+2 (Orrenius, S., 1989; McConkey, D. J., 1996) as a second messenger, increased nitric oxide in phagocytosis as a second messenger (Kogishi, J. I.,2000), free iron (Halliwell,1992) to catalyze OH• generation, and as a part of signal transduction (Finkel, T. 2000). ROS has been suggested to act as the second messenger to regulate the activity of protein kinases and phosphatase (Hensley, K., 2000). Contrary, at high concentration (as the result of imbalanced homeostasis) ROS can react with biological macromolecules, i.e. proteins, DNA, Lipid, carbohydrate, RNA, etc. This reaction leads the oxidative damage to these molecules and cellular dysfunction that causes the cell death by apoptosis (Stadtman, E. R., 1998; Nordberg, J., 2001)

OS is involved in a wide spectrum of diseases and linked with several pathologies (Figure 4).

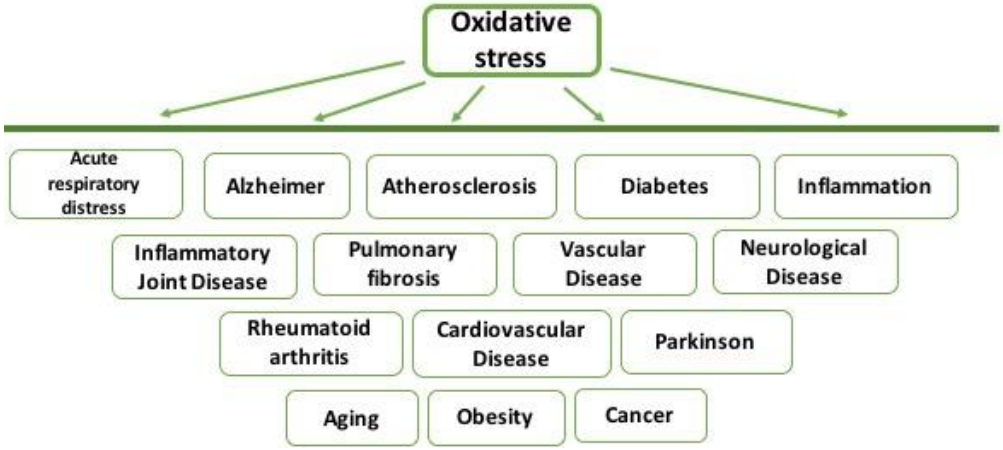



**Figure 4**: ROS involvement in different disease pathologies.

In 1956, Denham Harman suggested that free radicals produced during aerobic respiration cause cumulative oxidative damage, resulting in aging and

death. He noted parallels between the effects of aging and of ionizing radiation, including mutagenesis, cancer, and gross cellular damage (Harman D.1956; Hempelmann L. H.,1953). As per free radical theory of aging, it has been observed that species with high energy consumption by mitochondria and high metabolic rate ultimately results in unlimited O2•−production that linked to a faster rate of respiration, associated with an enormous generation of oxygen radicals and hastens aging which gives rise a shorter maximum lifespan potential (MLSP) (Beckman, K. B., 1998).

However, oxidative damages in such diseases are balanced by the antioxidant system in both enzymatic and non-enzymatic manner. The enzymatic oxidation system includes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx). Nonenzymatic antioxidants are derived from the plants, fruits and vegetables, i.e. lipid soluble α-tocopherol, carotenoids and many flavonoids and water-soluble glutathione, uric acid and ascorbic acids (Sies, H., 1997; Beckman, K. B., 1998). Besides, uric acids, β-carotene, and bilirubin are majorly considered as endogenous antioxidants produced in cells in-vivo (Halliwell, B., 1989).

### 1.3.2 Oxidative stress in AD brain

Many hypotheses were proposed to be involved in AD pathology which includes the amyloid cascade, excitotoxicity, oxidative stress, and inflammation hypotheses and all are centered on the role of Aβ, which ultimately cause the oxidative stress in the brain (Drake, J., 2003; Hardy, J., 2002). Therefore, studies suggested that oxidatively modified proteins are considered as a significant factor to contribute in etiology or pathogenesis of

many neurodegenerative diseases, including Alzheimer's disease (AD), and Parkinson's disease (PD) as well (Butterfield, D. A., 2007a; Butterfield, D. A., 2002; Cross, C. E., 1987; Markesbery, W. R.,1997). For many years it has been noticed that increased protein oxidation, protein nitration, and lipid peroxidation appear in neurofibrillary tangles and neuritic plaques of AD patients, and simultaneously the levels of oxidation products are also increased in cerebrospinal fluid of AD patients (Hensley, K., 1998).

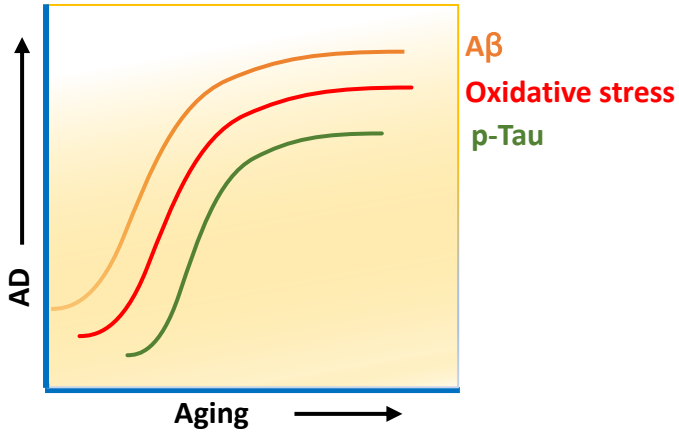



**Figure 5**: Aging and AD associated characteristics.

A brain is exclusively prone to secondary carbonylation via lipid peroxidation because it contains a large concentration of polyunsaturated fatty acids (PUFA) in the brain tissue. Lipid peroxidation process considered as a toxic due to the allylic hydrogen abstraction and occurrence of subsequent radical chain reactions and propagation on acyl chain of polyphenols. Such processes may spread between the membrane system and even adjacent cells (Butterfield, D. A., 2002; Butterfield, D. A., 2010a; Castegna, A., 2004). Status of lipid peroxidation in AD brain can be measured by the detection of elevated levels of free or protein-bound acrolein, HNE(4-

hydroxynonenal), isoprostane 8, 12-iso-iPF2α-VI, F2-isoprostane (F2-IsoP), and F4-neuroprostane (F4-NP) (Markesbery, W. R., 2005; Praticò, D., 2004; Butterfield, D. A., 2002; Butterfield, D. A., 2010a; Castegna, A., 2004).

Following the HNE abundance, many proteins/enzymes were further identified with protein nitration (3-nitrotyrosine) modification from early stage in AD brain (Butterfield, D. A., 2008). In particular for example BVR-A; one of the reductase enzyme and component of heme degradation pathway (HO/BVR-A) and kinase in insulin signaling pathway, has found with 3-NT (3-nitrotyrosine) modifications, resulted in reduced phosphorylated BVR-A which is necessary for its kinase activity in insulin signaling cascade (Barone, E., 2011a). Thus, oxidation derived changes to a protein could reduce the propensity of other PTMs and their functions as well. Lately, redox proteomics has become an advancement in the field to measure the oxidative stress-mediated total protein modification in AD brain (Butterfield, D. A., 2003) to provide an overview upon AD pathology.

Interestingly, few studies from the past revealed that increased OS has the significant impact on several signaling pathways; that not only causing damage because of the action of free radicals, but deranges signaling pathways leading to tau hyperphosphorylation, a hallmark of the disease and could involve in AD pathology. Evidently, both oxidative stress and reduced insulin signaling activation in the brain (known as brain insulin resistance), were proposed to favor Tau hyperphosphorylation in AD although the molecular mechanisms are still unclear ( Talbot, K., 2012; Steen, E., 2005; Rivera, E. J., 2005; Zhang, X., 2016; Morales-Corraliza, J., 2016; Sajan, M. P., 2016; Yarchoan, M., 2014; Bomfim, T. R., 2012; Butterfield, D. A.,2014). Tau phosphorylation due to increased OS has been highlighted by Zhu et al. in their study where they demonstrated the link between p38(one of

the tau kinase), tau phosphorylation, oxidative stress, and cell cycle-related events in Alzheimer disease (Zhu, X., 2000) and Lloret, A., et al. showed in their study that Aβ and tau toxicities in Alzheimer's are linked via oxidative stress-induced p38 activation (Giraldo, E., 2014). At the consequences, both aging and AD brain encountered with oxidative stress which further i) oxidizes the protein through PTMs mechanism and ii) alters signaling pathways including kinases led to ultimate Tau phosphorylation.

### 1.3.3 BVR-A/HO-1 System

Increased quantity of pro-oxidants (ROS) in brain up regulates many anti-oxidant responses including; stress responses gene expression and enzyme activities. Biliverdin reductase-A and heme-oxygenase (BVR-A/HO) is evolutionary conserved and one of the major anti-oxidant systems to maintain the cellular redox homeostasis. It's up-regulation provides the earliest defense response to the stress (Poon, H. F.,2004). HO-1 is the very first rate-limiting enzyme which is responsible for the breakdown of heme moiety into equimolar molecules biliverdin, carbon monoxide (CO), and ferrous iron (Maines, M. D., 1997), this reaction latter catalyzes the biliverdin into bilirubin by biliverdin reductase (BVR). Exposing to ROS, this enzyme elevates the availability of physiological bilirubin, CO and ferritin compelling antioxidant, anti-apoptotic and anti-inflammatory properties, therefore HO-1 known as a key player in maintaining cellular homeostasis (Ryter, S. W.,2005). Typically two main isoforms of heme oxygenase, i.e. HO-1 and HO-2 has been recognized and categorized on the basis of expression of their genes and distribution in the distinct tissue (Ryter, S. W., J 2006; Abraham, N. G., 2008).HO-1 (about 32 kD) is encoded by gene

HMOX1 and HO-2 (36 kD ) encoded by HOMX2 respectively in the tissue (Gozzelino, R., 2010). HO-2 is considered to be a constitutive isoenzyme most highly expressed in neuronal tissues (Brain) and testis contributing to cell homeostasis, whereas HO-1 is thought to be an inducible form with relatively low expression in most tissues (Zhu, X., 2011; Ryter, S. W., J 2006; Maines, M. D., 1997), though HO-1 mainly regulates protection against several stress conditions; ischemia (Burger, D., 2009), hypoxia (Chang, A. Y., 2009) and ROS (Cooper, K. L., 2009) and also known as heat shock protein (Hsp32).

Expression of HO-1 at the HOMX1 gene level is regulated by transcription repressor Bach-1 under basal condition but under pro-oxidant state its upregulation at both gene and protein level controlled by two upstream enhancer regions, E1 and E2 (Sun, J., 2002), which contains multiple stress- responsive elements (SREs), also known as antioxidant-responsive elements (AREs). These emerging evidences suggest its up-regulation is highly dependent on ROS stimuli and represent an oxidative inducible nature of this protein (Barone, E., 2014). AREs share a consensus sequence (GCnnnGTA) with the Maf recognition element (MARE) (Stewart, D.,2003). The interactions between MAREs and heterodimers formed by a Maf protein (MafK, MafF, or MafG) and an NF-E2-related factor 2 (Nrf2), or activator protein 1 (AP-1) play a direct role in HO-1 induction (Maines, M. D., 2005a; Sun, J.,2002).

Up-regulation of HO-1 upon ROS induction includes double mechanisms; i) by inducing conformational modification of Bach1 structure, which leads to its translocation from the nucleus to the cytoplasm where

Bach1 is ubiquitinated and degraded thereby, releasing transcriptional repression; and ii) by promoting the ubiquitination and consequent degradation of Keap1, which under normal conditions sequesters Nrf-2 into the cytoplasm, avoiding its transcriptional activity (He, X., 2007; Zenke-Kawasaki, Y., 2007).

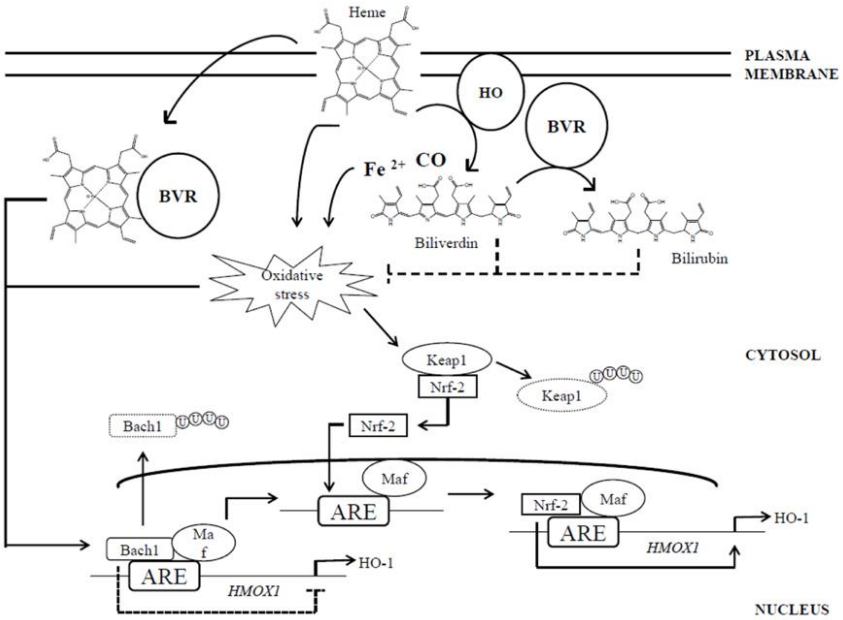



**Figure 6**: HO-1/BVR system and regulation.

### 1.3.4 Role of BVR-A in the regulation of cells stress response

Similar to heme oxygenase, BVR possesses two isoforms as well; BVR-A and BVR-B (Kapitulnik, J., 2009; Maines, M. D., 2005b; Pereira, P. J. B., 2001). At functional level both these enzymes generate BR, but only BVR-A reduces BV-alpha into the powerful antioxidant and anti-nitrosative molecule BR-IX-alpha (thereafter BR) (Barone, E., 2009; Stocker, R., 2004),

whereas BVR-B prefers the other BV isoforms, such as BV-β, BV-γ, and BV-δ (Kapitulnik, J., 2009; Maines, M. D., 2005b; Pereira, P. J. B., 2001). Both BVR-A and BVR-B were identified in humans, with age-dependent characteristics. Biliverdin reductase-A is the primary form detected in the adult (95–97% BR is found in the bile), whereas BVR-B is predominant (~ 87%) in the fetus (Cunningham, O., 2000). Under normal condition, Biliverdin reductase co-expresses with HO-1/HO-2 as heat shock inducible enzymes (Ewing, J. F., 1993).

BVR is not only a reductase enzyme but also has been identified as serine/threonine/tyrosine kinase involved in various cellular function (Kapitulnik, J., 2009; Maines, M. D., 2005b). However, activation of BVR-A through phosphorylation at Ser/Tyr/Thr sites are essential for its dual characteristic function (reductase and kinase activity) (Kapitulnik, J., 2009; Lerner-Marmarosh, N., 2005). From the studies, it is demonstrated that BVR-A is capable of autophosphorylation which is required for its so-called reductase activity (Lerner-Marmarosh, N., 2005) whether phosphorylation through other kinases, i.e. insulin receptor kinase (IRK) is necessary to activate its own kinase activity (Kapitulnik, J., 2009; Lerner-Marmarosh, N., 2008; Maines, M. D. 2007; Tudor, C., 2008). Highlighting BVR-A's activity, it does regulate many down-stream pathways like;

1. Catalyze the last step in the heme-degradation pathway by reducing the γ-meso (methylene) bridge of BV to BR (Kapitulnik, J., 2009).
2. Modulates the activity of members of conventional and atypical groups of PKC isozymes (PKC-βII and PKC-ζ, respectively) (Kapitulnik, J., 2009; Lerner-Marmarosh, N., 2007; Maines, M. D. 2007; Gibbs, P. E., 2012b).

3. Functions as a scaffold protein for the formation a ternary complex with MEK1 and ERK1/2, placing ERK in a position that enables its activation by MEK (Kapitulnik, J., 2009; Lerner-Marmarosh, N., 2008).
4. Regulate the expression of stress-responsive genes such as HO-1 (Tudor, C.,2008) and iNOS (Di Domenico, F., 2013; Gibbs, P. E., 2012a). (Demonstrated in the figure below).

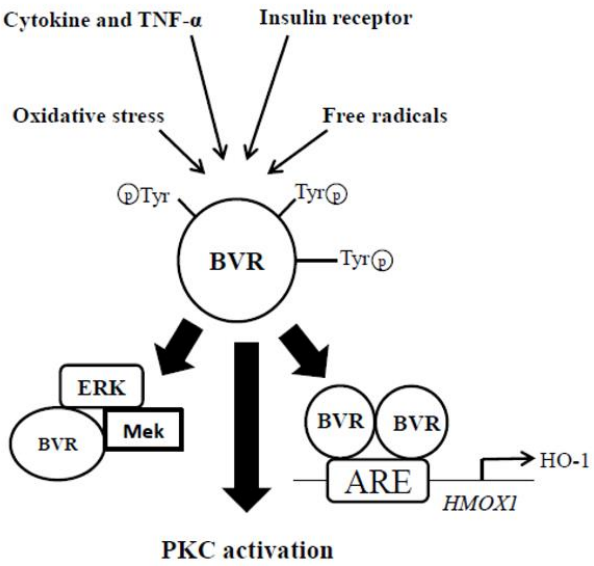



**Figure 7**: multifaceted Role of BVR-A.

Activated BVR-A (phosphorylated form) further regulates two central signaling pathways in insulin cascade; MAPK and phosphatidylinositol-3-kinase (PI3K) (Kapitulnik, J., 2009). Both the pathways synergistically play a significant role in neuronal development, neuronal plasticity and learning/memory consolidation (Horwood, J. M., 2006; Akter, K., 2011).

In recent years, published work from Maines group directs new insight on BVR-A's propensity as a scaffold protein in signaling pathways.They discovered that the phosphotyrosine binding motif $Y^{198}$MKM in BVR-A after being phosphorylated by IRK, interacts with NPXY motif of IRK. YMXM binding motif of any protein including IRS1/IRS2 and PI3K after phosphorylated by IRK, become docking site for src-homology domain-containing proteins and help in the assembly of a multiprotein complex (Miralem, T., 2016). A significant BVR fragment was found to activate insulin/IGF-1 signaling by its ability to interact with the intracellular kinase domain of the insulin receptor and to stimulate glucose uptake (Gibbs, P. E., 2014). As BVR and Akt both are downstream target in insulin signaling pathway and activated by IRK, they also share similarity at i) functional level: both targeted by ROS stimuli, IGF-1/2 insulin and hypoxia (Datta, S. R.,1999; Miralem, T.,2005; Salim, M., 2001; Gibbs, P. E., 2010), and ii) structural level: Akt isoforms and hBVR share certain similarities in their protein-protein interaction domain. The N-terminal pleckstrin homology (PH) domain of the Akt isoforms (aa1–113) (Coffer, P. J., 1998; Agamasu, C., 2015), and the C-terminal half of hBVR, which folds as a large 6-stranded β sheet (Kikuchi, A., 2001; Whitby, F. G., 2002), are the protein-protein interaction domains (Gibbs, P. E., 2012a).

Sequence identified in the β sheet of BVR is $^{225}$RNRYLSF which is identical to the canonical Akt interaction motif RxRxxS and this motif is also found in all Akt substrate, non-substrate binding proteins and Akt co-activator i.e members of proto-oncogenes TCL1 family (TCL1, MTCP1, TCL1b) (Laine, J.,2000; Brazil, D. P., 2002), noticeably the underlined amino acids are prominently required for proposed interactions between proteins.

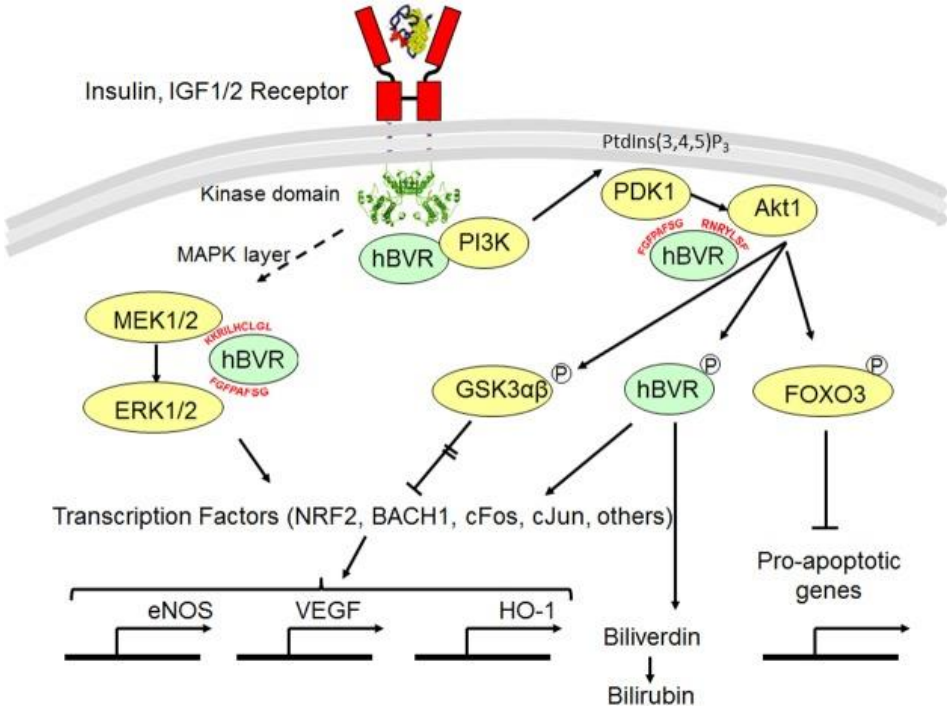



**Figure 8**: Schematic overview of BVR as a scaffold protein

Overall, activated BVR is recognized as a key player compelling its pleiotropic properties (Reductase, kinase and scaffold protein) in the cellular system under the various signal inductions.

Since Alzheimer disease has also partial involvement of increased OS within aging and imbalanced antioxidant system, many recent studies are focused on understanding the pivotal role of BVR-A in AD pathology because of its pleotropic functions and abundance in ROS signaling.

## 1.4 Glucose Metabolism in the Brain

*Nobody realizes that some people expend tremendous energy merely to be normal.*

*"Albert Camus, Notebooks 1942-1951*

Human brain depends on glucose as its primary source of energy. In the adult brain, neurons have the highest energy demand (Howarth, C., 2012), as per requiring continuous delivery of glucose from the blood. Humans brain accounts for ~2% of the body weight, but it consumes ~20% of glucose-derived energy making it the primary consumer of glucose (~5.6 mg glucose per 100 g human brain tissue per minute) in the entire body (Erbsloh, F., 1958). Glucose metabolism provides the fuel for physiological brain function through the generation of ATP, generates several neurotransmitters, and foundation for critical for brain physiology and disturbed glucose metabolism in the brain underlies several diseases affecting both the brain itself as well as the entire organism.

One of the prioritized function of glucose metabolism is to provide the substantial energy source, i.e. ATPs to the neurons to possess information, balance the action potential flow, postsynaptic potential, maintenance of ion gradients, the neuronal resting potential (Howarth, C., 2012; Erbsloh, F., 1958; Harris, J. J., 2012; Ivannikov, M. V., 2010).However, approximately 2–5% of total glucose is also utilized in the hexosamine biosynthetic pathway (HBP) to produce glucosamine-6-phosphate and, ultimately, UDP-N-acetylglucosamine (UDP-GlcNAc) (Liu, F., 2009; Copeland, R. J., 2013).

UDP-GlcNAc is the donor substrate for O-linked-ß-N-acetylglucosamine transferase (OGT), which catalyzes protein O-GlcNAcylation, a post-translation process transferring a single moiety of N-acetyl-D-glucosamine residues from UDP-GlcNAc to the hydroxyl side chains of serine/threonine

residues of proteins and similar to phosphorylation. The reversible attachment of O-linked GlcNAc to proteins is catalyzed by O-GlcNAcase (OGA), which regulates its removal. The activation of OGT is sensitive to UDP-GlcNAc substrate and, therefore, to altered intracellular glucose metabolism (F. liu 2009, C.X. Gong 2016, B.D. Lazarus 2009, K. Vaidyanathan 2014, Y. Yu, 2012).

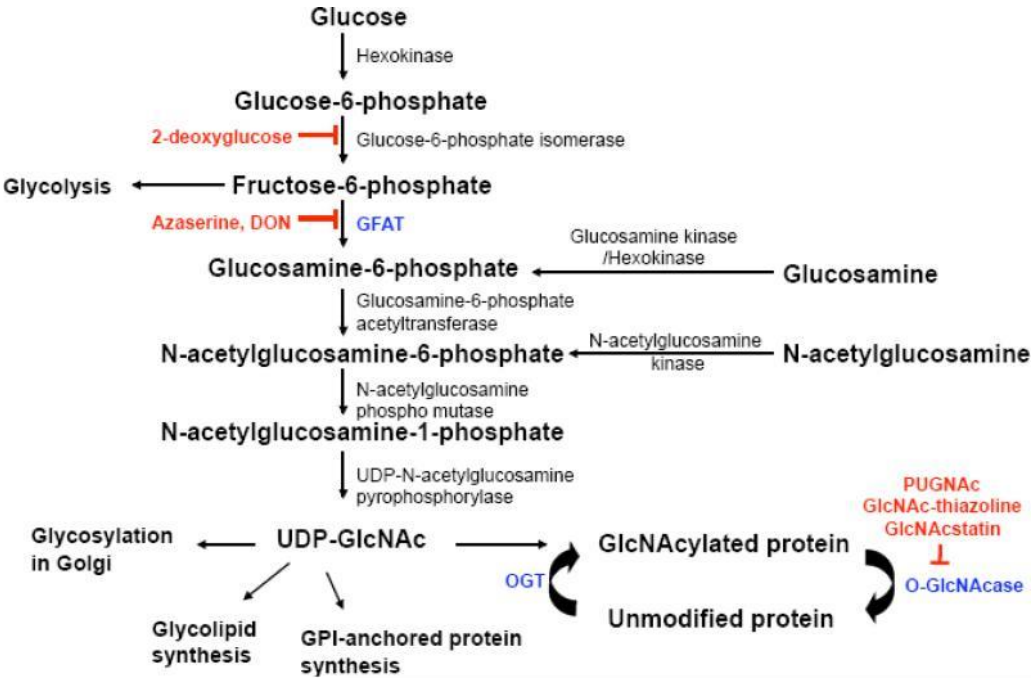



**Figure 9**: Hexosamine biosynthetic pathway (HBSP).

The synthesis of UDP-GlcNAc from glucose and enzymes involved in the process are shown and inhibitors of HBP and GlcNAcylation are shown in red (Figure 9). Interestingly, both the post-translation modification process; O-GlcNAcylation and phosphorylation are mutually related. Indeed, several studies reported that O-GlcNAc could either occur reciprocally to

serine (Ser) and threonine (Thr) phosphorylation or can interact with adjacent phosphorylated sites influencing their modification (Yu, Y., 2012, Deng, Y., 2008; Li, X., 2006; Trinidad, J. C., 2012). Interestingly altered glucose metabolism has been involved in several pathophysiological conditions, i.e. insulin resistance, which is one of the commons among the older people. Development of Insulin resistance in the peripheral system including pancreas, liver, white adipose tissue and brain generally thought to be primary etiological evident for glucose metabolism dysfunction and a significant decline in insulin signaling response in those tissues were observed with age (Salmon, A. B. 2012).

