Universitat Autònoma de Barcelona

The contribution of the PDK1/Akt signaling pathway to Alzheimer Disease analyzed by knock-in mutation

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Memoria de tesis doctoral presentada por Shaobin Yang para optar al grado de Doctor en Neurociencias por la Universitat Autònoma de Barcelona.

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SUMMARY

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases; it accounts for more than 80% of dementia cases worldwide in elderly people. Aging is the most important known nongentic risk factor for AD, which is accompanied by stereotypical structural and neurophysiological changes in the brain and different degrees of cognitive decline. The 3-Phosphoinositide-dependent protein kinase 1 (PDK1) activity is increased in human AD whole brains and in primary cultures derived from adult AD model mice. PDK1 triggers the phosphorylation and membrane depletion of TNF converting enzyme (TACE), which significantly influence the course of AD. Pharmacological inhibition and genetic depletion of PDK1 not only reduce amyloid-beta (Aβ) levels but also restore TACE-mediated soluble APPα levels and memory deficits in mice models for AD, which suggests that PDK1 may be activated by Aβ in AD. Of note, side-effects caused the dead of the mice after four months of treatment.

PDK1 acts downstream of phosphoinositide 3-kinase (PI3K)-mediated signaling, The binding of growth factors with their receptors cause the activation of PI3K, which phosphorylates the PtdIns[4,5]P_2 membrane lipid to synthesize the PtdIns[3,4,5]P_3 second messenger, which in turns induces the translocation of proteins to the membrane and its subsequent activation. Protein kinase B (PKB/Akt) and PDK1 bind to PtdIns[3,4,5]P_3 at the plasma membrane through their PH (Plekstrin Homology) domains, where Akt is phosphorylated at its activating residue threonine (Thr)308 by PDK1. PDK1 can also promote the phosphorylation and activation of a number of kinases through a PH-domain independent, docking site dependent mechanism. These include S6K, RSK, SGK and PKC isoforms. However, activation of the PI3K/Akt signaling pathway in brain by insulin-like growth factor 1 receptor (IGF-1R) and insulin receptor (IR) is markedly disturbed In AD. Among the different positively-charged lateral chains conforming the PDK1 PtdIns[3,4,5]P_3 binding pocket, mutating Lysine at position 465 to Glutamic acid abolishes the PDK1-phosphoinositide interaction by disrupting the conformation of this lipid-binding pocket. In order to analyse the importance of the PDK1-PtdIns[3,4,5]P_3 interaction,

homozygous PDK1$^{K465E/K465E}$ knock-in mice were generated, which express the PDK1 K465E mutant protein abrogating phosphoinositide binding resulting in an specific signalling lesion. Consequently, the mutant mice were shown to be smaller than control littermates, hyperinsulinemic and insulin resistant. These PDK1$^{K465E/K465E}$ mutant mice is a genuine model in which activation of Akt is only moderately reduced but not ablated, which has been proved instrumental in dissecting the contribution of Akt to PDK1 signaling. The moderate reduction of Akt activation was not rate-limiting for the phosphorylation of those Akt substrates governing neuronal survival and apoptosis such as FOXO and GSK3. Accordingly, neuronal survival responses were preserved in the mutant mice. In contrast, the moderate reduction of Akt activity in the PDK1$^{K465E/K465E}$ neurons markedly reduced phosphorylation of the PRAS40 and TSC2 substrates, leading to decrease mTORC1/S6K activation and reduced BRSK protein synthesis. As a consequence, the PDK1 mutant neurons in culture exhibited reduced neurite outgrowth, delayed polarization and deficient axonogenesis, which however did not translate onto overt abnormalities in ther patterning of the adult brain. By contrast, brain-specific knock-in mice expressing a mutant form incapable of docking site binding in which activation of most PDK1 substrates with the exception of Akt is blocked exhibited severes phenotypes.

Taken the phenotypes of the PDK1 knock-in models, I postulate that inhibition of Akt might have protected mice against Alzheimer Disease, whereas inhibition of the docking-site dependent PDK1 substrates might be responsible for the toxicity the treatments. In cortex and hippocampus, the phosphorylation of Akt at Thr308, the PDK1 site, was significantly reduced in the PDK1$^{K465E/K465E}$ mutant mice after 4 months and was then sustained for up to 21 months, since increased levels of BDNF synthesis compensate for the defects of the Akt signaling pathway. Importantly, TACE membrane localization, TACE activity and TNFR1 cleavage are increased in the PDK1$^{K465E/K465E}$ mutant mice, which render the mutant neurons resistant against TNFα-induced neurotoxicity, thereby providing strong evidence on the role of the Akt branch of the PDK1 signaling in regulating TACE trafficking and internalization.

Alzheimer's disease is characterized by the presence of amyloid plaques, neurofibrillary tangles and high Reactive Oxigen Species (ROS), which cause endoplasmic reticulum (ER)

stress contributing to exacerbate neuronal cell death. The cell main weapon against ER stress is the induction of the unfolding protein response (UPR). In the 3xTg-AD mice, the protein kinase RNA-like endoplasmic reticulum kinase (PERK) is hyperactivated and the eukaryotic initiation factor 2 (eIF2α) hyperphosphorylated. By contrast, in the $PDK1^{K465E/K465E}$ mice the age-induced phosphorylation of PERK and eIF2α is attenuated due to the deficient activation of Akt. Furthermore, in cultured primary neurons treated with tunicamycin or Aβ, the phosphorylation of PERK and eIF2α were reduced in the $PDK1^{K465E/K465E}$ cortical neurons compared to $PDK1^{+/+}$ wild type, which protected cells from both tunycamicin and Aβ-induced neurotoxicity.

In summary, this study show that reducing Akt activation can protect neurons against amyloid-beta induced-toxicity by restraining the reduction of TACE activity in the cell membrane and the increase of the unfolding protein response.

ABBREVIATIONS

AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
AGC	cAMP-dependent kinase, cGMP-dependent kinase and protein kinase C family of kinase
APP	Amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
BiP	Binding immunoglobulin protein
eIF2α	Eukaryotic initiation factor 2
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
FOXO	Forkhead box group O transcription factor
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GSK3	Glucogen systhase kinase 3
IGF-1R	Insulin-like growth factor 1 receptor
IRE1	Inositol-requiring enzyme 1
LTD	Long-term depression
MARK	Microtubule affinity-regulating kinase
mTOR	mammalian target of rapamycin
NFTs	Neurofibrillary tangles
NDRG1	N-myc dowmstream regulated gene 1
PH	Plekstrin Homology
PDK1	3-Phosphoinositide-dependent kinase 1
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PI3K	Phosphoinositide 3-kinase (also PI 3-kinase 1)
PIP2	Phosphatidylinositol-4,5-bisphosphate (also PtdIns(3,4,5)P_2)

PIP3	Phosphatidylinositol-4,5-bisphosphate (also PtdIns(3,4,5)P_3)
Akt	Protein kinase B
PKC	Protein kinase C
PRAS40	Proline-rich Akt Substrate of 40 kD
Raptor	Regulatory-associated protein of mTOR
Rheb	Ras homologue enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
ROS	Reactive Oxigen Species
S6K	p-70 ribosomal S6 protein kinase
TACE	TNF converting enzyme
TNFR1	Tumor Necrosis Factor Receptor 1
Trk	Tropomyosin-related kinase
TSC	Tuberous sclerosis complex
ULK	unc-51 like autophagy activating kinase 1
UPR	Unfolding protein response

Less frequently used abbreviations are defined upon their first use in the text

INTRODUCTION

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases, representing more than 80% of dementia cases worldwide in elderly people. AD is characterized by a progressive loss of mental, behavioral and functional decline and ability to teach (Anand *et al*., 2014). The major risk factor for Alzheimer's disease is age, with a sharp increase in incidence after 60 years (Kawas *et al*., 2000). AD now affects almost 50% of adults over the age of 85 years, and this figure can only grow as the average life expectancy rises. Typically, more than a decade after the illness should take its course patients would die in a completely helpness state. It is estimated that AD have cost the world $604 billion in 2010 alone. These costs are huge, particularly in light of predictions that the worldwide number of AD cases, currently estimated in 36 million, will increase to the triple by 2050 (Wimo and Prince, 2010). At present, the treatments prescribed by US Food and Drug Administration (FDA) include five drugs that are used to improve the cognitive functions of AD patients: AChEIs–rivastigmine (Exelon), galantamine (Razadyne, Reminyl), tacrine (Cognex), donepezil (Aricept) and the NMDA receptor antagonist memantine (Namenda) (Auld *et al*., 2002). These treatment strategies only delay the progression of symptoms associated with AD (Farlow *et al*., 2008). Much effort is being directed towards the discovery of disease-modifying therapies which can block the progression of the disease (i.e. clinical symptoms) by developing drugs targeting various molecular pathways.

1. PATHOPHYSIOLOGY OF ALZHEIMER'S DISEASE

Since Dr. Alois Alzheimer observed first the neuropathological features of the amyloidal plaques and neurofibrillary tangles (NFTs) from Alzheimer's Disease patient in 1907, several hypotheses have been promoted over the years to explain this multifactorial disorder, like the genetics, cholinergic, Aβ, tau and inflammation hypothesis (Huang and Mucke, 2012) (Fig.1).

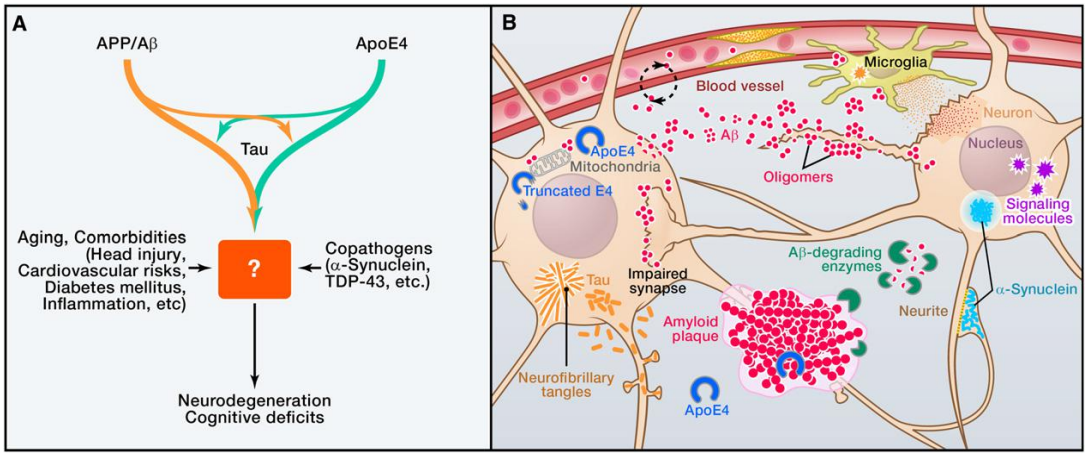


Figure 1. Multifactorial Basis of Alzheimer's Disease Pathogenesis (A) Alzheimer's disease (AD) is likely to be caused by copathogenic interactions among multiple factors, including APP/Aβ, apoE4, tau, α-synuclein, TDP-43, aging, and various comorbidities. How exactly they conspire to impair neuronal functions and survival remains to be determined. (B) Accumulation of Aβ in the brain may result from its increased production. Aβ oligomers impair synaptic functions and downstream signaling pathways, changing neuronal activities, and trigger the release of neurotoxic mediators from glial cells. When expressed within stressed neurons, apoE4 is cleaved, into neurotoxic fragments that disrupt the cytoskeleton and impair mitochondrial functions. Tau, which is normally mostly abundant in axons, becomes mislocalized to the neuronal soma and dendrites and forms inclusions called neurofibrillary tangles (NFTs) (Huang and Mucke, 2012).

1.1. ALZHEIMER'S DISEASE GENE: APOE

AD is probably caused by multiple genetic factors; mutation in three genes: amyloid precursor protein (APP), presenilin-1 (PS) and presenilin-2 (PS2) account for less than 1% of AD cases. These mutations affect APP processing, which can lead to altered production of different Aβ peptides (Campion *et al*., 1999; Bertram *et al*., 2010).

In addition, apolipoprotein (apo) E4 was identified as a genetic risk factor for late-onset (>60 years) cases of familiar AD. ApoE is a polymorphic protein with three common isoforms, apoE2, apoE3, and apoE4. The three isoforms differ one from the other by single-amino acid substitutions (Mahley *et al*., 2006). In *vitro* and in *vivo* studies suggest that astrocyte-derived apoE has isoform-specific effects on Aβ clearance or deposition (Kim *et al*., 2009), neurite outgrowth (Holtzman *et al*., 1995), and behavioral performance (Hartman *et al*., 2001). When incubated with Aβ peptides, apoE3 and apoE4 produce different results, where ApoE3 binds the Aβ peptide with a 20-fold greater affinity than apoE4. Interestingly, decreasing apoE lipidation by knockout of the ABCA1 gene, a major regulator of cellular cholesterol and phospholipid homeostasis, significantly increases brain

amyloid loads in mice expressing mutant human amyloid precursor protein (hAPP), whereas increasing apoE lipidation status by overexpression of ABCA1 decreases amyloid levels (Kim *et al*., 2009). Thus, altering apoE lipidation changes its ability to mediate Aβ clearance or deposition in brain. ApoE4 clearly increases Aβ accumulation and amyloid plaque formation in both humans and transgenic mouse models, although this process uncertainly contributes to cognitive deficits in AD. ApoE4 also impairs synaptogenesis and decreases dendritic spine density in *vivo* in apoE transgenic and gene-targeted mice as well as in *vitro* in primary neuronal cultures (Brodbeck *et al*., 2011). Since there is no Aβ accumulation in any of these apoE4 mouse models, the role of apoE4 in causing neuronal and behavioral deficits in *vivo* must be Aβ-independent. Some data suggest that neuronal apoE3, but not apoE4, prevents the age-dependent neurodegeneration seen in apoE-null mice as well as excitotoxicity-induced neurodegeneration (Buttini *et al*., 2010).

1.2. APP PROCESS AND ITS FUNCTION

The APP gene is located on chromosome 21 in humans with three major isoforms APP695, APP751 and APP770. APP751 and APP770 are expressed in most tissues and contain a 56 amino acid Kunitz Protease Inhibitor (KPI) domain within their extracellular regions; while APP695 is mainly expressed in neurons and lacks the KPI domain. APP belongs to a protein family that in mammals includes APP like protein 1 (APLP1) and 2 (APLP2) (Wasco *et al*., 1992 and 1993; Coulson *et al*., 2000), which are type-I transmembrane proteins processed in a similar fashion. Cellular localization of APP is dynamic, APP moves from the endoplasmic reticulum (ER) to the plasma membrane through the secretory pathway. During this process, APP is post-translationally modified by phosphorylation, tyrosine sulfation, and N-or O-linked glycosylation. APP processing is dependent on secretase enzymes, which finally leads to its release into the extracellular space. APP processing is generally divided into two pathways, non-amyloidogenic and amyloidogenic (Fig. 2).

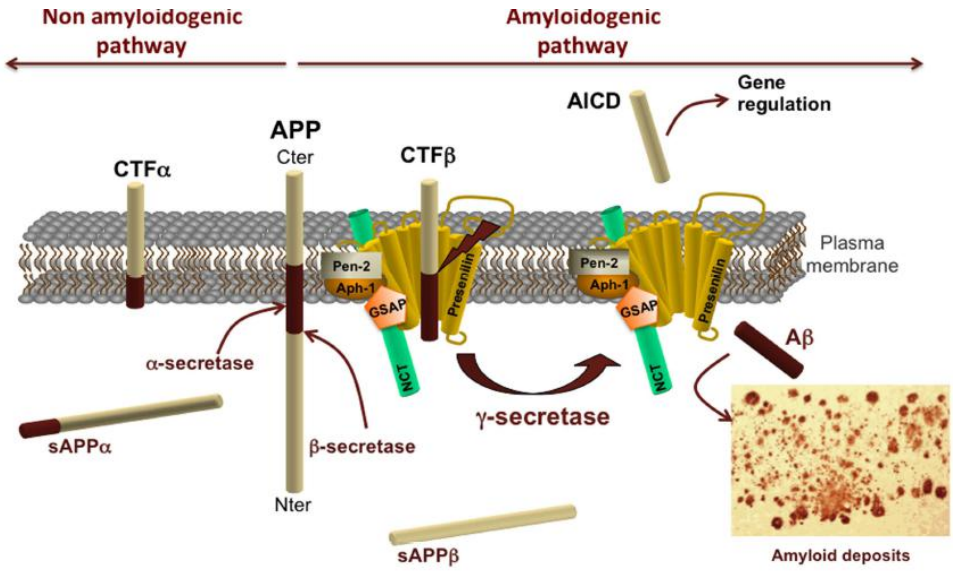


FIGURE 2. Amyloid protein precursor structure and metabolism. Schematic representation of APP processing by α-, β-, and γ-secretases. APP processing by secretase activities is divided into the non-amyloidogenic pathway on the left and the amyloidogenic pathway on the right. α and β-secretase activities cleave APP in its extracellular domain to release respectively a soluble fragment sAPPα or sAPPβ in the extracellular space and generate carboxy-terminal fragments CTFα or CTFβ. These CTFs can subsequently be processed by γ-secretase complex to generate AICD and Aβ. The γ-secretase complex is composed of presenilin, nicastrin (NCT), γ-secretase activating protein(GSAP), pen-2, and aph-1 (Vingtdeux et al., 2012).

