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Review

Aberrant protein phosphorylation in Alzheimer disease brain disturbs pro-survival and cell death pathways

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ABSTRACT

Protein phosphorylation of serine, threonine, and tyrosine residues is one of the most prevalent post-translational modifications fundamental in mediating diverse cellular functions in living cells. Aberrant protein phosphorylation is currently recognized as a critical step in the pathogenesis and progression of Alzheimer disease (AD). Changes in the pattern of protein phosphorylation of different brain regions are suggested to promote AD transition from a presymptomatic to a symptomatic state in response to accumulating amyloid β-peptide (Aβ).

Several experimental approaches have been utilized to profile alteration of protein phosphorylation in the brain, including proteomics. Among central pathways regulated by kinases/phosphatases those involved in the activation/inhibition of both pro survival and cell death pathways play a central role in AD pathology.

We discuss in detail how aberrant phosphorylation could contribute to dysregulate p53 activity and insulin-mediated signaling. Taken together these results highlight that targeted therapeutic intervention, which can restore phosphorylation homeostasis, either acting on kinases and phosphatases, conceivably may prove to be beneficial to prevent or slow the development and progression of AD.

1. Introduction

Alzheimer disease (AD) is an irreversible cognitive disorder that affects the integrity of the central nervous system and is the leading cause of dementia in the elderly. Slow and progressive degeneration of neurons in brain regions involved in learning and memory results in gradual cognitive decline, loss of memory, personality changes, and impairment of normal social and emotional behaviours. Extensive synapse and neuron loss in selective brain regions are key hallmarks of AD along with two other main histological lesions, amyloid plaques and neurofibrillary tangles.

Senile plaques are extracellular deposits resulting from the aggregation of amyloid-β (Aβ) peptides surrounded by dying neuritis [1], while neurofibrillary tangles [2] result from the intracellular accumulation of hyperphosphorylated tau, a microtubule-associated protein that is involved in stabilizing microtubules, necessary for axonal transport and other functions [3].

Growing evidence indicates that the progressive atrophy of the AD brain is due to cell and synaptic loss, however the precise mechanisms/types of cell death have to be clarified yet. Considering that AD is an age dependent neurodegenerative disease it is reasonable to speculate that neuronal death is the result of the accumulation of multiple "chronic" insults that alone are insufficient to lead to disease onset [4]. Among putative pathways, it is likely that disturbance of cell cycle machinery and/or increasing oxidative stress conditions make neurons vulnerable to further insults. If from one side low levels of ROS can activate physiological intracellular signaling and induce compensatory changes, from the other side with "chronic" progressive stress the majority of neurons become irreversibly damaged and may undergo neuronal loss [4]. Indeed, increased levels of oxidative stress are associated with mitochondrial dysfunction, sustained inflammation, axonal degeneration and impairment of synaptic transmission that contribute to neuronal death, possibly by apoptosis. Neuronal loss is particularly evident in vulnerable brain areas such as entorhinal cortex, hippocampus and association regions of the neocortex.

Cell survival and cell death pathways are mainly regulated by orchestrated signaling cascades due to a number of post-translational modifications (PTMs) of selected protein targets, among which phosphorylation/de-phosphorylation is the most prominent. The addition of phosphate groups to proteins is a major modification for initiating, coordinating and controlling complex cellular functions

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such as energy production, cell growth and survival. Phosphorylation can have profound effects on protein activity, function and localization in the cell, both in health and disease.

Because phosphorylation is fast, reversible, and highly specific, it is the most efficient way to temporarily modulate protein function, i.e. induce or inhibit enzyme activity, facilitate or disrupt protein interactions, alter protein conformations, or target proteins for clearance. Protein phosphorylation and dephosphorylation are catalyzed by over 500 kinases and 100 phosphatases and are themselves regulated by phosphorylation, revealing a complex crosstalk among multiple signaling pathways [5].

However, despite this great number of kinases and phosphatases in the brain, it is probably the aberrant regulation of a few that represent the triggering events leading to the spread of an aberrant signaling in AD. In particular, the role of the kinases regulating learning and memory is noteworthy of mention. In this context, the dysregulation of the calcium/calmodulin-dependent kinase II (CaMKII), the calcium/calmodulin kinase cascade, extracellular signal regulated kinase 1 and 2 (ERK1/2), cAMP-dependent protein kinase A (PKA), cGMP-dependent protein kinase G (PKG), the phosphatidylinositol 3-kinase (PI3K) pathway, and protein kinase M z (PKMz) could represent the starting signal, which promotes neurotoxic outcomes [6,7]. Indeed, these kinases have a main role in the regulation of a number of different molecular mechanisms involved either in the control of neuronal functions or in the maintenance of neuronal structures including: (1) regulation of ion channel density and/or conductivity, which impacts on synaptic transmission (e.g., regulation of AMPA receptor trafficking); and (2) regulation of gene transcription and/or local translation, which impact on structural growth of existing synapses and/or synaptogenesis (reviewed in [7]). This picture is further complicated by the fact that, a given kinase may regulate different processes. Furthermore, it should be noted that other than the kinases described above, a long list of other kinases play key roles in the regulation of neuronal plasticity and have been found to be altered in AD [8,9]. For example, PKMzeta and iota, another form of PKC, have specific roles in spine plasticity. The receptor tyrosine kinase TrkB, binding the neurotrophin BDNF, has been implicated in regulating spine plasticity through CREB-dependent transcription and local protein synthesis. Cdk5, a predominantly neural-specific serine/threonine kinase, regulates spine plasticity through phosphorylating NR2A subunits of NMDA receptors, and seems pivotal in the regulation of spine morphology by inhibiting, indirectly, actin polymerization and reducing the number of stubby-shaped spines [6,7].

A crucial kinase in AD pathology is FYN kinase, that belongs to the Src family of nonreceptor tyrosine kinases [15]. Fyn activity, like that of other Src family kinases, is regulated by intramolecular interactions upon tyrosine phosphorylation and de-phosphorylation [16]. Fyn possesses diverse biological functions such as T-cell receptor signaling, cell division and adhesion, platelet function, synaptic function and plasticity, and central nervous system myelination [17]. Of particular interest for AD is the link between Fyn kinase and synaptic function. Shirazi and Wood reported that a subset of neurons from AD brain exhibited strong Fyn immunoreactivity compared with control brains, and that these neurons were also positive for abnormally phosphorylated Tau protein [18]. Conversely, Fyn negative cells are protected against Aβ-induced neurotoxicity [19]. Further, the finding that tau-negative cells are also protected against Aβ-induced neurotoxicity leads to speculation that the interaction between Fyn and tau has a crucial role in the neurodegenerative process [15]. Thus, Fyn represents a promising therapeutic target in AD as it is involved both in Aβ signal transduction and also has major functional interactions with Tau, thereby unifying the two pathological hallmarks of AD.

In addition, dysregulation of protein phosphatases is of extreme importance due to the fact that: i) their activity is often regulated by kinase-dependent phosphorylation; and ii) they are responsible of protein de-phosphorylation. Although tau-associated phosphatases are widely investigated in AD [10], other phosphates could be of relevance to AD, especially because they regulate the activity of the kinase cited above. These include, e.g., CD45 [11], PTP1B [12] PTEN [13], PP2A and PP2B [14], which have been also identified as possible target for AD treatment.

Aberrant phosphorylation of several proteins occurs in the brain of AD patients and also in its prodromal phase, amnestic mild cognitive impairment (MCI) [20,21]. Several experimental approaches have been utilized to profile alteration of protein phosphorylation in the brain, including proteomics platforms and top-down approaches, suggesting that the protein phosphorylation/dephosphorylation system might be dysregulated in AD brain. Among central pathways regulated by kinases/phosphatases those involved in the activation/inhibition of either pro-survival or cell death pathways play a central role in disease pathology, both in cancer and in neurodegeneration.