In the line, it has also shown that increased oxidative stress in the brain can directly promote insulin resistance (Barone, E., 2011a) and alter the glucose metabolism that promotes severe cognitive decline. Thus, impairment in glucose metabolism and insulin resistance in peripheral organs including brain in elder age could be a part of AD-like dementia. However, how this oxidative stress and impaired glucose metabolism participates at the synergistic level in AD-like dementia, have discussed in the next subtitle.

### 1.4.1 Impaired glucose metabolism, oxidative stress (OS) and Alzheimer

Symptom severity in Alzheimer progression was only limited to the NFTs and Aβ depositions until the series of studies provided some intriguing evidence that has confirmed the involvement of early metabolism dysfunction in the pathology. NFTs and Aβ depositions are profoundly has been seen in healthy aging and non-demented patients (mentioned before) as well which is not distinguishable at pathological status. PET analysis of AD patients has revealed the reduced cerebral metabolic rate for glucose

(CMRglc) (Mosconi, L., 2008). As per the required energy for functional synapses and axonal transportation are usually provided through glucose consumption and ATP production along with balanced ROS homeostasis and clearance pathways in normal aging but disturbed ROS homeostasis results in increased oxidative stress and strongly associated with stress-mediated abnormalities in the brain (Mattson, M. P., 2006). Nevertheless, reduced glucose uptake principally minimizes the generation of superoxide anions which in turn results in lower the level of $H_2O_2$ and OH• and lessen the damage to proteins and other biomolecules. (Mattson, M. P., 2006). Contrary to the reduction in glucose uptake which promotes the lower OS in the brain, aging deliberately causes ROS generation by other means i.e. perturbation of ER, peroxisome and mitochondria (Zorov, D. B., 2006, Circu, M. L., 2010) and abnormal NFTs/ Aβ depositions which elevate the risk of higher ROS in the brain (Lambert, M. P., 1998). Further many studies in cell culture and ex-vivo rat muscle showed that this increased OS reduces insulin-stimulated glucose uptake, reduction of the downstream insulin signaling mediators proteins, i.e. Akt, IRS-1(insulin receptor), GSK-3β (summarized mechanism in figure 3) and reduced glucose transporter translocation to the cellular membrane (Salmon, A. B., 2012). Therefore, it's very well predicted that age-associated increased OS and metabolic dysfunction (impaired glucose uptake) in the brain is a significant contributor to the pathophysiological status of AD patients.

Positron emission tomography (PET) imaging with 2-[18F] fluoro-2-deoxy-D-glucose (FDG) as the tracer has long been used to track AD-related brain changes by providing qualitative and quantitative estimates of the cerebral metabolic rate of glucose (CMRglc). This study brought the significant contribution of glucose metabolism forefront in the AD pathology

when a reduction in glucose uptake has confirmed in AD patients compared to the age-matched healthy patients (Mosconi, L., 2008).

Besides these observations as mentioned above, many other studies are focused on identifying the other alternative mechanism that could further be a leap in AD pathology. To shed light on this concern, lately, it has been discovered in a few studies that reduced glucose availability in the brain could raise the probability of occurrence of protein phosphorylation than glycosylation simultaneously. The balanced interplay between these two post-translation modifications could be one of the key mechanisms to rescue the NFTs formation and tangles in AD brain. All these studies taken together provide us an overview that reveals an important link between oxidative stress, impaired glucose metabolism and AD pathology underlining to a different stage of aging.

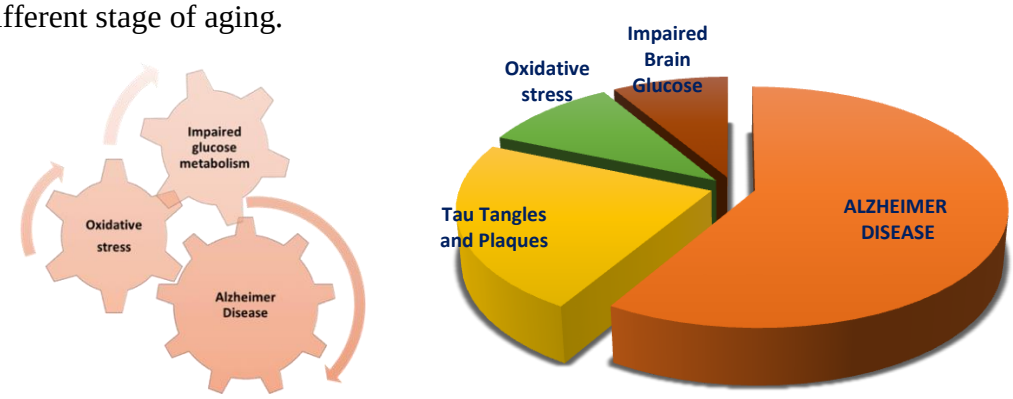



**Figure 10:** Relationship among the oxidative stress, Alzheimer disease and impaired glucose metabolism.

### 1.4.2 Cross talk between *O- GlcNAcylation* and phosphorylation in Alzheimer disease: A link between AD pathology and impaired glucose uptake

These two PTM (phosphorylation and glycosylation) mechanism for any protein, shares reciprocal manner of regulation in which inhibition of protein kinase (Protein Kinase A, Protein C) and inhibition of O-GlcNAcase increase the global O-GlcNAc levels while shows the decreased phosphorylation for many events involved in cell development signaling or homeostasis (Griffith, L. S., 1999; Wang, Z., 2007). Interestingly, the site-specific target for O-GlcNAc and phosphorylation could occur on A) same site (Ser/Thr) B) competitive

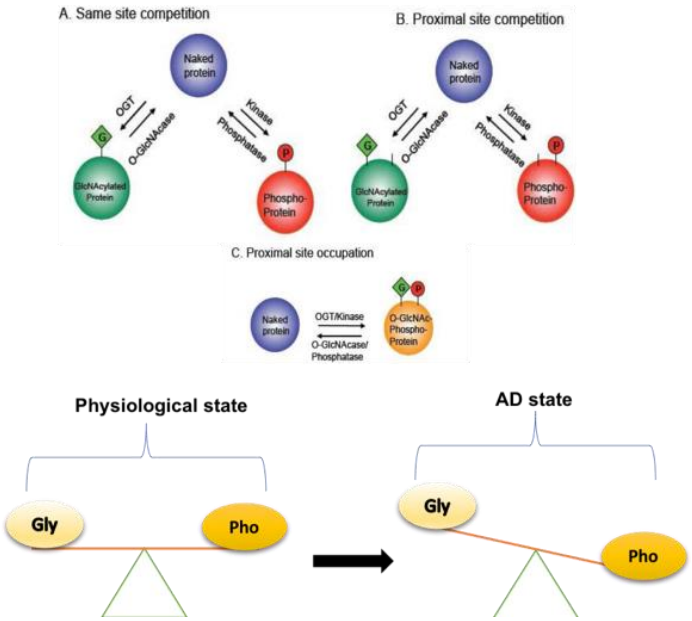



**Figure 11**: Dynamic interplay between of GlcNAcylation and phosphorylation

proximal site of Ser/Thr C) Occupied both proximal sites (Chutikarn Butkinaree 2010). This interplay between *O-GlcNAclytion* and phosphorylation can be more complicated than just a competitive mechanism for any cell signaling and the balance between these two PTM at the same time on the same protein could achieve the cell fate in cell survival decision. Interestingly, AD brain identified with aberrant glycosylated Tau other than being hyperhosphorylated (Liu, F., 2002) and this study further supports the idea that impaired glucose uptake in AD brain favors the reduced glycosylation modification on Tau protein but coincides with increased abnormal phosphorylation.

### 1.4.3 *O-GlcNAcylation* of Tau and APP in Alzheimer disease

Brain Amyloid deposition and Tau aggregates are the clear evidence for the AD, but impaired glucose metabolism coincides in AD brain and responsible for synaptic dysfunctionality as a result of Aβ neurotoxicity. However, impaired glucose metabolism (Hypometabolism) becomes clinically evident in a significant manner for many different disorders. Alterations of brain glucose metabolism seem to occur also in subjects affected by mild cognitive impairment (MCI), before the appearance of clinical signs of dementia. Therefore, it is suggested this event is a cause, rather than a consequence of AD (Gong, C. X., 2016; Yu, Y., 2015). In past three decades PET (positron emission topography) analysis of AD brain has revealed its importance as an early onset biomarker in AD pathology (Blennow, K.,2006), validating the coexisted AD and insulin resistance together. The new hypothesis suggested the limited glucose uptake gives rise reduced GLUT1, GLUT 3 in neuronal cells supporting the increased insulin resistance in the AD (Calvo-Ochoa, E.,

2015; Correia, S. C., 2012; de la Monte, S. M., 2009). The occurrence of O-GlcNAcylation to any protein is UDP-GlcNAc (a substrate for OGT) dependent via Hexosamine biosynthetic pathway (HBP), but the failure of O-GlcNAcylation is now considered as a sensor for limited nutrition supply to the brain. Therefore, glucose hypometabolism within diseases, i.e., diabetes and AD favors the reduced O-GlcNAclytion and reciprocally increased phosphorylation. Post-translation glycosylation of several nuclear and cytoplasmic proteins has reported by Torres and Hart including cytoskeleton protein Tau and membrane-associated protein APP (Hart, G. W., 2007).

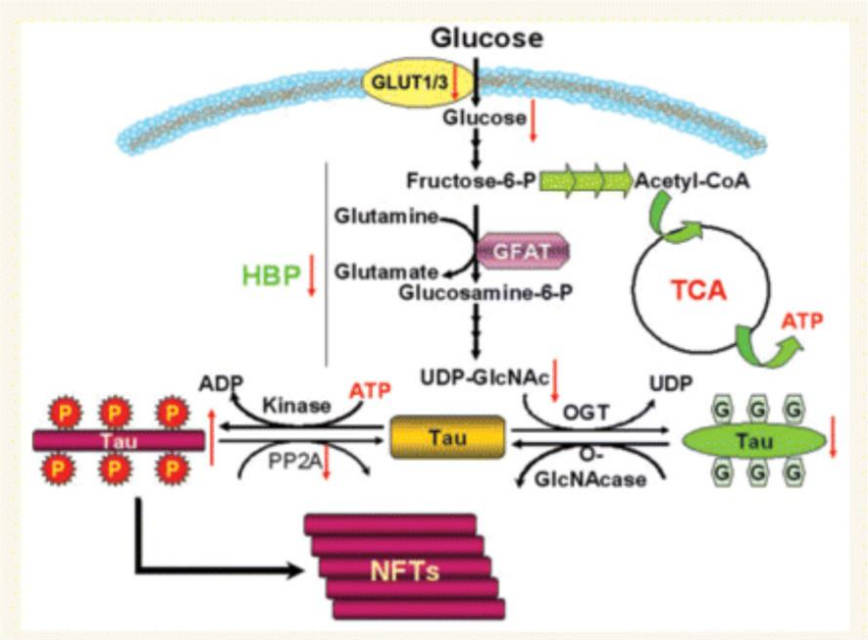



**Figure 12:** Impaired brain glucose metabolism contributes to neurofibrillary degeneration in Alzheimer's disease

Mass spectrometry results of Tau aggregates in AD patients show many sites occupied for O-GlcNAcylation and less for phosphorylation. In the Study

from Lefebvre group mention the Inhibiting phosphatase activity results in increased phosphorylation and vice versa O-GlcNAcylation (Lefebvre, T., 2003). Data from fasting mice demonstrate decreased O-GlcNAcylation and increased phosphorylation (Li, X., 2006).

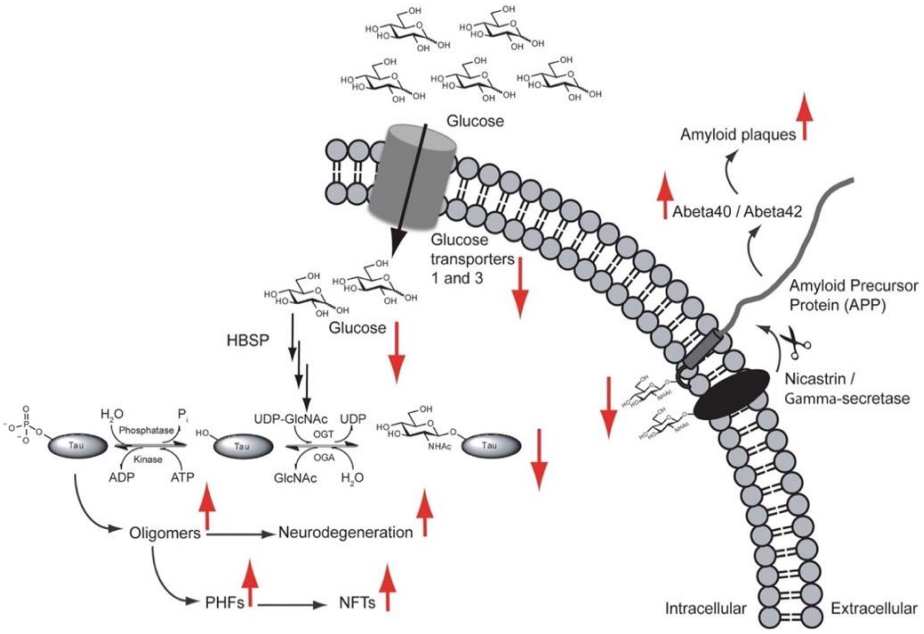



**Figure 13**: Potential protective role of *O*-GlcNAcylation within the healthy brain, which may become deficient in the AD brain.

Impairments in glucose utilization (may perhaps by decreased cell-surface glucose transporters) as indicated above in the figure would result in reduced flux down the hexosamine biosynthetic pathway (HBSP) and ultimately lead to reduced O-GlcNAc on Tau, APP, and nicastrin (a non-catalytic component of the -secretase complex), which could result in the increased production of NFTs and amyloid plaques, the hallmark features of the AD brain.

Intriguingly, during AD progression brain O-GlcNAc levels appear to decrease while phosphorylation increases. Indeed, samples of frontal cortex from AD patients displayed a significant reduction in global O-GlcNAc levels but increased Tau hyperphosphorylation as compared to controls (Gatta, E., 2016; Yuzwa, S. A., 2014; Liu, F., 2004). However, the reduction of total protein O-GlcNAcylation in the AD is still a matter of controversy since other studies reported an inverse trend (Förster, S., 2014; Frenkel-Pinter, M., 2017; Griffith, L. S., 1995). The abnormal phosphorylation/O-GlcNAcylation ratio of Tau is critical to neurodegeneration, as increased phosphorylation promotes Tau aggregation into tangles, while higher levels of Tau O-GlcNAcylation are protective against tangles formation (Gong, C. X., 2016; Li, X., 2006; Liu, Y., 2009; Zheng, B. W., 2016). Immunofluorescent studies on human brain samples revealed an inverse relationship between Tau O-GlcNAcylation and phosphorylation (Liu, F., 2009, Liu, Y., 2009). Further for the reason that reciprocity sustains the O-glycosylation and phosphorylation, many studies have confirmed the increased O-GlcNAclytions and decreased phosphorylation with selective OGA inhibitors in-vitro. Treatments using inhibitors of OGA has been tested on different mouse models of AD or tauopathy showing in general that increasing the levels of O-GlcNAcylation can reduce disease hallmark and lower cognitive decline (Jacobsen, K. T., 2011; Kim, C., 2013; Yuzwa, S.A., 2012; Graham, M. E., 2011).

The APP, a membrane-bound protein that results in Aβ deposition during AD development (as already mentioned in 1.3.1 in the beginning) has been observed with decreased O-GlcNAcylation modification comparatively phosphorylation. When T2DM has found being associated with Aβ toxicity

in AD model, its considered that impaired glucose metabolism can contribute in the development of AD through drop off in a total O-GlcNAcylated APP (Griffith, L. S.,1995). Griffith et al. first discovered Post-translation modification (O-GlcNAcylation) of APP and APP associated proteins became the attention for its role in APP processing. Following this observation, it has been found that increased O-GlcNAcylation of APP leads to decreased production of Aβ (40) in cultured SH-SY5Y cells with non-selective OGA inhibitors (Kim 2013). Surprisingly study in 5xFAD Aβ mice model (conceive amyloid pathology), shows a decreased level of Aβ (40) and Aβ (42), limited plaques, less neuroinflammation and recovered cognition ability in the brain while exposing to long-term selective OGA inhibitors (Macauley, M. S., 2010). In the case of site-specific O-GlcNAcylation, both APP and APP processing enzyme (nicastrin-non-catalytic components of secretase complex) were spotted on Ser-708 being O-GlcNAcylated. (Kim, C., 2013). Regardless of the known pathway of γ-secretase for Aβ generation, it is believed that decreased O-GlcNAcylation on nicastrin induces APP processing and produces Aβ fragments. Reconciling the glucose hypometabolism with increased APP processing refer to the hypothesis of overlapping T2DM/insulin resistance with hyperphosphorylated Tau and excess Aβ depositions in AD.

# Chapter 2: *Background and Aim*

In a past decades neurofibrillary tangles (NFTs) and Aβ plaques has spotted in brain specific region as a hallmark of AD, that gradually causes cognitive impairment. PET analysis together with biochemical analysis has confirmed the significance of NFTs and plaques in the AD pathology but we further need to strength on the causes that lead to cognitive impairment before it distinguishes in severe AD symptoms.

Interestingly, Insulin resistance together with impaired glucose metabolism and increased ROS production in the brain has been linked with AD development (Barone, E., 2011a; 2011 b; 2014, and 2015; Butterfield, D. A., 2014; Hart, G. W., 2011). These alterations in the signaling pathways have seen very often in a large population of old people with increasing in numbers every year. Thus, in the current situation the biggest challenge is not only to discover the biomarkers but also to understand the molecular mechanism of pathology at early-onset AD and late-onset as well.

For the first-time, the link between oxidative stress and impaired glucose transport has been evidenced in cultured neurons, which is followed by a decrease in cellular ATP levels due to a dysfunctional respiratory chain reaction in mitochondria (Keller, J. N.,1997; Luo, Y., 1997). Further studies suggested that altered glucose metabolism is a very early change in the AD (De Leon, M. J., 2001; Mosconi, L., 2008; Small, Gary W., 1995; Reiman, E. M., 1996; Mosconi, L., 2006) and legitimately correlates the clinical disabilities with dementia (Blass, J. P., 2002). Occurrence of insulin resistance in early AD due to impaired glucose metabolism concomitantly stimulates the oxidative stress in the AD brain and have recognized as early

symptoms. This alteration can be an indication for the transition of pre-clinical AD (PCAD) to the MCI stage. Although one study suggests that OS occurs before the onset of AD symptoms and that oxidative damage is found before robust Aβ plaque formation (Butterfield, D. A., 2014). Thus, insulin resistance, oxidative stress and impaired glucose metabolism promotes a feedback mechanism and involved as a vicious cycle in early phase of AD development.

It is known that OS and impaired brain metabolism affects downstream targets; i) ***GSK-3β*** and, ii) ***BVR-A/HO-1***, which plays a significant role in several signaling and among all OS mediated Akt activation and GSK-3β inhibition is a part of neuroprotective mechanism in the AD brain (Talbot, K., 2012; Steen, E., 2005; Rivera, E. J., 2005; Zhang, X., 2016; Morales-Corraliza, J., 2016; Sajan, M. P., 2016; Yarchoan, M., 2014; Bomfim, T. R., 2012; Butterfield, D. A.,2014). When it comes to understand the role of HO-1/BVR-A system in the AD, one of the studies from Butterfield and the group has been reported an astonishing observation which revealed that oxidative/nitrosative post-translation modification of HO/BVR system associated with its decreased phosphorylation forms (BVR-A activity) in MCI and AD brain (Barone E 2011a and 2011b). On the other hand, based on its scaffold properties, another study from Maines group has reported the BVR-A/Akt mediated GSK-3β activation (Miralem, T., 2016) and also data from our group showed that BVR-A plays a significant role as a scaffold protein in CK1 mediated BACE-1 phosphorylation and resulting in Aβ depositions in aging model (Triani, F., 2018). So further BVR-A is a central attraction in my study because previously our group has shown that reduced total BVR-A level and its activity proportionally associated with the increased levels of OS in AD and MCI brain (Barone, E., 2011a; Barone, E.,

2011b; Barone, E., 2014) and 3xTg-AD mice (Barone, E., 2016). Therefore, it would be surprising if downstream target i.e., Akt and GSK-3β gets affected by reduced BVR-A levels, instead by merely insulin resistance mediating signaling. Alteration in BVR-A associated signaling may further lead to development of tauopathy in AD brain through GSK-3β regulation which is a prominent kinase for Tau hyperphosphorylation. Therefore, while the presence of BVR-A in the human brain could contribute in several kinases (i.e., GSK-3β) mediated or scaffold-mediated signaling i.e., BVR-A/Akt and BVR-A/CK1, we further hypothesized that its impairment could prevent those regulatory implications and leads to pathology in the brain including insulin resistance and Alzheimer disease.

To the betterment of our understanding in AD pathology, in our **first project,** we combine all the previous observation and propose the role of BVR-A in Akt-mediated GSK-3β inhibition. *We hypothesize that the loss of BVR-A could prevent the BVR-A/Akt complex formation and the Akt mediated GSK-3β inhibition which may lead to Tau hyperphosphorylation in the AD brain.* To this aim we performed our experiments with; i) 3xTg-AD mice (AD mice model) /Non-Tg mice at the early age (6 months) and late age (12 months); ii) Human MCI and AD subjects and iii) in-vitro HEK cells.

Moreover, early insulin resistance and BVR-A impairment further causes reduction in glucose availability to the brain and lasts till the late phase in the AD. Less glucose consumption favors impairment of HBP pathway and glycosylation; a typical post-translation modification (PTM) that responsible for protein functionalities. Interestingly, glycosylation and phosphorylation share the same target site (Ser/Thr) on protein in reciprocal manner. Speaking

about AD, dysregulation of any of these PTM indicates the pathophysiological status of the AD brain. Several preceding studies were focused on understanding the crosstalk between phosphorylation and O-GlcNAcylation in disease pathophysiology as they compete for the same site (Hart, G. W., 1997).

Recent advanced proteomic that includes the selective enrichment of low -abundance O-GlcNacylated species (proteins) from a complex mixture, followed by high-technology mass spectrometric (MS) method have allowed the mapping and quantification of multiple *O-GlcNAcylation* sites (Khidekel, N., 2007). This proteomic analysis suggests that *O-GlcNAcylation* parallels phosphorylation concerning all dynamics, abundance, and impact on protein function. On the basis of these observations, we can presume that limited glucose uptake (impaired glucose metabolism) veils the occurrence of *O-GlcNAcylation* but would slightly increase the chances of phosphorylation. These results, with the agreement of our first hypothesis would further clarify the accumulation of hyperphosphorylated Tau in AD brain.

Therefore, further our interest focused on brain glucose impairment in late phase AD and to understand the crosstalk between phosphorylation and glycosylation in AD pathology. With the help of proteomic approach, in the second study we identified and validated O-GlcNAcylated proteins involved in different pathways and pathologies (i.e. Alzheimer). In our **second project,** *we highlight the synergistic relationship between phosphorylation and glycosylation to reveal the involvement of impaired brain glucose metabolism in Tau like pathology in AD brain.*

# Chapter 3: Project 1

***Loss of biliverdin reductase-A limits the oxidative stress-induced Akt-mediated inhibition of GSK-3β: implications for Alzheimer disease***

## 3.1 Material and Methods

### 3.1.1Animals

Six- and 12- month-old 3×Tg-AD male mice (n=6 per group) and their male littermates Non-Tg (n=6 per group) were used in this study. The 3×Tg-AD mice harbour 3 mutant human genes ($APP_{Swe}$, $PS1_{M146V}$, and $tau_{P301L}$) and have been genetically engineered by LaFerla and colleagues at the Department of Neurobiology and Behaviour, University of California, Irvin. Colonies of homozygous 3×Tg-AD and WT mice were established at the vivarium of Puglia and Basilicata Experimental Zooprophylactic Institute (Foggia, Italy). The 3×Tg-AD mice background strain is C57BL6/129SvJ hybrid and genotypes were confirmed by PCR on tail biopsies. The housing conditions were controlled (temperature 22°C, light from 07:00 –19:00, humidity 50%–60%), and fresh food and water were freely available. All the experiments were performed in strict compliance with the Italian National Laws (DL 116/92), the European Communities Council Directives (86/609/EEC). All efforts were made to minimize the number of animals used in the study and their suffering. Animals were sacrificed at the selected age and the hippocampus was extracted, flash-frozen, and stored at −80 °C until total protein extraction and further analyses were performed.