1.2.1. α-secretase and α-processing

Within the Aβ domain, cleavage of APP by α-secretase precludes Aβ generation and releases a large soluble neuroprotective ectodomain named sAPPα, which has an important role in neuronal plasticity and survival acting against excitotoxicity (Furukawa *et al*., 1996; Mattson *et al*., 1997). Several members of the ADAM (a disintegrin and metalloproteinase) family possess α-secretase-like activity and three of them are known as the α-secretase: ADAM9, ADAM10, and ADAM17. ADAM17 (also called tumor necrosis factor-α converting enzyme, TACE) has a major role in TNFα proteolytic cleavage and release of its extracellular soluble domain (Black *et al*., 1997). ADAM17 can perform α-cleavage of APP and influence in this manner Aβ generation; in ADAM17-deficient cells, α-cleavage of APP is abolished, therefore ADAM17 is likely the α-secretase responsible for APP cleavage (Buxbaum *et al*., 1998). Overexpression and RNAi studies with ADAM9 demonstrate that ADAM9 has no effect on sAPPα generation and it only is involved in regulated α-cleavage function. Other studies suggest that ADAM10 is the constitutive

α-secretase that is active at the cell surface, and has some functional redundancy in α-cleavage among the ADAM family (Black *et al*., 1997).

1.2.2. β-secretase and β-processing

The first step in Aβ generation is the cleavage of APP by the β-secretase; several groups concomitantly identified BACE1 (also called Asp2 or memapsin 2) as the major β-secretase (Sinha *et al*., 1999; Vassar *et al*., 1999). BACE is a membrane-bound aspartyl protease with a characteristic type I transmembrane domain near the C-terminus. Some results provide convincing evidence that BACE1 is the β-secretase involved in APP metabolism (Cai *et al*., 2001), and BACE1 activity is thought to be the rate-limiting factor in Aβ generation from APP. As a consequence, a number of studies investigated if BACE1 could be used a therapeutic target; in AD mice model, BACE1 deficiency has been shown to rescue cholinergic dysfunction, neuronal loss and memory deficits, with a dramatic reduction in Aβ40/42 levels (Luo *et al*., 2001; Ohno *et al*., 2004 and 2007). Moreover, BACE1 protein and activity levels are elvated in AD correlated brain regions (Ohno *et al*., 2007). However, some studies have found several phenotypic abnormalities in BACE1 KO mice, which showed hyperactive behavior and died in the first weeks after birth (Dominguez *et al*., 2005). BACE1 is therefore likely not as safe drug target as first assumed.

1.2.3. γ-secretase and γ-processing

After α- and β-cleavage, the carboxyl terminal fragments (CTFs) of APP, known as αCTF and βCTF, respectively, remain membrane-associated and will be further cleaved by γ-secretase to generate p83 fragment and Aβ peptide. The p83 is quickly degraded and widely believed to not to play any important function. γ-secretase-mediated cleavage is unique because the cleavage takes place within the transmembrane domain. γ-cleavage can produce the most abundant species Aβ40, and the more amyloidogenic species Aβ42, as well as release the intracellular domain of APP (AICD). Some evidences have shown than γ-secretase activity resides in a high molecular weight complex consisting of two presenilin homologs, PS1 and PS2. Mutations in these two genes, particularly PS1, are

involved in the majority of Familiar AD (FAD) cases. PSs include two highly conserved aspartate residues indispensable for γ-secretase activity. The PS1 N-terminus and C-terminus Fragments heterodimers (NTF/CTF) are bound on transition-state by analogue γ-secretase inhibitors, suggesting that PSs are the crucial catalytic components of the γ-secretase (Kimberly *et al*., 2003; Takasugi et al., 2003).

The APP is normally cleaved by α-secretase and unnormally processed by β and γ-secretases leading to an imbalance between production and clearance of Aβ peptide (Salomone *et al*., 2012). Next, Aβ peptides spontaneously aggregate into soluble oligomers and form fibrils with insoluble beta-sheet conformation and eventually are deposited in diffuse senile plaques (Farlow *et al*., 2008). Mounts of evidence demonstrate that overproduction of Aβ results in synaptic dysfunction, formation of intraneuronal fibrillary tangles and eventual neuronal loss in brain areas related with AD symptomatology (Shankar *et al*., 2009). Compared to Aβ40, Aβ42 is more hydrophobic and prone to fibril formation, so elevated levels of Aβ42 are critical for AD pathogenesis (Burdick *et al*., 1992). Recently, some studies have suggested that Aβ42 oligomers are produced by cooperative activities of both neurons and neighboring astrocytes (Dal *et al*., 2014). It has been postulated that Aβ42 oligomers lead to toxic effects on synapses and mitochondria (Kurz *et al*., 2011; Kumar *et al*., 2008). Aβ42 plaques can also attract and activate microglia, resulting in production and release of pro-inflammatory cytokines, which stimulate the nearby astrocytes and neurons to increase further Aβ42 production. Aβ peptide is considered to be responsible for the neuronal and vascular degeneration in AD brains, which also results in oxidative stress (Galim *et al*., 2013). However, some studies show that Aβ42 activate the neuroprotective signaling of insulin-like growth factor-1 receptor (IGF-1R), glycation end products (RAGE), Frizzled receptor, NMDA-glutamate receptor, p75 neurotrophin receptor (p75NTR) and cellular prion protein (PrPc) also can act as Aβ oligomer receptor (Xu *et al*., 2012; Armato *et al*., 2013). Recently, it has been observed a cross-connection of Aβ42 oligomers and hyperphosphorylated tau oligomers on synapses of closely-associated target cells. Moreover, Intracerebral (i.c.) administration of brain extract from AD patients or Aβ-APP transgenic mice induces production of Aβ and related pathologies in a time- and concatenation-dependent manner (Eisele *et al*., 2010).

1.3. TAU AND NEUROFIBRILARY TANGLES

In AD, multiple proteins assume potentially pathogenic conformations and accumulate separately or together within the brain. AD is not only associated with the abnormal accumulation of amyloid plaques, but also related with neurofibrilary tangles (NFTs), which are intracellular aggregates of tau bearing abnormal posttranslational modifications like increased phosphorylation and acetylation (Cohen *et al*., 2011; Iqbal *et al*., 2010; Min *et al*., 2010). Recently, several findings in cells and mouse models suggest that tau may normally facilitate or enhance excitatory neurotransmission via regulating the distribution of synaptic activity-related signaling molecules (Morris *et al*., 2011). However, when it is abnormally modified and undertakes pathogenic conformations, tau forms enriched clumps in dendritic spines where it can disturb neurotransmission (Hoover *et al*., 2010). Aβ oligomers can promote this postsynaptic enrichment of tau through a process that involves members of the microtubule affinity-regulating kinase (MARK) family (Zempel *et al*., 2010). Interestingly, reducing tau can prevent Aβ production and ameliorate neuronal deficits in cells and APP transgenic mice (Morris *et al*., 2011). APP/PS1 mutations increase Aβ accumulation, which in turn cause AD with amyloid plaques and NFTs in the brain (Bertram *et al*., 2010). By contrast, tau mutation do not cause NFTs neither amyloid plaques nor AD. These conclusions mean that Aβ acts upstream of tau and its effects depend in good part on tau.

1.4. AGING CONTRIBUTION TO ALZHEIMER'S DISEASE PATHOGENESIS

Aging is the most important non-genetic known risk factor for AD, and it is accompanied by stereotypical structural and neurophysiological changes in the brain and different degrees of cognitive decline. In particular, multiple studies provide evidence of increased amyloid-β accumulation and reduced mitochondrial function in aged brain.

The free-radical theory of aging proposes that increased reactive oxygen species (ROS) induces mitochondrial DNA damage and impairs mitochondrial functions. Some studies suggested that ROS increase may be a factor leading to age-related AD (Zhu *et al*., 2004). Clinical and animal studies showed an age-dependent decrease of brain bioenergetic metabolism, with an impaired redox homeostasis in parallel with a trend towards a more

oxidized state (Yin *et al*., 2014). Even before the appearance of the hallmarks of Alzheimer's disease, Aβ plaques and neurofibrillary tangles, brain hypometabolism and oxidative stress are observed at early stages of AD.

Mitochondria provide the universal cellular fuel by ATP generation. When mitochondria fulfill their physiological functions, however, they can also act as a double-edged sword: on one hand, it produces the energy necessary for cell survival, on the other hand, it induces the formation of ROS (Adam and Chinopoulos, 2006; Turrens, 2003). In physiological conditions, ROS are involved in a number of functions such as immune response, inflammation, synaptic, plasticity, learning and memory (Giorgio *et al*., 2007; Kishida and Klann, 2007; Veal *et al*., 2007). However, if ROS production overwhelms the endogenous antioxidant system it can lead to shutdown of energy production, decrease of antioxidant defense, mitochondrial dysfunction and apoptosis (Wallace, 2005). Mitochondria are the first target of ROS toxicity because the long polyinsaturated fatty acid chains of mitochondrial membranes are easily susceptible to oxidation and lead to membrane depolarization and consequently to mitochondrial impairments (Goldstein and Merenyi, 2008; Harper *et al*., 2004). This oxidative stress has been implicated in aging and AD (Fig.3). For example, a decrease in the GSH/GSSG ratio (glutathione reduced/oxidized form) was found in the brain of 21 months-old wild-type mice, especially in regions involved in the loss of cognitive function such as cortex, hippocampus and striatum. In these regions, the decrease of the GSH/GSSG ratio was paralleled by a shift of the redox potential to a pro-oxidized state (Rebrin *et al*., 2007). In rat brains, a progressive decrease in MnSOD (Mn-Superoxide Dismutase) activity was observed at 24 months compared to 7 months of age, with a parallel loss of electron transport chain complex I and IV activity (Navarro and Boveris, 2004). Generally, brain aging is marked by a decrease in the mitochondrial oxidative phosphorylation activity, affecting the protein level and activity of complexes involved in the electron transport chain, particularly complexes I, III, IV and ATP synthase.

Numerous studies have been performed in *vitro* and *vivo* showing that the overexpression of APP and Aβ accumulation induced a decrease in ATP production, weaken mitochondrial membrane potential (MMP), as well as a reduction in mitochondrial complex IV activity,

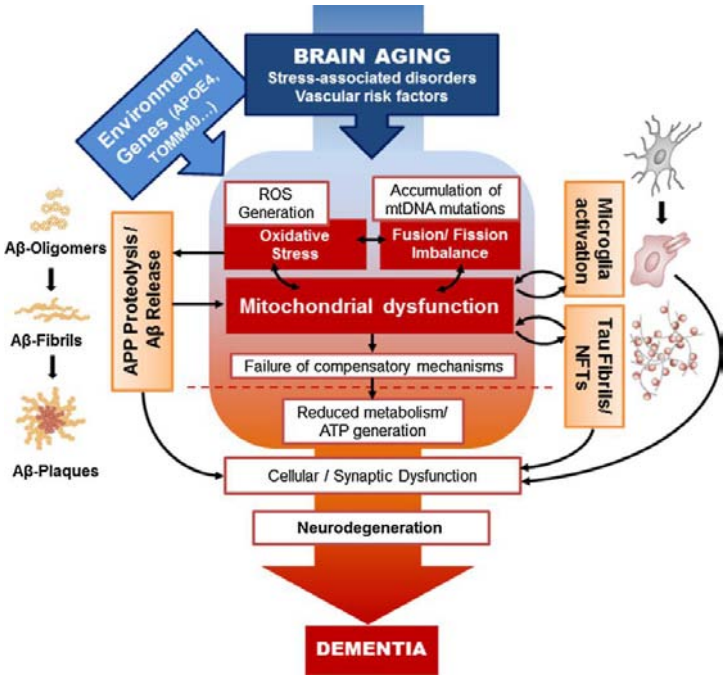


Figure 3. Conceptual representation of sequential events linking brain aging and sporadic Alzheimer's disease. Aging is marked by a gradual increase in brain oxidative stress and consequent damages. The causes and/or consequences of mitochondrial dysfunction may be exacerbated by genetic and environmental factors and may lead to impaired mitochondrial bioenergetics and dynamics that, in turn, increase ROS production. This vicious cycle may involve Aβ production, tau hyperphosphorylation and microglial activation (Grimm *et al*., 2016).

corresponding with an increase in ROS levels (Crouch *et al*., 2005; Gillardon *et al*., 2007; Grimm *et al*., 2015). Recently, some evidences show that ROS themselves cause Aβ accumulation by enhancing the amyloidogenic pathway. Mitochondrial complex I defects were associated with brain aging, since the inhibition of this complex by rotenone mimicked the age-associated mitochondrial dysfunction in *vitro* (Leuner *et al*., 2012). Other studies showed that ischemia in neuroblastoma cells, or energy deprivation in rodents, increased ROS levels leading to an up-regulation of BACE1 expression and activity leading to increased Aβ generation (Guglielmotto *et al*., 2009; Tamagno *et al*. 2008, 2005). Aβ load increase was also observed in mice having a complex I defect or in APP mutant mice treated with complex I inhibitors (Leuner *et al*., 2012). Beside Aβ, abnormally hyperphosphorylated tau protein may trigger mitochondrial dysfunction in AD synergistically with Aβ. Injection of Aβ fibrils in the brain of mutant P301L tau transgenic mice displayed an enhanced vulnerability of isolated mitochondria, with a fivefold increase in NFT pathology (Eckert *et al*., 2008).

2. TACE AND ITS FUNCTION

ADAM enzymes (a disintegrin and metalloproteinase) are Zn^{2+}-dependent, modular cell surface proteins which belong to the adamalysin protein family. ADAMs have both adhesive and proteolytic properties and therefore participate in cellular adhesion and proteolytic cleavage of various cell surface molecules, which determine cellular fate, proliferation, and growth (Blobel, 2005). ADAMs are contributors to physiological and pathophysiological processes and may be potential therapeutic targets in most of diseases. ADAM-17 (TACE) is one of the most well studied ADAM enzymes; it is widely expressed in most of tissues, such as brain, heart, kidney and skeletal muscle. TACE is a multi-domain protein of 824 amino acids (aa) starting with the catalytic domain sequence (1-17 aa), also called metalloenzyme, that has the typical HEXXHXXGXXH (X being any aa) sequence. TACE function is to cleave ectodomains of various transmembrane proteins; this process usually occurs at the membrane-adjacent part of the substrate molecule, like TNFα and its receptor TNFR1, as well as the amyloid precursor protein. After cleavage, the molecules may bind to their receptor on the same cell (autocrine effect)or to neighboring cell receptors (paracrine effect). Usually, the Golgi apparatus provides a reserve for the shedded ligand, and ligand shedding itself can promote ligand movement to the membrane (Wang *et al*., 2003). TACE regulates cellular signaling dependent on the cellular context. For example, TACE can cleave heparin-binding epidermal growth factor (HB-EGF) and induce proliferation of mesangial cells (Gooz *et al*., 2006), and induce macrophage colony-stimulating factor receptor cleavage (M-CSFR), resulting in the down-regulation of macrophages activation (Rovida *et al.*, 2001). TNFR1 mutation leads to receptor accumulation on the cell surface due to inefficient shedding; this increases cell susceptilility to TNFα, leading to inflammatory responses with concomitant fever (McDermott *et al.*, 1999). The receptor shedding can also occurs before the ligand binding, then the soluble receptor can serve as a decoy and inhibit the ligand binding to cell surface receptors (Brodowicz *et al.*, 1997).

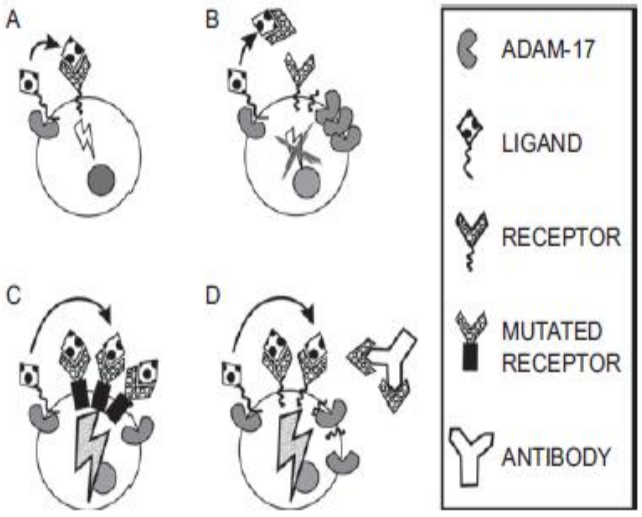


Figure4. Consequences of protein ectodomain shedding. (A) Cleaved substrate binds to its receptor and initiates downstream signaling events. (B) Receptor cleavage stops ligand-initiated signaling. (C) Failure to downregulate receptor hypersensitizes the cells for receptor agonist. (D) Shedded receptor serves as decoy for antibody and contributes to resistance in cancer. (Gooz, BMB (2010))

TACE suffers post-translational modifications, the most importants being glycosylation and phosphorylation. There are six potential N-linked glycosylation sites on the mature form: three are located on the metalloenzyme module, two are located on the disintegrin module, and one is present on the cysteine-rich domain. The glycosylation of TACE affects activity and transport of the enzyme. Another modificattion of TACE is the cytoplasmic domain phosphorylation on serine and theronine residues. Some studies show that the gastrin releasing peptide, a GPCR agonist, induces activation of Src-PI3K-PDK1, and then PDK1 acts as the enzyme that directly phosphorylates TACE both on threonine and serine residues (Zhang *et al*., 2006). The agonist carbachol can also induce threonine 735 and tyrosine 702 phosphorylation of TACE in HEK 293 cells (Tellier *et al*., 2006). It is also possible that TACE becomes phosphorylated on different residues depending on the cell type and stimulation. Recent data suggest that after and during furin cleavage, active TACE is localized separately from its substrate in cholesterol-rich membrane domains or lipid rafts, and ERK-dependent threonine 735 phosphorylation is necessary for TACE to reach the secretory pathway during maturation (Soond *et al*., 2005).