Emerging evidence obtained from brain tissue derived from patients with chronic neurodegenerative diseases and animal models implicates the p53 tumor suppressor protein in the regulation of neuronal cell death. Recent observations suggest that p53 plays a crucial role in aging and in neurodegenerative disorders. However, there is still an open debate whether brain aging is due to a programmed process or is the consequence of failed mechanisms for regeneration and/or repair. Although p53 promotes longevity by decreasing the risk of cancer through activation of apoptosis or cellular senescence, several findings suggest that the uncontrolled increase of its activity may have deleterious effects leading to “abnormal” aging phenotypes [22–24].

Phosphorylation cascades also are crucial in transducing pro-survival responses. Among these, key survival pathways mediated by several kinases are controlled by insulin and insulin growth factor (IGF1). In the brain, insulin contributes to synaptic maintenance, neuronal outgrowth and survival, learning and memory, as well as weight and sexual maintenance and regulation [25]. Increasing studies report that aberrant insulin signaling contributes to neurodegeneration in AD by affecting all the above-mentioned pathways [26].

In the sections below, we report proteomics results showing aberrant phosphorylation of specific proteins in AD brain that suggest the impairment of several neuronal functions. In addition, we will discuss in detail how aberrant phosphorylation could contribute to dysregulation of p53 activity and insulin-mediated signaling. Taken together, these results highlight that therapeutic intervention that can restore phosphorylation homeostasis, either acting on kinases and phosphatases, may prove to be beneficial to prevent or slow the development and progression of AD.

2. Phosphoproteomics: a tool to identify aberrant signaling in Alzheimer brain

Protein phosphorylations is one of the most prevalent PTMs that mediates diverse cellular functions in living cells [27]. It is estimated that at least one-third of eukaryotic proteins are phosphorylated, however only a subset these are modified by any given stimulus [28–31]. A highly dynamic network of about 500 kinases and 100 phosphatases regulates protein phosphorylation. The complex cooperation between the different classes of kinases and phosphatases triggers the dynamics of phosphorylation cycles regulating major cellular processes like proliferation, differentiation, apoptosis, protein subcellular localization and degradation [32,33]. Aberrant protein phosphorylation is generally accepted as a critical step in the onset or progression of neurodegenerative disorders such as AD [34,35]. Changes in the pattern of protein phosphorylation of different brain regions is likely crucial to promote AD transition from a presymptomatic to a symptomatic state in response to accumulating amyloid β-peptide (Aβ) [36]. Indeed, phosphorylation changes contribute to disturbance of multiple signaling pathways and, in part, contribute to the transition to a pathological state postulated to be necessary for cognitive decline [37]. The hyperphosphorylation of tau, which leads to the formation of

neurofibrillary tangles, is one of the most extensively studied aberrant phosphorylation event in neurodegenerative disease. Tau phosphorylation represents an early event in AD progression and strongly correlates with impairment of episodic memory and cognitive decline [38]. Tau hyperphosphorylation is a primary example of "phospho-pathology", in which progressive alterations in phosphorylation status alter the capacity of tau to stabilize microtubules thereby impairing axonal transport [39]. However, tau hyperphosphorylation has been found to be associated also with other neurotoxic effects in AD, thus highlighting multiple roles for this protein [40,41]. Among these (1) iron accumulation [42], (2) dysfuntion of motor proteins [43], (3) impairment of the long-term depression (LTD), which results in a deficit in spatial reversal learning [43], (4) NMDA receptor hypofunction [44], (5) impaired neuronal hyperexcitability [45] and (6) reduced Fyn-induced Src family kinase activity [46] have been detected following tau dysregulation.

Aberrant phosphorylation of several other proteins also has been found to be associated with AD [29,31,47–49]. The imbalance between kinase- and/or phosphatase- function seems the primary cause of aberrant protein phosphorylation. Indeed, in AD brain, a growing list of kinases such as Akt, extracellular signal-regulated kinase 1 and 2 (ERK1/2), cAMP- dependent protein kinase (PKA), glycogen synthase kinase-3β (GSK-3β), p70S6 kinase, and cyclin-dependent protein kinases 5 (Cdk5) was found with increased expression or activity, whereas decreased activity was observed for protein phosphatases such as PP1, PP2A and PP5 [50–52].

Several hypotheses have been proposed to clarify the altered phosphorylation events in AD. A role has been suggested for insulin signaling that appears impaired in AD brain [26,53]. The binding of insulin to insulin receptor leads to its autophosphorylation and activation of its kinase activity, and this in turn phosphorylates different substrates activating various downstream signaling partners and signaling cascades such as MAPK pathway [54]. Recent studies posted that the reduction of brain glucose metabolism contributes to the pathogenesis of AD also through the modulation of the phosphorylation pattern of brain proteins [55,56]. Impaired brain glucose metabolism decreases hexsosamine biosynthesis pathway flux and, consequently, alters O-GlcNAcylation, leading to abnormal phosphorylation [55,57–60]. The recent development of novel phosphoproteomics approaches, applied to the analysis of the AD brain proteome, has been crucial in identifying the alteration of key signaling networks and of their cross-talk, thereby providing to acquire additional insights into the onset and progression of the pathology [21,34,61–64] (Table 1).

In 2008 Xia et al. described the phosphoproteome analysis of post-mortem AD brain tissue proteins using calcium phosphate precipitation (CPP) enrichment directly coupled with the LC-MS/MS approach. Before CPP and LC-MS/MS the protein samples were resolved by one-dimensional polyacrylamide gel electrophoresis and subjected to gel excision and in-gel digestion [61]. A total of 466 phosphorylation sites on 185 proteins, including tau protein, were identified. The further selection of the 20 proteins most frequently identified, among which were present the microtubule-associated protein 1B, syntaxin 1A and HSP90, reported the identification of a number of novel phosphorylation sites [61].

In 2010 Rudrabhatla and co-workers applied the isobaric tag for relative and absolute quantitation (iTRAQ) technique to the quantitative phosphoproteomic approach for the characterization of the high-molecular-weight neurofilament protein (NF-H) and the medium-molecular-weight neurofilament (NF-M) phosphorylation sites in AD brain [65]. The authors identified 13 hyperphosphorylated sites of NF-M; 9 Lys-Ser-Pro (KSP) sites; 2 variant motifs, Glu-Ser-Pro (ESP) Ser-736 and Leu-Ser-Pro (LSP) Ser-837; and 2 non-S/T-P motifs, Ser-783 and Ser-788. All the Ser/Thr residues were phosphorylated at significantly greater abundance in AD brain compared with control brain. In addition, ten hyperphosphorylated KSP sites were identified on the C- terminal tail domain of NF-H, with greater abundance of phosphorylation in AD brain compared with control brain. This study

Table 1
Summary of phosphoproteomics studies on human and on mouse model brains from early and late pathological stages of AD.