### 3.1.2 Human samples

Brain tissue was provided by Sanders-Brown Center on Aging of the University of Kentucky. All the studies were performed on the inferior parietal lobule (IPL) of non-disease control, MCI or AD cases. Clinical diagnosis of disease stage was made as described previously. Age and gender are listed in the Table 1. The post-mortem interval range was between 2 and

4 h and was comparable between the three groups. The degree of cognitive impairment was assessed using the Mini Mental State Examination (MMSE) (Table 1).

### 3.1.3 Cell culture and treatments

The HEK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (20 units/ml) and streptomycin (20 mg/ml), (GIBCO, Gaithersburg, MD, U.S.A.). Cells were maintained at 37°C in a saturated humidity atmosphere containing 95% air and 5% $CO_2$. Cells were seeded at density of $40x10^3/cm^2$ in 6 wells culture dishes for the subsequent treatments. In a first set of experiments HEK cells were treated with $H_2O_2$ (Sigma–Aldrich, St Louis, MO, USA) 1-500 μM for 24 h to select the best dose of $H_2O_2$ to be used in the other experiments. In a second set of experiments, aimed to demonstrate that the effects of $H_2O_2$ on GSK-3β were really due to the increase of intracellular oxidative stress, HEK cells were pre-treated with bilirubin (BR) 0.1-5 μM for 2 h, then the medium was discarded and cells were treated with 100 μM $H_2O_2$ μM for a further 24 h. Bilirubin (Sigma–Aldrich, St Louis, MO, USA) was dissolved in sodium hydroxide (0.1 M) at a concentration of 10 mM and further diluted in double-distilled water as previously described(Mancuso C, 2008). Bilirubin solutions were freshly prepared before each experiment and protected from light. In a third set of experiments to test the effects produced by silencing *BVR-A*, HEK cells were seeded at density of $40x10^3/cm^2$ in 6 wells culture dishes. After 24 h medium has been replaced with DMEM with 10% FBS, without antibiotics. Following, cells have been transfected with 10 pmol of a small-interfering RNA (siRNA) for BVR-A (Ambion, Life Technologies, LuBioScience

GmbH, Lucerne, Switzerland, #4392420) using Lipofectamine® RNAiMAX reagent (Invitrogen, Life Technologies, LuBioScience GmbH, Lucerne, Switzerland, #13778-030) according to the manufacturer's protocol, and then treated with 100 μM $H_2O_2$ μM for 24 h. At the end of each treatment, cells were washed twice with PBS, collected and proteins were extracted as described below.

### 3.1.4 MTT assay

Cell viability was measured by the MTT reduction assay. Briefly, HEK cells were plated in 96-well microplates at $1.5\times10^4$/well, eight replicas per condition. After overnight incubation, the medium was replaced with fresh medium containing $H_2O_2$ at concentrations ranging from 0–500 μM and further incubated for 24 h. Then, MTT was added at a final concentration of 1.25 mg/ml. After 2 h of incubation at 37°C, medium was discarded, and the reduced insoluble dye was extracted with 0.04 N HCl/isopropanol. Cell viability was evaluated by the absorbance ($A_{540–750}$) measured in a microplate reader (Labsystem Multiscan MS).

### 3.1.5 Samples preparation

Total protein extracts were prepared in RIPA buffer (pH 7.4) containing Tris-HCl (50mM, pH 7.4), NaCl (150mM), 1% NP-40, 0.25% sodium deoxycholate, EDTA (1mM), 0.1% SDS, supplemented with proteases inhibitors [phenylmethylsulfonyl fluoride (PMSF, 1mM), sodium fluoride (NaF, 1mM) and sodium orthovanadate ($Na_3VO_4$, 1mM)]. Before clarification, brain tissues were homogenized by 20 passes with a Wheaton tissue homogenizer. Both brain tissues homogenates and collected cells were clarified by centrifugation for 1 hr at 16,000× g, 4°C. The supernatant was

then extracted to determine the total protein concentration by the Bradford assay (Pierce, Rockford, IL)

**3.1.6 Slot Blot analysis**

For the analysis of total Protein Carbonyls (PC) levels hippocampal total protein extract samples (5 µl), were derivatized with 5µl of 10mM 2,4-dinitrophenylhydrazine (DNPH) (OxyBlot™ Protein Oxidation Detection Kit, Merck-Millipore, Darmstadt, Germany) in the presence of 5 µl of 10% sodium dodecyl sulfate (SDS) for 20 min at room temperature (25° C). The samples were then neutralized with 7.5 µl of neutralization solution (2M Tris in 30% glycerol) and loaded onto nitrocellulose membrane as described below.

For total (i) protein-bound 4-hydroxy-2-nonenals (HNE) and (ii) 3-nitrotyrosine (3-NT) levels: hippocampal total protein extract samples (5 µl), 12% SDS (5 µl), and 5 µl modified Laemmli buffer containing 0.125 M Tris base, pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol were incubated for 20 min at room temperature and then loaded onto nitrocellulose membrane as described below.

Proteins (250 ng) were loaded in each well on a nitrocellulose membrane under vacuum using a slot blot apparatus. The membrane was blocked in blocking buffer (3% bovine serum albumin) in PBS 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 1 h and incubated with an anti-2,4-dinitrophenylhydrazone (DNP) adducts polyclonal antibody (1:100, EMD Millipore, Billerica, MA, USA, #MAB2223) or HNE polyclonal antibody (1:2000, Novus Biologicals, Abingdon, United Kingdom, #NB100-63093) or an anti 3-NT polyclonal antibody (1:1000, Santa Cruz, Santa Cruz, CA, USA, #sc-32757) in PBS containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 for 90 min. The membrane was washed in PBS following

primary antibody incubation three times at intervals of 5 min each. The membrane was incubated after washing with an anti-rabbit IgG alkaline phosphatase secondary antibody (1:5000, Sigma–Aldrich, St Louis, MO, USA) for 1 h. The membrane was washed three times in PBS for 5 min each and developed with Sigma fast tablets (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate [BCIP/NBT substrate]). Blots were dried, acquired with Chemi-Doc MP (Bio-Rad, Hercules, CA, USA) and analyzed using Image Lab software (Bio-Rad, Hercules, CA, USA). No non-specific binding of antibody to the membrane was observed.

### 3.1.7 Western blot

For western blots, 20 µg of proteins were resolved on Criterion TGX Stain-Free 4-15% 18-well gel (Bio-Rad Laboratories, #5678084) in a Criterion large format electrophoresis cell (Bio-Rad Laboratories, #1656001) in TGS Running Buffer (Bio-Rad Laboratories, #1610772). Immediately after electrophoresis, the gel was then placed on a Chemi/UV/Stain-Free tray and then placed into a ChemiDoc MP imaging System (Bio-Rad Laboratories, #17001402) and UV-activated based on the appropriate settings with Image Lab Software (Bio-Rad Laboratories) to collect total protein load image. Following electrophoresis and gel imaging, the proteins were transferred via the TransBlot Turbo semi-dry blotting apparatus (Bio-Rad Laboratories, #1704150) onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA, #162-0115) and membranes were blocked with 3% bovine serum albumin in 0.5% Tween-20/Tris-buffered saline (TTBS) and incubated overnight at 4 °C with the following antibodies: anti-BVR-A (1:5000, abcam, Cambridge, United Kingdom, #ab90491), anti-BVR-A (1:1000, Sigma-Aldrich, St Louis, MO, USA, #B8437), anti-Akt (1:1000, 1:1000, Bio-Rad Laboratories,

#vma00253k), anti-phospho(Ser473)-Akt (1:1000, Cell Signaling, Bioconcept, Allschwill, Switzerland, #193H12), anti-GSK-3β (1:500 Santa Cruz, Santa Cruz, CA, USA,#sc-9166 ), anti-phospho(Ser9)-GSK-3β (1:500 Santa Cruz, Santa Cruz, CA, USA,#sc-11757), anti-phospho(Tyr216)- GSK-3β (1:500 Santa Cruz, Santa Cruz, CA, USA,#sc-135653), anti-Tau (1:1000, Santa Cruz, Santa Cruz, CA, USA, #sc-5587), anti-phospho(Ser404)-Tau (1:500, Santa Cruz, Santa Cruz, CA, USA, #sc-12952), anti-phospho(Ser416)-Tau(1:1000,Cell Signaling, Bioconcept, Allschwill, Switzerland, #15013), anti 3-NT polyclonal antibody (1:1000, Santa Cruz, Santa Cruz, CA, USA, #sc-32757). After 3 washes with TTBS the membranes were incubated for 60 min at room temperature with anti-rabbit/mouse/goat IgG secondary antibody conjugated with horseradish peroxidase (1:5000; Sigma–Aldrich, St Louis, MO, USA). Membranes were developed with Clarity ECL substrate (Bio-Rad Laboratories, #1705061) and then acquired with Chemi-Doc MP (Bio-Rad, Hercules, CA, USA) and analyzed using Image Lab software (Bio-Rad, Hercules, CA, USA) that permits the normalization of a specific protein signal with the β-actin signal in the same lane or total proteins load.

### 3.1.8 Immunoprecipitation

The Immunoprecipitation experiment was performed using SureBeads™ Protein A Magnetic Beads (BioRad SureBeads™ Protein G #161-4023). Briefly,100 µl (100 µg) Surebeads were magnetized and washed with a solution of PBS/Tween20 0.1% v/v. as per manufacturer instructions, and then incubated for 30 min with 1 µg anti- GSK-3β polyclonal antibody (1:200, Santa Cruz, Santa Cruz, CA, USA,#sc-9166 ) at room temperature. After incubation, beads were washed 3 times with PBS/Tween20 0.1% v/v.

After that, the beads/antibody complexes were recovered and incubated with 100 µg of protein extracts for 1 h at room temperature. Immunoprecipitated complexes were then pulled down and washed 3 times with PBS/Tween20 0.1% v/v. Immunoprecipitated GSK-3β was recovered by re-suspending the pellets in 2x Laemeli sample buffer and electrophoresing them on 12% gels, followed by western blot analysis. Total GSK-3β was used as a loading control.

### 3.1.9 Immunofluorescence

For immunocytofluorescent staining, cells were plated on coated glass coverslips for 24 hours. Cells were washed three times with PBS and fixed in 4% paraformaldehyde for 30 min. After fixation, cells were washed twice with PBS and permeabilized for 30 min with permeabilization buffer composed by 0.2% Triton-X100 and PBS. Cells were blocked for 1 hour with a solution containing 3% normal goat serum and 0.2% Triton X-100 in PBS and then were incubated overnight at 4°C with following antibodies: anti-phospho(Ser404)-Tau (1:100, abcam, Cambridge, United Kingdom #ab92676), anti-phospho(Ser9)-GSK-3β (1:500 Santa Cruz, Santa Cruz, CA, USA,#sc-11757).Cells were washed with PBS and then incubated with Alexa Fluor -488nm and -594nm secondary antibodies (Invitrogen Corporation, Carlsbad, CA, USA) at 1:1000 for 1 h at room temperature. Cells were then washed again and incubated with DAPI solution. For each group of treatment staining was performed by omitting primary antibodies to establish nonspecific background signal. Cover slips were placed using a drop of Fluorimount (Sigma-Aldrich, St Louis, MO, USA).

All slides were imaged using Zeiss AXiocam (Carl Zeiss, Oberkochen, Germany). All immunolabeling acquisition intensities, field

sizes, and microscopy settings were kept consistent across all images. Image montages for Figures were collated in Illustrator and Photoshop Cs6 (Adobe System, San Josè, CA, USA) software programs and were based upon cells that most closely approximated the group means.

### 3.1.10 Statistical Analysis

Statistical analysis of all data obtained from image lab software for western blot were performed using Student's t-test. Significance for all data were accepted if the p value $< 0.05$ (*), 0.01(**) and 0.001(***). All data presented in this study are presented as the mean ± SEM. All statistical analysis was performed using GraphPad Prism 6.0 software.

## 3.2 Result

Earlier, it is discovered by Butterfield and group that AD brain consists of the higher level of OS and several dysfunctional proteins. BVR-A is one of the enzymes play a critical role in the antioxidant system in AD brain, but at the severe level of pathology BVR-A shows impairment

As mentioned earlier that BVR-A has the pleiotropic functions in PI3K/Akt and Insulin signaling but also, it's impairment has been significantly observed in AD brain at early onset (Barone, E., 2016). While Impaired BVR-A in AD brain is associated with increased oxidative modified proteins that could alter their functions at molecular levels, interestingly BVR-A also shows scaffold protein-like properties to interact with other proteins in signaling pathways and regulate their functionality by means of activation/inactivation. Further to understand its involvement at protein-protein interaction levels, we used the inferior parietal lobule (IPL) region of human MCI and AD brain and hippocampus of 3x Tg-AD mice at 6 M and 12 M for our study to decipher the oxidative stress-mediated GSK-3β inhibition while BVR-A is impaired. In this study, we have investigated the role of BVR-A as a scaffold protein to explain the molecular mechanism in which oxidative stress mediated GSK-3β inhibition is regulated by BVR-A/Akt/ GSK-3β axis.

### 3.2.1 Early reduction of BVR-A protein levels is associated with reduced GSK-3β inhibition independently on Akt activation in the hippocampus of 3xTg-AD mice.

We evaluated changes of BVR-A and oxidative stress levels and how they were associated with alterations of (i) Akt protein and activatory phosphorylation (Ser473) levels as well as (ii) changes of GSK-3β protein levels and inhibitory (Ser9) and activatory (Tyr216) phosphorylations in the hippocampus of young (6 months) and old (12 months) 3xTg-AD mice compared to Non-Tg mice. Furthermore, downstream GSK-3β we looked at changes of Tau protein levels along with Tau phosphorylation at Ser404 (target of GSK-3β( Leroy, A., 2010; Hanger, D. P., 2011) and Ser416 (non-target of GSK-3β  β (Leroy, A., 2010; Hanger, D. P., 2011) residues.

We found that BVR-A protein levels were significantly reduced both at 6 months (-57%, $p<0.05$, Figure 14 B) and 12 months (-45%, $p<0.05$, Figure 14 B) of age, in the hippocampus of 3xTg-AD mice with respect Non-Tg mice. In parallel, increased oxidative and nitrosative stress levels were observed both at 6 months (3-NT, +11%, $p<0.05$, Figure 14 C) and 12 months (PC, +10%, $p<0.05$; 3-NT +47% $p<0.001$; 4-HNE , +20%, $p<0.001$, Figure 14 D) of age in the hippocampus of 3xTg-AD with respect to Non-Tg mice.

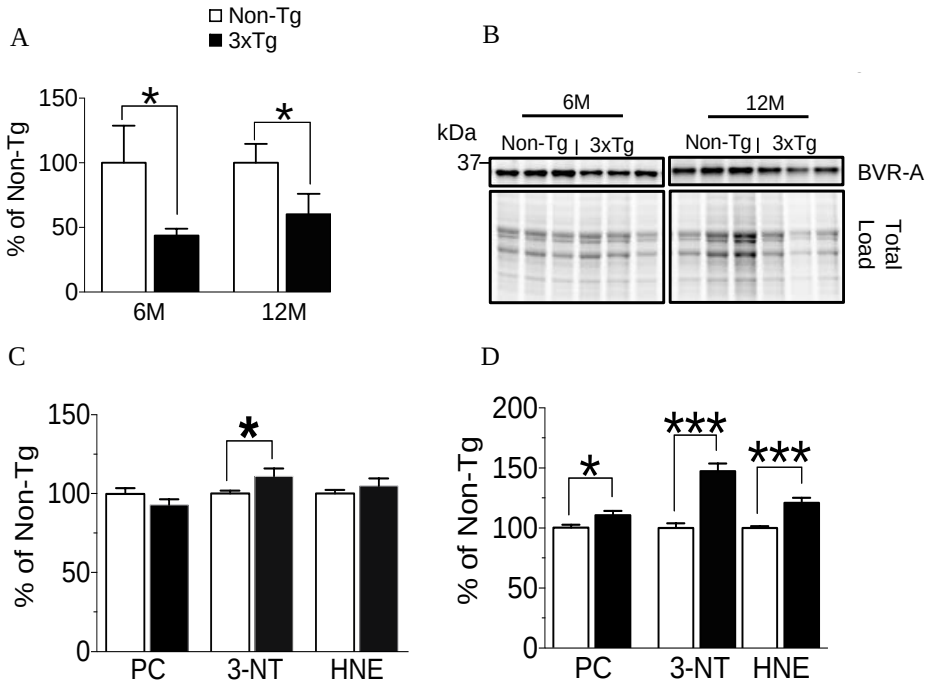



**Figure 14: Early impairment of BVR-A and increased oxidative/nitrosative stress levels in hippocampus of 3xTg mice.** A) Densitomteric analysis were evaluated for total BVR-A levels in the hippocampus of 3x Tg mice and non Tg at 6M and 12M. B) immunoblot image of BVR-A levels at 6 M and 12 M in 3xTg mice.Densitometric analysis of oxidative stress markers protein carbonyl (PC), 3-nitotyrosine (3-NT), 4-Hydroxy 2- noenal (HNE) levels were evaluated in the hippocampus of 3xTg mice at 6M C), and D) at 12M age, comparative with their age matched controls. All densitometric analysis values are percentage of controls (non Tg) set as 100%. Data are presented as means ± SEM (n=6 mice/group). All the data were significantly analysed with student t-test. *p>0.05, **p<0.01, ***p<0.001.

The analyses of Akt protein revealed no significant changes at 6 months of age between 3xTg-AD and Non-Tg mice (Figure 15 B), while a significant increase of Akt protein levels (+23%, $p<0.05$) along with a significant decrease of Akt activation (Ser 473/Akt, -22%, $p<0.05$) were

observed in the hippocampus of 3xTg-AD with respect to Non-Tg mice at 12 months of age (Figure 15 B).

Downstream of Akt, no changes of GSK-3β protein levels were observed either at 6 months or 12 months of age in 3xTg-AD with respect to Non-Tg mice (Figure 15 C). GSK-3β inhibition (Ser9/GSK-3β) was significantly reduced both at 6 months (-26 %, $p<0.05$, Figure 15 C) and at 12 months (-37%, $p<0.05$, Figure 15 C), without consistent changes of GSK-3β activation (Tyr216/GSK-3β) (Figure 15 C). At the net of these changes we found that GSK-3β inhibition/activation ratio (Ser9/Tyr216) was significantly decreased at 12 months (-51%, $p<0.001$, Figure 15 C) in 3xTg-AD with respect to Non-Tg mice.

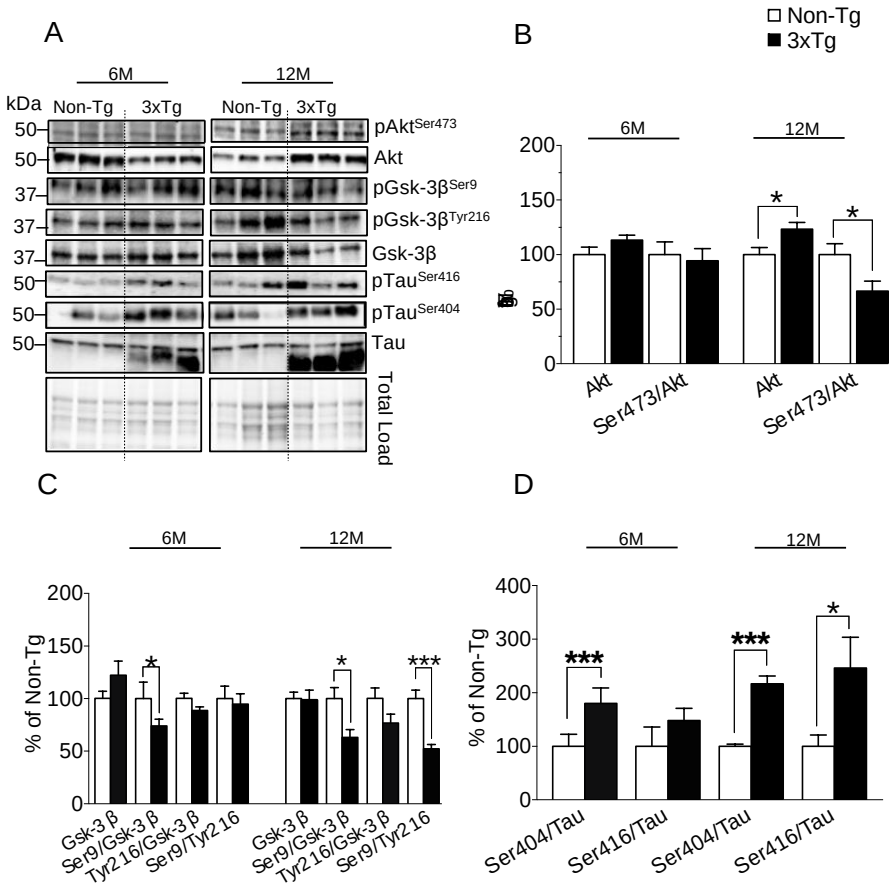


**Figure 15**: Reduced BVR-A levels are associates with i) Gsk-3 beta inhibition ii) Tau hyperphosphorylation iii) Akt inhibition in hippocampus of 3x Tg -AD mice. A) representative Immunoblot images. Densitometric analysis were evaluated for A) total Akt level, p-Ser 473/Akt at both 6M and 12M. B) total Gsk-3 beta level, p-Ser9/Gsk-3 beta, p-Tyr216/Gsk-3 beta and respective ratio of p-Ser9/p-Tyr216 expression over total p-Try216/Gsk-3 at 6 months and 12. D) total tau levels p-Ser 404/Tau, p-Ser416/Tau both at 6M and 12M. All densitometric values are percentage of controls (Non-Tg), set as 100%. Data are presented as means ± SEM (n=6 mice/group). All the data were significantly analysed with student t-test. *p>0.05, **p<0.01, ***p<0.001.

With regard Tau protein, we found that Tau Ser404 phosphorylation was significantly increased both at 6 months (Ser404/Tau, +60%, $p<0.05$, Figure 15 D) and 12 months (+116%, $p<0.01$, Figure 15 D) of age, while Tau Ser416 phosphorylation was significantly increased only at 12 months (Ser416/Tau, +146%, $p<0.05$, Figure 15 D) in the hippocampus of 3xTg-AD with respect to Non-Tg mice.

Overall, these results suggest that reduced BVR-A protein levels are associated with reduced GSK-3β inhibition in the hippocampus of 3xTg-AD mice. This phenomenon is of particular interest at 6 months of age, when reduced GSK-3β inhibition occurs independently from changes of Akt activation and results in an early increased Tau Ser404 phosphorylation.

### 3.2.2 Reduced BVR-A protein levels are associated with reduced GSk-3β inhibition in post-mortem samples from human MCI inferior parietal lobule.

To test whether the above-reported changes might be considered pathological features of AD, we analyzed inferior parietal lobule (IPL) post-mortem samples collected from amnestic mild cognitive impairment (MCI, a prodromal stage of AD (Petersen, R. C. 2003), AD and age-matched control

subjects. The choice of IPL was based on previous studies from the Butterfield group, showing that this brain region was characterized by increased oxidative stress along with increased Akt phosphorylation in both MCI and AD subjects although the inhibition of GSK-3β was evident only in AD subjects with respect to age-matched controls (Tramutola, A., 2015; Aluise, C. D., 2011; Sultana, R., 2010).

| *Ref. N°* | Groups | Age | Sex | Race | APOE | MMSE | PMI |
|---|---|---|---|---|---|---|---|
| *1* | **AD** | 86 | M | White | 3/4 | 9 | 3.25 |
| *2* | **AD** | 87 | F | White | 3/3 | 0 | 2.67 |
| *3* | **AD** | 95 | F | White | 3/3 | 17 | 2.1 |
| *4* | **AD** | 90 | M | White | 3/4 | 12 | 3.25 |
| *5* | **AD** | 93 | F | White | 3/3 | 0 | 2.75 |
| *6* | **AD** | 83 | M | White | NA | 15 | 2.77 |
| | *Average* | **89** | | | | **8.8** | **2.8** |
| | *SE* | **1.2** | | | | **3.4** | **0.4** |
| *7* | **MCI** | 88 | F | White | 3/3 | 28 | 3 |
| *8* | **MCI** | 87 | M | White | 3/3 | 27 | 2,75 |
| *9* | **MCI** | 96 | F | White | 3/3 | 27 | 2,42 |
| *10* | **MCI** | 91 | M | White | 3/2 | 28 | 2,33 |
| *11* | **MCI** | 84 | M | White | 3/4 | 24 | 3,5 |
| *12* | **MCI** | 84 | F | White | 3/3 | 26 | 2,5 |
| | *Average* | **88.3** | | | | **26.7** | **2.8** |
| | *SE* | **1.9** | | | | **0.6** | **0.2** |
| *13* | **Control** | 85 | F | White | 3/3 | 30 | 2.12 |
| *14* | **Control** | 87 | M | White | 3/3 | 29 | 2.42 |
| *15* | **Control** | 92 | M | White | 3/3 | 30 | 3.75 |
| *16* | **Control** | 88 | M | White | 3/3 | 30 | 2.08 |
| *17* | **Control** | 84 | F | White | 3/3 | 30 | 2.42 |
| | *Average* | **87.2** | | | | **29.8** | **2.5** |
| | *SE* | **1.3** | | | | **0.2** | **0.3** |

**Table 1**: Demographic information of Human AD, MCI and age matched control's autopsy

Reduced BVR-A protein levels in both MCI (-20%, $p<0.01$, Figure 16 B) and AD (-14%, Figure 16 B) subjects with respect to age-matched controls (Figure 3 B) were observed. No significant changes were observed for Akt protein levels among the 3 groups of subjects, although a trend of increased Akt activation was observed in both MCI and AD subjects with respect to age-matched controls (Figure 16 C).

Downstream Akt, we found a significant reduction of GSK-3β protein levels (-33%, $p<0.05$, Figure 16 D) along with a significant increase of GSK-3β inhibition (Ser9/GSK-3β, +33%, $p<0.05$, Figure 16 D) only in AD subjects with respect to age-matched controls. No significant changes were observed for GSK-3β activation (Try216/GSK-3β) in MCI and AD subjects with respect to controls. Taken together, a significant reduction of the GSK-3β inhibition/activation ratio in MCI subjects (-57%, $p<0.05$, Figure 16 D) was observed, whereas a significant increased inhibition/activation ratio was found in AD subjects (+161%, $p<0.05$, Figure 16 D), with respect to age-matched controls.