2.1. TACE FUNCTION IN THE CENTRAL NERVOUS SYSTEM

TACE plays an importance role in learning and memory; some experiments also showed that TACE participates in glutamate receptor 1/5-induced long-term depression (LTD) (Cho *et al*., 2008). TACE can also cleave neuronal pentraxin (NPR), which is a necessary step for LTD in hippocampal and cerebellar synapses. TACE has an important role in AD, as the described before, since TACE act as α-secretase to cleave the amyloid precursor protein to produce a soluble, non-amyloidogenic fragment, APPsα. APP also can be cleaved by β-secretase, which produces the amyloidgenic Aβ. This peptide can induce AD pathological features, which is manifested by microglia and astrocyte activation around amyloid plaques (Sastre *et al*., 2008). It is also suggested that DRAL, a TACE cytoplasmic domain partner, is necessary for APPsα formation. The pro-inflammatory cytokine interleukin-1 is able to enhance TACE activity and soluble APP formation in astrocytes, which decreases Aβ production (Tachida *et al*., 2008). Recently Pietri et al showed a new kinase regulating TACE availability at the cellular membrane that influences the proteolytic machinery responsible for the physiological cleavage of both βAPP and PrP^{C}. Indeed, PDK1 was shown to acts downstream of Src-mediated signaling by triggering the phosphorylation and membrane depletion of TACE, which significantly influence the course of both AD and prion disease. Pharmacological inhibition and genetic depletion of PDK1 not only reduce Aβ and membrane-bound PrP^{Sc} levels but also restore TACE-mediated sAPPα levels, which suggests PDK1 may be a common signaling node activated by Aβ and PrP^{Sc} in AD and prion disease (Fig.5).

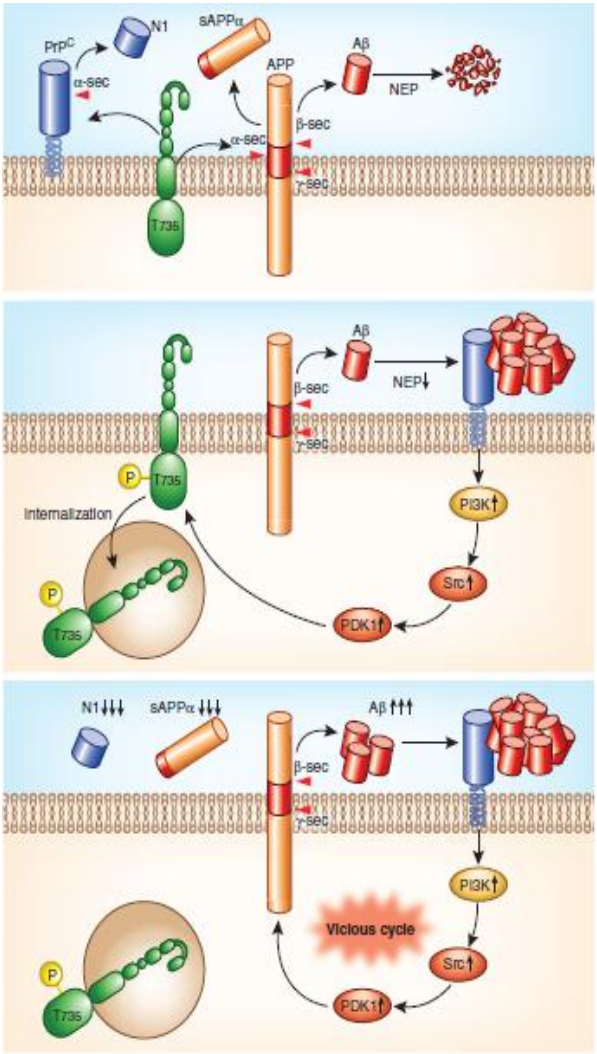


FIG5. PDK1-mediated phosphorylation of TACE triggers its membrane depletion and augments Aβ levels, leading to Alzheimer's disease–like pathology and prion infection. In physiological conditions (top), TACE triggers the α-secretase cleavage of both βAPP transmembrane protein and PrPC, thereby yielding sAPPα and N1, respectively. Concomitantly, βAPP undergoes minor cleavage by combined β- and γ-secretases that release Aβ peptides. In Alzheimer's disease, as well as in normal aging, NEP expression is reduced, e resulting in deficient Aβ degradation and accumulation (middle). After Aβ aggregates, it forms oligomers that interact physically with PrP^C, eliciting a molecular cascade that activates PI3K, Src and PDK1, which then phosphorylates TACE. This process induces TACE internalization in caveolae, thereby reducing TACE-mediated production of sAPPα and N1. This preserves whole PrP^C at the membrane, which is necessary for both scrapie PrPSc infection and for its interaction with Aβ oligomers (bottom) (Pietri *et al*, 2013).

2.2. TNFR1 FUNCTION IN ALZHEIMER'S DISEASE

Tumor necrosis factor (TNF), a ligand cytokine, is involved in systemic inflammation. TNF and its type 1 receptor (TNFR1) are not only involved in AD-related brain neuroinflammation, but contribute to amyloidogenesis by β-secretase regulation. As previously explained, TNFα is synthesized as a membrane-bound pro-protein comprising 233 amino acids and is cleaved by TACE to generate mature TNFα. The major source of TNFα comes from cells in the immune system, such as monocytes and macrophages. In the brain, TNFα is expressed by neurons and glia, and promotes inflammatory responses by recruiting microglia and astrocytes to lesion sites, leading to glial cell activation (Lee *et al*., 2009). There are two receptor subtypes for TNFα: TNFR1 and TNFRII; both differ

considerably in their amino acid sequences, with 24% homology in the extracellular domain region and approximately 10% homology in the intracellular domain (Paganelli *et al*., 2002). TNFR1 contains an intracellular death domain that activates nuclear factor NF-kB signaling pathways leading to apoptosis. TNFR2 has a trophic or protective role in neuronal survival (Yang *et al*., 2002).

Under pathological conditions in which glial cells in the brain are activated, TNFα is secreted and TNFRs become activated. $TNFRII^{-/-}$ mice are more vulnerable to TNFα than are neurons from $TNFRI^{+/-}$ or wild type animals. However, at lower doses of TNFα, LDH release from $TNFRI^{-/-}$ hippocampal neurons did not differ significantly from that of wild type mice. These suggest that neuronal death induced by TNFα through TNFRI involves an apoptotic process. In brain, Aβ peptide can induce neuronal apoptosis through TNFR1, which is mediated via alteration of apoptotic protease-activating factor (Apaf-1) expression (Li *et al*., 2004). Other studies have shown that TNFα not only induces cytotoxic effects and apoptosis, but also has antiviral activity (Lim *et al*., 2000). In AD mouse models, TNFα expression is prepathologically up-regulated; that excessive and unopposed TNFα signaling portends intracellular Aβ accumulation and Tau pathology that leads to neuronal cell death and enhances AD-associated pathology (Oddo *et al*., 2003; Janelsins *et al*., 2008). So TNFα levels reflect the pathologic progression of AD.

2.3. CYTOKINES AND CHEMOKINE FUNCTION IN ALZHEIMER'S DISEASE

The inflammatory cytokines and chemokines may play a vital role in the occurrence and development of AD. Immunogens formed by abnormal deposition of Aβ in AD patientsresults in the activation of microglia and astrocytes, which release inflammatory cytokines, leading to neuronal damage through direct or indirect toxic effects elicited by this chronic immune response. Microglia, the resident phagocytes of the CNS, is ubiquitously distributed in the brain. Microglia constantly use highly motile processes to survey their assigned brain regions for the presence of pathogens and cellular debris, and simultaneously provide factors that support tissue maintenance and plasticity of neuronal circuits, thereby contributing to the protection and remodelling of synapses. This protective

and remodelling action is mediated by the release of trophic factors, including brain-derived neurotrophic factor (BDNF), which contributes to memory formation. Once activated by pathological triggers, such as neuronal death or protein aggregates, microglia extends their processes to the site of injury and migrates to the lesion, where it initiates an innate immune response. On the other hand, pathological responses of astrocytes include reactive astrogliosis, a complex, multistage and pathology specific reaction different from astrocytes remodelling, which is generally aimed at neuroprotection and recovery of injured neural tissue (Sofroniew, 2009). Next to activated microglia, hypertrophic reactive astrocytes accumulate around senile plaques and are often seen in post-mortem human tissue from patients with Alzheimer's disease and in animal models of the disorder (Olabarria *et al*., 2010). Glial cell activation might occur early in Alzheimer's disease, even before Aβ deposition. Reactive astrocytes are characterized by the increased expression of glial fibrillary acidic protein (GFAP) and signs of functional impairment; however, astrocytes do not seem to lose their domain organization, and no evidence of scar formation exists (Fig.6).

Microglia activation have dual effects on AD progression: on one side, activation of microglia via microglia's scavenger receptors (SRs) plays a beneficial role by reducing Aβ accumulation by increasing its phagocytosis, clearance and degradation in early stages of AD, which prevents the formation of amyloid plaques in the brain and hinder the AD progression (Solito *et al*., 2012; Yang *et al*., 2011). On the other side, prolonged microglia activation leads to the release of pro-inflammatory cytokines, which initiates a pro-inflammatory cascade and subsequently contributes to neuronal damage and losses. In AD, the microglia has the capacity to respond to various stimuli, including amyloid peptides, their precursor protein (APP), and neurofibrillary tangles (Zilka *et al*., 2012).

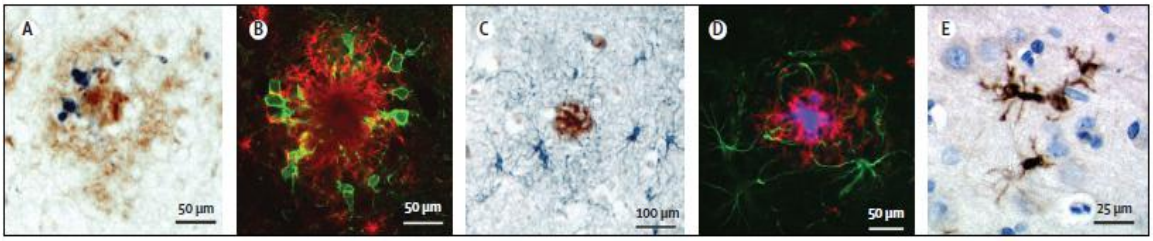


Figure 6.Changes in microglia and astroglia in Alzheimer's disease. Microglia and astroglia are key players in the inflammatory response: changes in microglia and astroglia are evident in the post-mortem brains of patients with Alzheimer's disease and in animal models of the disorder. (A) CD11b-positive microglia (blue) within an amyloid β (Aβ) deposit (brown) in the parietal cortex of a brain section from a patient with Alzheimer's disease. (B) Activated, IBA1-positive microglia (green) at an Aβ plaque site (red) in a brain section from an APP/PS1 transgenic mouse. (C) GFAP-positive astrocytes (blue) surround the site of Aβ deposition (brown) in the parietal cortex of a brain section from a patient with Alzheimer's disease. (D) GFAP-positive astrocytes (green) at an Aβ plaque site (red) in a brain section from an APP/PS1 transgenic mouse. (E) Interleukin-1β-positive microglia (brown) in the frontal cortex of a brain section from a patient with Alzheimer's disease (Heneka *et al*., 2015).

Cytokines contribute to nearly every aspect of neuroinflammation, including proinflammatory and anti-inflammatory processes, bystanding neuronal injury, chemoattraction, and response of microglia to Aβ deposits. Increases in Aβ concentration in ageing AD model mice are associated with increased doses of proinflammatory cytolines, like TNFα, interleukin 6 (IL-6), interleukin 1 (IL-1) and GM-CSF. So, the accumulation of Aβ is a key factor that drives neuroinflammatory responses in AD (Stoeck *et al*., 2014). Additionally, exposure of microglia to preaggregated Aβ also increases production of proinflammatory cytokines, such as IL-1, IL-6, MIP-1 and M-CSF (Lee *et al*., 2009; Qian *et al*., 2006; Minnerup *et al*., 2008). Aβ binding to the microglial cell surface induces pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, which lead to tau hyperphosphorylation and neuronal loss (Bernhardi *et al*., 2010). In addition, several studies suggest that sustained systemic inflammation can also lead to raises in the levels of pro-inflammatory cytokines in the CNS through the blood brain barrier (BBB) that can contribute to cognitive decline in AD patients. Some studies tried to elucidate the pathways for the transport of pro-inflammatory cytokines to the brain from systemic circulation (Holmes *et al*., 2009). It seems that the inflammatory response within the brain tissue will be associated with pro-inflammatory cytokines dysfunction, raising the potential use of

pro-inflammatory cytokines measurement as a surrogate marker for local inflammatory response in AD (Fig.7).

Chemokines are small heparin-binding proteins; some of them are considered to be pro-inflammatory and can be induced during an immune response to recruit cells of the immune system to a site of infection; other chemokines are considered to be homeostatic and are involved in controlling the migration of cells during normal processes of tissue maintenance or development. Some evidences show that chemokines play a role in the physiology of the nervous system, including neuronal migration, cell proliferation, and synaptic activity, and are important in mediating neuroinflammation. In physiological conditions, chemokines and other inflammatory mediators acting on microglia and astrocytes contribute to memory, learning, brain development, and synapse formation. In pathophysiological inflammatory contexts, chemokines expression is increased from the periphery to the brain (Liu *et al*., 2014).

Significant differences of the levels of chemokines and their receptors in serum, cerebrospinal fluid, or even brain tissue are observed in AD patients compared to normal people. Some studies suggest that prodromal AD patients within the highest tertile of CSF MCP-1 levels exhibited a significantly faster cognitive decline and developed dementia within a shorter time period compared to those that with the lowest tertile levels (Westin *et al*., 2012). Chemokines and chemokine receptors play multiple roles in the occurrence and development of AD; on one hand, inflammatory chemokines under pathological conditions actively participate in the inflammatory response by attracting immune cells to the site of inflammation in AD; on the other hand, chemokines can regulate the migration of microglia, neurons and neural progenitors to sites of neuroinflammation. Evenly the effects of chemokines can aggravate Aβ deposition whilst some are supposed to reduce Aβ deposition.

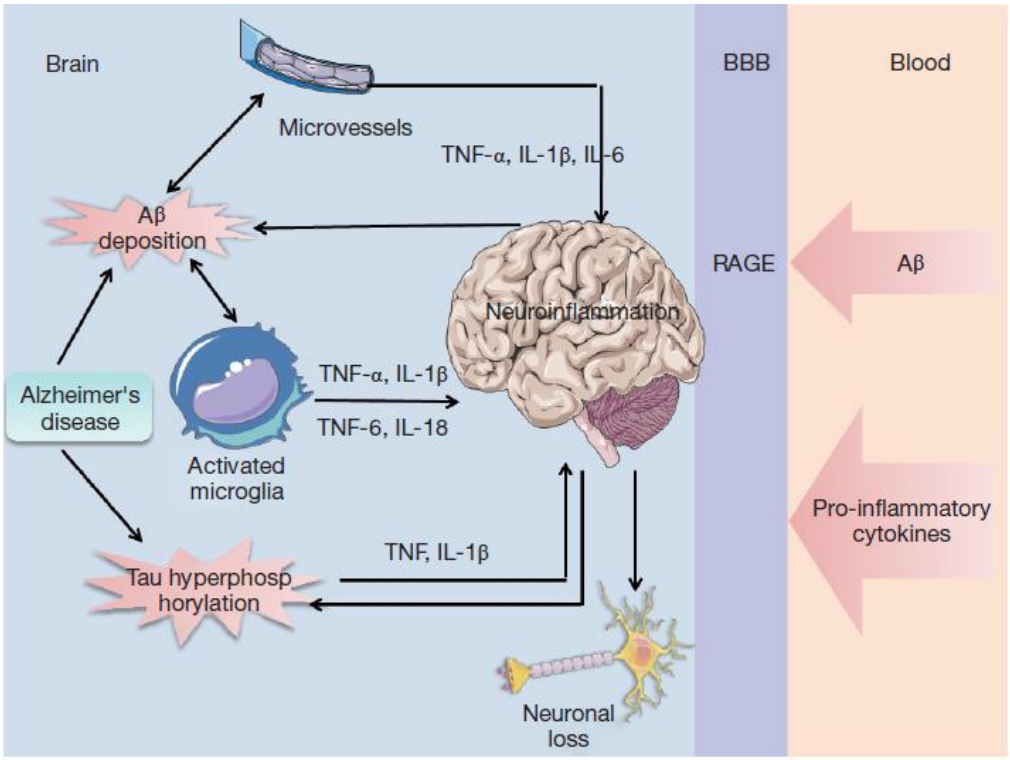


Figure 7. Speculative model of dysregulation of pro-inflammatory cytokines in the AD brain. A vicious circle between microglia activation, pro-inflammatory cytokines production, and Aβ accumulation and and tau hyperphosphorylation in AD brain; AD cerebral microvessels participates in a destructive cycle of events where inflammation precedes Aβ deposition and Aβ in turn promotes release of proinflammatory cytokines; pro-inflammatory cytokines and Aβ could cross the BBB from the periphery into brain. (Wang *et al*., 2015)

3. SIGNALING IN THE CENTRAL NERVOUS SYSTEM

3.1. BRAIN-DERIVED NEUROTROPHIC FACTOR IN AGING AND ALZHEIMER'S DISEASE

Brain-Derived Neurotrophic Factor (BDNF) belongs to the neurotrophins family, is synthesized by both neurons and glia, and play important roles in the brain to support cell survival and synaptic plasticity (Lu *et al*., 2008). BDNF is synthesized as a 35-kD pro-BDNF, and then converted into the 14-kDa mature form. BDNF released from cells exerts its functions by binding to TrkB (a tyrosine kinase belonging to the trk family of receptors) or $p75^{NTR}$ receptor (Matsumoto *et al*., 2008). BDNF and its receptors are broadly expressed in the CNS neurons.