Samples	Method of analysis	Proteins aberrantly phosphorylated	Biological function	References
Human AD total brain	Calcium phosphate precipitation + LC-MS/MS	185 proteins (e.g. MAPs NF3, NF-H, Adducin 1–2, Ankyrin 2, Syntaxin 1, GAP-43, BASP1, AKAP12, MARKS, NCAM1, HSP90)	Neuronal structure, molecular chaperones, synaptic transmission.	Xia et al. [61]
Human AD total brain	iTRAQ + MS/MS	NF-M, NF-H	Neuronal structure	Rudrabhatla et al. [65]
Human AD total brain	LC-MS/MS	Tau, MAP1B, MAP2	Neuronal structure	Rudrabhatla et al. [64]
Human AD hippocampus	2DE proteomics + ESI-MS/MS	MEK1, GDI2, GNAO1, GAPDH, ENOA-G, FBA, CRMP2, IMMT, GFAP, VPC, CKB, SIRT2, NDUFS3, 6PGD, GRHPR, QDPR.	Signal transduction, energy metabolism, anabolic pathways, neuronal structure and trafficking.	Di Domenico et al. [34]
Human AD cortex and substantia nigra	2DE proteomics + Q-ToF MS	FBA, GAPDH, TPI, CRMP2, GFAP, MDH, AAT, APEH, FTH.	Energy metabolism, neuronal structure and transport, iron homeostasis.	Zahid et al. [72]
TgCRND8 AD mouse model hippocampus	Ti^{4+}- IMAC + nanoflow LC-MS/MS.	139 phosphopeptides, 36 directly interacting proteins (e.g. SHANK3, CRMP1–5, MAP1A, tau)	Synaptic transmission, neuronal structure, signal transduction	Wang et al. [36]
APP-Tg2576, PS1-dE9, PS2-M1, 5xFAD and P301S mouse models brain + human AD occipital lobe	2D LC–MS/MS	ACON, ADDB, ATPA-B, NFH-L, SPTA2, BASP1, CLH, G3P, GPRIN1, MARCKS, NEUM, SRRM2,HS90A,Marcksl1, SYT1 (common to >2 AD model/sample)	Synaptic transmission, energy metabolism, neuronal structure, spine formation	Tagawa et al. [63]
Human AD frontal cortex	IMAC + LC-MS/MS	253 phosphopeptides (e.g. tau, HSP27, CRYAB)	Neuronal structure, molecular chaperones, synaptic transmission, cell junction	Dammer et al. [73]
Human, AD, MCI and PCAD inferior parietal lobule	2DE proteomics + ESI-MS/MS	PCAD vs. CTR: HSP70, Gsn, SMP30, SP2, BVR-B, LDHB, VDAC1, CNP1. MCI vs. CTR: EFhd1, CS, VCP, SMP30, Gsn. AD vs. CTR: VDAC1–2, LDHB, GNAO, Efhd1, Gsn, SMP30, CRMP2, Stm, Stxbp, SOD1, CBR1, Prdx1.	Energy metabolism, cellular signaling, Neuronal structure, oxidative stress response, proteostasis network	Triplett et al. [59]

provided the direct evidence that NF- M/H are hyperphosphorylated in AD compared with control brain and suggested a role for both proline-directed and non-proline-directed protein kinases in AD [65]. The same authors also performed LC-MS/MS of NFT tryptic digests [64]. The phosphoproteomics of NFTs clearly identified NF-M phosphopeptides corresponding to Ser685, and to Ser736, and an NF-H phosphopeptide corresponding to Ser942. Mass spectrometry revealed Tau phosphopeptides corresponding to Thr231, Ser235, Thr181, Ser184, Ser185, Thr212, Thr217, Ser396, and Ser403. And finally, phosphopeptides corresponding to MAP1B (corresponding to Ser1270, Ser1274, and Ser1779) and MAP2 (corresponding to Thr350, Ser1702, and Ser1706) were identified. In corresponding matched control preparations of PHF/NFTs, none of these phosphorylated neuronal cytoskeletal proteins were found [64].

In 2011 a study conducted by Di Domenico and co-workers employed a multiplexed 2DE proteomics approach to analyze alterations of the AD hippocampus phosphoproteome compared to healthy subjects (CTR) [34]. The methodology took advantage of a fluorescent phosphosensor, Pro-Q Diamond, capable of sensitive detection of phosphoserine-, phosphothreonine-, and phosphotyrosine-containing proteins. The data obtained by MS/MS protein identification confirmed the altered phosphorylation in AD samples compared to CTR, detecting seventeen proteins with either increased or decreased phosphorylation [34]. The proteins identified belong to critical neuronal processes and could be classified according their function into four groups including: signal transduction, energy metabolism, anabolic pathways and neuronal structure and trafficking. Map kinase kinase 1 (MEK1) and Rab GDP dissociation inhibitor beta (GDI2), found aberrantly phosphorylated in AD, are part of the MAPK and GDI family involved in neuronal signaling in response to growth factor stimulus [66,67]. The altered phosphorylation of metabolic enzymes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enolase (ENO), and fructose bisphosphate aldolase (FBA), contribute to the impairment of energy metabolism and reduced glucose utilization occurring in AD [68]. Moreover, the altered phosphorylation of structural proteins, such as collapsin response mediator protein 2 (CRMP2), inner membrane protein, mitochondrial (IMMT), glial fibrillary acidic protein (GFAP) and transitional endoplasmic reticulum ATPase (VCP) observed [34], could contribute to the known altered neuronal architecture and the altered neurotransmission in AD [69,70]. It is interesting to note that also a number of proteins found with altered phosphorylation are known to be also targets of oxidative modification in AD, suggesting a potential cause-effect relationship between different PTMs [71].

In 2012 Zahid and colleagues applied the same 2D multiplexed approach to the cortex and the substantia nigra of AD patients compared to healthy subjects [72]. Altered phosphorylation was found for 9 proteins involved in cell metabolism, signal transduction, cytoskeleton integration, and synaptic function. This study confirmed the aberrant phosphorylation of GAPDH, FBA, enolase and DRP2 (CRMP2) reported in [34] as a characteristic feature of AD brain. Moreover the identification of further proteins with impaired phosphorylation pattern such as aspartate aminotransferase (AAT), triosephosphate isomerase (TPI), malate dehydrogenase (MDH), ferritin heavy chain (FECH) and acylamino-acid-releasing (ACY1) enzyme that suggested their alteration could contribute to AD progression [72].

In 2013 Wang et al. utilized an unbiased proteomic approach to compare the phosphoproteome of brain from TgCRND8 mouse model of AD, identifying the disruptions of a network of signaling pathways implicated in the manifestation of behavioural indices of learning and memory impairment [36]. Methodologically, the authors applied phosphopeptide enrichment with triple isotopic dimethylation labeling combined with online multidimensional separation and MS-to-profile phosphoproteome changes to the analysis of the hippocampus from presymptomatic (2-month-old) and symptomatic (6-month-old) Tg mice compared to non-Tg littermates. A total of 1026 phosphopeptides, representing 1168 phosphorylation sites (83% pS, 15% pT, and 2% pY) from 476 unique proteins were identified [36]. Of these, 595 phosphopeptides from 293 unique proteins were reliably quantified and 139 phosphopeptides were found to change significantly in the hippocampus of TgCRND8 mice following conversion from a presymptomatic to a symptomatic state. Taken together, these data further identify the subset of phospho-signaling targets that are altered during the transition to symptomatic state of Tg mice. By network analysis the authors identified a subset of 36 phosphoproteins that directly interact each others and whose phosphorylation status is consistently altered upon conversion from a presymptomatic to a symptomatic state. This protein interaction network comprises changes in proteins involved in synaptic function and in cytoskeletal maintenance such as SH3 and multiple ankyrin repeat domains protein 3 (SHANK3), CRMP1–5, MAP1a and tau, thus supporting once again that the aberrant phosphorylation of proteins involved in neuronal structure and architecture is a key feature in this AD model [36].

In 2015 Tagawa and colleagues screened phosphoproteins and phosphopeptides in four types of AD mouse models: APP-Tg2576 (KM670/671NL), PS1-ΔE9 (Exon9-deleted), PS2-M1 (N141I) and 5xFAD (APP, KM670/671NL, I716V, V717I. + PS1, M146L, L286V.), one type of Tau (P301S) transgenic mice and human AD postmortem brains using 2D LC–MS/MS analysis [63]. The changes in the phosphoproteome observed in mouse models and AD brain were investigated using super computer analysis and methods of systems biology. Interestingly, the authors identified seventeen phosphoproteins altered among the groups of comparison (Tg mice vs. WT mice), known to be involved in energy production, axonal and dendritic spine formation, synapse vesicle function and the cytoskeleton. By using experimentally verified protein – protein interaction databases authors found that twelve of seventeen proteins were directly connected and three proteins, were linked via one independent protein, therefore all forming a functional network linked to synaptic spine formation [63]. The changes of the identified core network of phosphoproteins started at a preclinical stage even before histological Aβ deposition, suggesting a primary role in neurodegeneration. Moreover, the authors identified phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS) by overactivated kinases are an initial event to trigger synapse pathology at the earliest stage of mouse AD pathology far before clinical onset and even before histological Aβ deposition [63]. A subsequent two-photon microscopy analyses revealed that genetic and pharmacological manipulations targeting MARCKS and candidate kinases is able to recover the loss of spines in the mouse models employed in the study supporting the hypothesis formulated by the authors and based on phosphoproteome data [63].