Total Tau protein levels were significantly reduced in both MCI (-23%, $p<0.05$, Figure 16 E) and AD (-66%, $p<0.01$, Figure 16 E) subjects, while Tau phosphorylation levels (Ser404/Tau) were significantly increased in MCI (+89.1%, $p<0.05$, Figure 16 E) and in AD (+ 212%, $p<0.01$, Figure 16 E) with respect to age-matched controls.

These results suggest that reduced BVR-A levels are associated with an increased activation of GSK-3β, which favors Tau Ser404 phosphorylation also in humans. This effect seems prominent during the early stage of the pathology, i.e., MCI, since similar alterations in AD were not observed

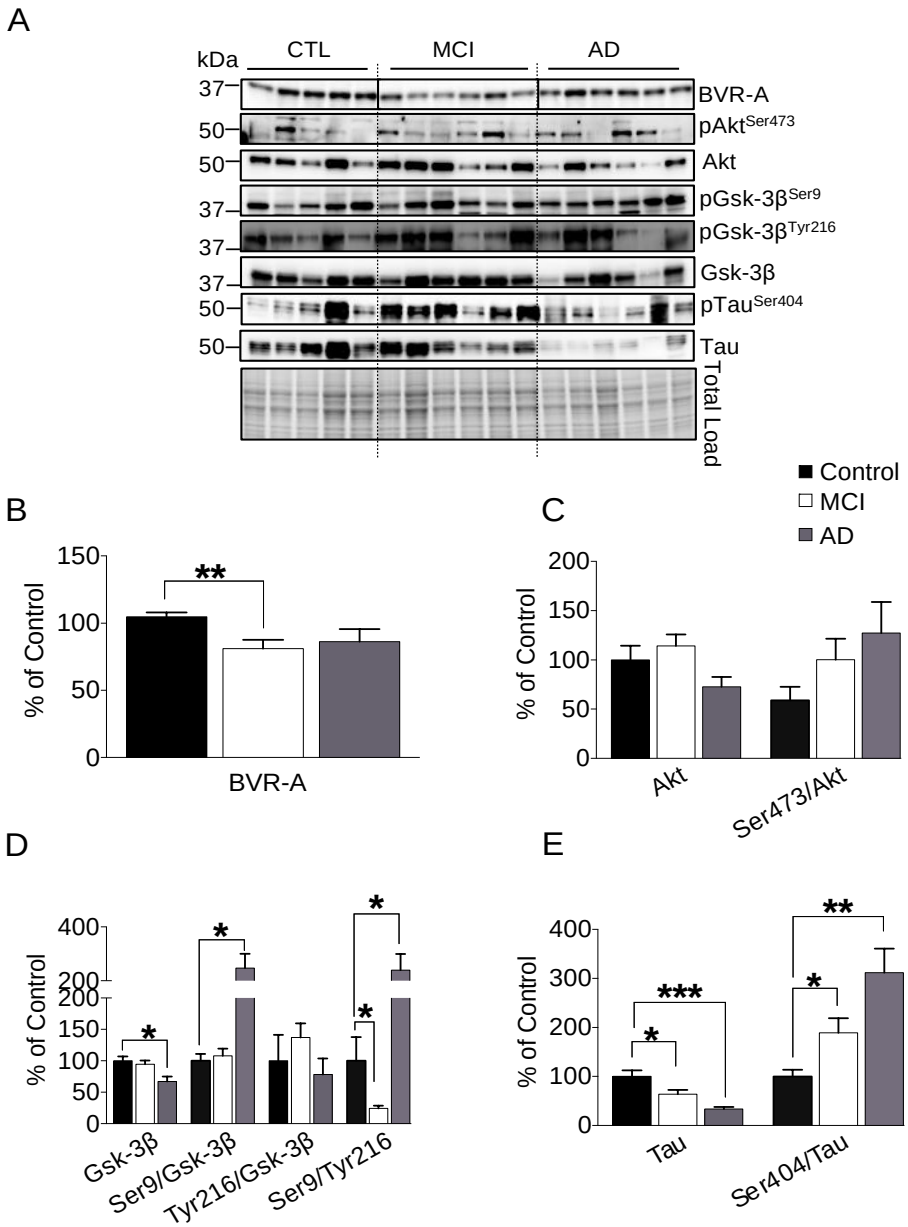



**Figure 16**: Reduced BVR-A levels are associated with i) Gsk-3 beta inhibition ii) increased Tau phosphorylation in inferior partial lobule (IPL) of human AD brains**.** Post mortem human IPL (inferior parietal lobule) were received from control, MCI and AD subjects as described in the table (1). A) representative immunoblot image. Densitometric analysis for B) total BVR-A levels in all subjects C) Total Akt and p-Ser 473/Akt levels. Activation of

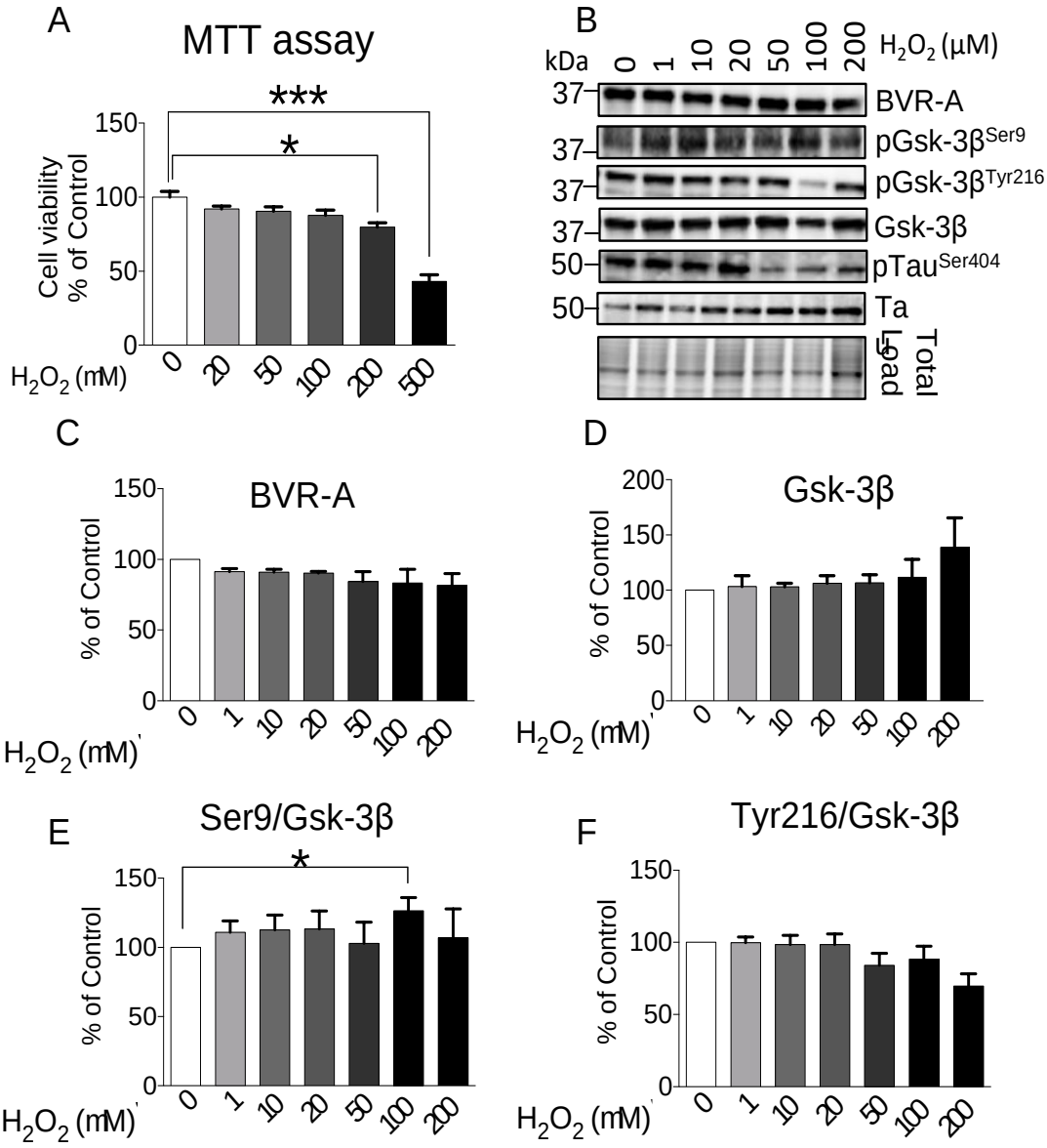
Akt does not show any significant changes in control, MCI and AD subjects D) Total Gsk-3 beta levels, p-Ser 9/Gsk-3 beta levels, p-Tyr216/Gsk-3 beta levels and respective ratio of p-Ser9/p-Tyr216 expression over total p-Tyr216/Gsk levels. E) Total Tau levels and p-Ser (404)/Tau levels in all the subjects.All densitometric analysis of the phosphorylated proteins are shown as ratio between total protein and phosphorylated form, considering the fact that how much phosphorylated form of given proteins are expressed over total protein expression. Densitometric values are percentage of controls set as 100%. Data are presented as means ± SEM (n=5-6/group). All the data were significantly analysed with student t-test. *p>0.05, **p<0.01 ***p<0.00

### 3.2.3 Loss of BVR-A promotes oxidative stress-mediated GSK-3β activation *in vitro*.

To clarify the molecular mechanisms responsible for the increase of GSK-3β activation during the early phase of AD, we used HEK cells to evaluate whether reduced BVR-A protein levels limit the oxidative stress-induced Akt-mediated inhibition of GSK-3β.

In a preliminary set of experiments, we tested the effects of increased oxidative stress levels on (i) cell viability (ii) BVR-A protein levels, (iii) GSK-3β levels and activation and (iv) Tau Ser404 phosphorylation levels by exposing HEK cells to increasing doses of $H_2O_2$ (1-500 µM) for 24 h. Among the tested doses we selected 100 µM found to promote a significant increase of GSK-3β inhibition along with reduced Tau phosphorylation without affecting cell viability (Figure 17).

We confirmed the pro-oxidant effect mediated by 100 µM $H_2O_2$ by showing that $H_2O_2$-treated HEK cells were characterized by increased 3-NT levels (+31%, $p<0.01$, Figure 18 A) and increased GSK-3β Ser9 phosphorylation (-29%, $p<0.05$, Figure 18 D) and that pre-treatment with increasing doses of bilirubin (BR, 0.1-5 µM for 2 h) – one of the most potent endogenous antioxidant (Barone, E., 2009; Mancuso, C., 2012) – abolished the effects of $H_2O_2$ (Figures 18 A, D and F)


A
MTT assay
***
*
Cell viability
% of Control
150
100
50
0
H2O2 (mM)
0
20
50
100
200
500
B
kDa
0
1
10
20
50
100
200
H2O2 (μM)
37
BVR-A
37
pGsk-3βSer9
37
pGsk-3βTyr216
37
Gsk-3β
50
pTauSer404
50
Ta
Total Load
C
BVR-A
% of Control
150
100
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H2O2 (mM)
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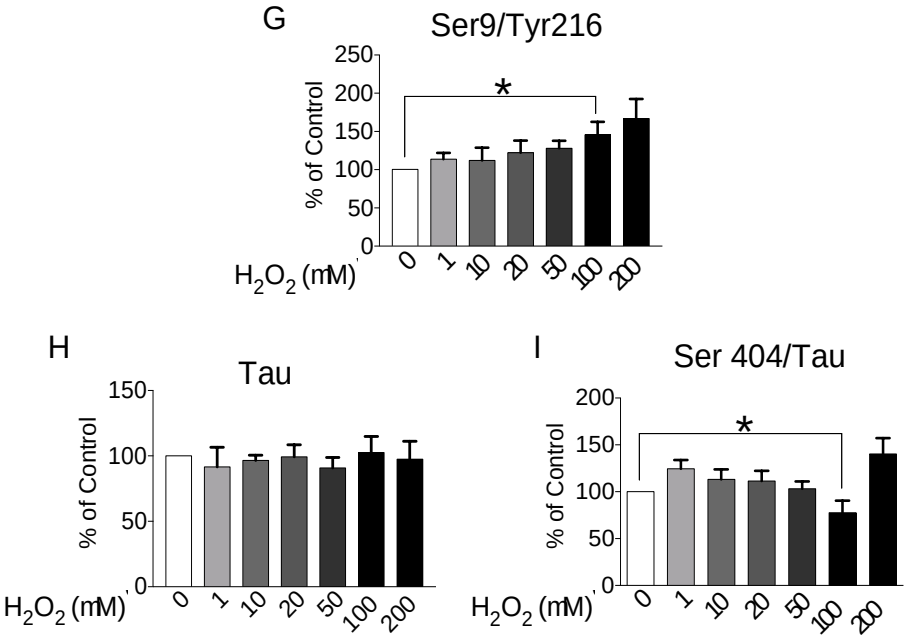



**Figure 17**. $H_2O_2$ treatment promotes the inhibition of GSK-3β in HEK cells. (A) MTT assay. In (B) representative western blot images and densitometric evaluation of (C) BVR-A protein levels; (D) GSK-3β protein levels; (E) GSK-3β Ser9 phosphorylation (F) GSK-3β Tyr216 phosphorylation, (G) GSK-3β Ser9 /Tyr216 ratio; (H) Tau protein levels; and (I) Tau Ser404 phosphorylation evaluated in HEK cells (n=3 independent cultures/group) treated with $H_2O_2$ (1-200 μM, for 24 h). BVR-A, GSK-3β and Tau protein levels were normalized per total protein load. GSK-3β-, and Tau-associated phosphorylations were normalized by considering the respective protein levels and are expressed as the ratio between the phosphorylated form and the total protein levels: Ser9/GSK-3β, Tyr216/GSK-3β, Ser404/Tau. All densitometric values are given as percentage of Controls cells set as 100%. Data are presented as means ± SEM, *p>0.05, **p<0.01, and ***p<0.001 (Student's t-test).

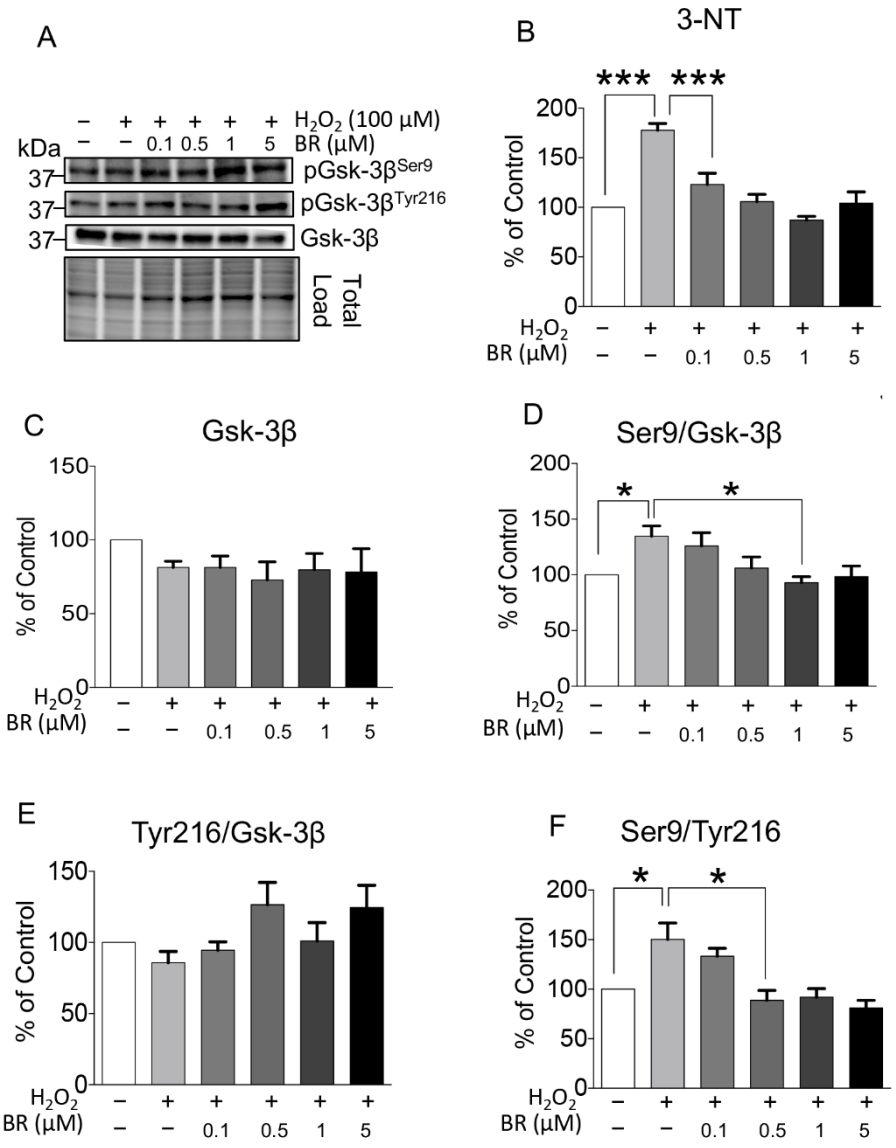



**Figure 18**. Bilirubin prevents the increase of oxidative stress levels in $H_2O_2$-treated cells. (A) Densitometric evaluation of 3-nitrotyrosine (3-NT) levels in HEK cells pre-treated with bilirubin (BR) 0.1-5 μM for 2 h and then exposed to 100 μM $H_2O_2$ for 24 h (n=3 independent cultures/group). In (B) Representative western blot images and densitometric evaluation of (C) GSK-3β protein levels; (D) GSK-3β Ser9 phosphorylation and (E) GSK-3β Tyr216 phosphorylation and (F) GSK-3β Ser9 /Tyr216 ratio evaluated in HEK cells pre-treated with

bilirubin (BR) 0.1-5 μM for 2 h and then exposed to 100 μM $H_2O_2$ for 24 h (n=3 independent cultures/group). GSK-3β protein levels were normalized per total protein load. GSK-3β-associated phosphorylations were normalized by taking into account total GSK-3β protein levels and are expressed as the ratio between the phosphorylated form and the total protein levels: Ser9/GSK-3β, Tyr216/GSK-3β. All densitometric values are given as percentage of Controls cells set as 100%. Data are presented as means ± SEM, *p>0.05, **p<0.01, and ***p<0.001 (Student's t-test).

Starting from these observations, we evaluated the effect of oxidative stress on GSK-3β and Tau protein in cells lacking BVR-A. Indeed, this condition would mimic what we have observed both in mice and humans. The results show that while 100 μM $H_2O_2$ for 24 h promote increased Akt Ser473 phosphorylation (+12%, $p<0.05$ Figure 19 C) along with increased GSK-3β inhibition (+27%, $p<0.01$, Figure 19 D) and a significant reduction of Tau Ser404 phosphorylation (-36%, $p<0.05$, Figure 19 G) in control cells, these changes do not occur in cells lacking BVR-A. Indeed, in siRNA-treated cells, 100 μM $H_2O_2$ for 24 h led to a significant reduction of Akt Ser473 phosphorylation (-27%, $p<0.05$, Figure 19 C), a concomitant decreased GSK-3β inhibition (-47%, $p<0.01$, Figure 19 D) and a significant increased Tau Ser404 phosphorylation (+28%, $p<0.001$ Figure 19 G), with respect to control cells treated with $H_2O_2$. No changes were found for total protein levels (Figure 20).

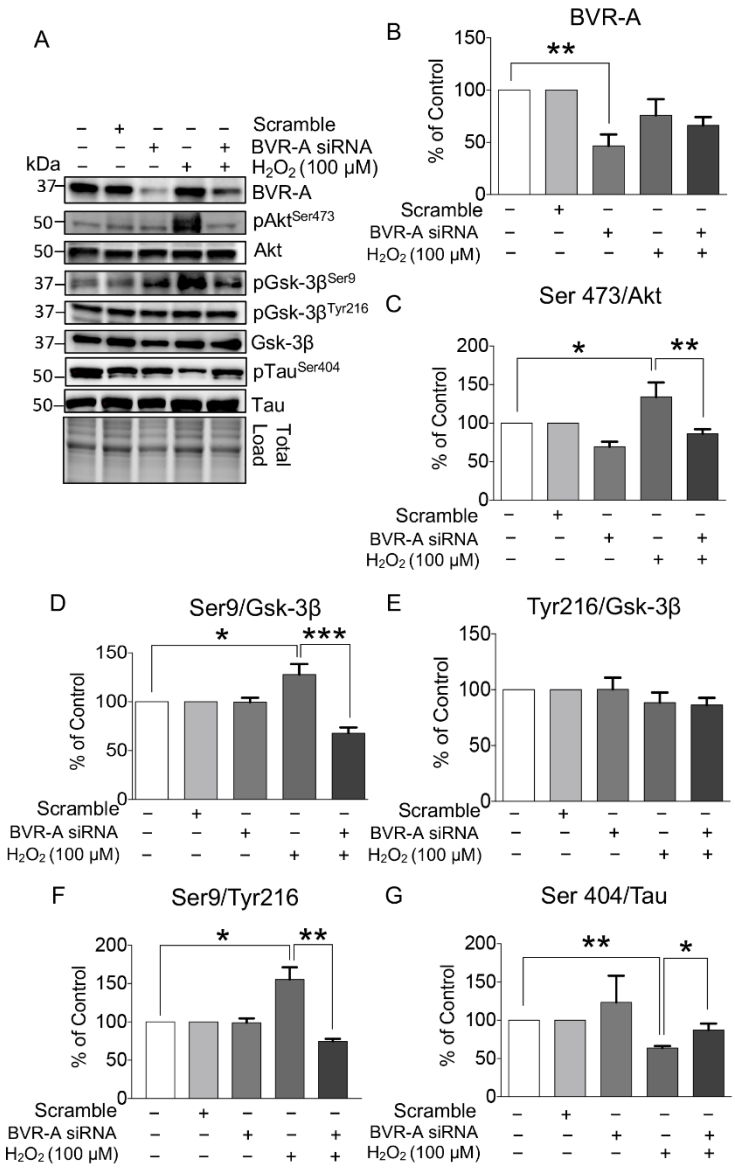



**Figure 19**. Loss of BVR-A and increased oxidative stress levels exert a synergistic effect in mediating GSK-3β activation and Tau phosphorylation in HEK cells. (A) Representative western blot images and densitometric evaluation of **(B)** BVR-A protein levels**; (C)** Akt Ser473 phosphorylation; **(D)** GSK-3β Ser9 phosphorylation **(E)** GSK-3β Tyr216 phosphorylation, **(F)** GSK-3β Ser9 /Tyr216 ratio; and **(E)** Tau Ser404 phosphorylation evaluated in HEK cells (n=3 independent cultures/group) treated with $H_2O_2$ (100 μM, for 24 h) in the presence or not of BVR-A, whose silencing has been obtained through the use of a

specific si-RNA. BVR-A protein levels were normalized per total protein load. Akt-, GSK-3β-, and Tau-associated phosphorylations were normalized by taking into account the respective protein levels and are expressed as the ratio between the phosphorylated form and the total protein levels: Ser473/Akt, Ser9/GSK-3β, Tyr216/GSK-3β, Ser404/Tau. All densitometric values are given as percentage of Controls cells set as 100%. Data are presented as means ± SEM, *p>0.05, **p<0.01, and ***p<0.001 (Student's t-test).

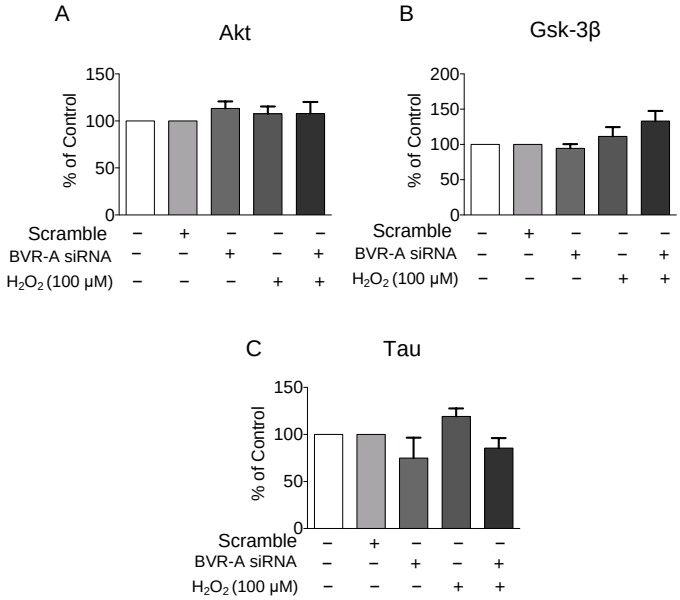



**Figure 20.** Densitometric evaluation of **(A)** Akt **(B)** GSK-3β and **(C)** Tau protein levels evaluated in HEK cells (n=3 independent cultures/group) treated with $H_2O_2$ (100 µM, for 24 h) in the presence or not of BVR-A, whose silencing has been obtained through the use of a specific si-RNA. Protein levels were normalized per total protein load. All densitometric values are given as percentage of Controls cells set as 100%. Data are presented as means ± SEM.

Reduced GSK-3β inhibition and increased Tau Ser404 phosphorylation in cells lacking BVR-A and treated with $H_2O_2$ for 24 h were also confirmed by immunofluorescence analyses (Figure 21).