Mechanistically, BDNF protection may be elicited through multiple signalling mechanisms, such as the mitogen-activated protein kinase (MAPK), the phospholipase Cγ (PLCγ) and the phosphoinositide-3 kinase (PI3K) pathways. TrkB phosphorylation on tyrosine residue 515 enhances Src homologous and collagen-like protein (Shc) recruitment and leads to its phosphorylation. Growth factor receptor-bound protein 2/son of sevenless (Grb/SOS) is then recruited to the activated Shc, which results in the activation of rat sarcoma (Ras). (Rapid Accelerated Fibrosarcoma (Raf), (mitogen-activated protein)/(extracellular-regulated kinase) kinase (MEK), and extracellular-regulated kinase (ERK) constitute a MAPK signalling cascade which is all phosphorylated following Ras activation sequentially (Huang and Reichardt, 2003) (Fig.8). Exogenous BDNF protects primary cortical neurons from apoptosis in a dose-dependent manner. Also, BDNF increases the activation of PI3K and Akt, and the inhibition of the PI3K pathway by LY294002 restricts the neuroprotective effects of BDNF in primary cortical neurons (Hetman *et al*., 1999). Similarly, BDNF causes a significant increase of extracellular-signal regulated kinase (ERK) 1/2 phosphorylation and activation, and inhibition of ERK 1/2 pathway with the PD98059 compound markedly reduced the BDNF neuroprotective effects. Moreover, intracerebroventricular (i.c.v.) administration of BDNF to postnatal day 7 rats resulted in the phosphorylation of ERK1/2 and Akt quickly (Han and Holtzman, 2000). BDNF plays a crucial role in learning and memory, as it can regulate long-term

depression (LTD) and long-term potentiation (LTP), synaptic plasticity, axonal sprouting, proliferation of dendritic arbor and neuronal differention (Murer *et al*., 2001). BDNF modulate biological processes in aging via interaction with different receptors (Silhol *et al*., 2008). In the aging process, the gradual impairment of cognitive abilities is associated with cortical and hippocampal alterations (Tapia-Arancibia *et al*., 2008). Some studies show that a chronic BDNF shortage leads to age-dependent learning deficit in old animals, and a positive correlation between hippocampus BDNF levels and learning performance is evidenced (Petzold *et al*., 2015). The reduced BDNF levels during aging might be due to impaired CREB-mediated transcription of the BDNF gene, since the DNA-binding activity of CREB is greatly decreased in several different regions of aged rats compared with young adult's rats (Asanuma, 1996). In aged rats, CREB expression was also decreased in the cerebral cortex and hippocampus neurons (Baquet *et al*. 2004).

A number of studies have demonstrated the role of BDNF in AD both in animal models and patients. BDNF protein and mRNA levels are decreased in samples of hippocampal and cortical tissue from AD patients compared with age-matched control subjects (Phillips *et al*. 1991). By contrast, increased serum levels of BDNF were also reported in both AD and Mild Cognitive Impairment (MCI) patients (Angelucci *et al*., 2010). Increased expression of TrkB was found in the CA1 region of the hippocampus of patients in early stages of AD (Kao *et al*., 2012). Beyond BDNF, a study show platelet GSK3β activity and lymphocyte CREB activity increased in AD, which can be explained by the presence of low BDNF levels (Platenik *et al*., 2014). These changes in the levels of BDNF may be well related to the several stages of AD pathology. Decreased levels have been found in later stages and an increase in the initial stages, which may be a mechanism to counteract memory loss. Some reports have demonstrated increased BDNF levels in AD transgenic mice or upon injection of mice with the amyloid-β peptide. The treatment with caffeine in AD transgenic mice increased BDNF levels, and in turn improved cognitive impairment (Prakash *et al*., 2014). Pioglitazone (a hypoglycemiant drug) restores BDNF concentration and attenuates the inflammatory markers in AD model mice. Also, the oral fingolimod can improve cognitive impairment by enhanced BDNF levels in mice (Prakash *et al*., 2014).

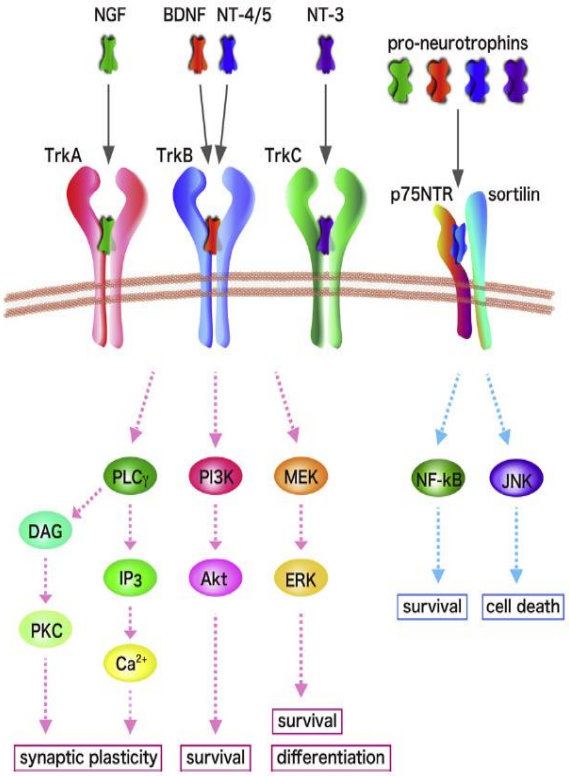


Figure 8. Signaling pathways evoked by neurotrophins and their receptors. Mammalian neurotrophins – NGF, BDNF, NT-3, and NT-4/5 – stimulate intracellular signaling through binding to Trk receptors and a common receptor p75NTR. The mature neurotrophins bind to their specific Trks receptors with high affinity, while the pro-neurotrophins interact with p75NTR with high affinity. Activation of Trks stimulates three main signaling cascades including PLCγ, MAPK/ERK, and PI3K/Akt to exert beneficial effects on neurons. Activation of p75NTR is capable of inducing cell death or survival. Sortilin, which recognizes the pro-domain structure of neurotrophins, is assumed to make a complex with p75NTR and pro-neurotrophin during apoptosis (Numakawa *et al*., 2013).

3.2. PI3K SIGNALING PATHWAY IN ALZHEIMER'S DISEASE

The phosphoinositide 3-kinase (PI3K) family contains eight mammalian isoforms grouped into three classes. Class 1A has a catalytic subunit and a regulatory subunit. The catalytic subunits consist of p110α, p110β, and p110δ isoforms and the regulatory subunits include p85α, p85β and p55γ isoforms. Class 1B consists of only a catalytic subunit p110γ and two regulatory subunits, p87 and p101 (Cantley, 2002). Class 2 PI3Ks are monomeric proteins, which comprise three isoforms, PI3K2α, PI3K2β and PI3K2γ, whereas class 3 has only one isoform, hVps34 (Fyffe *et al*., 2013). Class 1 PI3K are activated by G-protein-coupled receptors as well as growth factor receptors; once activated, they phosphorylate the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PtdIns[4,5]P_2) at carbon 3′ within the inositol group to produce phosphatidylinositol-3,4,5-trisphosphate (PtdIns[3,4,5]P_3). PtdIns[3,4,5]P_3 plays a role as a second messenger by inducing the translocation of proteins to the membrane and its subsequent activation. Modular domains, such as the pleckstrin homology (PH) domains, can interact with 3′-phosphorylated lipids; this function is an important step forward in the PI3K pathway. The most marked example of PtdIns[3,4,5]P_3-dependent protein activation is the serine/threonine protein kinase B

(PKB, also named Akt). Akt contains a PH domain that binds to PtdIns(3,4,5)P_3 and promote the translocation of Akt to the plasma membrane, where it is phosphorylated at threonine (Thr)308 within the activation loop or T-loop by 3-Phosphoinositide-dependent protein kinase 1 (PDK1), which itself is also anchored to the membrane by PH domain-dependent binding to PtdIns(3,4,5)P_3. Both PDK1 and Akt are therefore key effectors of the PI3K signaling pathway (Fyffe et al., 2013).

PDK1, like Akt, p70 ribosomal S6K kinase (S6K), p90 ribosomal S6K kinase (RSK), serum- and glucocorticoid-induced protein kinase (SGK) and isoforms of protein kinase C (PKC) belong to the AGC family of serine/ threonine kinases, which show a sequence homology in their catalytic domain to cAMP-dependent protein kinase (PKA), cyclic guanosine monophosphate-dependent protein kinase (PKG) and calcium-dependent protein kinases (PKC), thereby the name of the family. Many AGC kinases require phosphorylation at two sites in order to be activated: one in the activation loop, which is located within the kinase domain; another in the hydrophobic motif, which is located in a site adjacent to the catalytic domain (Alessi *et al*., 1997). Activation of PI3K signaling and phosphorylation of these sites leads to increasing kinase activity and enzymatic full activation. AGC kinases are activated downstream of a wide range of extracellular stimuli by different mechanisms. PDK1 acts as the upstream activator phosphorylating a specific Thr or Ser residue located at the T-loop of the kinase domain of at least 23 members of the AGC family (Bayascas, 2010).

3.2.1. PDK1, mechanisms of activation and its physiological functions

PDK1 was discovered as the kinase responsible for the phosphorylation of the Akt at residue Thr308, which is essential for enzyme activation. PDK1 acts downstream of PI3K because Akt phosphorylation at Thr308 is dependent on PtdIns(3,4,5)P_3 presence in *vitro*. PDK1 kinase is a protein of 556 amino acids that comprises an N-terminal catalytic domain, a C-terminal PH domain and a nuclear export sequence, which possesses four amino acids that are essential for exporting PDK1 from cell nuclear to the cytoplasm (Fig. 9). PDK1 possesses a phosphorylation site within the activation loop, Ser241, which is required for PDK1 activation but is not affected by growth factor stimulation. By contrast,

PDK1 is a constitutively active enzyme capable of autophosphorylating at various sites including Ser241 within the activation loop. It occurs at multiple sites through *cis* and *trans* mechanisms, which suggests that dimerization and trans-phosphorylation might serve as mechanisms to regulate PDK1 activity in cells (Wick *et al*., 2003).

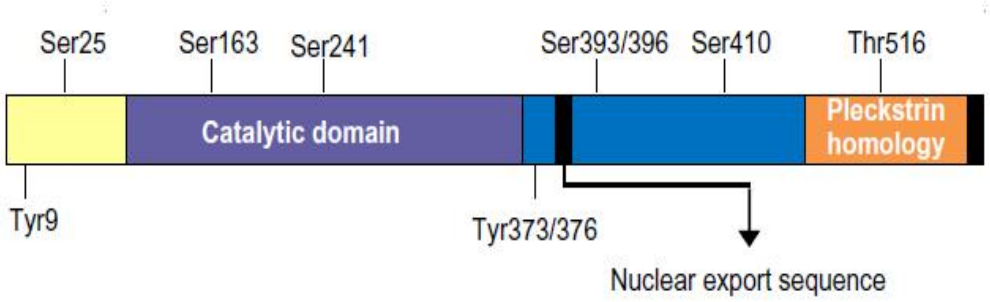


Figure 9. Schematic representation of PDK1 structure. PDK1 kinase is a protein of 556 amino acids that possesses an N-terminal cata lytic domain, a C-terminal PH domain, and a nuclear export sequence The nuclear export sequence is a short sequence of four amino acids that are essential for exporting PDK1 from the cell nucleus to the cytoplasm through the nuclear pore complex using nuclear transport (Fyffe et al., 2013).

PDK1-activated signaling regulation involves different mechanisms. PDK1 is constitutively localized at the plasma membrane since its PH domain can also interact with other phospholipids different from PtdIns(3,4,5)P_3 with high affinity. PI3K activation PtdIns(3,4,5)P_3 production mediates the recruitment of Akt to the plasma membrane and promote phosphorylation of Akt by resident PDK1 (Raimondi *et al*., 2011; Currie *et al*., 1999).

A part from Akt, many other kinases are known to be downstream substrates of PDK1, such as SGK, S6K, RSK and PKC isoforms, which are phosphorylated at specific serine/threonine residues of their activation loop by PDK1. The mechanism of activation of these kinases differs from the Akt activation mechanism. S6K and SGK lack of PH domain and are phosphorylated by PDK1 at the same rate in the presence or absence of PtdIns(3,4,5)P_3, but they have a Ser/Thr residue at the hydrophobic motif site, whose phosphorylation is essential for maximal activation of these enzymes. All hydrophobic motifs consist on a conserved Phe-Xaa-Xaa-Phe/Tyr-Ser/Thr-Phe/Tyr sequence, where the Ser/Thr represents the site of phosphorylation. Some findings indicated that the

hydrophobic motif of AGC kinases could serve as a substrate-docking site which is recognized by an specific groove within the small lobe of the PDK1 catalytic domain termed the PIF-pocket, enabling PDK1 to dock and then phosphorylate their substrates. Moreover, phosphorylation of the hydrophobic motif of AGC kinases might enhance the ability of PDK1 to interact with their substrates (Balendran *et al*., 1999). So PI3K promotes the activation of S6K and SGK by controlling hydrophobic motif phosphorylation of these enzymes. This phosphorylation does not directly activate S6K or SGK but regulates their interaction with PDK1 and hence activation (Fig.10).

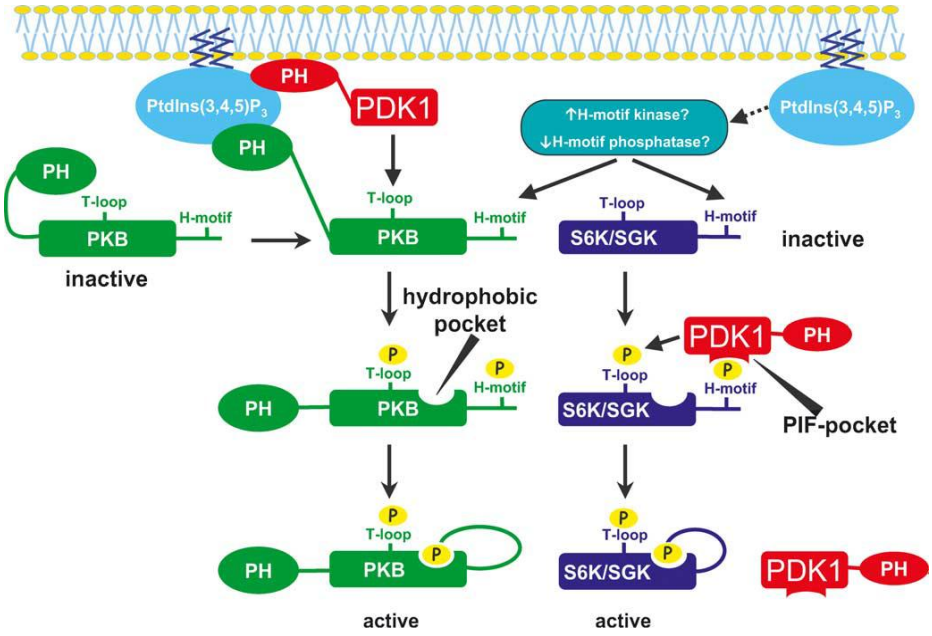


Figure 10. Mechanism of activation of Akt, S6K and SGK by PDK1. Akt is activated through binding of its PH domain to PtdIns(3,4,5)P_3. In contrast, for S6K and SGK, is activated through its PIF-pocket, hence permitting the T-loop phosphorylation of these substrates (Mora *et al*., 2004)

3.2.2. Akt, the key mediator of PI3K pathway

Akt is a serine/threonine protein kinase of 60kD which attracts a major focus of attention because of its critical regulatory role in diverse cellular processes. Since its discovery as a proto-oncogene, Akt has been widely studied and revealed as a key effector of the PI3K pathway. There are three different Akt isoforms existing in mammals, each of them is encoded by a separated gene but shares 85% amino acid sequence identity and analogous structural organization in three domains: N-terminal Pleckstrin Homology domain, catalytic kinase domain and a carboxyl-terminal regulatory domain including the hydrophobic motif. This structure is conserved from fly, worm, mouse, to human. Both the

C-terminal regulatory domain and hydrophobic motif are highly preserved among the AGC group, including PKC, S6K, RSK and (SGK). In order to reach the full activation of Akt, both the T308 site in the activation loop of the catalytic domain and the S473 site in the hydrophobic motif of the regulatory domain need to be phosphorylated. The crystal structure study provides us a chance to understand how these two phosphorylation sites contribute to enzymatic activation of the kinase. The catalytically active conformation is requires the phosphorylation of T308, whereas the phosphorylation of S473 could enhance the stabilization of the active conformation by intramolecular interactions between the hydrophobic motif and corresponding acceptor structure within the catalytic domain, termed as hydrophobic groove (Mora et al., 2004).

Akt is involved in many cellular processes and pathologies, such as metabolism, proliferation, survival, growth, cancer and neurodegenerative disorders. Activated Akt phosphorylates serine/threonine residues on downstream target proteins at a recognition sequence Arg-X-Arg-X-X-Ser/Thr-Hyd, where X means any amino acid and Hyd means bulk hydrophobic residue (Alessi *et al*., 1996), which mediate the physiological processes in response to Akt activation. The requirement of Arg residues at the -3 and -5 terminal on peptides phosphorylated by Akt distinguishes it from other AGC kinases, which prefer the Lysine residue at the same site (Manning and Cantley, 2007).