In the same year Dammer and colleagues employed immobilized metal ion affinity chromatography (IMAC) followed by LC-MS/MS to identify phosphopeptides from postmortem human brain tissues of AD patients and age-matched control [73]. The researchers identified 5569 phosphopeptides in frontal cortex across all 16 cases in which phosphopeptides represented 80% of all MS/MS spectra. Comparative marker selection software analysis identified 253 significantly altered phosphopeptides by precursor intensity, changed by at least 1.75-fold relative to controls. Approximately 21% of all significantly altered phosphopeptides in AD tissue were derived from tau [73]. The other 142 proteins hyperphosphorylated in AD, were mainly part of HSPs, membranes, synapses and cell junction structures. Of these, the authors validated differential phosphorylation of HSP27 and crystallin-alpha-B as hyperphosphorylated in AD [73]. In addition, it was highlighted that sites of increased phosphorylation of small HSPs highly correlated and potentially co-regulated with kinase phosphorylation events during AD, thus supporting the notion that a number of kinases are regulating and/or are regulated by the small HSP folding network [73].

A parallel study by Tan and co-workers analyzed AD phosphoproteome by a pilot TiO_2 enrichment coupled with high resolution LC-MS/MS [62]. Using this method, the authors analyzed the phosphoproteome of 1 mg of digested AD brain lysate, identifying 5243 phosphopeptides containing 3715 non-redundant phosphosites on

1455 proteins, including 31 phosphosites on the tau protein. This study, beyond supporting the use of an effective and robust method for dissecting the phosphoproteome, identified also a number of novel phosphorylated sites of AD post mortem brain proteins [62].

Recently, Triplett et al. evaluated changes in protein phosphorylation states in the inferior parietal lobule of subjects with AD, amnestic MCI, pre-clinical AD (PCAD), and control brains [59] using the 2D multiplexed approach described before [34]. These analyses led to the identification of 19 proteins differentially phosphorylated in the disease states. In particular: 8 proteins were found to have significantly altered phosphorylation levels in the comparison of PCAD and control brains (Heat shock protein70, gelsolin, regucalcin, L-lactate dehydrogenase, voltage dependent anion channel 1, septin 2, flavin reductase [NADPH] (also known as BVR-B) and 2, 3-cyclic nucleotide-3-phosphodiesterase); 5 proteins were found to have significantly altered phosphorylation levels in the comparison of MCI and control specimens (citrate synthase, VCP, gelsolin, regucalcin and EF-hand domain-containing protein D1); 14 proteins were found to have significantly altered phosphorylation levels in the comparison between AD and control brains (voltage-dependent anion-selective channel protein 1, voltage dependent anion-selective channel protein 2, gelsolin, L-lactate dehydrogenase, CRMP2, guanine nucleotide-binding protein G (o), EF-hand domain-containing protein D1, VCP, Cu/Zn superoxide dismutase, peroxiredoxin 1, regucalcin, stathmin, syntaxin-binding protein 1 and carbonyl reductase 1) [59]. Interestingly, some of the proteins are common to two or more groups of comparison (e.g. gelsolin; regucalcin) suggesting their involvement in disease progression from early stages of AD, while others such as CRMP2 or VCP are common to other phosphoproteomic studies [34,72], thus supporting their involvement in AD progression. Overall the authors suggest that aberrant phosphorylation in different AD stages mainly target proteins that contribute to altered Ca^{2+} signaling, energy metabolism, neuronal plasticity, signal transduction, and oxidative stress response. These findings illustrate the impact and contribution that the diseased-state phosphoproteome has on the brain starting in the early non-demented PCAD stage that may lead to progressive cognitive decline in MCI and escalates to dementia of AD [59].

So far, phosphoproteomics studies of AD samples (brain from human and mouse models) have been crucial in identifying the alterations of protein phosphorylation pattern, which results in the impairment of neuronal function, in aberrant neuronal architecture, and in increased neuronal death, thereby contributing to cognitive decline.

3. Cell death pathway in Alzheimer disease: role of p53 and Pin1

The crosstalk between kinases and phosphatases regulates the dynamics of phosphorylation cycles [74], which in turn switch on/off a plethora of cellular processes including proliferation, differentiation, apoptosis, protein subcellular localization and degradation. In the brain of healthy subjects, the number of neurons in different regions is relatively constant and originates during early developmental stages through complex interactions and tuned signaling [75]. Moreover, under physiological conditions, cell death plays a central role in establishing the number of connections among neurons during development.

A major regulator of cell death pathways is p53, at the hub of numerous signaling pathways that are initiated in response to particular stresses (Fig. 1). Thus, p53 and its down-stream mediators contribute to induction of apoptosis or cell-cycle arrest in response to DNA damage, thereby maintaining genetic stability by transcriptional and non-transcriptional mechanisms [76]. The demonstration that p53 promotes apoptosis has important implications for the central nervous system (CNS), where cell death is observed normally during development, in response to injury, and during neurodegeneration, including AD. A possible role for p53-related modulation of neuronal viability has been suggested by the finding that p53 expression is elevated in damaged neurons in acute models of injury such as ischemia and epilepsy and

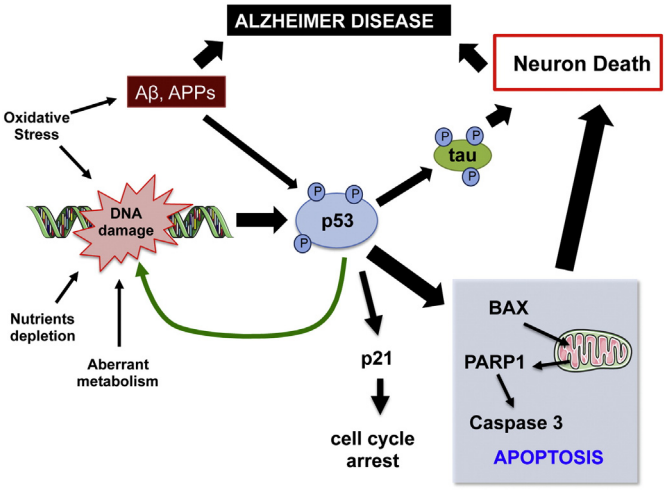

Fig. 1. Sustained activation of p53 pathway contributes to neuronal death. Accumulation and transcriptional activation of p53 occurs in response to different stresses such as DNA damage, oxidative stress, excitotoxicity, etc. p53 undergoes PTMs that critically control its stability and function among which phosphorylation plays a crucial role in the activation of p53 transcriptional activity. It is likely that chronic, toxic stimuli, including APP-Aβ-OS–insult, damage neurons and may be responsible for increased susceptibility of neurons to death. p53 is able to integrate different signals that may lead to a pro-apoptotic phenotype that contributes to the development of AD.

in brain tissue samples derived from patients with chronic neurodegenerative diseases [77]. Moreover, the absence of p53 has been shown to protect neurons from a wide variety of toxic insults by upregulating antioxidant and other protective systems [23,78].