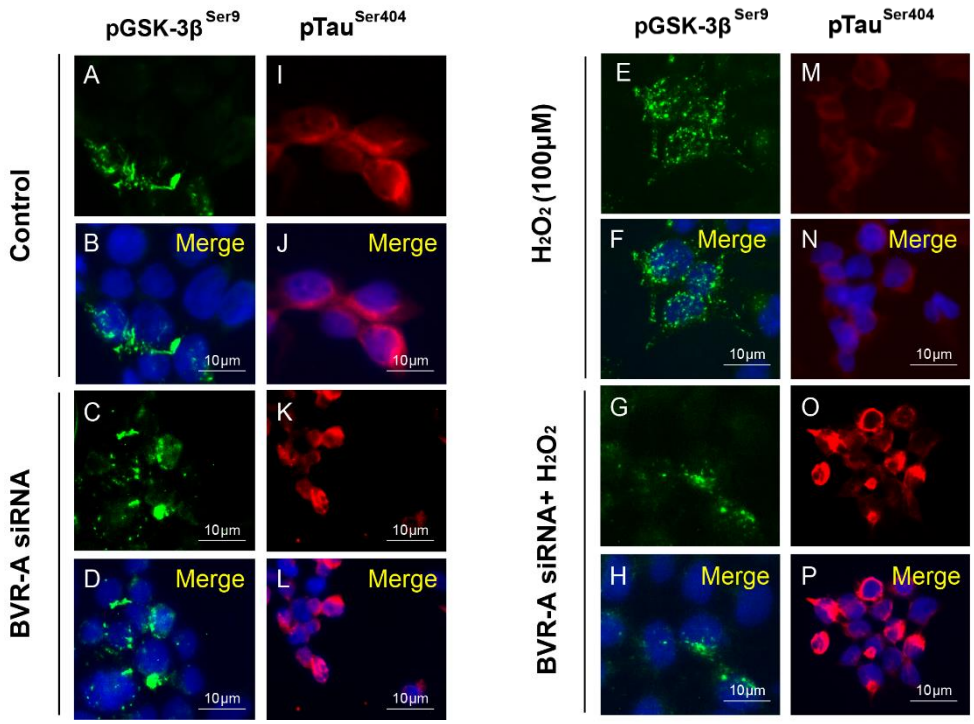



**Figure 21**. Reduced GSK-3β inhibition along with increased Tau Ser404 phosphorylation in response to increased oxidative stress in HEK cells. Representative immunofluorescence images (40X objective) from fixed (4% PAF) HEK cells stained with anti-pGSK-3 Ser9 (green) and pTauSer404 (red) in the following conditions: control (A-B and I-J); BVR-A siRNA (C-D and K-L); H2O2 (100 µM, for 24 h) (E-F and M-N); and BVR-A siRNA + 100 µM H2O2 (G-H and O-P). DAPI (blue) was used to identify cell nuclei. All images represent on the scale bar 10 µm.

### 3.2.4 Reduced BVR-A protein levels impair the interaction between Akt and GSK-3β

While the reduction of GSK-3β Ser9 phosphorylation observed in old 3xTg-AD mice (12 months) and in siRNA-treated cells exposed to $H_2O_2$ could be explained, at least in part, through the parallel reduction of Akt activation, it remained to understand whether BVR-A could play a role in the

uncoupling of the Akt/GSK-3β axis observed in young 3xTg-AD mice and in MCI brain. Moreover, examination of the results collected in siRNA-treated cells and exposed to $H_2O_2$, revealed that an additional mechanism had to exist, other than the mere reduction of Akt activation. Indeed, the extent of the reduction of GSK-3β inhibition was much higher than the reduction of Akt activation in siRNA-treated cells exposed to $H_2O_2$ with respect to similarly exposed control cells.

For that reason, based on the well-known role for BVR-A to work as a scaffold protein (Triani, F., 2018; Kapitulnik, J., 2009; Miralem, T., 2016; Gibbs, P. E., 2012a), we hypothesized that reduced BVR-A protein levels would impair the interaction between Akt and GSK-3β under oxidative stress conditions.

We observed that $H_2O_2$ treatment in HEK cells promotes the formation of the Akt/GSK-3β complex (+50%, $p<0.05$, Figure 22 A), while this does not occur in cells lacking BVR-A (-57%, $p<0.01$, Figure 22 A).

Similarly, a significant reduction of the Akt/GSK-3β complex levels in 3xTg-AD mice at 6 months of age with respect to Non-Tg (-58%, $p<0.05$, Figure 22 B) was found, while increased levels were observed in 12 months old 3xTg-AD mice (+50%, $p<0.05$, Figure 22 B).

Interestingly, a significant reduction of the complex was observed in MCI samples with respect to age-matched controls (-36%, $p<0.05$, Figure 22 C), but not in AD samples.

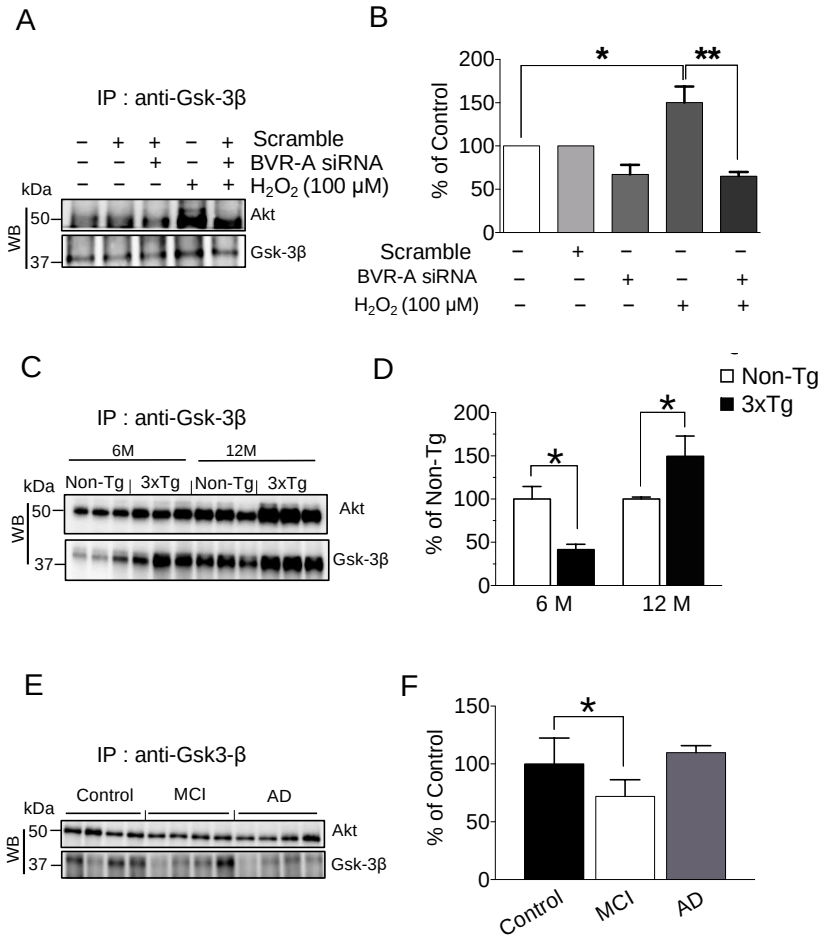



**Figure 22**. Loss of BVR-A and increased oxidative stress levels impair the association between Akt and GSK-3β both in vitro and in vivo. (A, C and E) Representative western blot images and densitometric evaluation of the Akt/GSK-3β complex isolated from (B) HEK cells (n=3 independent cultures/group) treated with $H_2O_2$ (100 μM, for 24 h) in the presence or not of BVR-A, whose silencing has been obtained through the use of a specific si-RNA; **(D)** hippocampal samples collected from 3xTg-AD and Non-Tg mice at 6 (n=6/group) and 12 (n=6/group) months of age; and IPL samples collected from Control (n=6), MCI(n=6) and AD (n=5) subjects. Akt levels bound GSK-3β were normalized by using total GSK-3β as loading control. All densitometric values are given as percentage of (B) Controls cells, (D) Non-Tg mice and (F) Control subjects set as 100%. Data are presented as means ± SEM, *p>0.05, **p<0.01, and ***p<0.001 (Student's t-test).

These observations indicate that loss of BVR-A is associated with an impairment in the formation of the Akt/GSK-3β complex, which could explain why we observed a reduction of GSK-3β inhibition in spite of no changes of Akt activation.

## 3.3. Discussion

In this study we demonstrate for the first time that BVR-A is required to mediate the oxidative stress-induced activation of Akt, which is a key regulator of GSK-3β activity.

Indeed, activation of the GSK-3β pathway has been shown to affect neuronal survival and synaptic plasticity by promoting Tau phosphorylation (Pei, J. J., 2009; Mandelkow, E. M., 1992; Anderton, B. H., 2001). In the brain from AD subjects and mouse model, altered localization of GSK-3β is associated with NFTs formation (Lucas, J. J., 2001; Ishizawa, T., 2003; Baum, L., 1996; Pei, J. J., 1997). During the early stage of AD development, GSK-3β accumulates with Tau in the cytoplasm of pre-tangle neurons (Pei, J. J., 1997), whereas in mature NFTs, the colocalization with GSK-3β is reduced (Baum, L., 1996; Harr, S. D., 1996; Shiurba, R. A., 1996). In addition, overexpression of active GSK-3β results in an AD-like phenotype (Brownlees, J., 1997), that can be reversed by reducing GSK-3β levels( Lucas, J. J., 2001; Engel, T., 2006; Farr, S. A., 2014).These lines of evidence, therefore, indicate that the activation of GSK-3β is a key, early event promoting Tau phosphorylation in AD (Rockenstein, E., 2007).

Our findings strengthen the above-cited data and suggest a molecular mechanism favoring the activation of GSK-3β and the consequent increase of Tau phosphorylation in early stage AD. In particular, we demonstrate that loss of BVR-A is associated with the uncoupling of the Akt/GSK-3β proteins in response to oxidative stress, thus contributing to the pathological activation of GSK-3β. This aspect is fascinating because the Akt/GSK-3β complex is target of multiple signalling cascades activated in response to different pro-survival stimuli (Hermida, M. A., 2017; Manning, B. D., 2017) and

found to be altered in AD (Zhang, Y., 2018). In parallel, BVR-A was demonstrated to participate in a number of intracellular processes regulated by Akt including energy metabolism, gene expression, cell proliferation, and survival (Barone, E 2014; Kapitulnik, J., 2009), thus suggesting a mutual interaction.

Previous studies from our group showed that reduced BVR-A activation triggers the development of brain insulin resistance in 3xTg-AD mice (Barone, E., 2016). We demonstrated that oxidative stress leads to reduced BVR-A Tyr phosphorylation, thus making BVR-A less active. In turn, reduced BVR-A activity is responsible for IRS1 hyper-activation, which is not associated downstream with a parallel activation of Akt (Barone, E., 2016; Barone, E., 2018). To note, intranasal insulin administration prevents BVR-A impairment and the alterations of IRS1/Akt axis (Barone, E., 2018).

The above-cited observations together with current findings support the idea, that BVR-A is at the cross-road between (1) the activation of the insulin signalling cascade and (2) the cell stress response in AD, both having Akt as molecular target (Manning, B. D., 2017). In this picture, we provide novel evidences about the loss of Akt-mediated inhibition of GSK-3β in the hippocampus of 3xTg-AD mice and in MCI, which agree with the proposed role for GSK-3β in driving Tau phosphorylation in AD.

The increased activation of GSK-3β observed in the hippocampus of 3xTg-AD mice both at 6 and 12 months of age, is effectively due to a reduction of Ser9 phosphorylation rather than an increase of Tyr216 phosphorylation (Figure 15), thus strengthening the hypothesis about the loss of Akt-mediated inhibition (Manning, B. D., 2017). This latter, is further confirmed by the increase of Tau Ser404 phosphorylation, which is significantly elevated at 6 months and persists in old 3xTg-AD mice at 12

months of age (Figure 15). Indeed, GSK-3β is able to phosphorylate Tau at multiple sites) (Liu, S. J., 2004; Llorens-Marítin, M., 2014; Godemann, R., 1999) although the most prominent phosphorylation sites are Ser396 and Ser404 (PHF-1 epitope) (Godemann, R., 1999; Agarwal-Mawal, A., 2003; Lovestone, S., 1996; Sun, W., 2002). Phosphorylation at Ser396 and Ser404 seems to precede phosphorylation at the other sites on Tau (Godemann, R., 1999). Although GSK-3β preferentially phosphorylates many of its substrates after they have been pre-phosphorylated by other kinases, the stretch of amino acids in Tau that includes the phosphorylatable residues, Ser396, Ser400, and Ser404, can be directly phosphorylated by GSK-3β without the prior activity of other kinases (Leroy, A., 2010; Hanger, D. P., 2011). However, phosphorylation at Ser404 is critical to this process and substitution of this residue by alanine ablates phosphorylation of both Ser396 and Ser400. It appears, therefore, that the primary phosphorylation of Ser404 by GSK-3β can itself serve as a primed residue for the subsequent sequential phosphorylation of Tau at Ser400 and Ser396 by GSK-3β (Leroy, A., 2010; Hanger, D. P., 2011).

Data collected in MCI (Figure 16), showing reduced GSK-3β inhibition without significant changes of Akt activation (Figure 16) despite increased oxidative stress (Aluise, C. D., 2011), reinforce the idea that increased GSK-3β activation represents an early event during the progression of AD pathology (Steen, E., 2005; Nicolia, V., 2017).

Noteworthy, under oxidative stress conditions loss of BVR-A impairs the Akt-mediated inhibition of GSK-3β via two mechanisms: (1) by precluding the activation of Akt; and (2) by avoiding the physical interaction between Akt and GSK-3β. The first mechanism is consistent with previous data from Maines' group (Miralem, T., 2016) and helps to explain the results collected in old 3xTg-AD mice in which reduced Akt activation is associated

with reduced GSK-3β inhibition. The second mechanism is completely novel and conceivably can explain the uncoupling of Akt and GSK-3β in young 3xTg-AD mice (Figure 15) as well as in MCI (Figure 16).

Our data demonstrate that oxidative stress promotes the phosphorylation of Akt and the inhibition of GSK-3β in control cells but not in cells lacking BVR-A (Figure 19). Increased Akt phosphorylation following $H_2O_2$ is a physiological mechanism through which cells counteract toxic effects of oxidative stress (Chong, Z. Z., 2005), also by inhibiting GSK-3β (Chong, Z. Z., 2005). Accordingly, treatment with a GSK-3β inhibitor protected against $H_2O_2$-induced mitochondrial dysfunction and apoptotic DNA fragmentation in HEK cells (Shin, S. Y., 2004). Conversely, the effect of $H_2O_2$ becomes detrimental if it is associated with a reduction of BVR-A (Figure 19). Indeed, loss of BVR-A blocks $H_2O_2$-induced activation of Akt and precludes the association between Akt and GSK-3β, thus impeding the physiological inhibition of GSK-3β. This results in a dysregulated phosphorylation of Tau Ser404 (Figure 19), which would become more prone to aggregate as NFTs (Wang, Y., 2015).

The proposed mechanism is thought to occur in the early phases of AD pathology, in both humans (MCI, Figure 16) and mice (Figure 15 and Figure 14), where elevated oxidative stress is associated with (i) reduced BVR-A levels and (ii) reduced GSK-3β inhibition. In contrast, increased Akt phosphorylation along with increased GSK-3β inhibition without changes in their interaction in late stage AD (Figure 6) agree with others and our previous studies (Griffin, R. J., 2005; Yarchoan, M., 2014; Pei, J. J., 2003; Tramutola, A., 2015). Surprisingly, we observed increased Akt/GSK-3β complex levels in the brain of old 3xTg-AD mice (Figure 22). Despite of that, the interaction appears not successful since reduced Akt activation along

with reduced GSK-3β inhibition were observed (Figure 16), in agreement with our hypothesis.

Based on these observations we propose a regulatory network in which BVR-A dysfunction alters the physiological response to oxidative stress and insulin, thus resulting in increased Tau phosphorylation. Indeed, we found in 3xTg-AD mice that the reduction of BVR-A protein levels is evident at 3 months of age, even before significant increase of oxidative stress ( Barone, E., 2016). It is conceivable to hypothesize that reduced BVR-A levels lead to alterations of the insulin signaling cascade, which would trigger metabolic defects favoring increased oxidative stress (de la Monte, S. M. 2009; Neumann, K. F., 2008; de la Monte, S.M. 2014). In turn, oxidative stress would impair BVR-A kinase activity, favoring the pathological activation of IRS1(Barone, E., 2016). At this point, despite increased oxidative stress and IRS1 activation, reduced BVR-A protein levels would impede the correct activation of Akt and the following inhibition of GSK-3β. As result, an increase of Tau phosphorylation occurs (Figure 23).

This paradigm is consistent with previous studies showing an association among oxidative stress, brain insulin resistance and increased Tau phosphorylation in AD (Butterfield, D. A., 2014; Chatterjee, S., 2018; Schubert, M., 2003; Schubert, M., 2004; Cheng, C. M., 2005).

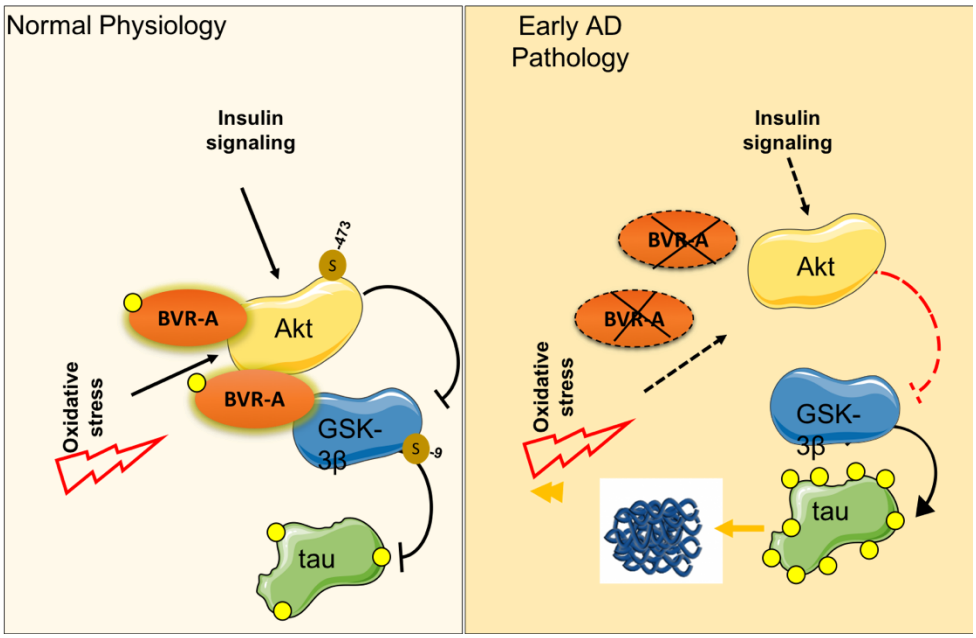



**Figure 23: Schematic diagram of proposed mechanism.** Early impairment of BVR-A in 3xTg-AD mice at 6 months and in human MCI subjects under oxidative stress and insulin signaling shows failure of BVR-A/Akt complex formation and mediating GSK-3β inhibition. This mechanism further supports the evidences of aberrant Tau hyperphosphorylation at earl onset of AD progression and consistently following in late onset AD as well. Although oxidative stress mediated Akt activation appears when there is no loss of BVR, instead with impaired BVR-A. Thus, loss of BVR-A at early stage shows pathological significance in AD brain.

# Chapter 4: Project 2

***Proteomic identification of altered protein O-GlcNAcylation in in a triple transgenic (3xTg) mice model of Alzheimer's disease***

## 4.1 Material and Method

### 4.1.1 Animals

Experiments were conducted on 6- and 12-month-old 3×Tg-AD (B6;129-Psen1$^{tm1Mpm}$ Tg(APPSwe, tauP301L)1Lfa/Mmjax) male mice (6 mice per group) and on the corresponding non-Transgenic (Non-Tg) male littermates (6 mice per group). The 3xTg-AD mice harbor 3 mutant human genes (APP KM670/671NL (Swedish), MAPT P301L, PSEN1 M146V) and have been genetically engineered by La Ferla and colleagues (S. Oddo 2003)". The background strain of the mice is the C57BL6/129SvJ hybrid. The presence of mutations was confirmed using a PCR approach as previously report (Cassano, T., 2011; Romano, A., 2015). Colonies of homozygous 3xTg-AD and Non-Tg mice were established at the vivarium of Puglia and Basilicata Experimental Zooprophylactic Institute (Foggia, Italy). The housing conditions were controlled daily (temperature 22 °C, light from 07:00–19:00, humidity 50%–60%) and fresh food and water were freely available. All the experiments were performed in strict compliance with the Italian National Laws (DL 116/92), the European Communities Council Directives (86/609/EEC). All efforts were made to minimize the number of animals used in the study and their suffering. Animals were sacrificed at the selected age and the brain was extracted and divided according to its differential experimental utilization (1/2 brain, the left hemisphere was used for immunohistochemical analysis and therefore fixed in paraformaldehyde, while the other 1/2, the right hemisphere, was dissected and processed for biochemistry and molecular biology analysis). Fresh tissues were flash-frozen and stored at -80 °C until total protein extraction and further analyses were performed

### 4.1.2 Sample preparation

Hippocampi from 3×Tg-AD and Non-Tg mice (n= 6 mice per group) were thawed in Media 1 lysis buffer (pH 7.4) containing: 320 mM Sucrose, 1% of 1 M Tris-HCl (pH = 8.8), 0.098 mM MgCl2, 0.076 mM EDTA, the proteinase and phosphatase inhibitors cocktail (Sigma-Aldrich, St Louis, MO, USA), PUGNAc (O-(2-Acetamido-2-deoxy-D-glucopyranosylidenamino) N-phenylcarbamate) [100 µM] and Benzyl-2-acetamol-2-deoxy- α -D-galactopyranoside [100 µM] (Sigma-Aldrich, St Louis, MO, USA), respectively OGA and OGT inhibitors. The brains were homogenized and sonicated and the resulting homogenates were centrifuged at 14,000×g for 10 min to remove debris. The supernatant was extracted to determine the total protein concentration by BCA method (Pierce Biotechnology, Rockford, IL, USA).

### 4.1.3 Two-dimensional (2D) electrophoresis and 2D blots

For the first-dimension electrophoresis, approximately 200 µl of sample (100 µg of proteins) from 3×Tg-AD and Non-Tg mice at 12 months of age were applied to 110-mm pH 3–10 IPG® ReadyStrip (Bio-Rad, Hercules, CA, USA). The strips were then actively rehydrated in the protean isoelectric focusing (IEF) cell (Bio-Rad, Hercules, CA, USA) at 50 V for 18 h. The isoelectric focusing was performed in increasing voltages as follows; 300 V for 1 h, then linear gradient to 8000 V for 5 h and finally 20000 V/h. Strips were then stored at −80 °C until the 2D electrophoresis was to be performed. For the second dimension, the IPG® Strips, were thawed and equilibrated for 10 min in 50 mM Tris–HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 15 min in the same buffer containing 4.5% iodacetamide

instead of dithiothreitol. Linear gradient precast criterion Bis-Tris gels (12%) (Bio-Rad, Hercules, CA, USA) were used to perform second dimension electrophoresis. Precision Protein™ Standards (Bio-Rad, Hercules, CA, USA) were run along with the samples at 200 V for 50 min. After running the gels were incubated in fixing solution (10% acetic acid, 40% methanol) for 40 min and stained overnight at room temperature with 50 mL SYPRO Ruby gel stain (Bio-Rad, Hercules, CA, USA). The SYPRO ruby gel stain was then removed and gels stored in deionized water.

For 2D blots, gels were blotted on nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and glycoproteins were detected on the membranes. Briefly, membranes were blocked for 1 h with 3% albumin in T-TBS, incubated with a mix of two primary antibodies: *O*-GlcNAc RL-2 (#sc-59624, mouse, 1:1000; Santa Cruz, Biotechnology, Dallas, TX, USA) and CTD110.6 (#9875, mouse, 1: 500, Cell Signaling, Danvers, MA, USA) overnight at 4°C. After washing with T-TBS three times for 10 min, membranes were further incubated at room temperature for 1 h with the secondary antibody alkaline phosphatase-conjugated anti-mouse IgG (1:5000; Sigma-Aldrich, St Louis, MO, USA). Membranes were then washed with T-TBS three times and developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution (BCIP/NBT).

### 4.1.4 Image analysis

SYPRO ruby-stained gel and Blot images were obtained using a Chemidoc MP System (Bio-Rad, Hercules, CA, USA). All the images were saved in TIFF format. 2D gels and 2D blots images (12 gels and 12 blots) were analyzed by PD-Quest 2D Analysis (7.2.0 version; Bio-Rad, Hercules, CA, USA). PD-Quest spot-detection software allows the comparison of 2D

gels as well as 2D blots, from 3×Tg-AD and Non-Tg groups. Briefly, a master gel was selected followed by normalization of all gels and blots (3×Tg-AD and Non-Tg) according to the total spot density. Gel-to-blot analysis was then initiated in two parts. First, manual matching of common spots was performed, that could be visualized among the differential 2D gels and 2D blots. After obtaining a significant number of spots the automated matching of all spots was then initiated. Automated matching is based on user-defined parameters for spot detection. These parameters are based on the faintest spot, the largest spot, and the largest spot cluster that occur in the master gel and are defined by the user. This process generates a large pool of data, approximately 400-800 spots. Only proteins showing computer-determined significant differential levels between the groups being analyzed were considered for identification.

To determine significant differential levels of proteins, analysis sets were created using the analysis set manger software incorporated into the PD-Quest software. The numbers of pixels that occur in a protein spot were computed by the software corresponding to an increase/decrease in protein level. The image analysis was conducted first on blot and then on Sypro Ruby-stained expression gels. The two analyses were compared by software to normalize glycosylation value to expression value for each spot matched.