3.2.3. PI3K/PDK1/Akt signaling pathway in AD

The PI3K/Akt pathway mediates the IGF-1R and IR signaling, which are expressed at high levels mostly in neurons within the brain. The function of the PI3K/Akt pathway is distinct in the developing embryonic brain, because the neurons are still mitotic. During this process, the IGF-1 and insulin-activated PI3K/Akt pathway serves as a potent neuronal survival and division signal, which contributes to the survival/death balance that sculpts and wires the brain, as well as contributing to neuronal migration, myelination and establishment of neuronal polarity. In adult brain, PI3K/Akt pathway has significant functional impact on synaptic plasticity, neuronal polarity, neurotransmission, proteostasis, metabolic control and stress responses (Kitagishi *et al*., 2014).

In AD brain, the activation of the PI3K/Akt signaling pathway by IGF-1R and IR is markedly disturbed compared with those controls of the same age. Indeed, Akt activation is increased and its subcellular localization in hippocampal and cortical neurons altered with concomitant inactivation of PTEN (Griffin *et al*., 2005; Moloney *et al*., 2010; Sonoda *et al*., 2010). This sustained activation of Akt cause neurons to become increasingly and ultimately totally resistant to both insulin and IGF-1 in AD, a process which is mediated by the inflammatory JNK-TNFα pathway (Moloney *et al*., 2010; Neill *et al*., 2012; Talbot *et al*., 2012). Aβ contributes both to over-activate the PI3K/Akt signaling pathway at several levels and to induce insulin and IGF-1 resistance. Aβ binding to IRs lead to it internalization and removal from the neuronal dendrites (Townsend *et al*., 2007; Zhao *et al*., 2008). Moreover, Aβ blocks IR activation in *vitro* and can also inactivate IRS-1 by promoting its phosphorylation at serine residuesby JNK (Bomfim *et al*., 2012; Ma *et al*., 2009). Importantly, Aβ-inducedPI3K/Akt signaling is linked to cognitive decline (Bhaskar *et al*., 2009). Aβ peptides may compete for the IGF-1R/IR signaling system in AD neurons via inappropriately increased activation of the PI3K/Akt signaling as well as the JNK-mediated negative feedback on the pathway. This presents a detrimental “dual hit” where the extent of regular insulin and IGF-1 responses are reduced to a degree that is not compatible with healthy brain function and longevity. This sustained activation of the PI3K/Akt disallows the protective effects of FOXO activation and mTOR inactivation that further exacerbate Aβ, tau and synaptic pathology and cognitive decline (Fig. 11).

Tau is abnormally hyper-phosphorylated and aggregated in AD; Aβ induces Tau protein hyper-phosphorylation, which is the main event responsible for NFT formation. Akt inhibition may contribute to the decrease of Tau phosphorylation (Kyoung *et al*., 2004). Akt phosphorylates and inhibits GSK-3β, which is a major upstream kinase for Tau phosphorylation. The PI3K/Akt signaling pathway dyfunction leads to inefficient GSK-3β inhibition and Tau hyperphosphorylation. Tau phosphorylation at the proline-rich region, which is located upstream of the microtubule-binding domains, moderately inhibits its microtubule assembly activity and slightly promotes its self-aggregation. Abnormally hyper-phosphorylated Tau disengages from microtubules and then increased cytosolic concentrations of unbound Tau occur, resulting in NFT. Hyperphosphorylated Tau is the

major component of the paired helical filaments that accumulate in degenerating neurons in AD and other neurodegenerative diseases (Cowan *et al*., 2010; Lippens *et al*., 2007). Protein phosphatase 2A (PP2A) is the major protein phosphatase in the brain that can dephosphorylate Tau at several phosphorylation sites, thereby stopping the ability of Tau to inhibit microtubule assembly and to self-assemble into helical filaments and NFTs (Liu *et al*., 2005). GSK3β is antagonized by PP2A in regulating many Tau phosphorylation sites; this regulatory interaction between PP2A and GSK3β seems to be controlling the equilibrium of Tau phosphorylation levels (Meske *et al*., 2008). Sustained PI3K/Akt signaling is therefore the first candidate effector to transmit pathophysiological responses from Aβ to Tau.

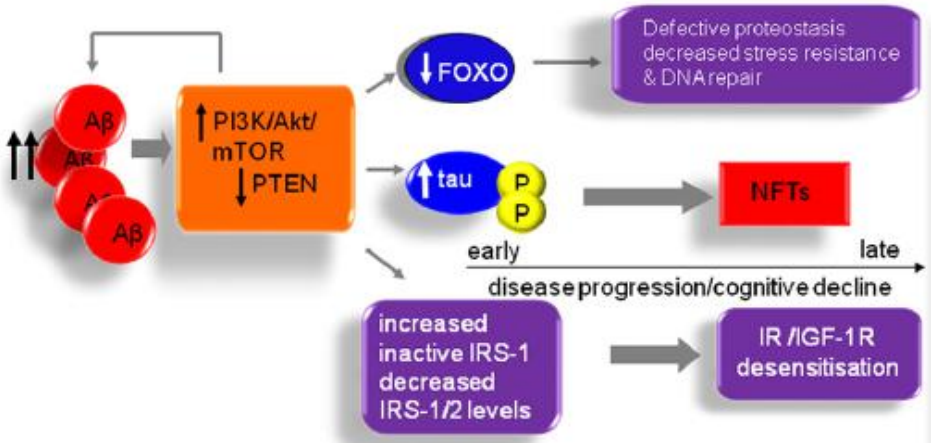


Figure 11. A model outlining the mechanisms by which progressive and eventually sustained activation of the PI3-kinase pathway in the aging brain causes insulin and IGF-1 resistance, pathological Aβ and tau disfunction, leading to the development and progression of synaptic and cognitive decline (Cora, 2013)

3.3. MAMMALIAN TARGET OF RAPAMYCIN SIGNALING IN AGING AND IN ALZHEIMER'S DISEASE

The mammalian target of rapamycin (mTOR) is a 289-kD serine/threonine multi-domain protein with a kinase domain and a FKBP12 binding domain regulating many physiological processes. Insulin, growth factors, AMPK, PI3K/Akt, and GSK-3 are mTOR upstream signal components. mTOR is involved in many diseases, such as aging, cancer and neurodegenerative diseases. Some evidences have shown that the activation of the

mTOR signaling is related to AD pathology and associated with the presence of its two hallmarks: Aβ and NFTs (Perluigi *et al*., 2015).

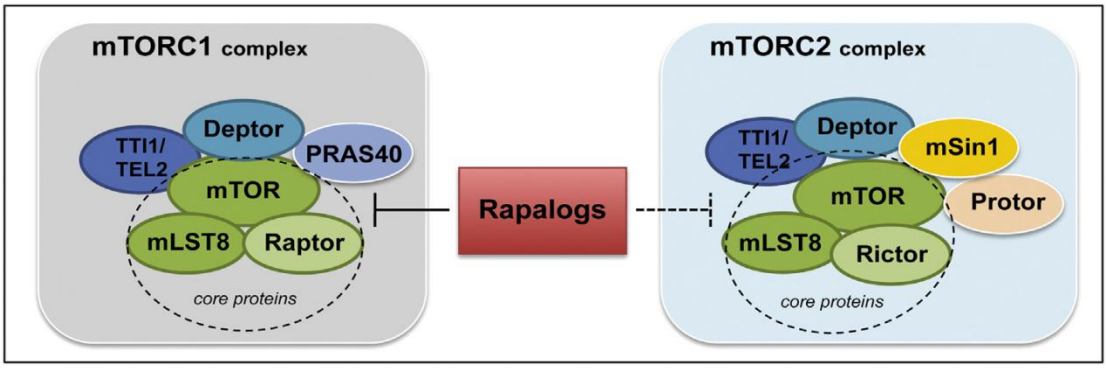


Figure 12. mTOR complexes. mTOR Complex 1 (mTORC1) is composed of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with sec-13 protein 8 (mLST8) (core proteins) and the non-core components proline-rich Akt substrate 40 kDa (PRAS40), the DEP domain containing mTOR-interacting protein (Deptor) and the TTI1/TEL2 complex. This complex functions as a nutrient/energy/redox sensor and controlling protein synthesis. mTOR Complex 2 (mTORC2) is composed of mTOR, rapamycin-insensitive companion of mTOR (Rictor), mLST8, and the non-core proteins mammalian stress-activated protein kinase interacting protein 1 (mSin1), Deptor, TTI1/TEL2 and protein observed with rictor 1 and 2 (Protor). The mTORC2 signaling pathway is less defined than the mTORC1 signaling pathway (Perluigi *et al*., 2015)

mTOR is known to be part of two different protein complexes: mTORC1 and mTORC2, which differ in some components, in upstream and downstream signaling, and in responsiveness to rapamycin treatment. Both mTOR complexes share the catalytic mTOR subunit, the mammalian lethal with sec-13 protein 8 (mLST8), the DEP domain containing mTOR interacting protein (Deptor), and the Tti1/Tel2 complex (Wullschleger *et al*., 2006). However, mTORC1 has two specific components, the regulatory-associated protein of mammalian target of rapamycin (Raptor) and the proline-rich Akt substrate 40 kDa (PRAS40). In contrast, mTORC2 is formed with the specific rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated map kinase interacting protein 1 (mSin1), and the protein observed with rictor 1 and 2 (Protor) (Laplante and Sabatini, 2012; Takei and Nawa, 2014). Raptor is the main characteristic component of mTORC1, that binds to the TOR signaling motif-containing substrates p70S6Ks, 4EBPs and STAT3, carrying them to the mTOR catalytic domain to phosphorylate them (Hoeffer and Klann, 2010). PRAS40 regulates the interaction between mTOR and Raptor and negatively

regulates mTOR signalling by blocking mTORC1 access to its substrates. While mTORC1 is activated, it is able to directly phosphorylate and reduce PRAS40 and DEPTOR function (Dunlop and Tee, 2009). mTORC2 plays a role in the organization of the actin cytoskeleton, and can phosphorylate Akt, which activates mTORC1 and inhibits FOXO nuclear recruitment (Crino, 2011). Tuberous sclerosis 1 and 2 (TSC1 and TSC2) are key upstream regulators of mTORC1. TSC1/2 functions to constitutively inhibit mTORC1 by inhibiting the mTORC1 activator Rheb (Ras-homolog expressed in brain) (Fig.12).

Some research studies have shown that the activation of mTORC1 causes the failure of Aβ removal from the brain due to an mTORC1-triggered dysfunction of autophagy that affects both the process of Aβ generation and weakens its clearance. Autophagy is an intracellular catabolic process in which cytosol fractions, organelles and macromolecules are degraded through the lysosome. This function includes the degradation of aggregates of neurodegenerative-associated proteins such as tau and polyglutamine-expanded proteins (Martinez-Vicente *et al*., 2007). Lysosomes are the essential catalytic component of the autophagic degradation system; lysosomes are single-membrane vesicles that contain a large variety of cellular hydrolases such as proteases, lipases, nucleotidases and glycosidases. These hydrolases reach their higher enzymatic activity at the acidic pH of the lysosomal lumen. The lysosomal proteolytic activity leads to the conversion of proteins into small di- and tri-peptides and free aminoacids that are released into the cytosol through permeases of the lysosomal membrane and either used to obtain energy or recycled to synthesize the novo proteins (Mizushima *et al*., 2011). Autophagy is modulated by many signaling pathways such as AMPK, Sirtuin 1 (SIRT1) and mTORC1.

A chronic deterioration of the autophagy pathway is an important factor in the failure of Aβ removal in the AD brain. Normally, Aβ is mostly degraded by the autophagy/lysosome pathway that plays a role in the protein quality control and in the removal of aberrant forms of protein. The immaturity of autophagolysosomes results in a huge accumulation of autophagic vacuoles (AVs), which become sites for Aβ generation (Boland *et al*. 2008). A failure of the Aβ clearance generating from the accumulation of AVs that were colocalized within Aβ deposits causes the Aβ accumulation and the formation of amyloid plaque in AD. Inducing or inhibiting autophagy by regulating mTORC1 signaling leads to the

corresponding changes in AVs proliferation and Aβ generation (Yu *et al*., 2005). Some study has demonstrated that chronic rapamycin intervention retards the progression of AD-like deficits and decreases Aβ levels by autophagy enhancement in AD model mice (Pierce *et al*., 2013).

mTORC1 regulates Aβ generation through several signaling pathways, such as PI3K/Akt, GSK3, insulin/IGF-1 and S6K. It has been shown increased Akt activation, mTOR phosphorylation and tau hyperphosphorylation in AD cortical neurons, with significantly reduced and altered PTEN phosphorylation (O’ Neill, 2013). The sustained activation of neuronal PI3K/Akt/mTORC1 signaling in AD brain was reported to cause insulin receptor substrate 1 (IRS1) inhibition, disabling normal activation of PI3K/Akt by insulin (Gupta and Dey, 2012; Neill *et al*., 2012). The interplay between the PI3K/Akt/mTORC1 pathway and the autophagic process in neurodegeneration is complex; there is a direct association of hyperactive PI3K/Akt/mTORC1 pathway with the inhibitory effect on the autophagic process in AD. Recently some studies suggest that the PI3K/Akt/mTORC1 pathway may be disrupted and downregulated in the earlier stages of the AD pathology, but when the disease and the cellular damage progress, the PI3K/Akt/mTORC1 pathway may be hyperactivated to counteract the damage, to promote survival, or due to a more generalized dysregulation of cellular function that ultimately could impair autophagy. Arctigenin (extracted from *Fructus arctii,* can both inhibit Aβ production by suppressing BACE) inhibits phosphorylation of ULK1 and S6K by mTORC1, activates autophagy, promotes Aβ clearance, and ameliorates memory impairment in the APP/PS1 transgenic mice (Zhu *et al*., 2013). S6K, the most well-known downstream components of mTORC1, is significantly hyperactivated in AD model mice, and can phosphorylate tau protein and promote Aβ generation.

4. UNFOLDED PROTEIN RESPONSE IN ALZHEIMER'S DISEASE

The unfolded protein response (UPR) is a protective cellular response induced during periods of endoplasmic reticulum (ER) stress, which reduces unfolded protein load and return protein-folding homeostasis. The ER is the largest membrane-bound organelle in the cell, and it possesses a diverse range of signaling and homeostatic functions. As well as the synthesis, folding/maturation, and trafficking of all secretory/transmembrane proteins, it also synthesizes lipids, plays a critical role in Ca^{2+} homeostasis, and is essential for compartmentalization of the nucleus and the structure of chromatin. The different secreted and transmembrane proteins are managed by the ER as unfolded proteins to be properly gathered or to be targeted for degradation (Ellgaard *et al*., 2003). During ER stress, proteins are waiting to be folded and exported, which leads a backlog that is stressful to the cell. Binding immunoglobulin protein (BiP) binds to exposed hydrophobic domains of unfolded proteins that can be detected in the ER (Gething, 1999). The UPR is an adaptive mechanism coping with protein folding alterations in the ER. Under moderate misfolded protein accumulation, the UPR transduces information from the ER to the nucleus and cytosol and thereby inhibits protein translation, expands the ER membrane, recruits ER chaperones to aid in the correct folding of misfolded proteins and promotes protein degradation in order to reduce the load of unfolded or misfolded protein (Hetz, 2012). The UPR has three arms, the transcription factor 6 (ATF6), the protein kinase RNA (PKR)-like ER kinase (PERK), and the inositol-requiring enzyme 1 (IRE1) (Ron *et al*., 2007), which promote (1) downregulation of protein translation, (2) enhanced expression of ER chaperone proteins that promote protein refolding, and (3) activation of proteases involved in the degradation of misfolded proteins. GRP78/BiP is proposed to be the master initiator of the UPR. BiP is an ER molecular chaperone that plays vital roles in preventing aggregation while also regulating signaling within the UPR. BiP keeps the UPR proteins in an inactive state until it binds hydrophobic residues in the unfolded regions of proteins, which result in the UPR activation.

PERK dissociated from BiP homoligomerizes and autophosphorylates, PERK-P then phosphorylate and inhibits eIF2α, which is a vital component of a ternary complex involved in the initiation of translation. This process attenuates protein translation and increases the load of mRNA onto the ribosomes (Zhang *et al*., 2002).

IRE1 is an ER-transmembrane protein that normally exists in its monomeric form. Under ER stress conditions, IRE1 monomers homoligomerize and trans-autophosphorylate to become active endoribonuclease (Harding *et al*., 2000). The cleaced of a particular mRNA by IRE1 leads to a frame shift, creating a stable and potent transcription factor that induces the expression of genes involved in protein folding and translocation into ER. Apart from that, mammalian IRE1 activates the Jun N-terminal kinase signaling pathway and interacts with the cell death machinery.

ATF6 is an ER-transmembrane protein that is concomitantly activated through translocation from the ER to the Golgi and proteolysis by S1P (site 1 protease) When active, it translocates to the nucleus to lead the expression of XBP1, BIP and CHOP (Cox *et al*., 1993). eIF-2α regulation of protein synthesis plays an important role in relation to memory impairment. Some studies have shown that both GCN2 and PERK can phosphorylate eIF2α leading to the inhibition of ATF4 mRNA translation (Halliday and Mallucci, 2015) (Fig.13).