The regulatory events that affect the amount, stability and activity of p53 are the result of different PTMs, including phosphorylation, ubiquitination and acetylation [79]. One of the first events that stimulate p53 activity are the DNA damage and various toxic insults that could cause genomic instability of cells, such as oxidative stress, hypoxia, oncogene activation, changes in metabolism, aberrant intracellular signaling and also depletion of nutrients, among others (Fig. 1).

In normal cells, p53 protein has a relatively short half-life and is degraded by a ubiquitin-proteasome dependent pathway through the action of E3 ubiquitin ligases including MDM2, PirH2, COP-1, and CHIP [80]. Following stress, p53 is phosphorylated at multiple residues, thereby modifying its biochemical functions required for increased activity as a transcription factor. The biochemical functions include sequence-specific DNA binding and protein-protein interactions. Among multiple phospho-acceptor sites reported on p53 only three (Ser15, Thr18, Ser20) are highly conserved among humans [81]. Biochemical and genetic studies show that phosphorylation can activate p53 function. However, the many other sites of covalent modification on p53 also likely play important roles in p53 function or regulation [81]. p53 activity could also be impaired as consequence of a conformational change. p53 may lose its transcriptional activity due to an unfolded tertiary structure which determines a reduction in its affinity for specific DNA target sequence. Recent observations confirm that p53 structural changes can play a central role in aging and in AD.

Among stress conditions, increased oxidative stress plays, at least, two distinct roles in the p53 pathway [4]. First, reactive oxygen species (ROS) are important activators of p53 through their capacity to induce DNA strand breaks [82]. Second, they regulate the DNA-binding activity of p53 by modulating the redox state of a critical set of cysteines in the DNA-binding domain, which in turn induces conformational changes [83,84].

Several studies investigated the relevance of p53 as a marker/effector of AD pathology. Kitamura et al. reported a consistent increase in p53 expression in the pathological temporal cortex areas, and more precisely at the glial level [85]. This astrocytic localization was partly confirmed by a concomitant study showing that indeed, p53 detection

by in situ immunolocalization revealed enhanced p53 expression not only in astrocyte and oligodendrocyte populations of frontal and temporal lobes but also in numerous cortical neurons [86]. The increased p53 expression was also accompanied by enhanced DNA fragmentation and Fas expression, suggesting that p53 could be responsible, at least in part, for cell death observed in AD brain [86]. Interestingly, the same results were also collected by analyzing Down syndrome (DS)-affected brains [87], which are characterized by AD-like histological alterations [88], likely due to the extra copy of chromosome 21.

Studies from our group recently reported the increased acetylation and phosphorylation of p53, coupled to reduced MDM2/p53 complex level and lower levels of SIRT1 [89]. Further, the activation of p53 was associated with a number of targets (BAX, PARP1, caspase-3, p21, heat shock proteins and PGC1α) that were modulated in both DS and DS/AD compared with age-matched controls. In particular, the most relevant changes (increased p-p53, acetyl-p53 and reduced formation of MDM2/p53 complex) were found to be modified only in the presence of AD pathology in DS. Taken together, these data suggest that a pro-apoptotic phenotype may contribute to accelerated development of AD neuropathology in DS population [89].

Data obtained in the superior temporal gyrus showed enhanced levels of p53 [90] as well as in the inferior parietal lobule of AD-affected brains [91]. Interestingly, the enhanced p53 expression was correlated with markers observed in oxidative stress-induced neuronal cell death [92]. The same authors, in agreement with studies from others, proposed that p53 could be conformationally altered in AD [93]. The first evidence of immunological characterization of unfolded p53 were obtained from fibroblasts of AD-affected patients at early stages of the disease [94]. These structural alterations of p53 translate into functional changes such as altered DNA binding properties and impairment of its transcriptional activity [95]. However, it should be noted that such unfolded p53 species were not detected in the brain and that they were observed very early in the blood of MCI-affected patients. Therefore, it is likely that unfolded p53 could be a peripheral and early signature of AD, independent of the cell death observed in the central nervous system.

Recent evidence suggests that enhanced susceptibility of neurons to p53-mediated cell death is exacerbated by genetic mutations associated with AD, such as amyloid precursor protein (APP) [96]. A differentiated neuronal cell line, that express wt human APP, are reportedly protected from cell death in response to elevated p53 expression induced by UV irradiation, staurosporine treatment and p53-adenovirus [97]. Conversely, mutant forms of APP, known to be associated with familial-early onset forms of AD failed to be neuroprotective. Though p53 protein levels or p53 nuclear translocation were not altered in both conditions, wild-type APP, in contrast to mutant APP, suppressed p53-mediated transcriptional activation from a p53-responsive promoter. The mechanism by which APP-mediated signaling altered p53 activation has not yet been identified. In addition, it has also recently been observed that Aβ itself ($A\beta_{1\text{-}42}$), binds the p53 promoter and enhances transcription [98].

p53 was found to induce phosphorylation of human 2N4R tau at the tau-1/AT8 epitope in HEK293a cells [90]. Pathological and prolonged expression of p53 in adult brain might contribute to tau hyperphosphorylation, which is critical in the formation of NFTs. Interestingly, the same authors previously observed that an other member of the p53 family, TAp73, induces tau phosphorylation in HEK293a cells at the tau-1 site and at the PHF-1 epitopes [99].

A key "supplementary" role in the regulation of phosphorylation/dephosphorylation cycle is played by Pin1. Phosphorylation of the amino acids serine or threonine that precede proline (pSer/Thr-Pro) is a central signaling mechanism that regulates many cellular processes [100]. This proline-directed phosphorylation is catalyzed by a large family of enzymes, such as cyclin-dependent protein kinases (CDKs) and a large family of stress-activated protein kinases. Intriguingly, Pin1 is a unique enzyme that, through its WW domain, changes the shape of target proteins by acting on the prolyl residue of the target protein to change its conformation from cis to trans and vice versa. This conformational change affects protein function and is a major signaling and regulatory mechanism in the cell [100].

Abnormal regulation of Pin1 has been associated with aging and several pathological processes, including AD [101]. Age-related Pin1 deregulation provides a link between Aβ and Tau abnormalities as well as neuronal loss. In the brain of people with AD, Pin1 has lower activity; consequently, tau, with cis proline conformation, is accumulated in early stages of AD. Further, APP also is a target of Pin1, and cis proline conformation at residue 669 leads to an increased production of beta-amyloid. When in their trans-conformations, tau and APP are healthy and functional. Therefore, Pin1 can lead to production of both tangle and plaque pathology when damaged. As a result, Pin1-deficient mice develop age-dependent tau and Aβ pathologies and neuronal degeneration and loss [101].

Increasing evidence suggests that Pin1 activity is regulated by multiple PTMs [102]. Pin1 is oxidatively modified, which inhibits its PPIase activity [103]. Moreover, the oxidized Pin1/total Pin1 levels is elevated in the early stage of AD pathology [104]. Since Pin1 regulates activities of a critical tau kinase (GSK-3β) and a phosphotau phosphatases (PP2A), oxidation of Pin1 likely contributes to the hyperphosphorylation of tau and subsequent deleterious pathology in MCI and AD brain [51].

Oxygen/glucose deprivation can trigger partial inhibition of Pin1 enzymatic activity and also increase Ser16 phosphorylation. The fact that Pin1 is highly expressed in neurons and is oxidized and inactivated in the hippocampus of patients with MCI and AD suggests that it may take part in the early response to oxidative stress. Taken together, these data support the view that down regulation and/or inactivation of Pin1 may perturb healthy aging and promotes neurodegenerative phenomena.

Taken together, these findings suggest that phosphorylation plays a central role in regulating protein activity, being the most abundant PTM within the cells. However, considering that many proteins undergo multiple PTMs at the same time, among which some of these may compete for the same residue, the picture becomes quite complex and understanding if these concomitant events are cooperative or antagonist requires further knowledge.