### 4.1.5 In-gel trypsin digestion/peptide extraction

Protein spots identifies as significantly altered from the comparison of two groups (3×Tg-AD and Non-Tg mice) were excised from 2D-gels and transferred to individual Eppendorf microcentrifuge tubes for trypsin digestion as described previously. In brief, DTT and IA were used to break, and cap disulfide bonds and the gel plug was incubated overnight at 37°C

with shaking in modified trypsin solution. Tryptic peptide solutions were reconstituted in water and stored at −80° C until MS/MS analysi

### 4.1.6 RP-HPLC-high resolution MS/MS characterization of tryptic peptides

High-resolution HPLC-ESI-MS/MS experiments were carried out by an Ultimate 3000 RSLC nano system coupled to an LTQ Orbitrap ELITE apparatus (Thermo Fisher Scientific, Waltham, MA, USA). Zorbax 300 SB-C18 (3.5 μm particle diameter; column dimension 1 mm × 150 mm) (Agilent Technologies, Santa Clara, CA, USA) was used as chromatographic column. The following eluents were used: (A) 0.1% (v/v) aqueous FA and (B) 0.1% (v/v) FA in ACN/water 80/20 v/v. The applied gradient was: 0-2 min 5% B, 2-40 min from 5 to 70% B (linear), 40-45 min from 70 to 99% B (linear), at a flow rate of 50 μL/min with a total run of 65 min. MS spectra were collected with 120,000 resolution and m/z range from 350 to 2000. In data-dependent acquisition mode the five most intense multiply-charged ions were selected and fragmented in ion trap by using CID 35% normalized collision energy. Tuning parameters were: capillary temperature 300 °C, source voltage 4.0 kV.

### 4.1.7 MS data analysis

MS/MS data were elaborated by Proteome Discoverer software (version 1.4.1.14, Thermo Fisher Scientific, Waltham, MA, USA), based on SEQUEST HT cluster as search engine against UniProtKB mouse database (released on 28 of February 2017, mus musculus 16839 entries). The search parameters were 10 ppm tolerance for precursor ions and 0.5 Da for-product ions, 2 missed cleavage, carbamydomethylation of cysteine (+57.02 Da) as

fixed modification, oxidation of methionine (+15.99 Da), *O*-GlcNac (+203,1950) as variable modification on Serine and Threonine. Protein characterization was set with the identification of a minimum of two peptides per protein and two unique peptides by applying the high confidence filter

### 4.1.8 Immunofluorescence

Half mouse brain (left hemisphere) from each animal was removed and immersed in 4% paraformaldehyde 48 h. Fixed brains were cryoprotected in successive 72 h in 20% sucrose solution as described previously. Brains were frozen on a temperature-controlled freezing stage, coronal sectioned (20 μm) on a sliding microtome and stored in a solution of PBS containing 0.02% $NaN_3$ at 4°C. Brain sections were mounted on glass slides with medium (30% ethanol in PBS). Once dry, sections were blocked and permeabilized in blocking buffer (10% normal goat serum and 0.2% Triton X-100 in TBS) for 2h. Slides were then incubated overnight at 4°C with following antibodies: *O*-GlcNAc RL-2 (#sc-59624; mouse 1:250, Santa Cruz, Biotechnology, Dallas, TX, USA) and CTD110.6 (#CST-9875; mouse 1:250, Cell Signaling, Danvers, MA, USA), total p-Thr (#sc-5267, mouse 1:250, Santa Cruz, Biotechnology, Dallas, TX, USA) and total p-Ser (#AB-9332, rabbit 1:500, Abcam, Cambridge, UK). Slides were washed with TBS and then incubated with Alexa Fluor 488nm secondary antibody (#A10680, Fisher Molecular biology, Rome, Italy) at 1:1500 for 2 h at room temperature. Slides were then washed again and incubated with DAPI (1:10.000, Immunological Science, Rome, IT). One section from 3×Tg-AD and Non-Tg were stained omitting primary antibodies to establish nonspecific background signal. Cover slips were placed using a drop of Fluorimount (Sigma-Aldrich, St Louis, MO, USA).

### 4.1.9 OGA assay

OGA activity assay was performed using the synthetic substrate p-nitrophenyl N-acetyl-β-D-glucosaminide (pNP-GlcNAc) as described by Zachara et al. [40]. Briefly, samples were homogenized in isolation buffer without EDTA, EGTA and GlcNAc, and crude cytosolic fractions were prepared as described above. The samples were incubated with concanavalin A agarose for 30 minutes at 4°C to remove interfering hexosaminidases. Samples were then desalted using Zeba™ Desalt Spin Columns (Pierce Biotechnology, Rockford, IL, USA) and protein estimation was performed. Sample (25 µg) was mixed with activity assay buffer (final concentrations: 50 mM sodium cacodylate, pH 6.4, 50 mM N-acetylgalactosamine, 0.3% BSA, and 2 mM pNP-GlcNAc). Reactions were incubated at 37 °C for 2 h and stopped by the addition of 500 mM $Na_2CO_3$. Absorbance was read at 405 nm and OGA activity is reported as enzyme activity units where one unit catalyzes the release of 1 µmol pNP/min from pNP-GlcNAc.

### 4.1.10 Western blot

For western blot, 30 µg of proteins from hippocampus of 3×Tg-AD and Non-Tg at 6 and 12 months of age (n=6 mice per group) were separated on criterion gels, transferred to the membrane as already mentioned in project I. Further overnight incubation at 4°C with following primary antibodies: *O*-GlcNAc RL-2 (#sc-59624, 1:500, Santa Cruz, Biotechnology, Dallas, TX, USA), CTD110.6 (#9875, 1:1000, Cell Signaling, Danvers, MA, USA), OGA, (#SAB-4200267, 1:1000, Sigma–Aldrich, St Louis, MO, USA), OGT, Drp-2, Ulk1, p-Ser636 and total IRS1 (#sc-74546, #sc-30228, #sc-390904, #sc-33957, #sc-8038, 1:1000, Santa Cruz, Biotechnology, Dallas, TX, USA),

Gapdh and Eno1 (#GTX-100118, #GTX-113179, GeneTex, Irvine, CA, USA), p-Ser473 Akt (#CST-4058, 1:1000, Cell Signaling, Danvers, MA, USA) and total Akt (#VMA-00253, 1:1000, Bio-Rad, Hercules, CA, USA). Secondary antibodies horseradish peroxidase-conjugated anti-mouse or rabbit IgG (1:5000; Sigma–Aldrich, St Louis, MO, USA) were incubated for 1 h at room temperature. Membrane was developed with the Super Signal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, MA, USA), acquired with ChemiDoc MP (Bio-Rad, Hercules, CA, USA) and analyzed using Image Lab software (Bio-Rad, Hercules, CA, USA).

**4.1.11 Immunoprecipitation**

The different samples-sets (100 µg of proteins) were incubated overnight with I.P. buffer (10mM Tris, pH 7.6; 140mM NaCl; 0.5% NP40 including protease inhibitors) and the following antibodies: OGT, Drp-2, Ulk1 (#sc-74546, #sc-30228, #sc-390904, 1:100, Santa Cruz, Biotechnology, Dallas, TX, USA), Gapdh and Eno1 (#GTX-100118, #GTX-113179, GeneTex, Irvine, CA, USA) followed by 2 h of incubation with Protein G beads (Sigma–Aldrich, St Louis, MO, USA) and then washed three times with RIA buffer (10mM Tris, pH 7.6; 140mM NaCl; 1% NP40). Proteins were separated by SDS-PAGE followed by immunoblotting on a nitrocellulose membrane (Bio-Rad, Hercules CA, USA). Membrane were incubated with the antibodies anti-*O*-GlcNAc RL-2 (#sc-59624, mouse, 1:500, Santa Cruz, Biotechnology, Dallas, TX, USA) and CTD110.6 (#9875, mouse, 1:500, Cell Signaling, Danvers, MA, USA), total p-Thr (#sc-5267, mouse 1:250, Santa Cruz, Biotechnology, Dallas, TX, USA) and total p-Ser (#AB-9332, rabbit 1:500, Abcam, Cambridge, UK) and then detected by the peroxidase-conjugated secondary antibody ( 1:5000; Sigma–Aldrich, St

Louis, MO, USA) with Super Signal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, MA, USA). Membranes were then acquired with ChemiDoc MP image system (Bio-Rad, Hercules, CA, USA) and analyzed using Image Lab software (Bio-Rad, Hercules, CA, USA). The IP results were normalized on the total amount of the proteins of interest.

### 4.1.12 Statistical analysis

Data obtained by PD-QUEST software were compared using Student's t-test. Significance was accepted if the p value $< 0.05$. All the data are expressed as mean ± SEM of 6 independent samples per group. All statistical analyses were performed using GraphPad Prism 5.0 software

## 4.2 Result

### 4.2.1 Protein levels and localization of *O*-GlcNAcylated and phosphorylated (Ser/Thr) proteins in hippocampus of 3×Tg-AD and Non-Tg mice

3×Tg-AD mice represent a valuable model for the study of *O*-GlcNAcylation since previous studies demonstrated that they develop an age-related reduction of brain glucose uptake, together with age-related insulin resistance and decreased expression of the type-3 neuronal glucose transporter (GLUT-3) (Ding, F., 2013; Nicholson, R. M., 2010). Further, those mice do not show neuronal loss suggesting that variation of protein *O*-GlcNAcylation should be driven by altered glucose metabolism (Oddo, S., 2003). Earlier findings by Gatta and collaborators reported that 12-month-old 3×Tg-AD mice show reduced Tau *O*-GlcNAcylation associated with increased tau hyperphosphorylation in the hippocampus (Gatta, E., 2016). Interestingly, the reduction of Tau *O*-GlcNAcylation is region-specific and did not occur in the cortex, as also observed by us (Fig. 24), or cerebellum. Further, the authors demonstrated that the selective reduction in tau *O*-GlcNAcylation, found in the hippocampus of 12-month-old 3×Tg-AD mice, was in line with the pattern of tau pathology observed in these mice.

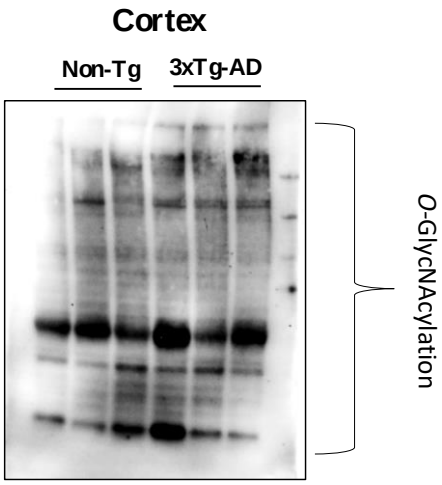



**Figure 24**. Total O-GlcNAcylated proteins in cortex of 3×Tg-AD and Non-Tg mice at 12 M. Representative western Blot showing the protein O-GlcNAcylation profile detected by RL2 and CTD110.6 antibodies used independently. Total protein O-GlcNAcylation in the cortex of 3×Tg-AD mice at 12 months compared with their age matched Non-Tg mice.

Before performing our proteomic analysis, we decided to explore the decrease of total protein *O*-GlcNAcylation and increased tau phosphorylation in 3×Tg-AD mice compared to Non-Tg mice at two different ages, 6 months and 12 months (Fig. 25). The age of 6 months represents an early phase of the neurodegenerative process in the 3×Tg-AD mice, while 12-month-old animals are crucial to demonstrate a robust alteration of *O*-GlcNAcylation and phosphorylation (Barone, E., 2016). Noteworthy, hippocampus homogenates were analysed in the presence of OGT and OGA inhibitors. We found that the use of inhibitors is a key step during sample preparation in order to obtain reliable and reproducible data (Zachara, N. E., 2011). Conflicting data, about *O*-GlcNAcylation levels in AD human and mouse brain, may depend on the accuracy of the sample preparation and the lack of specific inhibitors in the sample homogenate.

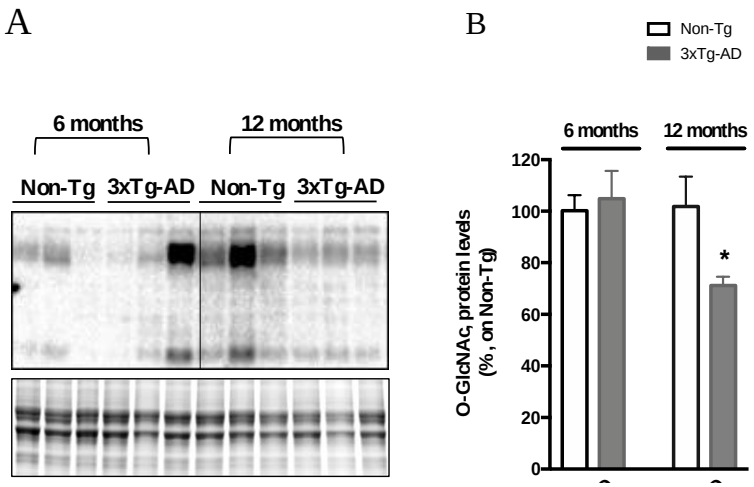



**Figure 25**. Total O-GlcNAcylated proteins in hippocampus of 3×Tg-AD and Non-Tg mice at 6 and 12 M Representative Western Blot (A) showing on the left the protein O-GlcNAcylation profile detected by RL2 and CTD110.6 antibodies used independently in the hippocampus from of 3×Tg-AD mice at 6 and 12 months compared with their age matched Non-Tg mice. Quantification of the Western Blot (B) indicate a significant reduction in O-GlcNAcylation in 3×Tg-AD mice at 12 months compared with Non-Tg.

In addition, to obtain a broad range of data on protein aberrantly glycosylated we decided to use a mix of two antibodies (CTD110.6 and RL2 recognizing both low and high molecular weight *O*-GlcNAcylated proteins only, as demonstrated by us (Fig.27 A, Fig. 26) and others (Förster, S.,2014; Gatta, E., 2016) As expected, western blotting data revealed a consistent decrease in total protein *O*-GlcNAc levels in 12-month-old 3×Tg-AD mice (about 0.3-fold vs. Non-Tg mice), which correlates with tau hyperphosphorylation (Fig. 27 A-E), while no difference between genotype was observed in 6-month-old mice (Fig. 25).

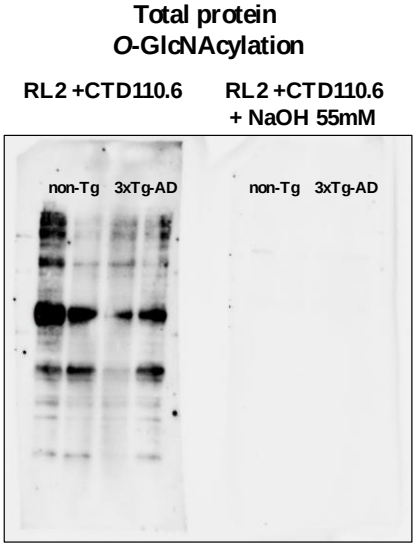



**Figure 26**. Total O-GlcNAcylated proteins in hippocampus of 3×Tg-AD and Non-Tg mice at 12 M identified by RL2 and CTD110.6 antibodies to detect both high and low molecular weight proteins on left side of membrane and treatment with 55mM NaOH on right side of membrane showed above.

Subsequently, we analysed total levels of *O*-GlcNAcylated proteins in parallel with total protein Ser/Thr phosphorylation by immunofluorescence in 12-month-old 3×Tg-AD mice brain (Fig. 27 D). Our data show decreased *O*-GlcNAc levels in the CA3 region of the hippocampus of 3×Tg-AD mice (Fig. 27 D 1-4), accompanied by an increase of serine and threonine phosphorylation (Fig. 27 D 5-8). These data support an evident degree of inverse relationship between *O*-GlcNAcylation and phosphorylation in the proteome of hippocampal neurons. Indeed, the correlation analysis between total protein *O*-GlcNAcylation and total protein Ser/Thr phosphorylation in 3×Tg-AD mice demonstrate a significant negative correlation (R squared=0.852; p=0.025) while no correlation is shown for Non-Tg animals (Fig. 27 E).

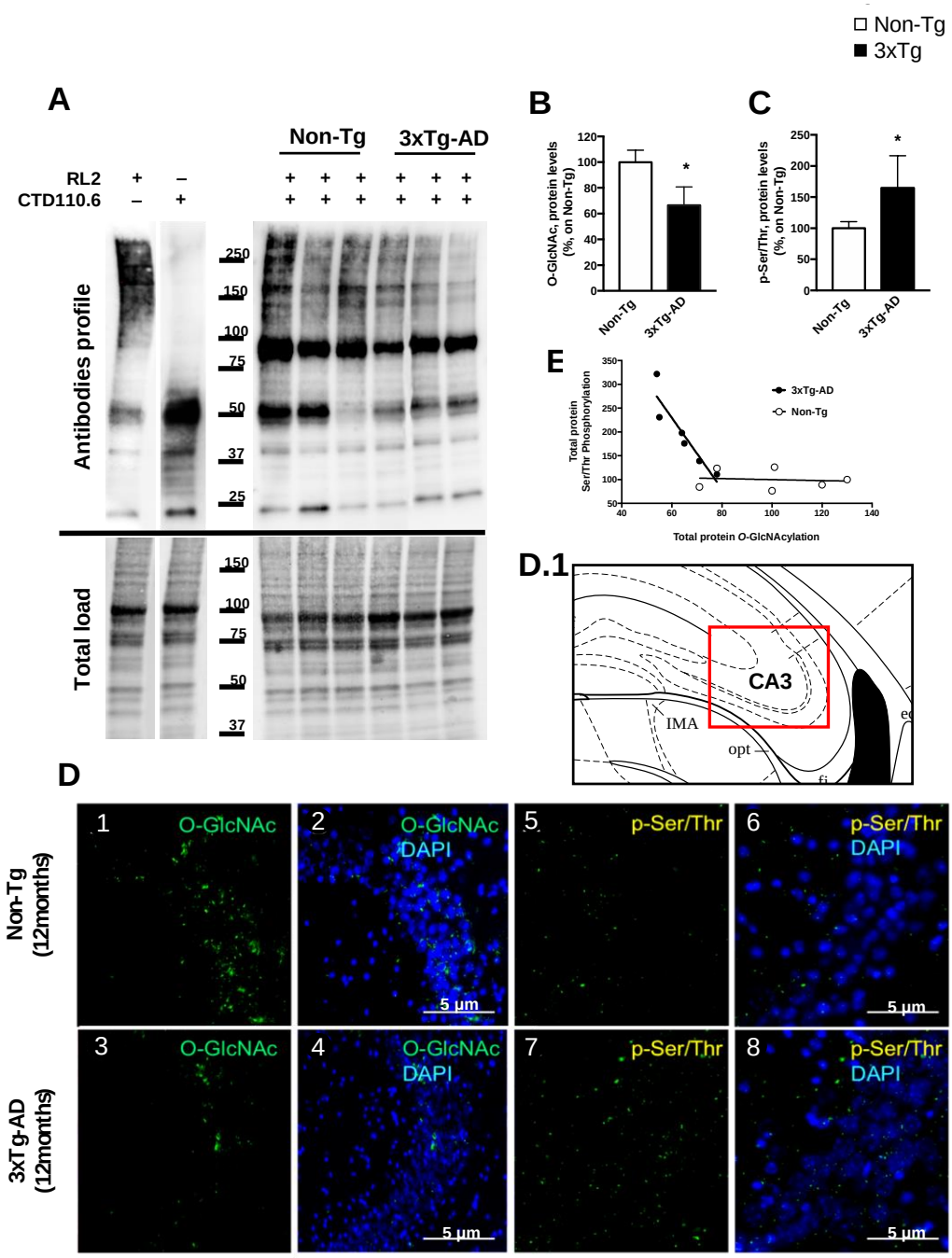



**Figure 27**. Protein levels and localization of O-GlcNAcylated and phosphorylated (Ser/Thr) proteins in hippocampus of 3×Tg-AD and Non-Tg mice. Representative western Blot (A)

showing on the left the protein O-GlcNAcylation profile detected by RL2 and CTD110.6 antibodies used independently and, on the right, the total levels of protein O-GlcNAcylation of the hippocampus from of 3×Tg-AD mice at 12 months compared with their age matched Non-Tg mice. Quantification of the Western Blot (B) indicate a significant reduction in O-GlcNAcylation in 3×Tg-AD mice at 12 months compared with Non-Tg. Representative immunofluorescence images (D) showing O-GlcNAc signal (1-4) and p-Ser/Thr (5-8) in the CA3 region of the hippocampus (D.1) of 3×Tg-AD and Non-Tg at 12 months. DAPI (blue in panel C, 1-8) was used to identify cell nuclei. Quantification of immunofluorescence staining (C) for p-Ser/Thr indicate a significant increase of total phosphorylation in the CA3 region of the hippocampus of 3×Tg-AD mice at 12 months compared with Non-Tg. Correlation analysis (E) between total protein O-GlcNAcylation and protein phosphorylation of 3xTg-AD (black) and Non-Tg (white) animals (3xTg-AD - R squared= 0.852, p= 0.025). Densitometric values shown in the bar graph are the mean ± SEM of 6 individual mouse samples per each group normalized on total load and are given as a percentage of Non-Tg, set as 100%. Statistical significance was determined using Unpaired t test (* $p<0.05$)

### 4.2.3 Protein levels and activation status of the enzymes involved in the *O*-GlcNAcylation process in the hippocampus of 3×Tg-AD and Non-Tg mice

In order to explore the causes that lead to reduced protein *O*-GlcNAcylation in AD mice we further investigated the protein levels and activation status of the enzymes involved in the process of removal and addition of *O*-GlcNAc residues to proteins: OGA and OGT (Fig. 28). The analysis of OGA demonstrated that protein levels did not change in 12-month-old 3×Tg-AD mice compared to age-matched Non-Tg mice, however in the same animals we showed an increase of OGA enzyme activity in the transgenic mice (Fig. 28 A, B). This result is in line with the observed reduction of protein *O*-GlcNAcylation. The analysis of OGT levels demonstrates, as well, no changes between AD mice compared to Non-Tg mice. As far as OGT, previous studies demonstrated no alterations in enzyme activity in 3×Tg-AD mice compared to Non-Tg (Gatta, E., 2016), We analysed its phosphorylation/*O*-GlcNAcylation ratio, which may control,

beyond enzyme activity, also its substrates recognition and/or its translocation from nucleus to plasma membrane (Kaasik, K., 2013; Bullen, J. W., 2014; Seo, H. G., 2016; Hart, G. W., 2011).

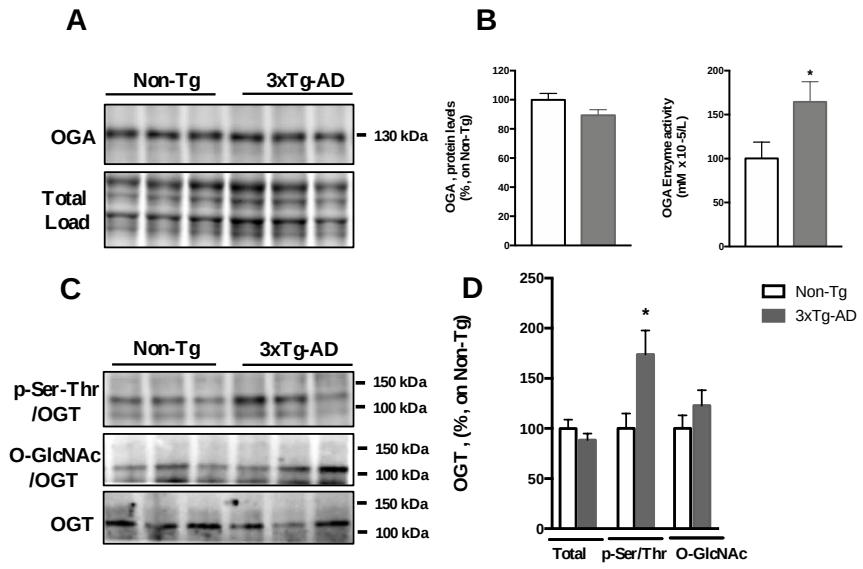



**Figure 28**. Protein levels and activation status of the enzymes involved in the O-GlcNAcylation process in the hippocampus of 3×Tg-AD and Non-Tg mice. Panel A and B: representative western blot (A) and quantification of total protein levels (B) and activity assay (B) of OGA in 3×Tg-AD mice at 12 months compared with Non-Tg. Panel C and D: representative western blot (C) obtained by immunoprecipitation assay for OGT (C). Quantification of O-GlcNAcylation, total phosphorylation on Ser/Thr residues and total protein levels (D) of OGT in 3×Tg-AD mice at 12 months compared with Non-Tg. Densitometric values shown in the bar graph are the mean ± SEM of 6 individual mouse samples per each group normalized on total load (A-B) and total OGT protein levels (C-D) and are given as a percentage of Non-Tg, set as 100%. Statistical significance was determined using Unpaired t test (* $p<0.05$)

Our results show an increase for OGT total Ser/Thr phosphorylation of about 2-fold but not significant alterations of specific *O*-GlcNAcylation of OGT in 12-month-old 3×Tg-AD mice (Fig. 28 C, D). Overall, the analysis of

OGA and OGT enzymes suggests the involvement of these two enzymes in the reduction of total protein *O*-GlcNAcylation in AD pathology.

### 4.2.4 Proteomic profile of all identified proteins that are differentially O-glycosylated in 3×Tg-AD mice compared with Non-Tg

After having confirmed the reduction of *O*-GlcNac in AD, driven by the aberrant activity of OGT and OGA enzyme activation, we performed proteomics analysis of *O*-GlcNAcylated targets in order to identify specific proteins with altered *O*-GlcNAc levels in AD mice compared to Non-Tg mice. Our analysis allowed the identification of 14 proteins differentially *O*-GlcNAcylated (normalized on protein levels) (Fig. 29 A and Table 2).