Both the expression of the ER chaperone, BiP, and the phosphorylation of PERK are significantly increased in the temporal cortex and hippocampus of AD individuals compared to controls (Hoozemans *et al*., 2005). A growing number of studies suggest that Aβ interferes with ER functioning leading to ER stress in the early stages of AD progression. For example, exogenous Aβ induces ER stress in primary neuronal cultures and activates mitochondria and ER-mediated cell death pathways (Hoozemans *et al*., 2005; Costa-Mattioli *et al*., 2010). High eIF2α levels correlate with elevated BACE1 levels in AD patient brains and with the upregulation of PERK, IRE1 and eIF2α in neurons of hippocampus in AD patients. PERK inhibition may therefore be a potential therapeutic target to prevent eIF2α phosphorylation and improve the synaptic plasticity and spatial memory deficits arising from reduced protein synthesis in AD. As a proof of concept, eIF2α kinases deletion in AD transgenic mice prevents deficits in protein synthesis, protects AD mice from AD-associated synaptic plasticity and spatial memory impairment. Also, decreased formations of AD-related senile plaques were reported in these mice. Interestingly, PERK-dependent eIF2α phosphorylation semms to be dominant in the AD brain, since only PERK deletion alone decreased the level of phospho-eIF2α, while

thedeletion of GCN2 kinase had no significant impact on the level of phosphorylated eIF2α in the AD brain (Ma *et al*., 2013).

CHOP activation was detected in neuronal cells treated with Aβ, increased levels of cleaved caspase-12, cleaved caspase-3, cleaved caspase-4, and CHOP were detected in the temporal cortex of AD patients (Lee *et al*., 2010a). In PC12 cells and knock-in mice expressing a mutant form of PS1, the protein levels of CHOP were up-regulated. In the hippocampus of the 3xTg-AD mice, increased levels of CHOP precede the increase in BACE and Aβ levels. Aβ-activated ER stress was correlated with the induction of Tau phosphorylation (Resende *et al*., 2008). However, UPR activation can induce Tau phosphorylation that may not activate ER stress (Sakagami *et al*., 2013). Change of the UPR can also be protective in AD cellular and animal models. PERK-eIF2α pathway may play a role in cell survival rather than apoptosis during ER stress. Some AD pathological events, such as neurofibrillary tangles, neuroinflammation, and excitotoxicity, were also linked to the occurrence of ER stress (Cornejo and Hetz, 2013). It has also been proposed that ER stress may interfere with the normal trafficking of APP through the normal secretory pathway, leading to the production of Aβ and subsequent toxicity (Plácido *et al*., 2014). The modulation of the ER-UPR could therefore be a suitable therapeutic strategy to avoid neuronal degeneration in AD; indeed, manipulating ER-associated quality control mechanisms through the stimulation of protective and adaptive responses or the inhibition of the apoptotic pathway associated with the UPR can be beneficial to treat or prevent AD. Ca^{2+} is an important player in neurodegeneration and many chaperones do not work properly when Ca^{2+} homeostasis is impaired. Targeting ER Ca^{2+} may allow the restoration of ER homeostasis and increase protein folding. The Ryanodine Receptor (RyR) calcium channel antagonist dantrolene, licensed for the treatment of spasticity, inhibits ER Ca^{2+} release and the activation of PERK, eIF2α and CHOP.

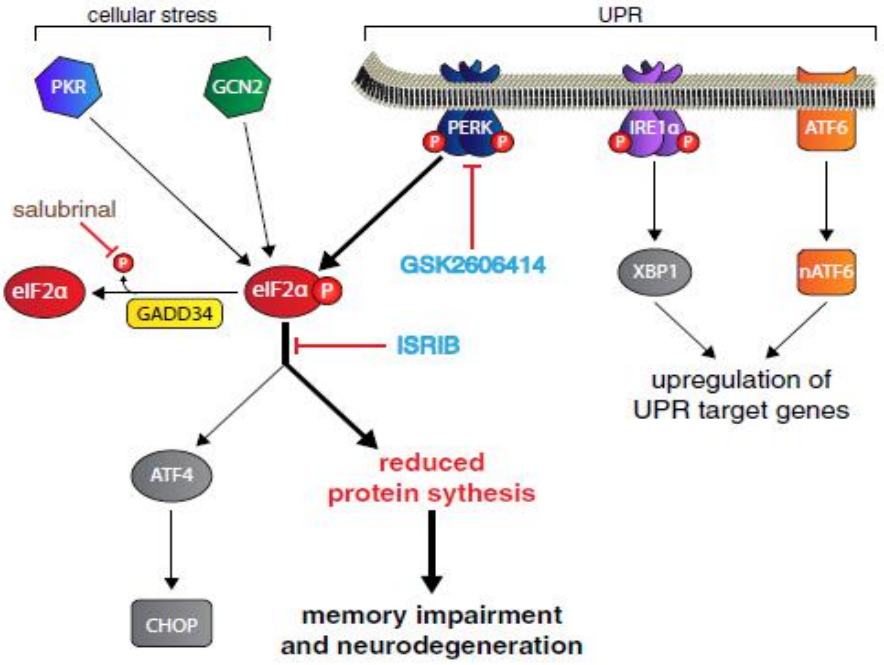


Figure 13. UPR and ISR signalling through eIF2α-P. Unfolded proteins induce the UPR, which transduces through PERK, IRE1 and ATF6. PERK phosphorylates eIF2α, leading to the rapid attenuation of protein synthesis. Chronic reduction in protein synthesis can lead to memory impairment and neurodegeneration. eIF2α-P also leads to the selective translation of some proteins such as ATF4 and the pro-apoptotic CHOP. Other kinases activated by cellular stress, such as PKR and GCN2, can also phosphorylate eIF2α. GADD34 dephosphorylates eIF2α-P, restoring translation to normal levels. The PERK inhibitor GSK2606414 and the compound ISRIB avoiding eIF2α inhibitory actions prevent neurodegeneration and improve memory, respectively. Salubrinal inhibits eIF2α-P dephosphorylation, exacerbating neurodegeneration in some, but not all models (Halliday and Mallucci, 2015).

5. PDK1/AKT MICE GENETIC MODELS

5.1. PDK1$^{K465E/K465E}$ PH-DOMAIN KNOCK-IN MICE

Crystal structure resolution of the PDK1 PH domain in complex with PtdIns(3,4,5)P_3 showed that this domain possesses a ligand-binding site that enables PDK1 to interact with phosphoinositides with high affinity. Among the different positively-charged lateral chains conforming this binding pocket, Lysine at position 465 establishes fundamental interactions with the phosphate groups at position D3 and D4 within the PtdIns(3,4,5)P_3 inositol ring, and the targeted mutagenesis of this Lysine 465 to Glutamic acid abolished the PDK1-phosphoinositide interaction by disrupting the conformation of this lipid-binding pocket (Komander *et al*., 2004). Interaction of both PDK1 and Akt with PtdIns(3,4,5)P_3 was meant to play fundamental roles in Akt activation. First, some studies show that it promotes the translocation of Akt to the plasma membrane (Andjelkovic *et al*., 1997). Second, the Akt PH domain bound to phosphoinositides undergoes a conformational change that lead to the switch of the kinase from the inactive to the active conformer, which then is phosphorylated and activated by membrane-localized PDK1 (Calleja *et al*., 2007; Milburn *et al*., 2003). By contrast, the role that the PDK1-PtdIns(3,4,5)P_3 interaction plays in this pathway was at the time not been well defined. In order to analyze the importance of PDK1-PtdIns(3,4,5)P_3 interaction, the homozygous PDK1$^{K465E/K465E}$ knock-in mice were generated that express the PDK1 K465E mutant protein abrogating PDK1-phosphoinositide binding which result in an specific reduction of Akt activation. Consequently, the mutant mice were shown to be smaller than control littermates, hyperinsulinemic and insulin resistant but otherwise viable and fertile (Bayascas *et al*., 2008). Notably, this study constituted the first genetic evidence in an animal model to highlight the fact that the binding of PDK1 to phosphoinositides is required for optimal activation of Akt, since the homozygous PDK1$^{K465E/K465E}$ mutant mice express a mutant form of PDK1 just incapable of phosphoinositide binding but with intact catalytic activityIndeed, the PDK1 protein is expressed at normal levels in the PDK1$^{K465E/K465E}$ mice and the catalytic activity of purified PDK1 is unaffected by the K465E mutation, as judged by the ability of the mutant PDK1 protein to phosphorylate the T308tide peptide (Bayascas

et al., 2008). So the PDK1$^{K465E/K465E}$ knock-in mice is a genuine model in which activation of Akt is only moderately reduced but not ablated, that has been proved instrumental in dissecting the PDK1 signaling (Bayascas *et al*., 2008).

Stereological analysis of embryonic brain sections showed that the PDK1$^{K465E/K465E}$ mice displayed reduced brain size due to a reduction in neuronal cell size rather than cell number, since the number of cortical and hippocampal neuronal populations between PDK1$^{+/+}$ and PDK1$^{K465E/K465E}$ was not significantly different, whereas the volume of the mutant neuronal soma was approximately 80% of the volume of the wild type neuronal soma (Zurashvili *et al*., 2013).

Stimulation of cortical neurons with BDNF induced a robust phosphorylation of the TrkB receptor followed by the phosphorylation of Akt at Thr308 in the PDK1$^{+/+}$ cells, which was blunted in the PDK1$^{K465E/K465E}$ neurons, whereas Akt phosphorylation at the mTORC2 site Ser473 proceeded normally in both type of cells. The moderate reduction of Akt activation was not rate-limiting for the phosphorylation of those Akt substrates governing neuronal survival and apoptosis such as FOXO and GSK3. In contrast, the moderate reduction of Akt activity in the PDK1$^{K465E/K465E}$ neurons markedly reduced phosphorylation of the PRAS40 and TSC2 substrates, leading to decrease mTOR1/S6K activation and also reduced BRSK protein synthesis. The PDK1 mutant neurons in culture exhibited reduced neurite outgrowth, delayed polarization and deficient axonogenesis. To establish the possible causal relationship between the Akt pathway defects and axon formation, the impact of specific pharmacological treatments with Akt and mTOR1 inhibitors on neuronal differentiation was assessed, which provided strong evidence that differentiation defects were due to reduced Akt activity and inefficient activation of the mTOR1 signalling. Moreover, the overexpression of BRSK isoforms rescued the axonogenesis defects of the PDK1$^{K465E/K465E}$ hippocampal cells (Zurashvili *et al*., 2013). The PDK1$^{K465E/K465E}$ mice exhibited neither an overt phenotype nor growth abnormalities in the architecture of the central nervous system, which showed normal cortical layering and connectivity (Zurashvili *et al*., 2013).

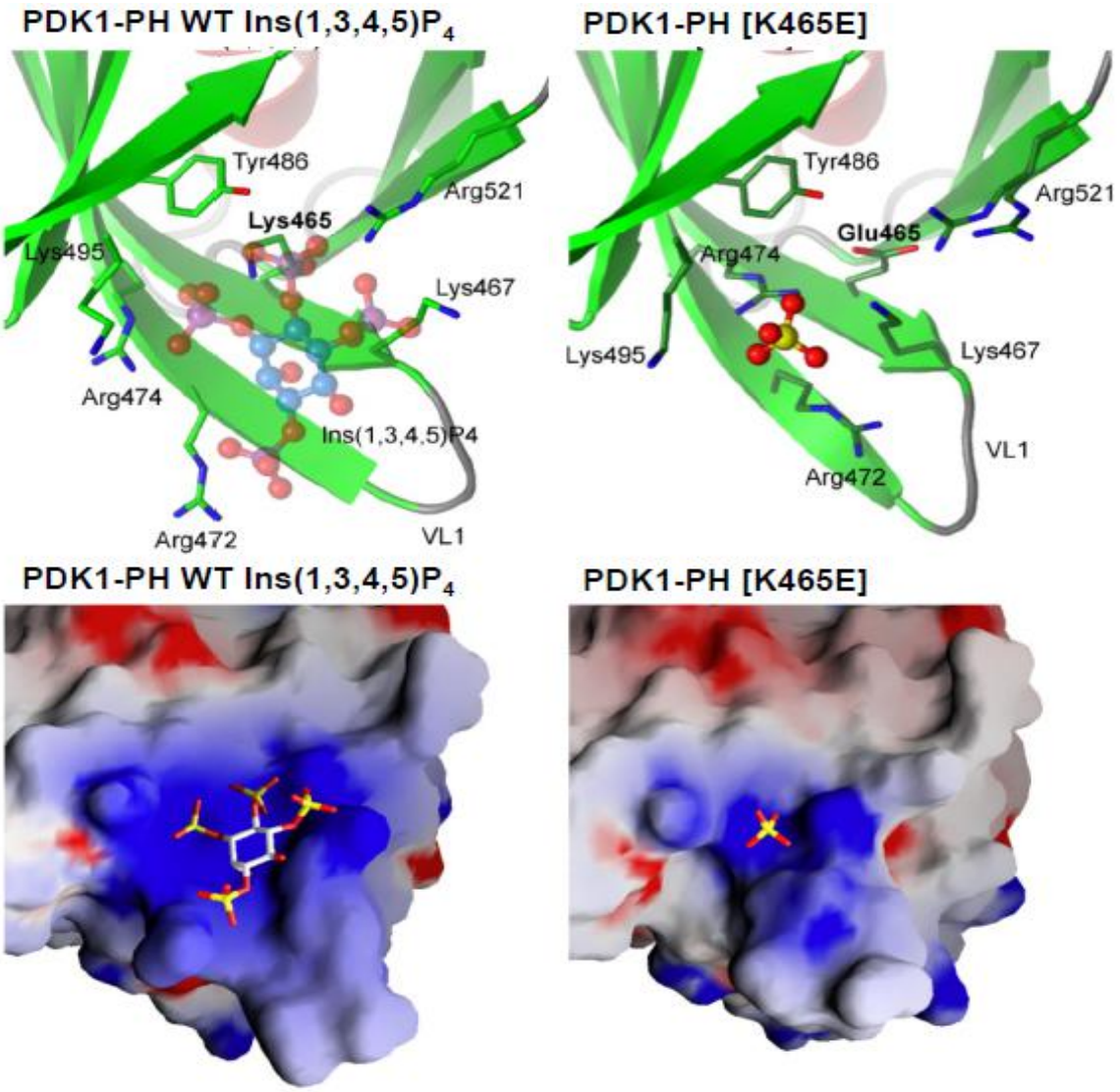


Figure 14. Crystal structure of the isolated PDK1[K465E] PH domain. (A) Comparison of the phosphoinositide-binding site of wild-type PDK1 (left) with that of the PDK1 K465E mutant abrogating phosphoinositide binding (right). A stick representation of the interactions of Ins(1,3,4,5)P_4 (blue, inositol ring; purple/red, phosphate groups) with protein residues (green) in the phosphoinositide binding site of the PDK1-PH domain is shown. Lys465 is the central residue in the back of the pocket, and its mutation to Glu does not affect the overall structure of the PH domain but leads to reorganization of the phosphoinositide-binding site by attracting surrounding positively charged residues. (B) An electrostatic surface representation of the phosphoinositide-binding site. Mutation of Lys465 to Glu alters the shape of the binding pocket (Bayascas *et al*., 2008).

5.2. PDK1 PIF POCKET KNOCK-IN MICE GENETIC MODELS

PDK1 possesses a hydrophobic pocket within the small lobe of its catalytic domain, called PIF (PDK1 Interating Frangment)-pocket. Mutation of the residue Leu155 to Glu within this pocket abolished the interaction of PDK1 with its substrates. As a consequence,

phosphorylation of S6K and SGK at their T-loops was abolished, while the phosphorylation of Akt was totally preserved since its regulation is not PIF-pocket but PH domain dependent (Biondi *et al*., 2000). The PDK1$^{L155E/L155E}$ mice failed to develop to term, as this mutant mice died and reabsorbed at E12,5. PDK1$^{L155E/L155E}$ embryos were retarded compared to control littermates of the same age, and displayed multiple abnormalities, including reduction in forebrain size and body axis defects. So a new strategy of transgenesis named tissue-specific conditional knock-in mice was engineered in which the expression of the mutant form of PDK1 was targeted to tissues expressing the Cre-recombinase under the control of a tissue-specific promoter. The muscle specific PDK1$^{L155E/L155E}$ conditional knock-in mice were viable and of normal size and displayed no abnormality in glucose homeostasis (Bayascas *et al*., 2008).

Recently, PDK1$^{L155E/L155E}$ neuronal-specific conditional knock-in mice have been generated, which were observed at birth at a three-fold reduced Mendelian frequency. The PDK1 L155E mutation abolished activation of S6K, RSK, SGK and PKC in neuronal cells and tissues; by contrast, Akt was normally activated. This observation demonstrate that Akt activation by PDK1 rely mostly on the phosphoinositide-mediated co-localization of both kinases at PtdIns(3,4,5)P_3-rich cellular membranes rather than docking site interaction. The neuronal polarization and axonal elongation in the PDK1$^{L155E/L155E}$ hippocampal neurons were inhibited, whereas neuronal survival was not affected. This caused altered cortical layering and reduced circuitry, leading to cognitive deficits and exacerbated disruptive behaviour combined with diminished motivation (Cordon-Barris *et al*., 2016).

AIMS

GENERAL AIM: Is the reduced activation of Akt signaling protective against AD-related pathology?

AIM 1.-To analyze the Akt-dependent signaling pathway in the PDK1$^{K465E/K465E}$ mutant mice brain.

AIM 2.-To analyze the PDK1 signaling pathway in the Alzheimer's disease model mice brain.

AIM 3.-To study whether the PDK1 K465E mutation can affect TACE subcellular localization and activity.

AIM 4.-To investigate the influence of the PDK1 K465E mutation on the unfolding protein response.