4. The pro-survival effect of the IR/BVR-A axis is disrupted in AD

Insulin and the insulin growth factor (IGF)-1 have intense effects in the CNS, regulating key processes such as energy homeostasis, neuronal survival, longevity, learning and memory [105]. Insulin and IGF-1 bind to tyrosine kinase receptors, IR, and IGF-1R, which share a high degree of identity in their structure and function [105]. IR and IGF-1R are selectively distributed in the brain with a higher density in the olfactory bulb, hypothalamus, as well as in two of the main brain areas affected by AD pathology, i.e., hippocampus and cerebral cortex [105]. According to the canonical pathway of the insulin signaling, binding of insulin or IGF-1 induces a conformational change of the receptor leading to their autophosphorylation on specific tyrosine residues on the β-subunit (Y1158, Y1162, Y1163) [106–108] with the consequent recruitment of the insulin receptor substrate-1 (IRS1) [105]. This latter, in turn, activates two main signaling pathways: (i) the PI3K pathway, which, among other functions, is involved in the maintenance of synaptic plasticity and memory consolidation [109], Aβ-induced memory loss [110], synthesis of nitric oxide (NO), which in turn plays a role in learning and memory processes [111]; and (ii) the MAPK cascade, which is responsible both for the induction of several genes required for neuronal and synapse growth, maintenance and repair processes, as well as serving as a modulator of hippocampal synaptic plasticity that underlies learning and memory [112] (Fig. 2).

However, IRS1, or even the other members of the IRSs family, are not the unique targets of the IR kinase activity. In fact, in 2005 Maines and her group reported for the first time that biliverdin reductase-A (BVR-

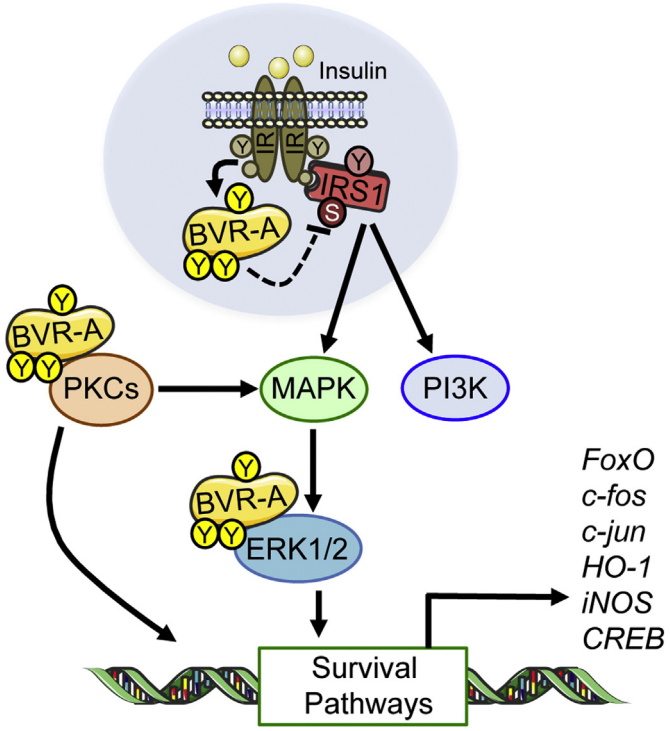

Fig. 2. Insulin signaling. Under normal conditions binding of insulin to the membrane resident insulin receptor (IR) promotes IR activation through IR dimerization and autophosphorylation of specific tyrosine (pTyr) residues. Stimulation of IR kinase activity is then followed by tyrosine phosphorylation of a variety of endogenous substrates, including the cytosolic insulin receptor substrate IRS-1. These events lead to the activation of multiple signaling pathways required for insulin's pleiotropic action, including: (i) the phosphoinositide-3-kinase (PI3K) pathway; and (ii) the mitogen-activated protein kinase (MAPK) pathway, which finally result in the induction of gene expression of cell survival pathways (FoxO, c-fos, c-jun, HO-1, iNOS, CREB).

A) – primarily known for its canonical activity (reductase activity) named for the reduction of biliverdin (BV) into the powerful antioxidant and antinitrosative molecule bilirubin (BR) [113,114] – is a direct substrate of the IR kinase activity [115]. Indeed, IR promotes the phosphorylation of specific Tyr residues of BVR-A (Y198, Y228 and Y291), responsible for the activation of BVR-A Ser/Thr/Tyr kinase activity [115]. Through this kinase activity, BVR-A controls either the activation of IRS1 or those of several members of the insulin signaling pathway of fundamental importance in the regulation of cell death and survival (MAPK and PKC) or cell stress response [c-fos, c-jun, heme oxygenase -1 (HO1), the inducible isoform of nitric oxide synthase (iNOS) and CREB/ATF-2] (reviewed in [116]).

With regard to IRS1, it is of interest to note that BVR-A represents an upstream regulator of IRS1 activation since BVR-A is able to phosphorylate IRS1 on inhibitory domains [human(h)/mouse(m): hSer307/mSer302, hSer312/mSer307 and hSer616/mSer612] critical for insulin signaling [115]. Interestingly, phosphorylation of IRS1 by BVR-A is increased when both proteins are available to IR, thus possibly reflecting a direct interaction of BVR-A and IRS1 [115]. This latter is not a rare case since changes in conformation of a kinase initiated by ligand binding can function both in directing proteins to subcellular targets and in modulating their activity [117–119]. In the case of BVR-A, because three tyrosines in the protein potentially can be phosphorylated by IR, a change in conformation of the protein caused by IRS1 binding may position a larger number of tyrosine residues for phosphorylation by IR [115].

Due to its neurotrophic functions [120–122], the insulin signaling pathway is therefore a perfect example of how phosphorylation of the different components could impact the progression of neurodegeneration, i.e. AD pathology, in the brain. Importantly, neurons are vulnerable to excitotoxic stress, and with some notable exceptions, there is a slow rate of neurogenesis in the brain. Hence, neurons remain postmitotic, and any increased stress or reduced repair mechanism can accumulate over time. The impairment of insulin signaling in the brain could well play a role in the development of neurodegenerative disorders, as it leaves neurons more exposed to toxic influences [123].

To note, several studies reported about the phenomenon of brain insulin resistance (BIR), whose onset and progression is dependent on changes of IR/BVR-A/IRS1 phosphorylation both during normal aging and AD [54,124,125]. Indeed, BIR – defined as the inadequate response to insulin by target cells [112] – is clearly characterized by a reduced IR/IGF-1R activation and/or an increased IRS1 inactivation [126–128].

Due to the complexity and the numerous mechanisms of regulation characterizing the insulin signaling cascade- whose analysis is out of the scope of this review- we will focus on the main changes affecting the IR/BVR-A axis and on how they correlate with the maintenance of neuronal survival in AD. In fact, despite a large literature that addresses the role of the insulin signaling in the brain, there is the tendency to mainly focus on the downstream targets of the pathway, e.g. PI3K, Akt or MAPK, which, although representing essential parts of the signaling cascade, are not exclusive to insulin signaling. Therefore, changes affecting these proteins also could be independent of insulin. Rather, changes occurring in upstream proteins of the pathway would be of great interest in order to clarify the onset of insulin resistance in AD. Furthermore, many papers already reviewed the importance if IRS1 inhibitory phosphorylation in the context of insulin resistance in AD [26,129].

As noted, neurons express both the IR and the IGF-1R, which are similar in terms of structure and activity [130]. Interestingly, although insulin and IGF1 could potentially bind both receptors, ex vivo tests revealed selective signaling effects of these hormones depending on the concentration [126]. Indeed, at low deses (around 1 nM) insulin and IGF-1 are selective for the respective receptor, whereas at higher doses (around 10 nM) they lack selectivity [126]. Although IR/IGF1-R auto-phosphorylation represents a central event in the insulin signaling cascade, because it is the starting point from which all the other downstream modifications derive, the evaluation of IR/IGF1-R phosphorylation in AD has been only partially analyzed.