Proteins identified by mass spectrometry were: Elongin-C (ELOC) (DNA and RNA regulation) with 4.1-fold decrease; Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) (Glucose metabolism) with 7.1-fold decrease; GTP-binding nuclear protein Ran (Ran) (Energy metabolism) with 5.2-fold decrease; Ser/Thr-protein kinase Ulk1 (autophagy) with 2.1-fold decrease; putative ATP-dependent RNA helicase TDRD9 (TDRD9) (DNA and RNA regulation) with 4.1-fold decrease; Myosin phosphatase Rho-interacting protein (Mprip) (Cytoskeletal network) with 5.0-fold decrease; Tubulin α-1C chain (Tuba1c) with 10.0-fold decrease; α-enolase (Eno1) (Glucose metabolism) with 9.0-fold decrease; Dihydropyrimidinase-related protein 2 (Drp-2) (Neurotransmission) with 2.0-fold decrease; malate dehydrogenase (Mdh) (Glucose metabolism) with 4.3-fold decrease; Contactin-associated protein like 2 (Cntnap2) (Neurotransmission) with 14.2-fold decrease; Coiled-coil domain-containing protein 63 (Ccdc63) (Cytoskeletal network) with 2.1-fold decrease; 14-3-3 protein epsilon (14-3-3 E) (cell signaling) with

6.25-fold decrease and Neurofilament light polypeptide (NF-L) (Cytoskeletal network) with 3.3-fold decrease. It is interesting to notice that all the proteins show a decrease of *O*-GlcNAc modification in agreement with the total reduction previously observed (Table 2).

| Spot N. | Protein | Uniprot N. | Fold decrease Tg vs nTg | p-value | O-GlcNac* | Score | Cvg | Pep. |
|---|---|---|---|---|---|---|---|---|
| 0303 | Elongin-C (Eloc) | P83940 | 4.1 | 0.031 | / | 2.01 | 17.86 | 1 |
| 0503 | Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | P16858 | 7.1 | 0.045 | / | 4.24 | 14.71 | 3 |
| 2103 | GTP-binding nuclear protein Ran (Ran) | P62827 | 5.2 | 0.017 | / | 1.98 | 13.43 | 3 |
| 4108 | Serine/threonine-protein kinase ULK1 (ULK1) | O70405 | | 0.033 | T327; Ser330 | 1.87 | 1.52 | 1 |
| 4502.1 | Putative ATP-dependent RNA helicase TDRD9 (TDRD9) | Q14BI7 | 4.1 | 0.041 | Ser1201; Ser1203 | 5.7 | 1.37 | 1 |
| 4502.2 | Myosin phosphatase Rho-interacting protein (Mprip) | P97434 | 5.0 | 0.013 | Ser485; Ser491 | 2.71 | 1.76 | 1 |
| 4804 | Tubulin alpha-1C chain (Tuba1c) | P68373 | 10.0 | 0.048 | / | 2.73 | 3.34 | 1 |
| 5407 | Alpha-enolase (Eno1) | P17182 | 9.0 | 0.044 | / | 73.2 | 41.9 | 16 |
| 5702 | Dihydropyrimidinase-related protein 2 (DRP2) | O08553 | 20.0 | 0.037 | / | 2.4 | 7.87 | 4 |
| 6203 | Malate dehydrogenase, (Mdh) | P08249 | 4.3 | 0.05 | / | 26.1 | 24.5 | 8 |
| 6802 | Contactin-associated protein-like 2 (Cntnap2) | Q9CPW0 | 14.2 | 0.028 | Ser543; Thr994 | 2.59 | 2.85 | 2 |
| 6905 | Coiled-coil domain-containing protein 63 (Ccdc63) | Q8CDV6 | 2.1 | 0.022 | Thr423; Ser432 | 2.42 | 3.05 | 1 |
| 8114 | 14-3-3 protein epsilon (14-3-3E) | P62259 | 6.25 | 0.044 | / | 23.39 | 27.45 | 7 |
| 9701 | Neurofilament light polypeptide (NF-L) | P08551 | 3.3 | 0.05 | / | 3.98 | 11.23 | 7 |

**Table 2**: List of identified protein with reduced O-Glycosylated

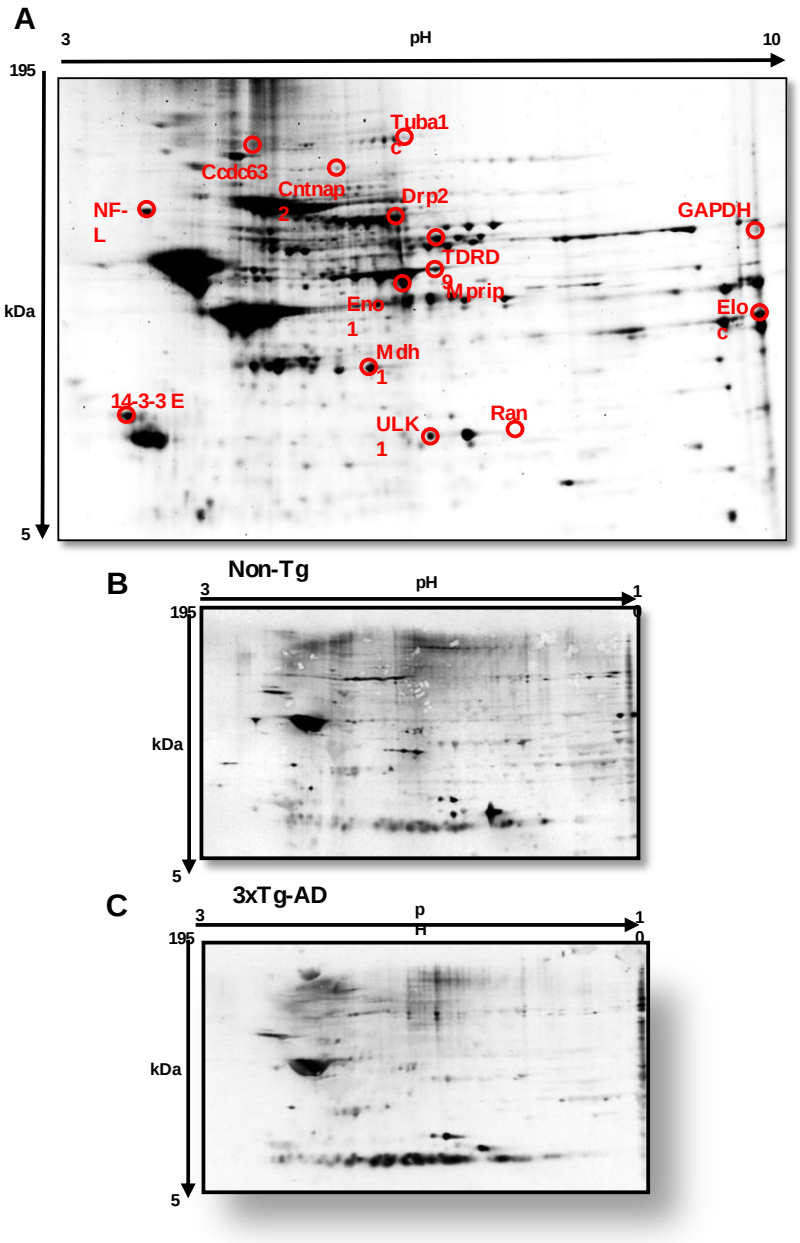



**Figure 29**. Proteomic profile of representative 2D-Blot with all identified proteins (A) that are differentially O-glycosylated in 3×Tg-AD mice compared with Non-Tg. In panel B and C a representative 2D-Blot of the glyco-proteomic profile in the hippocampus of Non-Tg and 3×Tg-AD mice.

### 4.2.5 Immunoprecipitation and quantification of Ulk1 and Drp-2 modified by O-GlcNAc and p-(Ser/Thr)

Further, to confirm that our method recognized *O*-GlcNAcylated proteins and that proteomics data are reliable, we performed validation analysis on four of the proteins found to be aberrantly *O*-GlcNAcylated, Drp-2, Ulk1, Eno1 and Gapdh (Fig 30). Samples were immunoprecipitated by specific antibodies and analysed for *O*-GlcNAc levels by western blot using both RL2 and CTD110.6 antibodies. The western blot confirmed proteomics analysis showing a reduction of *O*-GlcNAcylation for Drp-2, Ulk1, Eno1 and Gapdh (0.7 –fold, 0.75-fold, 0.4-fold and 0.3-fold respectively) (Fig. 30 A-D). Since reduced *O*-GlcNAc levels may affect protein phosphorylation on Ser/Thr residue, we analysed in parallel the total Ser/Thr phosphorylation levels of identified protein in 3×Tg-AD mice. Our data show no significant alteration for Drp-2, Ulk1 and Eno1 while a trend of increase (about 1.5-fold, not significant) might suggest a mutual relationship between O-GlcNacylation and phosphorylation (Fig. 30 A-D).

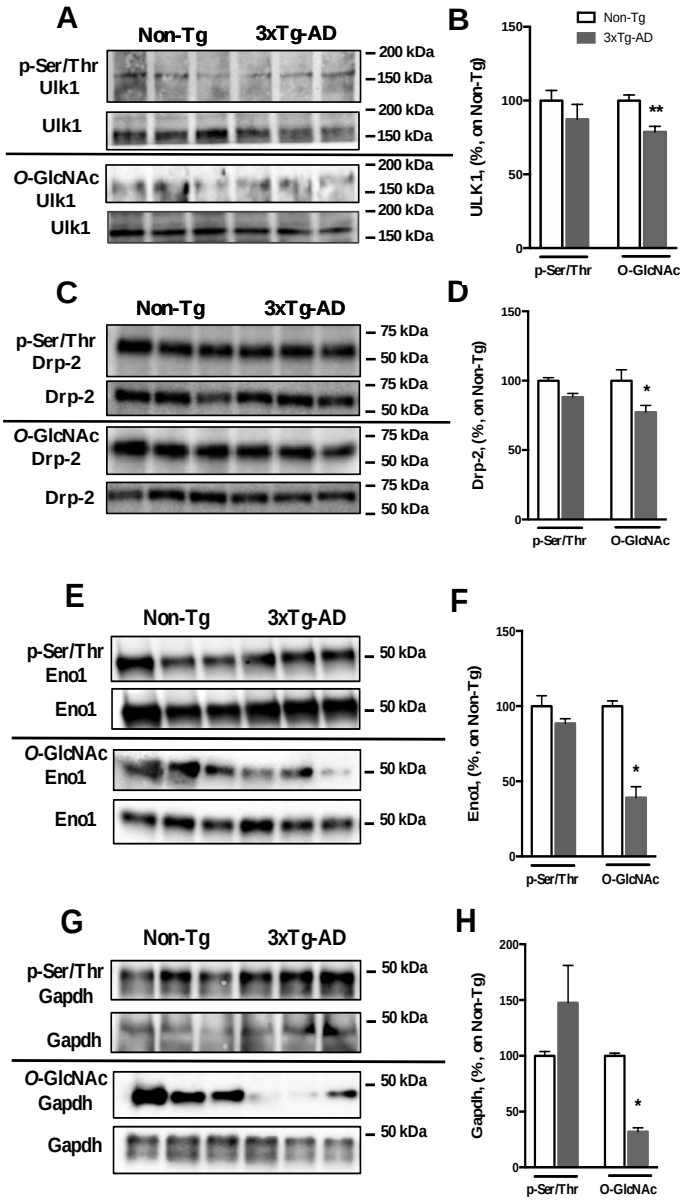



**Figure 30**. Immunoprecipitation and quantification of Ulk1 (A-B) and Drp-2 (C-D) modified by O-GlcNAc and p-(Ser/Thr). Protein total p-(ser/Thr) and O-GlcNAc of Ulk1 (A, B), Drp-2 (C, D), Gapdh (E, F) and Eno1 (G, H) were detected by immunoprecipitation of 3×Tg-AD mouse compared with Non-Tg. Densitometric values shown in the bar graph are the mean ± SEM of 6 individual mouse samples per each group normalized on Ulk1, Drp-2, Eno1 and

Gapdh total protein levels from IP experiments and are given as a percentage of Non-Tg, set as 100%. Statistical significance was determined using Unpaired t test (* $p<0.05$, ** $p<0.01$)

### 4.2.6 *O*-GlcNAcylation of insulin signaling components

Various studies have shown that *O*-GlcNAcylation of insulin signaling components is altered during AD and could be involved in energy failure, neuronal dysfunction and plaques and tangles formation (Hoyer, S. 2004; Yu, Y., 2015; Vosseller, K., 2002). IRS1 is a key player for insulin signaling and is a one of the downstream messengers of the insulin receptor. We found that *O*-GlcNAcylated IRS1 show a trend of decrease in the hippocampus of 12-month-old 3×Tg-AD compared to Non-Tg mice, which however is not significant (Fig 31). In parallel the analysis of IRS1 phosphorylation on an inhibitory residue, Ser636, demonstrate an increase of about 1.6-fold in 3×Tg-AD compared to Non-Tg mice. Our data demonstrate that increased phosphorylation of a single Ser residue of IRS1 is not coupled by the reduction of total Ser/Thr *O*-GlcNAcylation. (Fig. 31 A-C). Akt is a well-known Ser/Thr kinase enzyme involved in PI3K/Akt signaling pathway in the response of insulin signaling (Vosseller, K., 2002). Our western blot analysis shows surprisingly significantly increased *O*-GlcNAcylated Akt in the hippocampus of 12-month-old 3×Tg-AD mice compared to Non-Tg mice (about 2-fold, Fig 5), which negatively correlates with the decrease of the phosphorylation levels at Ser473 (about 0.5-fold) (Fig. 31 D-F). Overall, these findings suggest that high level of *O*-GlcNAcylation may modulate protein phosphorylation, as Akt activation, therefore the occurrence of *O*-GlcNAcylation event would likely to
contribute to the alteration of downstream regulatory pathway leading towards AD pathology.

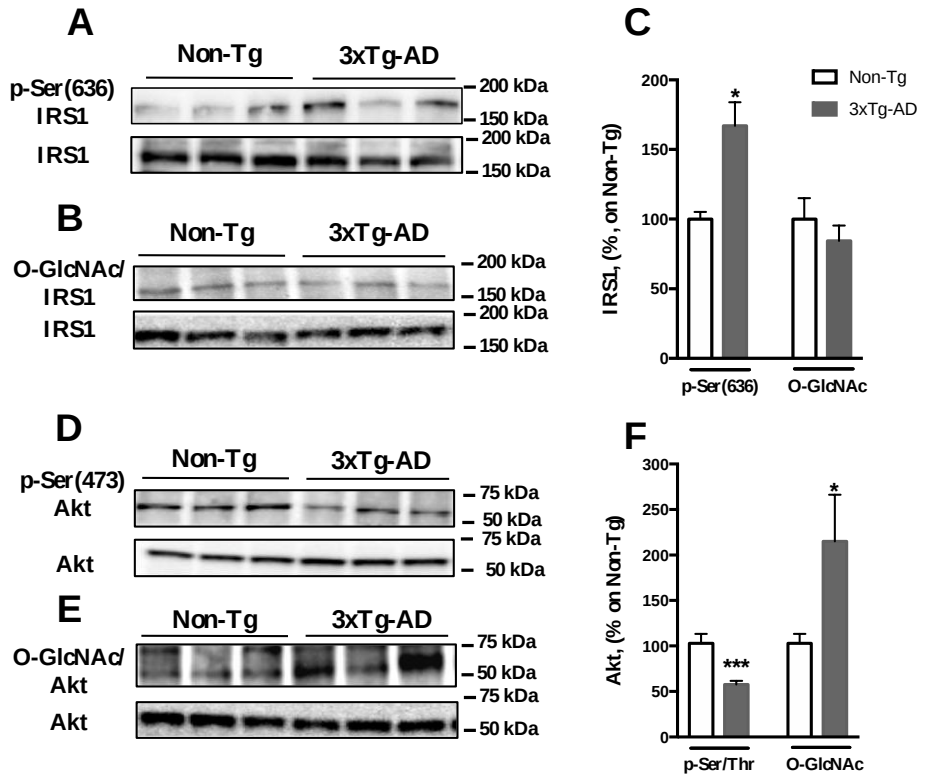



**Figure 31**. O-GlcNAcylation of insulin signaling components. Panel A, B and C: Representative western blot (A) showing phosphorylation of IRS1 on Ser 636 residue. Protein bound O-GlcNAc of IRS1 (B) are detected by immunoprecipitation in 3×Tg-AD compared with Non-Tg mice. Quantification of the Western Blot and the Immunoprecipitation (C) of IRS1 in the hippocampus of 3×Tg-AD and Non-Tg. Panel D, E and F: Representative western blot (D) showing phosphorylation of Akt on Ser 473 residue. Protein bound O-GlcNAc of Akt (E) are detected by immunoprecipitation in 3×Tg-AD compared with Non-Tg mice. Quantification of the Western Blot and the Immunoprecipitation (F) of Akt in the hippocampus of 3×Tg-AD and Non-Tg. Densitometric values shown in the bar graph are the mean ± SEM of 6 individual mouse samples per each group normalized respectively on IRS1 and Akt protein levels from IP and are given as a percentage of Non-Tg, set as 100%. Statistical significance was determined using Unpaired t test (*p<0.05, *** p<0.001).

## 4.3 Discussion

Protein *O*-GlcNAcylation is a ubiquitous PTM in living systems that appears to regulate more that 3000 proteins involved in transcription, protein translation and degradation, cell signaling among others (Copeland, R. J., 2013; Lazarus, B. D., 2009; Vaidyanathan, K., 2014; Zeidan, Q., 2010; Comer, F. I., 2009; Yang, Y. R., 2014). Remarkably, despite its abundance, *O*-GlcNAcylation is regulated primarily by the action of only two enzymes OGT and OGA whose expression is up to 10-times higher in brain compared to peripheral tissue, therefore suggesting that *O*-GlcNAcylation plays an important role in neuronal signaling (Zhu, Y., 2014). In a previous study performed on 12-months-old 3×Tg-AD mice, it was shown a hippocampus-specific reduction of protein *O*-GlcNAcylation compared with Non-Tg animals (Gatta, E., 2016). Our data confirm that total protein *O*-GlcNAcylation is reduced in 12-month-old 3×Tg-AD mice compared to Non-Tg with the concomitant increase of Ser/Thr phosphorylation levels. The analysis of OGT and OGA expression levels and regulation suggests the involvement of an aberrant activation of these enzymes during AD. Indeed, despite unchanged protein levels we observed an increase of OGA enzyme activity, which could translate as an increased removal of GlcNAc residues from proteins, as well as aberrant OGT phosphorylation/*O*-GlcNAcylation ratio which may regulate OGT activity, as well, as its substrate recognition or translocation to the plasma membrane (Bullen, J. W., 2014; Seo, H. G., 2016). Previous studies suggested a direct association between reduced glucose uptake, which has been observed in 3×Tg-AD mice (Gatta, E., 2016; Ding, F., 2013; Nicholson, R. M., 2010), altered insulin signalling and

aberrant OGT PTMs (Hart, G. W., 2011; Kreppel, L. K., 1999). Further, it was demonstrated that increased OGT phosphorylation may be related to GSK3ß kinase activity (Kaasik, K., 2013; Kreppel, L. K., 1999), which is increased in AD pathology comprising 3×Tg-AD mice (A. Romano 2014) or to AMPK activation (Bullen, J. W., 2014). Despite other did not observed altered OGT activity in 3×Tg-AD mice (Gatta, E., 2016), we suggest that its aberrant phosphorylation might result in altered nucleoplasmic localization or substrate binding.

The involvement of an aberrant HBP and protein *O*-GlcNAcylation in reduced glucose utilization and impaired insulin signaling has been extensively demonstrated by several authors in different tissues including the brain (Liu, F., 2009; Hoyer, S. 2004; Liu, Y., 2009; Kaasik, K., 2013;). Indeed, if on one hand the reduction of glucose entry in the cell, leads to reduced UDP-GlcNAc substrate availability and altered OGT activity, on the other side the reduction of protein *O*-GlcNAcylation can deregulate several proteins involved in glucose transport and catabolism (Liu, F., 2009; Hoyer, S. 2004; Yu, Y., 2015; Zeidan, Q., 2010; Dias, W. B., 2007; Liu, Y., 2009; Jahangir, Z., 2014). Interestingly, we identified by a proteomics approach the altered *O*-GlcNAcylation of three components of the glucose metabolic pathway, two enzymes involved into glycolysis, Gapdh and enolase, and one enzyme involved in Krebs cycle, Mdh. Gapdh has catalytic role in the formation of 1,3 di-phosphoglycerate from glyceraldehyde 3-phosphate. In addition, Gapdh also can exert a phosphotransferase/kinase activity (Butterfield, D. A., 2010). This enzyme presents in its amino acidic sequence several Ser/Thr residues that can be either *O*-GlcNAcylated or phosphorylated (Seo, J., 2008). Previous studies showed that Gapdh activity could be regulated by various cellular kinases including, protein kinase C,

epidermal growth factor kinase, and Ca2+/calmodulin-dependent protein kinase II, which may work in opposition to OGT in controlling the activation of the enzyme (Reiss, N., 1996; Reiss, N., 1986). Enolase catalyses the formation of phosphoenolpyruvate from 2-phosphoglycerate, while malate dehydrogenase catalyses the oxidation of malate to oxaloacetate. Likewise, enolase and Mdh contain in their amino acidic sequences several Ser/Thr residues that can be modified by either *O*-GlcNAcylation or phosphorylation (Bian, Y., 2014; Zhou, H., 2013); however, it has not been clarified yet how the modulation of these sites can influence Eno1 and Mdh function. Overall, our findings suggest in an AD model a potential role of *O*-GlcNAc modification in mediating Gapdh, Eno1 and Mdh activity during glycolysis and the Krebs cycle. It is tempting to speculate that the impairment of these glucose-related enzymes may contribute to the reduction of glucose metabolism and ATP production in the brain, which in turn leads to altered protein *O*-GlcNAcylation, therefore generating a vicious cycle of events that lead to neurodegeneration. However, further knowledge on the residues modified by *O*-GlcNAc is required in order to understand the exact mechanisms linking protein *O*-GlcNAcylation to altered protein activity.

Our data are supported by a previous study using iTRAQ proteomics approach on synaptosomes and post-synaptic densities isolated from human AD and control frontal cortex (Skorobogatko, Y. V., 2011). The authors demonstrated altered *O*-GlcNAcylation of Gapdh and Mdh enzymes, observing a decreased in AD samples compared to CTRs (Skorobogatko, Y. V., 2011). Considering the mutual relationship between *O*-GlcNAcylation and phosphorylation, an indirect confirmation of our results on Gapdh, Eno1 and Mdh can be obtained by published phosphoproteomics analysis. Indeed, previous studies from our laboratory showed, in the hippocampus of AD

patients compared to healthy subjects, increased phosphorylation levels for Eno1 and Gapdh suggesting a deregulation of such enzymes during neurodegeneration (Di Domenico, F., 2011). Further, phosphoproteomics analysis in AD human cortex and substantia nigra confirmed the hyperphosphorylation of Gapdh and proposed the hyperphosphorylation of Mdh enzyme (Perluigi, M., 2016; Zahid, S.,2012). In addition, it is also critical to note that components of the glycolysis pathways and of the Krebs cycle are targeted by reactive oxygen species during AD leading to protein oxidation and reduced energy metabolism (Butterfield, D. A., 2014). Within this context our data about the altered *O*-GlcNAcylation of enzymes involved in energy production, could add a further piece to the puzzle regarding the molecular mechanisms that lead to energy failure in AD. In agreement with our hypothesis, previous studies found the decreased *O*-GlcNAcylation of ATP5A in AD human tissue and in an AD mouse model (Cha, M. Y., 2015), supporting the involvement of aberrant *O*-GlcNAcylation in the reduction of ATP production.

Notwithstanding our proteomics analysis did not result in the identification of altered *O*-GlcNAcylation of any component of the insulin signaling pathway, due to the direct relationship between reduced glucose uptake and insulin signaling and to the pivotal role of insulin cascade in mediating synaptic plasticity in the brain (Hoyer, S. 2004; Liu, Y., 2009; Bedse, G., 2015; Pardeshi, R., 2017), we investigated the aberrant *O*-GlcNAcylation of IRS1 and Akt (Whelan, S. A., 2010). The identification of glucose transporters regulated by insulin on brain cells confirmed that metabolic control is essential for brain function (Brüning, J. C., 2000). In addition, previous studies suggested a role for OGT, when active, as a regulator of insulin signaling (Issad, T., 2010). High levels of glucose in the

cell lead to increased levels of OGT substrate UDP-GlcNAc, which in turn increase the activation of the OGT enzyme activity (Phoomak, C., 2017). OGT, which possesses a $PIP_3$ recognition sequence, is partially re-localized at the plasma membrane following PI3 kinase activation by insulin. At the plasma membrane, OGT induces *O*-GlcNAcylation of proximal elements of insulin signaling, such as IRS, PI3K, and Akt resulting in signal regulation (Issad, T., 2010). During AD, neurons face an opposite situation, in which reduced glucose metabolism leads supposedly to reduced OGT activity and reduced *O*-GlcNAcylation of insulin signaling components. The first component of the insulin signaling that we analysed was the insulin receptor substrate, which is involved in the activation of the PI3K pathway. IRS1 activity is regulated by its phosphorylation at multiple sites in either activating or inhibiting way. Previous studies from our laboratory demonstrated that triple transgenic AD mouse at 12 months of age showed increased phosphorylation of IRS1 at the inhibitory residue Ser 307, while no alteration in the activating residue tyrosine 632 was shown, supporting the reduced activity of IRS1 at this time point during AD pathology (Barone, E., 2016). We hypothesize that increased IRS1phosphorylation at the inhibitory Ser307 is accompanied by the reduction of *O*-GlcNAc levels due reduced glucose metabolism. Our data demonstrate indeed a parallel reduction of *O*-GlcNAcylated IRS1 Ser/Thr residues, which negatively correlates with the increase of IRS1 phosphorylation at Ser 636, a further inhibitory site. Predictive studies on alternate phosphorylation/*O*-GlcNAcylation levels of IRS1 support the mutual exclusive relationship in the inhibitory Ser residue 307 and 636, suggesting that the reduced *O*-GlcNAcylation of IRS1 may contribute to its increased phosphorylation on its inhibitory site and to its inactivation (Jahangir, Z., 2014, Ball, L. E., 2006).

A second key regulator of the insulin signaling is Akt, which is activated by PDK1 under the control of PI3K. Akt is a key signaling player in the neuron being involved in the regulation of GSK3β kinase activity, apoptosis, CREB and mTOR signaling, among others. The activity of Akt is regulated by phosphorylation at Ser 473; however, several studies showed that when *O*-GlcNAcylation occurs Akt activation is reduced (Vosseller, K., 2002; Whelan, S. A., 2010; Shi, J.,2015). Our data show that in 3×Tg-AD mice the increase of Akt *O*-GlcNAc glycosylation is concomitant with reduced phosphorylation of Akt at Ser 473, putatively resulting in reduced activity. Intriguingly, reduced activation of Akt is in line with the reported increase of GSK3β activation in 3×TgAD mice and in AD patients. A further intriguing characteristic of *O*-GlcNAcylation-mediated Akt regulation is the increase of N-acetylglucosamine levels of Akt despite reduced glucose metabolism, which has not been observed for any other protein during our study. Such behaviour may be explained with a probable regulatory role of OGT in Akt *O*-GlcNAcylation. Indeed, OGT, but not OGA, is recruited with Akt to the cytoplasmic membrane where it has direct access to Akt and can catalyses its *O*-GlcNAcylation (Shi, J., 2015; Shi, J.,2012).