AIM 5.-To investigate the potential relationship between Akt activity and inflammatory factors.

METHODS

1. MICE

The PDK1$^{K465E/K465E}$ knock-in mice were provided from the University of Dundee, Scotland, and maintained in the Animal House from Universitat de Lleida and the genotyping was described previously (Bayascas *et al*., 2008). The APP/PS1 transgenic mice and the 3xTg-AD mice were provided respectively by Marian Baltrons and Lydia Giménez lab. All animal procedures were performed in accordance with 2010/63/UE regarding the care and use of animals for experimental procedures. The study complies with the ARRIVE guidelines developed by the NC3Rs (Kilkenny *et al*., 2010).

2. PRIMARY CELL CULTURES

Primary cortical neuronal culture came from PDK1$^{+/+}$ and PDK1$^{K465E/K465E}$ littermate mice at embryonic day 15.5. Cerebral cortical tissues were dissected and the cells were enzymatically dissociated for 10 min at 37ºC in Solution 2, which consist on Krebs Ringer Buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 14.3 mM glucose), also containing 0.3% BSA, 0.03% $MgSO_4$ and 0.25 mg/ml of trypsin. The enzymatic digestion was stopped by adding solution 4 (Table 1). The disaggregated tissue was then pelleted by centrifugation for 0.5 min at 1500 rpm, the supernatant was discarded and the pellets were mechanically disgregated in Solution 3 (Krebs Ringer Buffer, 0.3% BSA, 0.03% $MgSO_4$, 0.08 mg/ml DNase and 0.52 mg/ml trypsin inhibitor) by gentle pipetting using fire-polished Pasteur pipette to leading a single cell suspension. Collected the cells and added to Solution 5 (Krebs Ringer Buffer, 0.3% BSA, 0.03% $MgSO_4$ and 0.0014% $CaCl_2$). Cells were then centrifuged for 5 min at 1000 rpm and resuspended, the Scepter^{TM} 2.0 Handheld Automated Cell Counter (Millipore) counted cells number and finally diluted in DMEM (Dulbecco' s Modified Eagle Medium, GIBCO # 31966-021) complemented with 30 mM glucose, 2 mM L-Glutamine, 250 U/ml penicillin-0.25 mg/ml streptomycin and 10% FBS (Fetal Bovine Serum, GIBCO # 10270). For Western blot analysis and cell viability studies, the cortical cells were plated onto poly-D-Lysine (50 μg/ml) coated 6-well or 24-well plates at a density of 2.0 x 10^5 cells/ml, whereas for immunocytochemistry studies, the cells were plated onto poly-D-Lysine (150 μg/ml)

SOLUTION	REAGENTS	QUANTITY
PBS (1X)	H_2O	430 ml
	PBS 10X	50 ml
	Glucose 30%	10 ml
	Penicillin 10000U/ml- Streptomycin 10mg/ml	12.5 ml
Krebs-Ringer Buffer (KRB) 10X	NaCl	70.70 g
	KCl	3.60 g
	KH_2PO_4	1.66 g
	$NaHCO_3$	21.40 g
	Glucose	25.70 g
	H_2O	till 1000 ml
Magnesium Stock 3.8%	$MgSO_4 \cdot 7H_2O$	19 g
	H_2O	to 10 ml
Calcium Stock 1.2%	$CaCl_2 \cdot 2H_2O$	0.12 g
	H_2O	to 10 ml

CELL CULTURE SOLUTIONS, FRESHLY PREPARED

Solution 1	KRB 1X	50 ml
	BSA Fraction V	0.15 g
	Magnesium Stock 3.8%	0.4 ml
Solution 2	Solution 1	10 ml
	Trypsin	2.5 mg
Solution 3	Solution 1	10 ml
	DNase	0.8 mg
	Trypsin inhibitor	5.2 mg
	Magnesium Stock 3.8%	0.1 ml
Solution 4	Solution 1	8.4 ml
	Solution 3	1.6 ml
Solution 5	Solution 1	5 ml
	Magnesium Stock 3.8%	40 µl
	Calcium Stock 1.2%	6 µl

TABLE 1. Solutions and media used for primary culture preparation and maintenance. A list of recipes for the preparation of solutions and mediums used in the primary cultures is provided.

coated 12-mm diameter glass cover slips in 24-well plates at a density of 7.5 x 10^4 cells/ml. It took two hours for cells to attach to the plate and then the medium changed with Neurobasal™ medium (GIBCO # 21103) including 30 mM glucose, 2 mM L-Glutamine, 250 U/ml penicillin-0.25 mg/ml streptomycin and 2% B27 Supplement (GIBCO # 17504). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2 in normoxia conditions.

3. DRUG TREATMENT

Experiments were always begun at day *in vitro* (DIV6). The inhibitors Akti-1/2 (Calbiochem # 124018), GSK2334470 (SML0217), TNFα and tunicamycin (all from Sigma) were dissolved in dimethylsulfoxide (DMSO). These drugs and soluble Aβ were added to cultures as indicated.

4. PREPARATION OF AMYLOID-β OLIGOMERS

Solution oligomers were prepared as shown previously (Manterola *et al*., 2013). 1 mg Aβ (BACHEM #1065068) was resuspended in 20 µl dimethylsulfoxide, after vortex 20 s, bathed 5 min at room temperature, 424 µl PBS was added to the peptide solution to a final concentration of 500 µM. Again vortex and bathed, the samples were incubated at 4°C for 24 hour, and then centrifuged at 14000 g 4°C for 10 min to remove insoluble pellets. The supernatant containing soluble $A\beta_{1-42}$ was collected and stored at - 80°C.

5. MEMBRANE FRACTIONS

Brain membrane fraction procedure was done with precooled reagents at 4°C. Brain regions were dissected into 10 volumes of cold homogenization buffer (50 mM Tris-HCl pH 7.4, 0.27 M sucrose, 1mM Na3VO4, 1mM EDTA, 1mM EGTA, 50 mM NaF) with 50 passes using glass potter homogenizer. Homogenates were centrifuged at 1000 g for 15 min at 4°C to remove cellular debris. The resultant supernatant was centrifuged at 20 000x g for 1 hour to yield cytosol; then resuspended pellet in the same volume of homogenization buffer again centrifuged at 20 000x g 1 hour to wash the pellet. Discard

supernatant and the membrane pellet was resuspended containing 1% SDS homogenization buffer.

6. WESTERN BLOT ANALYSIS

6.1. GENERATION OF PROTEIN EXTRACTS FROM CELLS AND TISSUES

Primary cortical neurons were cultured for six days, and then stimulated with the indicated agonists as described in the drugs process legends. The neurons were quickly lysed in ice cold Lysis Buffer (50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium beta glicerophosphate, 0.27 M sucrose, 1% (by mass) Triton-X 100, 0.1% (v/v) 2-mercaptoethanol, 1:100 Protease Inhibitor Cocktail) using the scraper for cell harvesting.

LYSIS BUFFER	**REFEERENCE**	**M.W.**	**grams/L**	**VOLUME FROM STOCK**
50 mM Tris-HCl, pH 7.5	Sigma T1503	121.14		50 mM Tris-HCl, 1M pH7.5
1 mM EGTA	Sigma E0396	380.35		4 ml EGTA 250 mM pH 7.5
1 mM EDTA	Sigma 443885	372.24		2 ml EDTA 500 mM pH 8.0
1 mM -orthovanadate	Sigma S6508	183.91		10 ml Na-OTV 100 mM
50 mM Na-fluoride	Sigma 7920	41.99	2.100	
5 mM Na-pyrophosphate	Sigma 221369	446.06	2.230	
10 mM Na-B-glycerol-P	Sigma G6501	216.11	2.161	
0.27 M sucrose	Sigma S9378	342.30	92.241	
1% Triton-X 100	Sigma X100			0.1 ml Triton-X 100 buffer
0.1% 2-mercaptoethanol	Sigma M7154			0.01 ml 2-mercapto buffer
Protease Inhibitor	Sigma P8340			0.1 ml Prot.Inhibitor buffer

STOCK	**M.W.**	**PREPARATION**
Tris-HCl, 1M, pH 7.5	121.14	60.7 g TRIS in 500 ml water, pH to 7.5 with HCl
EGTA	380.35	19.02 g EGTA in 200 ml water, pH to 7.5 with HCl
EDTA	372.24	93.06 g EDTA in 500 ml water, pH to 8.0 with HCl
Na-OTV	183.91	3.68 g Na-OTV in 200 ml water, pH to 7.0 with HCl

TABLE 2. Lysis Buffer. The composition and procedure to prepare lysis buffer are provided. Note:

Na-OTV should be microwaves to achieve yellow colour.

The lysates should be kept on ice about 30 min, and then centrifuged at 4°C for 15 min at 12500 rpm. The supernatants were collected into 1.5 ml eppendorf tubes, frozen rapidly and finally kept at -20 °C.

Tissue extracts were operated by homogenizing the tissue in a 10-fold mass excess of ice-cold Lysis Buffer using a pellet pestle for microcentrifuge tube (Sigma-Aldrich #Z359955-1EA), which can use for small size tissue, like cortex, hippocampus, and other brain parts of adult and old mouse brains, or Kinematica Polytron Homogenizer (PCU GmnH) for big parts, like whole brain. Firstly, the tissue samples were transferred into a tube on ice containing ice-cold Lysis Buffer. The homogenates were left on ice for 30 min, and then centrifuged at 4°C for 15 min at 12500 rpm; the supernatants were collected and kept at -20°C. Protein concentrations were determined by the Bradford method (Coomassie protein assay reagent, Pierce #23200) using bovine serum albumin (BSA) as a standard.

6.2. PDK1 PULL DOWN AND PROTEIN IMMUNOPRECIPITATION

For PDK1 pull down, 0.5 µg of bio peptide (Biotin-REPRILSEEEQEMFRDFAYIADWC) was conjugated with 10 ul of beads (Streptavidin Sepharose, GE Healthcare, #71-5004-70). For protein immunoprecipitation, 1 µg of the corresponding antibody was conjugated with 10 µl of beads (Protein G Sepharose, GE Healthcare, #17-0618-01). Firstly, the sepharose beads and peptide or antibody were incubated at 4°C for 1 hour on a tube rotator mixer. Beads were washed three times with washing buffer (Lysis Buffer plus 0.15 M NaCl). Secondly, the beads conjugated with the corresponding peptide or antibody were added to lysates containing 300 µg of protein and this immune complexes were allowed to form overnight at 4°C on a tube rotator mixer. The second day beads were collected by centrifugation at 4°C for 1 min, the supernatants were collected, and the beads were washed three times with wash buffer. Finally, the immune complexes were added Laemmli-SDS buffer and boiling 5 min. The assay was measured by Western blot with SDS-PAGE. The supernatants were loaded with immunoprecipitated served as control.

6.3. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

6.3.1.SDS-PAGE gel preparation

Polyacrylamide gels were prepared in BIO-RAD mini PAGE (polyacrylamide gel electrophoresis) system (10 well, thickness 1mm) using solutions list in table 3

SOLUTIONS (ml)	**RUNNING GEL**						**STACKING GEL**	
PERCENTAGE (%)	**5%**	**6%**	**7.5%**	**10%**	**12%**	**15%**	**3.6%**	**5%**
dH_2O	11.6	10.4	10	8.3	7	5	5.8	3.8
30% acryl 0.8 bis	3.4	4	5	6.7	8	10	1.3	0.85
1.5M Tris pH 8.8	5	5	5	5	5	5	-	-
0.5M Tris pH 6.8	-	-	-	-	-	-	2.5	0.325
20% SDS	0.25	0.25	0.25	0.25	0.25	0.25	0.05	0.025
10% APS	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.05
TEMED	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.005

TABLE 3 .Table of solutions used in the preparation of SDS-PAGE running and stacking BIO-RAD gels. SDS: sodium dodecyl sulphate; TEMED: N, N, N', N'-tetramethylethylenediamine; APS: ammonium persulfate.

6.3.2. Running a SDS-PAGE gel

5X Laemmli sample buffer was added to normalized lysate samples, then which were boiled for 10 min at 95°C, loaded on the SDS-PAGE gel alongside acting as protein markers (250-10kDa Bio-Rad #161-0373). Gels were run on 1X Tris-Glycine Buffer 0.1% (w/v) SDS at 150 V for 60-100 min depending on the nature of the gel and the marker protein.

LAEMMLI-SDS SAMPLE BUFFER

CONCENTRATION	5X	1X	For 100 ml of 5X
Tris-HCl pH 6.8	125 mM	25 mM	25 ml Tris-HCl 0.5 M pH 6.8
Glycerol	50 %	10 %	50 ml
SDS	10 %	2 %	10 g
Bromophenol Blue	0.01%	0.0025%	12.5 mg
β-Mercaptoethanol		1 %	Freshly added

TRIS-GLYCINE SDS ELECTROPHORESIS BUFFER (10X)

CONCENTRATION	1X	10X	For 1 litter of 10X
Tris base	25 mM	250 mM	30.3 g
Glycine	192 mM	1.92 M	144.1 g
SDS	0.1 % (w/v)	1 % (w/v)	10 g

TRIS-GLYCINE TRANSFER BUFFER (10X)

CONCENTRATION	1X	10X	For 1 litter of 10X
Tris base	25 mM	250 mM	30.3 g
Glycine	192 mM	1.92 M	144.1 g

TBS BUFFER (10X)

CONCENTRATION	1X	10X	For 1 litter of 10X
Tris base	25 mM	250 mM	30.3 g
NaCl	150 mM	1.5 M	87.6 g

TABLE 4. Western Blot Buffers. Recipes of western blot buffers are provided as well as the concentration of their components

6.4. TRANSFER OF PROTEINS TO NITROCELLULOSE MEMBRANES

Finished SDS-PAGE, proteins on gels were transferred to nitrocellulose membrane (Whatman GmbH) using the wet transfer system (Bio-Rad). The paper/gel/membrance like "sandwich", from the bottom to the top orderly: three 3MM papers (WhatmanTM #3030917) soaked in transfer buffer, membrane, gel and another three 3MM papers. Air bubbles were carefully removed after every layer of interface added. The transfer was performed at 100 V for 90 min.

After the transfer, membranes were blocked in 10% skimmed milk prepared by 1X TBST for 1 hour at room temperature. Primary antibodies were diluted in 5% milk (1X TBST) or in 0.5% BSA (1X TBST) as described in Table 4 and incubated overnight at 4°C.

Concentration of dilution for primary antibodies was according to manufacturer´ instructions. The second day, the membrane was washed three times for 5 min in 1X TBST and then incubated with corresponding secondary antibodies coupled to horseradish peroxidase diluted 1:5000 in 5% milk/1X TBST. Detection of proteins was performed by the enhanced chemiluminescence (ECL) system (table 5). Membranes were treated with the ECL reagent mixture for 1min before exposed to Super RX Fujifilm (MTB) in the dark room. The film was developed to produce the images shown in the figures.

SOLUTION	REAGENTS	FOR 50 ml
ECL1	Tris-HCl 1M pH 8.5 Luminol 0.5M in DMSO P-Coumaric Acid 79.2 mM in DMSO	5 ml 0.25 ml 0.25 ml
ECL2	Tris-HCl 1M pH 8.5 Hydrogen Peroxide 8.8M	5 ml 32 µl

TABLE 5 . Recipe of enhanced chemiluminescence (ECL) reagents.

6.5. Antibodies

Dario Alessi from the University of Dundee kindly provided the following antibodies, which were raised in sheep and affinity purified on the appropriate antigen. The Akt total antibody was raised against the sequence RPHFPQFSYSASGTA, corresponding to residues 466 to 480 of rat Akt; the total TSC2 antibody was raised against a sequence encompassing residues 1719 to 1814 of mouse TSC2; the total PRAS40 antibody was raised against the peptide DLPRPRLNTSDFQKLKRKY, corresponding to residues 238 to 256 of human PRAS40; and the total NDRG1antibody was raised against the recombinant human NDRG1 protein expressed in *Escherichia coli*. The rest of the primary and secondary antibodies used for western blot and immunofluorescence analysis were of commercial origin and are listed below (Table 6)

PRIMARY ANTIBODY	HOST	SUPPLIER	REFERENCE	DILUTION
Phospho-Akt Thr308	Rabbit	CST	# 4056	1:1000
Phospho-Akt Ser473	Rabbit	CST	# 4060	1:1000
Phospho-S6K Thr389	Rabbit	CST	# 9205	1:1000
Phospho-S6 Ser235/236	Rabbit	CST	# 2211	1:1000
Total S6K	Rabbit	CST	# 9202	1:1000
Total S6	Rabbit	CST	# 2217	1:1000
Phospho-GSK3α/β Ser21/9	Rabbit	CST	# 9331	1:1000
GSK3α/β	Mouse	SCB	# sc-7291	1:1000
Phospho-FOXO-1 Thr24	Rabbit	CST	# 9464	1:1000
Phospho-FOXO-1 Ser256	Rabbit	CST	# 9461	1:1000
Total FOXO-1	Rabbit	CST	# 2880	1:1000
Phospho-TSC2 Thr1462	Rabbit	CST	# 3611	1:1000
Phospho-PRAS40 Thr246	Rabbit	CST	# 2997	1:1000
Phospho-NDRG1 Thr346	Rabbit	CST	# 5482	1:1000

Total TrkB	Rabbit	CST	# 4603	1:1000
Acetylated-Lysine	Rabbit	CST	# 9814	1:1000
Total PDK1	Rabbit	CST	# 3062	1:1000
Phospho-ULK1 Ser757	Rabbit	CST	# 6888	1:1000
Total ULK1	Rabbit	CST	# 4773	1:1000
Phospho-PDK1 Ser241	Rabbit	CST	# 3061	1:1000
TACE	Rabbit	QED	# 1131	1:1000
TNFR1	Rabbit	MBL	# jm-3125-100	1:1000
BiP	Rabbit	CST	# 3177	1:1000
IRE1α	Rabbit	CST	# 3294	1:1000
CHOP	Mouse	CST	# 2895	1:1000
Total PERK	Rabbit	CST	# 5683	1:1000
PDI	Rabbit	CST	# 3501	1:1000
Phospho-PERK Thr980	Rabbit	CST	# 3179	1:1000
Phospho-elF2α Ser51	Rabbit	CST	# 3597	1:1000
ERK1/2	Rabbit	CST	# 9102	1:1000
GAPDH	Mouse	Abcam	# ab8245	1:25000
Phospho-Threonine	Rabbit	CST	# 9381	1:1000

SECONDARY ANTIBODY	HOST	SUPPLIER	REFERENCE	DILUTION
Anti-Rabbit HRP-conjugated	Goat	Thermo	# 31460	1:10000
Anti-Mouse HRP-conjugated	Goat	Thermo	# 31430	1:10000
Anti-Sheep HRP-conjugated	Rabbit	Thermo	# 31480	1:10000
Anti-Rabbit Alexa Fluor 594	Goat	Invitrogen	# A11072	1:400
Anti-Rabbit Alexa Fluor 488	Goat	Invitrogen	# A11017	1:400

TABLE 6. Primary and secondary antibodies for western blot and immunofluorescence analysis. A list of primary and secondary antibodies is provided, as well as their source and the dilution that was used for western blot and immunofluorescence analysis. CST: Cell Signaling Technology, SCB, Santa Cruz Biotechnology.