After the first evidence about changes of IR density in the brain of AD was reported [131], the evaluation of IGF1-R levels several years later, did not reveal any significant change with respect to control brain, thus proposing a potential compensatory role of IGF1-R in AD [132]. However, evidence for normal or increased IR/IGF1-R levels also exists [126,133]. Rather, the evaluation IR/IGF1-R phosphorylation clearly revealed a reduction of tyrosine kinase activity in AD [126,134], probably mediated by Aβ peptides, which compete for insulin binding to the insulin receptor, thus preventing IR auto-phosphorylation [135,136].

A detailed analysis of brain/neuron-specific insulin receptor knockout (NIRKO) mice revealed that IR activation mediates anti-apoptotic effects in neurons [137]. These data confirm the fact that insulin is capable of inhibiting apoptosis and demonstrate that this response is completely dependent on the presence of the IR, whose activation seems to be important for the phosphorylation and activation of the anti-apoptotic Akt [137]. In addition, NIRKO mice are characterized by tau hyperphosphorylation at sites associated with neurodegenerative disease [137]. These observations provide an in vivo molecular mechanism in which altered insulin signaling in the brain leads to one of the hallmarks of AD and demonstrate how neuronal insulin resistance potentially predisposes the brain for the development of neurodegeneration [137]. Similarly, the block of IGF1-R activity in rat brain led to brain amyloidosis, cognitive disturbance and hyperphosphorylated tau deposits together with other changes found in AD such as gliosis and synaptic protein loss [138]. While these disturbances were mostly corrected by restoring receptor function, blockade of the IGF1-R exacerbated AD-like pathology in old mutant mice already affected by brain amyloidosis and cognitive derangement [138].

Efforts to study the effect of insulin resistance in the brain have been also based on the use of streptozotocin (STZ) known to impair insulin secretion. Grünblatt et al. found that i.c.v. administration of STZ led to an unchanged or even elevated phosphorylation of the IR tyrosine residues in 3-month old rats brain, which may point to imbalances between the generation of proteins and their turnover and between protein phosphorylation and dephosphorylation under pathological conditions [139]. Indeed, these changes were associated with an increased tau phosphorylation and an impairment of cognitive functions [139]. In addition, quite recently, King and colleagues, also showed that STZ-induced insulin resistance in the hippocampus of rats and that constant insulin administration (0.1–1 nM) can improve IR phosphorylation and protect neurons from neuropathology [140].

Among the mechanisms responsible for the reduced IR/IGF1-R phosphorylation in AD, Zhao et al., for the first time, showed that signal transduction by neuronal IR is strikingly sensitive to disruption by soluble Aβ oligomers [136]. Aβ oligomers caused a rapid and substantial loss of neuronal surface IRs specifically on dendrites bound by Aβ oligomers [136]. Removal of dendritic IRs was associated with increased receptor immunoreactivity in the cell body, indicating redistribution of the receptors. Furthermore, Aβ oligomers caused a marked decrease of IR auto-phosphorylation and thus a loss of neuronal IR functions [136]. Interestingly, Aβ oligomers interactions with IR were relatively specific, as the oligomers did not bind to IGF1-R nor affect IGF1-R activity [136].

However, another group reported that Aβ oligomers facilitated IGF-1R phosphorylation together with an increased expression of the p75 neurotrophin receptor (p75NTR), whereas the co-administration of an IGF1-R kinase inhibitor, blocked Aβ oligomers-induced p75NTR expression [141]. This aspect appears of interest because the p75NTR is a member of the tumor necrosis factor receptor superfamily and controls survival, shape and function of neurons especially in the early stage of the life [142]. Conversely, with aging the activation p75NTR can also promote cell death as observed in various pathological conditions, including epilepsy, axotomy and neurodegeneration [141,142]. Interestingly, 6-month-old AβPPswe/PS1ΔE9 AD model mice showed significantly increased IGF1-R phosphorylation together higher p75NTR expression than age-matched wild-type mice. These observations all together possibly indicate that Aβ oligomers could stimulate the p75NTR protein expression in the hippocampus through the IGF1-R signaling [141].

Although these latter results seem to be different than those proposed by Zhao et al. [136], it has to be highlighted that they have been obtained by using different models (cells vs. animals) and therefore this aspect would require further elucidations. Notwithstanding, it is conceivable that Aβ oligomers at low doses could promote an increased phosphorylation of IR or IGF1-R followed by a reduction of their activation, which develops with Aβ accumulation in the brain. Whether Aβ mediates these effects directly of indirectly is still under investigation. However, Aβ-induced oxidative/nitrosative stress levels increase could play a major role in driving these molecular changes, since it has been previously reported that oxidative and nitrosative stress can promote the phosphorylation of the IR at low levels or in a short-time period whereas it becomes toxic at higher levels [54,143–145]. Furthermore, data collected in the hippocampus of another mouse model of AD pathology, i.e., the 3xTg-AD mouse, revealed a hyper-activation of the IR in young mice followed by a reduced IR phosphorylation in older animals, which progress with both Aβ and oxidative stress accumulation in the brain [54].

In addition, due to the fact that brain: i) is the organ with the highest energetic demands; ii) is the most susceptible to energy deficits and iii) is responsible for coordinating behavioural and physiological responses related to food foraging and intake, the effects of dietary interventions have been taken into consideration in order to extend lifespan and delay the appearance of age-related pathological conditions, associated with brain functional decline. Notably, Tg2576 mice fed with an high fat diet (known to promote insulin resistance [147]), were characterized by a 2-fold reduction of p-IR$^{Y1162/Y1163}$ without changes of total IR levels, which were associated with an increased gamma-secretase activity [148]. These data provided the first evidence that reduced insulin signaling could play a role in the shift from the non-amyloidogenic to the amyloidogenic pathway in the brain [148]. Conversely, Tg2576 mice that underwent a caloric restriction regimen for approximately 6 months showed an elevation of p-IR$^{Y1162/Y1163}$ in the brain, which are functionally associated with caloric restriction-mediated prevention of AD-type amyloid neuropathology through the regulation of the forkhead transcription factor FoxO3a [149]. Indeed, FoxO transcription factors are pivotal downstream targets of insulin/IGF- 1 signaling and regulate various downstream target genes involved in the cell cycle and cell death [150]. FOXO transcription factors have been postulated to influence longevity in part by conferring increased resistance to oxidative stress and slowing the accumulation of oxidative damage that might accelerate neurodegeneration [150].

As explained above, once phosphorylated, IR leads to the phosphorylation of BVR-A, which is essential for the correct functioning of the insulin signaling [54,115]. Interestingly, in 2011, our group reported for the first time alterations of the BVR-A activation state by showing decreased Tyr phosphorylation and increased oxidative/nitrosative post-translational modifications in the brain of subjects with AD and amnestic MCI [151]. These data shed light on an aspect often neglected in previous studies, that is, changes of BVR-A protein levels are not always associated with the same trend in terms of phosphorylation/activation. Indeed, at least in MCI and AD hippocampus, total BVR-A protein levels were increased while BVR-A phosphorylation was reduced, thus clearly showing an impairment of BVR-A activity [151]. Indeed, BVR-A undergoes nitrosative stress-induced modifications in AD as demonstrated by the elevation of 3-NT modification on BVR-A [151]. Since it is well known that oxidative/nitrosative post-translational modifications alter protein structure [152] and most often result in a reduced function [153–155], it is plausible to argue that the increased 3-NT levels on BVR-A could be responsible at least in part for the observed reduced phosphorylation/activation [151]. Indeed, nitration and phosphorylation processes occur on the same residues, i.e., Tyr residues [156]. Currently, it is not known if exactly the same Tyr residues are the substrate of these kinds of modifications, but it is conceivable that, due to the decreased Tyr phosphorylation and the increased Tyr nitration, a competition between nitration and phosphorylation processes could occur. Certainly, from a chemical point of view, steric hindrance of the NO_2 group on the 3-position of Tyr could significantly modulate activity of Tyr kinases for the 4-OH group. This notion strengthens the hypothesis that nitrosative stress prevents/inhibits Tyr phosphorylation on BVR-A [151].