A further interesting outcome of our proteomic analysis is the reduced O-GlcNAcylation of proteins implicated in the formation and maintenance of neuronal structure and architecture. As previously stated, the imbalanced regulation of tau *O*-GlcNAcylation/phosphorylation ratio is a key contributor to NTF formation during AD (Gong, C. X., 2016; Yu, Y., 2012; Liu, F., 2004; Iqbal, K., 2016). Our results report the altered *O*-GlcNAcylation of α-tubulin, which is directly involved, by interacting with β tubulin, in the organization of microtubules heterodimers. Microtubule structure is known to be impaired in AD and in addition to tau hyperphosphorylation, altered

PTMs of α- and β-tubulin have been observed. Previous analysis demonstrated that together with reduced α-tubulin levels, also acetylated tubulin, polyglutamylated tubulin, tyrosinated tubulin, and detyrosinated tubulin were significantly decreased in AD brain (Zhang, F., 2015). As far as phosphorylation/*O*-GlcNAcylation ratio of Ser/Thr residues heterodimers structure, only phosphorylation on Ser172 of β-tubulin has been recognized as crucial in the formation of microtubules, however several others Ser residues might be the target of *O*-GlcNAcylation or phosphorylation (Song, Y., 2015; Wloga, D., 2010

Another component of the neuron cytoskeleton identified with reduced *O*-GlcNAcylation by proteomics analysis in 3×Tg-AD mice is the neurofilament light chain (NF-L) that is part of the intermediate filaments family. Neurofilaments are believed to function primarily to provide structural support for the axon and to regulate axonal diameter (Deng, Y., 2008). Neurofilaments are composed by subunits that belong, in mature neurons, to three class of proteins, the light chain, the medium chain and the heavy chain. Those three class of subunits interacting together form the neurofilament triplets, which in mature neurons co-polymerized with α internexin, a fourth class of protein. Neurofilament proteins are extensively modified by both *O*-GlcNAcylation and phosphorylation. Altered phosphorylation of neurofilaments may precede their accumulation in axons [99], while increased *O*-GlcNAc levels may play, as discussed for tau, a protective role for NFT formation (Deng, Y., 2008). Our proteomics data are in line with previous reports supporting the reduced *O*-GlcNAcylation of NF-L in transgenic mice of AD.

Drp-2 is expressed in the nervous system during development and plays an important role in neurite extension and in axon guidance through the

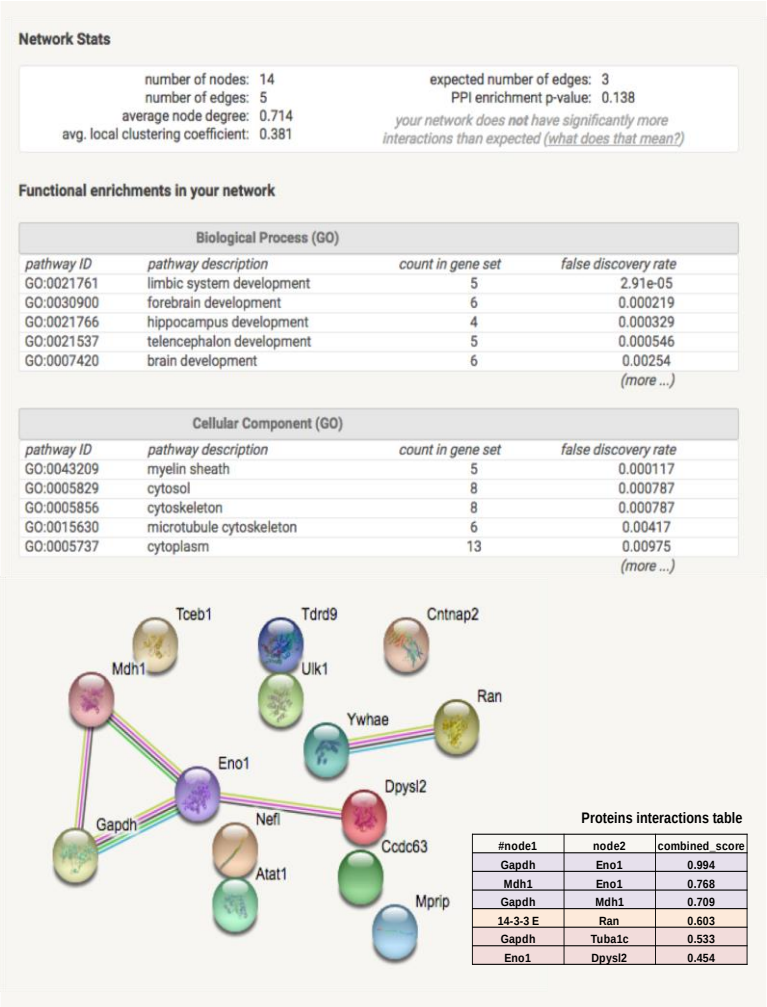
interactions with microtubules. In particular, Drp-2 is able to bind and copolymerize with tubulin heterodimers. Proteomics studies on rat synaptosomes demonstrated that during physiological condition Drp-2 is highly *O*-GlcNAcylated (Cole, R. N., 2001). In contrast, several phosphoproteomics studies highlighted that in AD brain, Drp-2 undergoes hyperphosphorylation and it is associated with NFT (Di Domenico, 2011; Perluigi, M., 2016; Triplett, J. C., 2016). Drp-2 hyperphosphorylation inactivates the protein by lowering the binding affinity to tubulin. In particular, Drp-2 phosphorylation at specific Ser/Thr residues has been linked to the degenerating neurites in AD (Petratos, S., 2007). Indeed, studies suggest that GSK3β and cyclin-dependent protein kinase 5 (Cdk5), highly expressed in AD, are some of the protein kinases responsible for inactivating Drp-2 in AD (Yoshimura, T., 2005). Unexpectedly, despite the reduction of *O*-GlcNAcylation, we did not observe an opposite increase of phosphorylation in our samples. This event may be explained with the high number of Ser/Thr residues that could undergoes to *O*-GlcNAcylation/phosphorylation mutual exchange or not. If the second case occurs, data will result in unaltered phosphorylation levels. However, a systematic analysis of all Drp-2 Ser/Thr residues would be needed.

A foster intriguing result of our proteomics analysis regards the altered *O*-GlcNAcylation of Ulk1 protein, a key component of the autophagy lysosome pathway (ALS). The induction of ALS, which is involved in degradation of protein aggregates and organelles, is largely known to be reduced in AD human samples as well as mouse models of the disease comprising the 3×Tg-AD (Tramutola, A., 2017; Di Domenico, F., 2017; Colacurcio, D. J., 2018). mTOR is the master regulator of autophagy and during physiological conditions mTOR responds to a nutrient-rich

environment by hyperphosphorylating Ulk1, leading to the inactivation of its complex, thus impeding autophagosome formation and autophagy flux (Perluigi, M.,2015). In the case of starvation, conversely, AMPK inhibits mTOR leads to the activation of the Ulk1 complex and induces the autophagy lysosome pathway (Wani, W.Y., 2015). During AD, a chronic hyperphosphorylation of mTOR and inactivation of Ulk1 has been observed (Tramutola, A., 2015). Ulk1 phosphorylation by mTOR is in line with its reduced *O*-GlcNAc levels observed in this study, suggesting that *O*-GlcNAcylation of Ulk1 could represent a protective mechanism for autophagy induction. In addition, previous studies demonstrated that in addition to Ulk1, other components of the autophagy pathway, such as LC3-II expression, are sensitive to modulation of *O*-GlcNAcylation levels (Zhu, Y., 2018; Wani, W. Y., 2015). Therefore, *O*-GlcNAcylation appears to be critical in regulating autophagy and may be involved in the increased proteotoxicity observed during neurodegenerative diseases.

Finally, we analysed our data by building a protein interaction map using STRING software (version 10.5) in order to understand the potential relationships among the proteins found altered in *O*-GlcNAcylation and the potential effects of their impaired function (Szklarczyk, D., 2017; Jensen, L. J., 2009). As expected, a strong interaction is evident between the three components of the glycolysis pathway and Krebs cycle suggesting their cross talk in mediating the failure of glucose metabolism in AD. Intriguingly, the interaction between Gapdh and α-tubulin has been previously postulated (Butterfield, D.A., 2010), suggesting that Gapdh mediates tubulin phosphorylation and catalyses microtubule formation and polymerization (details are reported in Fig.29).

overall, the proteins showing aberrant *O*-GlcNAcylation levels in 3×Tg-AD mice are involved in energy metabolism, insulin signaling, neuronal structure and autophagy among others. These pathways have pivotal roles in neuronal processes that are known to be impaired in AD; however, our data also allowed to gain further insights into the mechanisms that lead to their alterations. Within this context, our results may favour, conceivably, the identification of further pharmacological targets that can lead to reduced brain damage and cognitive decline.



**Network Stats**

number of nodes: 14
number of edges: 5
average node degree: 0.714
avg. local clustering coefficient: 0.381

expected number of edges: 3
PPI enrichment p-value: 0.138
*your network does **not** have significantly more interactions than expected (what does that mean?)*

**Functional enrichments in your network**

**Biological Process (GO)**

| *pathway ID* | *pathway description* | *count in gene set* | *false discovery rate* |
|---|---|---|---|
| GO:0021761 | limbic system development | 5 | 2.91e-05 |
| GO:0030900 | forebrain development | 6 | 0.000219 |
| GO:0021766 | hippocampus development | 4 | 0.000329 |
| GO:0021537 | telencephalon development | 5 | 0.000546 |
| GO:0007420 | brain development | 6 | 0.00254 |

*(more ...)*

**Cellular Component (GO)**

| *pathway ID* | *pathway description* | *count in gene set* | *false discovery rate* |
|---|---|---|---|
| GO:0043209 | myelin sheath | 5 | 0.000117 |
| GO:0005829 | cytosol | 8 | 0.000787 |
| GO:0005856 | cytoskeleton | 8 | 0.000787 |
| GO:0015630 | microtubule cytoskeleton | 6 | 0.00417 |
| GO:0005737 | cytoplasm | 13 | 0.00975 |

*(more ...)*



**Proteins interactions table**

| #node1 | node2 | combined_score |
|---|---|---|
| Gapdh | Eno1 | 0.994 |
| Mdh1 | Eno1 | 0.768 |
| Gapdh | Mdh1 | 0.709 |
| 14-3-3 E | Ran | 0.603 |
| Gapdh | Tuba1c | 0.533 |
| Eno1 | Dpysl2 | 0.454 |

**Figure 29. Protein-protein interaction network and enrichment analysis.** Combined screenshot from the STRING website, showing results obtained upon entering a set of 14 proteins that result differentially *O*-GlcNAcylated in 3×Tg-AD. In the top inset, two enriched function are described. The bottom inset is showing a reported enrichment of functional connections among the set of proteins, and statistical enrichments detected in functional subsystems. Different coloured edges represent the existence of different type of evidence. A green line indicates neighbourhood evidence; a blue line, gene co-occurrence; a yellow line, text mining; a purple line, experimental evidence. The software calculates an index of

interaction confidence from 0 to 1 based on predicted protein interactions analysis and experimentally published protein interaction data. In details, the table show stronger interactions for enzymes of glycolysis and Krebs cycle. A potential interaction is reported between 14-3-3 epsilon protein and Ran protein. The interaction between Gapdh and α-tubulin is also reported. Lastly, a predicted interaction is reported for Eno1 and Drp-2 proteins.

# Chapter 5

## *Conclusion*

Earlier biochemical analysis of post-mortem AD-brain demonstrated the involvement of insulin resistance, impaired brain glucose metabolism and excessive oxidative stress (OS) in the development of AD pathology. Complexity in the molecular mechanism of such signaling pathways now has become one of the major interests to be studied in neurodegeneration (i.e., Alzheimer). Nonetheless, lacking evidence and understandings, my work further postulated a mutual relationship between impaired brain glucose metabolism and OS mediated -Tau pathology. The study overall demonstrated;

- OS-mediated alteration in signaling pathway (i.e., Akt/GSK-3β axis) and associated hyperphosphorylated Tau in early AD development.
- Impaired brain glucose metabolism-mediated alteration in protein *O-GlcNAcylation* in late AD development.

Provided results from the first study clearly explain that increased OS and impaired BVR-A (a downstream target of insulin signaling cascade) favors the reduction in GSK-3β inhibition at 6 months (early AD phase) in 3xTg-AD mice model and human MCI brain. Because the previous study from different groups has shown; i) early impairment of BVR-A in an MCI and AD brain (Braone, E., 2015) and, ii) BVR-A functions as a scaffold for Akt activation and associated GSK-3β inhibition (Miralem, T., 2016), we further hypothesized an interlink between these two significant mechanisms to investigate the role of BVR-A in Tau hyperphosphorylation as an early event

in AD pathology. We found a novel mechanism in our 3xTg-AD mice, human MCI brain and BVR-A knocked out HEK cells, where BVR-A/Akt/GSk-3β axis is required for Akt activation and GSk-3β activity inhibition. To our knowledge, that impaired glucose metabolism being an early symptom of AD further leads to an early insulin resistance and inactivate the Akt but instead, Barone et al., has shown in their result that hyperphosphorylated IRS (insulin resistance) and reduced BVR-A does not further result in the downstream Akt inactivation (Barone, E., 2016). In this case in spite of Akt inactivation through insulin resistance, our data shows that increased OS-mediated impairment of BVR- A could also promote the Akt inactivation by eliminating the BVR-A/Akt complex formation. Thus, the reduction in Akt activation and GSK-3β inhibition results in enormous dysregulated Tau phosphorylation that can be accumulated persistently and become pathologically aberrant hyperphosphorylated Tau at the late phase of the AD.

Its known that insulin resistance and impaired brain glucose metabolism promotes the OS in feedback mechanism, but they also promote the reduction in brain glucose uptake which further followed by aberrant *O-GlcNAcylation* in the AD brain. Interestingly, aberrant *O-GlcNAcylation* has been coincided with 2-3-fold increased hyperphosphorylated proteins in the AD brain at late phase. Gatta et al. have demonstrated that reduced *O-GlcNAcylation* of Tau is associated with its increased hyperphosphorylation in the hippocampus of 3xTg-AD mice at 12 months but no significant changes at 6 months (Gatta, E., 2016). Therefore, we hypothesized in our second study that reduction in *O-GlcNAcylation* process at late phase could be a reason for increased phosphorylation of proteins and would alter its role in signaling. However, it

is not fully discovered whether proteins are more phosphorylated than glycosylated or vice versa. This cross-talk between *O-GlcNAcylation* and phosphorylation could be associated with several pathologies including Alzheimer disease where we have demonstrated that many proteins in the hippocampus of 3xTg-AD mice at 12 months are less *O-GlcNAcylated* but more hyperphosphorylated at the same time. These proteins have identified in energy metabolism (OGT), insulin signaling (IRS, Akt), neuronal structure (Drp-2, Tau) and autophagy (ULK-1) among others.

From our study we confirm that oxidative stress-mediated BVR-A impairment is responsible for reduction in GSK-3β inhibition at early phase of AD that further promotes Tau hyperphosphorylation. This hyperphosphorylated Tau in early phase will further accumulate over the time and together with increased hyperphosphorylated Tau in late phase due to reduced *O-GlcNAcylation* would precede to appear as a patho-clinical symptom in AD brain (Figure 32). Both our studies highlight the noteworthy interplay between impaired glucose metabolism and increased OS in the development of AD pathology which further provides a keen insight to understand the molecular events at different stages of the AD, i.e. early onset or late-onset AD. These molecular events could be a target for a therapeutic approach for AD in future.

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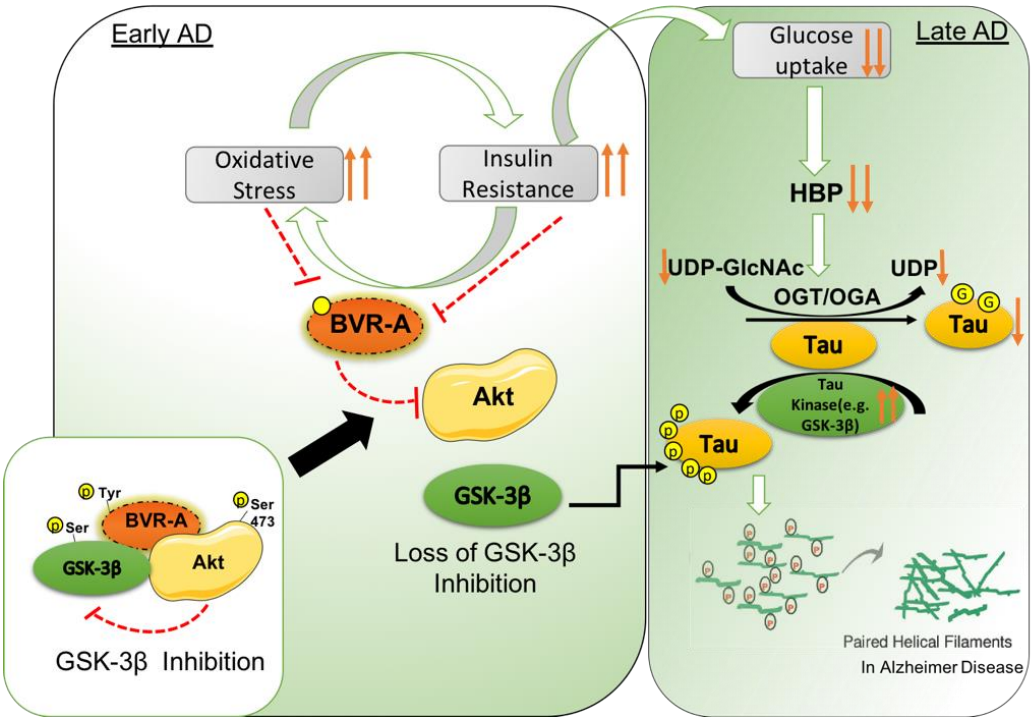


***project 1*** ***project 2***

**Figure 32:** Loss of biliverdin reductase-A (BVR-A) limits the oxidative stress-induced Akt-mediated inhibition of GSK-3β. This process is evident during the early phases of AD pathology and leads to increased Tau Ser404 phosphorylation, known to be more prone to aggregates into neurofibrillary tangles (NFTs) (***Project 1***). Such aggregates persistently accumulate and later become a pathology in the AD. Although, early impairment of BVR-A and insulin signaling is also responsible for impaired brain glucose uptake in the late phase of AD. This aberrant glucose uptake leads to the reduction in *O-glycosylation* (mechanism mentioned above) and increase the phosphorylation of several proteins involved in energy metabolism (i.e., Akt) or neuronal stability (i.e., Tau) ***(Project 2***). Thus, these two significant events occur respectively in the early phase and late phase of the AD and together contribute in a Tau pathology that leads to Alzheimer like dementia.

## APPENDIX A

### Loss of biliverdin reductase-A favors Tau hyper-phosphorylation in Alzheimer disease

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# APPENDIX B

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## Proteomic identification of altered protein O-GlcNAcylation in a triple transgenic mouse model of Alzheimer's disease

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ABSTRACT

PET scan analysis demonstrated the early reduction of cerebral glucose metabolism in Alzheimer disease (AD) patients that can make neurons vulnerable to damage via the alteration of the hexosamine biosynthetic pathway (HBP). Defective HBP leads to flawed protein *O*-GlcNAcylation coupled, by a mutual inverse relationship, with increased protein phosphorylation on Ser/Thr residues. Altered *O*-GlcNAcylation of Tau and APP have been reported in AD and is closely related with pathology onset and progression. In addition, type 2 diabetes patients show an altered O-GlcNAcylation/phosphorylation that might represent a link between metabolic defects and AD progression. Our study aimed to decipher the specific protein targets of altered *O*-GlcNAcylation in brain of 12-month-old 3×Tg-AD mice compared with age-matched non-Tg mice. Hence, we analysed the global *O*-GlcNAc levels, the levels and activity of OGT and OGA, the enzymes controlling its cycling and protein specific *O*-GlcNAc levels using a bi-dimensional electrophoresis (2DE) approach. Our data demonstrate the alteration of OGT and OGA activation coupled with the decrease of total *O*-GlcNAcylation levels. Data from proteomics analysis led to the identification of several proteins with reduced *O*-GlcNAcylation levels, which belong to key pathways involved in the progression of AD such as neuronal structure, protein degradation and glucose metabolism. In parallel, we analysed the *O*-GlcNAcylation/phosphorylation ratio of IRS1 and AKT, whose alterations may contribute to insulin resistance and reduced glucose uptake. Our findings may contribute to better understand the role of altered protein *O*-GlcNAcylation profile in AD, by possibly identifying novel mechanisms of disease progression related to glucose hypometabolism.

### 1. Introduction

The human brain constitutes only 2% of the body's mass but, under the basal condition, its metabolism accounts for up to 30% of the total body glucose uptake [1]. With the increase in age, brain glucose utilization declines to different degrees in different brain regions, and such decline is accelerated in Alzheimer disease (AD), for which aging is the most important risk factor [1, 2]. A broad number of studies have proven the reduction of glucose uptake in the brain of AD patients [3, 4]. Alterations of brain glucose metabolism seems to occur also in subjects affected by mild cognitive impairment (MCI), prior to the appearance of clinical signs of dementia. Therefore, it is suggested this event is a cause, rather than a consequence of AD [5, 6]. Most of the glucose in the brain is oxidatively metabolized to produce ATP, to maintain neuronal activities and functions. However, approximately 2–5% of total glucose is also utilized in the hexosamine biosynthetic pathway (HBP) to produce glucosamine-6-phosphate and, ultimately, UDP-*N*-acetylglucosamine (UDP-GlcNAc) [1, 7]. UDP-GlcNAc is the donor substrate for *O*-linked-β-*N*-acetylglucosamine transferase (OGT), which catalyses protein *O*-GlcNAcylation, a process transferring a single moiety of *N*-acetyl-D-glucosamine from UDP-GlcNAc to the hydroxyl side chains of serine and threonine residues of proteins. The reversible

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# APPENDIX C

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## Biliverdin reductase-A impairment links brain insulin resistance with increased Aβ production in an animal model of aging: Implications for Alzheimer disease

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ABSTRACT

Brain insulin resistance is associated with an increased Aβ production in AD although the molecular mechanisms underlying this link are still largely unknown. Biliverdin reductase-A (BVR-A) is a unique Ser/Thr/Tyr kinase regulating insulin signalling. Studies from our group, demonstrated that BVR-A impairment is among the earliest events favoring brain insulin resistance development. Furthermore, reported a negative association between BVR-A protein levels/activation and BACE1 protein levels in the parietal cortex of aged beagles (an animal model of AD), thus suggesting a possible interaction. Therefore, we aimed to demonstrate that BVR-A impairment is a molecular bridge linking brain insulin resistance with increased Aβ production. Age-associated changes of BVR-A, BACE1, insulin signalling cascade and APP processing were evaluated in the parietal cortex of beagles and experiments to confirm the hypothesized mechanism(s) have been performed in vitro in HEK293APPswe cells. Our results show that BVR-A impairment occurs early with age and is associated with brain insulin resistance. Furthermore, we demonstrate that BVR-A impairment favors CK1-mediated Ser phosphorylation of BACE1 (known to mediate BACE1 recycling to plasma membrane) along with increased Aβ production in the parietal cortex, with age. Overall, our results suggest that the impairment of BVR-A is an early molecular event contributing to both (I) the onset of brain insulin resistance and (II) the increased Aβ production observed in AD. We, therefore, suggest that by targeting BVR-A activity it could be possible to delay the onset of brain insulin resistance along with an improved regulation of the APP processing.

### 1. Introduction

Alzheimer disease (AD) is the most common cause of dementia and a primary age-related neurodegenerative disorder [1]. Aβ is thought to play a central role in the pathogenesis of AD: (i) high levels of Aβ small oligomers markedly reduce synaptic plasticity, thus favoring learning and memory deficits [2,3] (ii) accumulation of Aβ peptides into fibrillar deposits is associated with extensive neuronal loss [4] and (iii) Aβ impairs mitochondrial redox activity, increases the generation of reactive oxygen (ROS) and reactive nitrogen species (RNS), which in turn contribute to lipid and protein damage [5,6]. Impairment of brain insulin signalling, known as brain insulin resistance, has been proposed to have a role in the production and accumulation of Aβ in AD [7–10]. Consequently, as in a vicious cycle, increased Aβ oligomer generation, further exacerbates brain insulin resistance through the down-regulation the of the insulin receptor (IR) [11]. Subsequently, the activation of neuronal tumor necrosis factor-alpha (TNF-α) receptor along with the aberrant activation of stress-regulated kinases leads to insulin receptor substrate 1 (IRS1) inhibition [12,13].

From a molecular point of view, Aβ is generated following the sequential cleavage of the amyloid precursor protein (APP) by two enzymes, i.e., β-site APP cleaving enzyme 1 (BACE 1) and γ-secretase, which drive the amyloidogenic pathway [14,15]. BACE1 is the rate-limiting enzyme in the process leading to Aβ generation [15]. Among the mechanisms proposed to regulate BACE1 activity, it has been reported that mature BACE1 is internalized from the cell surface to early

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*-Nidhi Sharma*

*The Goodness of wisdom will never fade away*

*Rise your hopes high*

*Deep down we are passionate*

*Move forward till the last breath*

*Embrace the everlasting commitments*

*The love and compassion we own, is the only treasure in our life*

*We should make ourself independent and modest*

*We can rise and fall but should not loose the self esteem*

*Be strong to get milestones*

*Dont stop, we have got wings*

*We have got wide horizons*

*The troubles we have got, the hustles world has made , is just the gatway for our success*

*We only know that what we have earned and what we have won??*

*Wisdom is in you*

*Wisdom is for you*

***Nidhi Sharma***