Because the pivotal role of BVR-A in the insulin signaling cascade, the observation of nitrosative stress-associated reduced BVR-A phosphorylation in AD led to further analyses aimed to characterize the role of BVR-A in the onset of BIR in AD. These studies revealed that reduced BVR-A phosphorylation is an early event, which starts prior the accumulation of Aβ and tau pathology in the hippocampus of 3xTg-AD mice [54]. Reduced BVR-A phosphorylation is, therefore, firstly responsible for a sustained activation of IRS1, which then causes the stimulation of negative feedback mechanisms such as mTOR, aimed to turn-off IRS1 hyper-activity and thereby BIR results [54]. Similar alterations also characterize the normal aging process in mice, positing BVR-A impairment as one possible bridge in the transition from normal aging to AD [54].

Due to the fact that BVR-A, once phosphorylated by IR/IGF-1R, can act independently of the insulin signaling cascade [116], its dysfunction also possibly negatively impact survival pathways such as the MAPK signaling, thus worsening the already impaired neuropathological picture of AD brain. Indeed, phosphorylated BVR-A is an essential scaffold protein for the activation of ERK1/2 by MEK1/2 and then of Elk1 by ERK1/2 [157]. Therefore, the reduced phosphorylation of BVR-A coupled with the decreased interaction with ERK2 found in AD hippocampus

[151] lend support to the hypothesis that BVR-A could be responsible, at least in part, for the ERK1/2 dysregulation detected in this brain area in AD subjects [158] [35,36].

In addition, data collected to date have identified BVR-A as a key modulator of PKC isozymes including PKC-βII, PKC-ζ and PKC-δ [116]. This aspect is fascinating for two main reasons: 1) PKCs represent a bridge linking the two arms of the insulin/IGF-1 signaling cascade and 2) PKCs have a fundamental role in signaling mechanisms of differentiation, development, proliferation, apoptosis, stress-response-related protein synthesis, cell death and survival, and modulation of ion channels [116,159]. BVR-A, through its kinase activity, seems to have a role in regulating PKCs intracellular trafficking [116]. Age-related changes in PKC translocation have been linked to tau hyperphosphorylation and the phosphorylation of glycogen synthase kinase 3β (p-GSK3β) [160]. Stress-related dysfunction of PKC isoforms with age is linked to a progressive decline of memory and cognition with the potential for dementia and tau-related pathology [160]. Transgenic animals with a PKC-β or PKC-ζ knockout have disrupted memory formation as well as poor memory recall [161,162]. Furthermore, if PKCβ is not translocated, it can hyperphosphorylate tau and substantially contribute to AD pathology [163]. In contrast, restoration of the PKC cytosol-to-cell membrane translocation and activity decrease both NFTs and Aβ deposition in transgenic animal models [11]. In preclinical studies, PKC activators have been shown to increase the expression and activity of PKC isozymes, thereby restoring PKC signaling and downstream activity, including stimulation of neurotrophic activity, synaptic/structural remodeling, and synaptogenesis in the hippocampus and related cortical areas [164]. PKC activators also reduce the accumulation of neurotoxic Aβ and tau protein hyperphosphorylation and support anti-apoptotic processes in the brain [164]. These observations suggest that restoring PKC activity may rescue at least in part AD neuropathology. Whether reduced BVR-A phosphorylation/activation also affects PKCs in AD remains to be elucidated, even if previous data seems to agree with this possibility.

Summarizing this discussion, the impaired phosphorylation of the IR/BVR-A axis in AD emerges as a field to be investigated more deeply for its role in cell survival. Although BVR-A is an essential part of the insulin signaling cascade, its pleiotropic functions confer to this protein a pivotal role in neuroprotection. However, because BVR-A needs to be activated by IR/IGF-1R and both IR and BVR-A activation are reduced in AD, it appears evident that BVR-A functions are closely dependent on IR activation. Therefore, the impairment of the insulin signaling in AD not only produces negative effects related to its downstream targets, but also impairs BVR-A-associated effects on cell growth, apoptosis and stress-response.

5. Concluding remarks

Several observations indicate that senescent-related cognitive decline is due not only to neuronal loss, but is the result of functional changes occurring over time. Among age-dependent changes, disturbance of protein phosphorylation/de-phosphorylation cycles may contribute to neuronal dysfunctions. Indeed, protein phosphorylation regulates fundamental intracellular processes such as transcription and translation, regulation of the cell cycle, signaling within and among cells, synaptic function among others. Recent findings reveal how phosphorylation dependent signaling cascades may control lifespan with prime examples being the activation of p53 and the insulin-signaling pathway, as important regulators of lifespan, even if from opposite directions. Many of the functional changes that occur during aging such as impaired learning and memory and altered energy metabolism are controlled by protein phosphorylation and it is, therefore, important to understand how mechanisms of protein phosphorylation may either mediate aging or eventually exacerbate age-associated neurodegeneration. Increasing studies showed that abnormalities in protein phosphorylation contribute to the pathogenesis and progression of AD by impairing neuronal architecture, energy metabolism, cell survival and cell death pathways among others (Fig. 3).

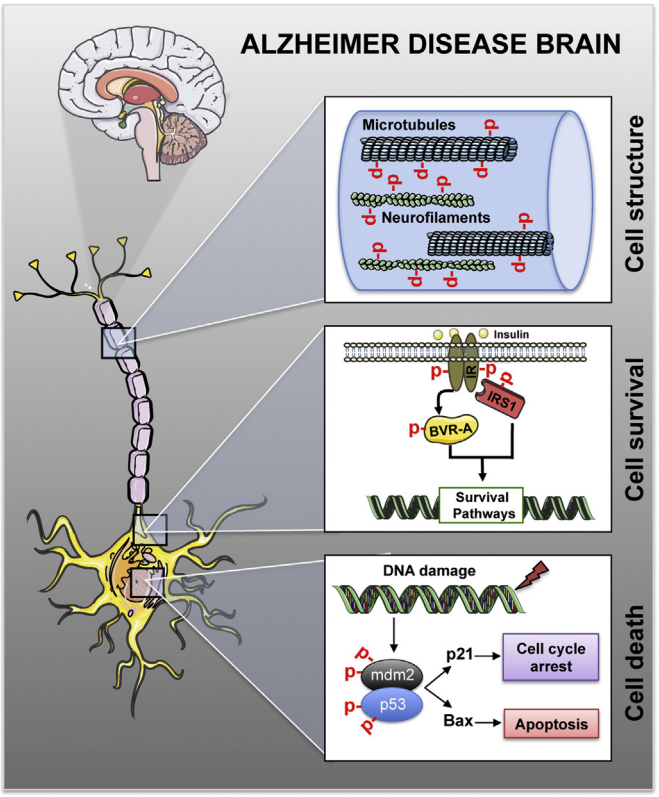

Fig. 3. Aberrant phosphorylation in AD brain leads to disturbance of cell structure integrity, cell survival and cell death pathways.

However, increasing evidence shows that regulation of protein function results not only from one single modification but also by multiple PTMs, among which some of these may compete for the same residue. Understanding if these concomitant events are cooperative or antagonist is difficult and further investigations are needed. Therapeutic intervention that can restore phosphorylation homeostasis, either acting on kinases and phosphatases, may prove to be beneficial to prevent or slow the development of AD.

Conflict of interest declaration

None of the authors of this manuscript have a conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

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