7. MORPHOLOGICAL ANALYSIS

7.1. IMMUNOHISTOCHEMISTRY

Three older PDK1$^{+/+}$ and PDK1$^{K465E/K465E}$ matched mice were used separately in the study. Mice were intraperitoneally anesthetized with 0.4 mg/g body weight of pentobarbital, and then an intracardiac injection of 70 heparin units was administered. Brains were collected and immediately frozen in dry ice for 10 min, initially stored at -80 °C. Then brains were embedded in O.C.T. Compound (Sakura Finetek, Tokyo, Japan), sliced into 10µm thick sagittal sections. Frozen sections were fixed by 4% PFA 40 min at 4ºC. For antigen retrieval, sections were boiled 10 min in 10 mM sodium citrate pH 6, cooled down for 30 min on ice then washed three times with Tris-buffered saline (TBS; 25 mM Tris pH7.5, 150 mM NaCl). Samples were blocked with buffer containing 5% goat serum and in 0.02% Triton in TBS for 30 min and incubated overnight at 4°C with primary antibodies diluted in the same blocking buffer. Sections were washed three times with TBS buffer, followed by incubation with the corresponding secondary antibodies diluted 1:400 in blocking buffer for 1.5 hours at room temperature and counterstained with 1 µg/ml Hoechst 33342 before mounting in Fluorsave reagent(Southern Biotec). Immunostained sections were photographed with a Nikon Eclipse 90i epifluorescence microscope, and the captured images were processed and analyzed with ImageJ 1.42q (Wayne Rasband, National Institutes of Health) and Fiji (http://pacific.mpi cbg.de/wiki/index.php/Main_Page) software.

7.2. IMMUNOCYTOCHEMISTRY

Dissociated cortical and hippocampal cells were cultured on cover-slips for indicated experimental days in vitro, and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Fixative solution was aspiratsed and cells washed three times with PBS for 5 min stored at 4°C until experiment day. The cells were washed four times with PBS for 5 min in slow rotation, and then the cells were permeabilized with 0.02% saponin in PBS for 7 min accurately at room temperature and incubated 15 min in 0.01% saponin,

10mM glycine in PBS, and then blocked in 0.01% saponin, 5% BSA, 10mM glycine in PBS for 1 hour at room temperature. Primary antibodies were diluted in PBS supplemented with 0.01% saponin, 1% normal goat serum according to manufacturer´ instructions, and incubated overnight at 4°C. Cells were then washed four times with PBS for 5 min agitating gently, corresponding secondary antibodies conjugated to Alexa594 or Alexa488 fluorescent dyes were used at a concentration of 1:400, and nuclei were stained with 1 µg/ml Hoechst 33342 for 90 min at room temperature. Cover slips were then mounted with FluoroSave Reagent on microscope slides for further analysis.

8. EVALUATION OF CELL VIABILITY

8.1. MTT REDUCTION ASSAY

Cell viability was determined by the MTT reduction protocol. Briefly, the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide salt (MTT, Sigma #M2128) was added to the cell culture at a final concentration of 0.5 mg/ml, and then returned to the incubator for 45-60 min. After this step, the medium also was aspirated completely and the resulting formazan crystals were dissolved by mixing with 300 µl of DMSO, Absorbance intensity was determined at 570 nm, with background measured at 690 nm, using a spectrophotometer running the Labsystem Multiskan software.

8.2. QUANTIFICATION OF APOPTOSIS

Cells were fixed in 2% paraformaldehyde, stained with 1 µg/ml of DNA dye Hoechst 33342, and then visualized under the fluorescence microscope. Apoptosis was quantified at each condition point by scoring the percentage of apoptotic cells in the adherent cell population. Cells exhibiting fragmented or condensed nuclei were scored as apoptotic whilst cells showing uniformly stained nuclei were scored as viable. At least 300 cells from 5 to 6 randomly selected fields per well were counted.

9. IMMUNOLOGY ANALYSIS

9.1. BDNF ELISA

Cortex and hippocampus BDNF levels were measured with the Quantikine human BDNF Immunoassay (Cat #: DBD00, R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer instructions. The optical density of each well was measured using an automated microplate reader (GloMax-Multi Microplate Reader, Promega). Data were calculated from the standard curves constructed with purified recombinant human BDNF for each plate and expressed as mean pg BDNF per mg of total protein.

9.2. sTNFR1 ELISA

Blood samples were clotted for 2 hours at room temperature and centrifuged for collection serum, which were aliquoted and stored at -80°C until the analysis was performed. After drugs processed, cell culture medium were frozen until used. Cortex, serum and cell medium concentrations were measured using an ELISA kit according to the manufacturer's instructions (Cat #: MRT10, R&D Systems, Inc., Minneapolis, USA). Serum needed to be diluted 10-fold and 40 μg cortex proteins were used for this assay.

9.3. MOUSE CYTOKINE/CHEMOKINE ASSAYS

Mouse Cytokine/Chemokine Magnetic Bead Panel (Cat #: MCYTOMAG-70K-PMX MILLIPORE) was utilized according to manufacturer's instructions and to assess the concentration of target factors using serum samples as used in the sTNFR1 assay. In brief, Wash Buffer was added into the plate shaker for 10 min at room temperature and then standard, control, and samples added into the appropriate wells. Samples were incubated with antibody-coupled beads with agitation on a plate shaker overnight at 4°C. Before incubation with detection antibody for 1 hour at room temperature, washing plate 2 times following instructions. Then Streptavidin-Phycoerythrin was added into wells on a plate shaker for 30 min at room temperature. Next well content was gently removed and plates were washed 2 times, drive fluid was added to resuspend and shock the beads for 5 min

prior to fluorescent measurement with MAGPIX, the data was obtained with the Xponent software. The cytokine/chemokine factor concentrations were calculated based on the corresponding standard curve. All samples were analyzed in duplicate.

10. TACE ACTIVITY

TACE activity was determined using the SensoLyte 520 TACE (á-Secretase) Activity Assay Kit (ANASPEC) following the manufacturer's instructions. Reaction was started by adding 40mM of the fluorophoric QXL520/5FAM FRET substrate continued for 60 minutes. 30 μg cortex and 20 μg cell lysate proteins were used for the protocol. Fluorescence intensity was quantified in a fluorescence microplate reader (FLx800, BIO-TEK Instruments, Winooski, VT) at an excitation of 490 nm and emission of 520 nm. Results are expressed as the percentage of change in fluorescence intensity compared with control samples.

11. Statistical analysis

Student's *t* test analysis were used to compare differences between categories, results were presented as mean ± standard error. Value of $P < 0.05$ and < 0.005 compared to controls as depicted in the figures were indicated statistical significance.

RESULTS

1. MUTATION OF THE PDK1 PH-DOMAIN IMPAIRS AKT ACTIVATION IN BRAIN

Recently our group showed that in the mutant PDK1$^{K465E/K465E}$ cortical neurons, the deficient BDNF-mediated activation of Akt was time and dose-dependent (Zurashvili *et al*., 2013). I next evaluated the contribution of the PDK1-PtdIns(3,4,5)P_3 interaction to Akt activation in the cortex and the hippocampus of PDK1$^{K465E/K465E}$ mice compared to PDK1$^{+/+}$ controls, at 4, 6-9, 12, 16 and 21 months of age. In the cortex, the phosphorylation of Akt at Thr308, the PDK1 site, was significantly reduced in the PDK1$^{K465E/K465E}$ mutant mice at 4 months of age. Unexpectedly, as the mice grew older, the phosphorylation of Akt at Thr308 was gradually recovered. By contrast, the phosphorylation of Akt at Ser473, the mTORC2 site, was not affected by the PDK1 mutation at any stage analyzed (Fig. 15A). Consistent with that, the phosphorylation of some Akt substrates at their specific Akt sites, such as PRAS40 at Thr246 and TSC2 at Thr1462, was also significantly reduced in the young mice and gradually recovered. Similarly, in the hippocampus the phosphorylation of Akt at Thr308 was significantly decreased until 16 months of age and by 21 months of age was fully recovered in the PDK1$^{K465E/K465E}$ mice compared to the PDK1$^{+/+}$ controls (Fig. 15B). The phosphorylation of PRAS40 at Thr246 and TSC2 at Thr1462 paralleled that of Akt thereby indicating that the deficient phosphorylation of Akt by PDK1 at Thr308 had consequences for Akt activation. All together, these results demonstrate that the PDK1 PH domain mutation attenuated Akt signaling in an age-dependent manner and that some kind of compensatory mechanism exists in the older ages.

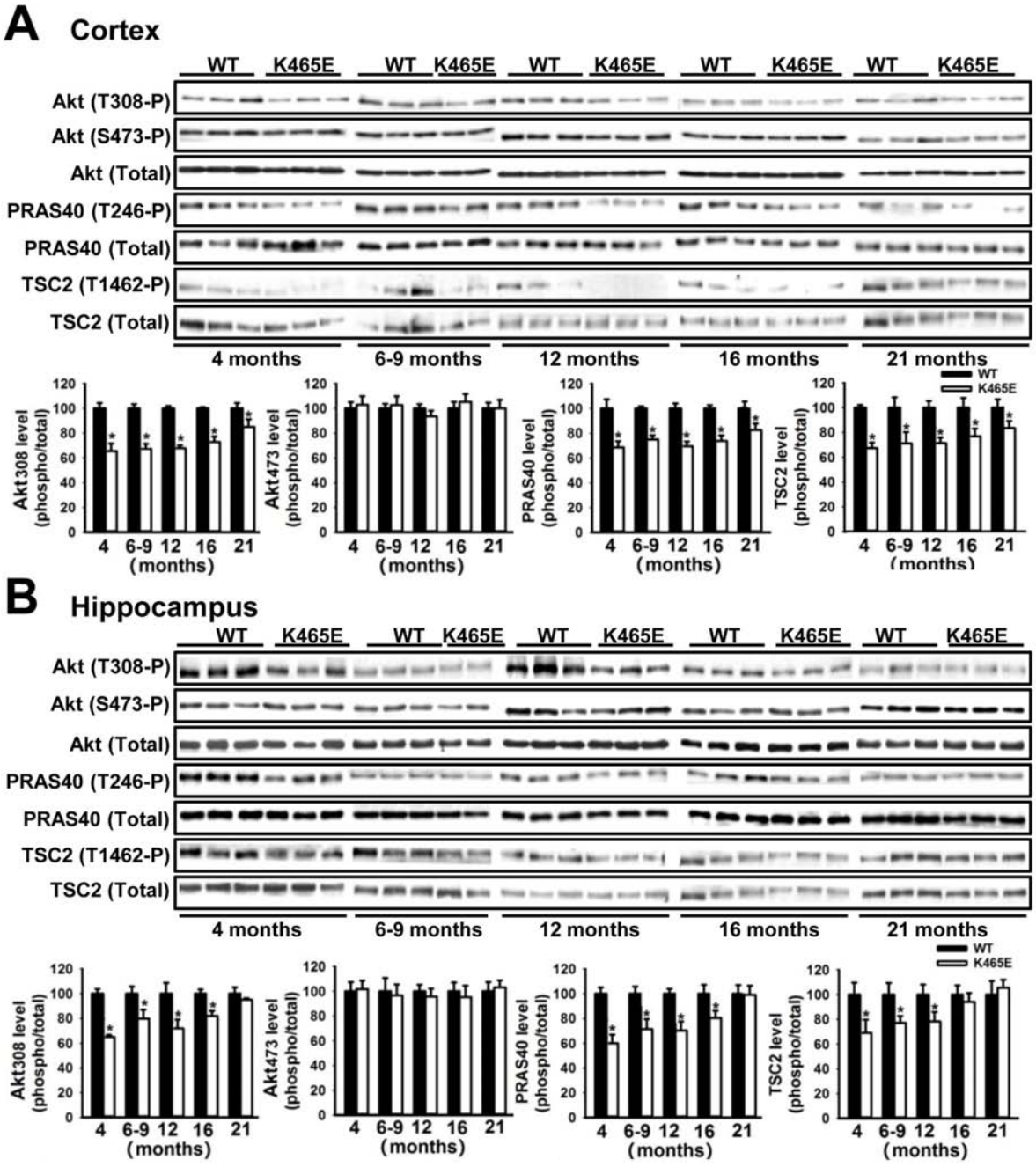


FIGURE 15. Deficient Akt phosphorylation and activation in the PDK1$^{K465E/K465E}$ brain. (A) Cortical and (B) hippocampal tissues from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) mice of the different depicted ages in months were subjected to immunoblot analysis with the indicated phospho and total antibodies. Each lane corresponds to a sample derived from a different mouse. Band densitometry quantification of the ratio between phosphorylated and total protein levels is shown at the bottom. Values are presented as percentage of the controls for each dataset. The data are represented as the mean ± SEM for at least three different mice per genotype. * $p < 0.05$ (Student' s *t* test) compared to controls.

1.1. BDNF SYNTHESIS COMPENSATE FOR THE DEFECTS IN THE ACTIVATION OF THE AKT SIGNALING PATHWAY

BDNF is an important neurotrophic factor and has a neuroprotective role in the brain; its actions are mediated among others through the activation of the TrkB-PI3K-Akt signalling pathway. In order to identify whether BDNF modulated Akt activation in the PDK1$^{K465E/K465E}$ mutant mice, the levels of BDNF were measured from brain tissue extracts. In the cortex, BDNF levels were reduced gradually with the time in the PDK1$^{+/+}$ mice, with a concentration of BDNF at 21 months 25% lower than at 4 months, which might be a result of aging (Fig. 16). The BDNF concentration showed no great differences between 4 and 12 months in both wild and mutant mice samples, but remarkably these levels were greatly increased in the PDK1$^{K465E/K465E}$ mice versus the control mice in the aged 16-21 months old mice. In the hippocampus, BDNF was maintained at similar levels in both genotypes until 16 months, but markedly enhanced in the the PDK1$^{K465E/K465E}$ mice at 21 months, consistently with the results of cortex. These data suggest that in the cortex and the hippocampus of the old PDK1$^{K465E/K465E}$ mice, the increased levels of BDNF synthesis compensate for the defects of the Akt signaling pathway.

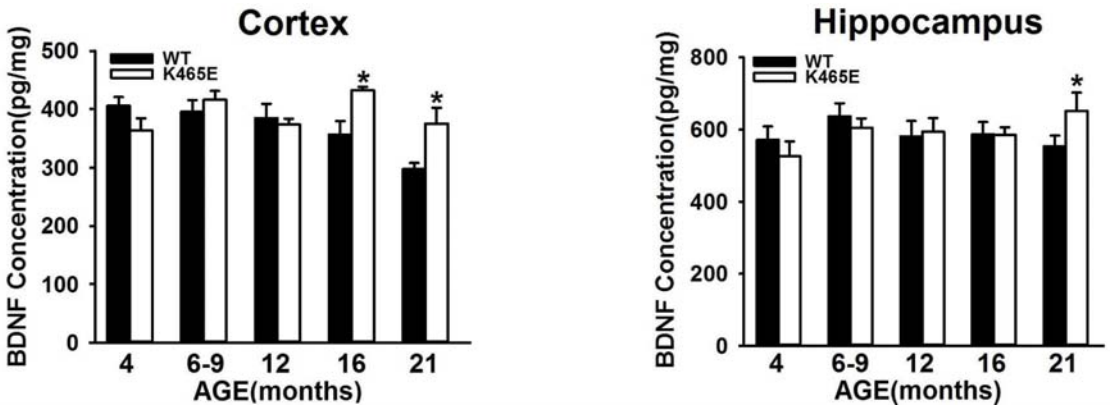


FIGURE 16. the levels of BDNF are increased in the PDK1$^{K465E/K465E}$ brain. The concentration of BDNF was measured on cortical and hippocampal tissues from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) mice at the indicated age in months. The data are represented as the mean ± SEM for at least three different mice per genotype. * $p < 0.05$ compared with wild types as obtained by the Student' s *t* test.

2. NORMAL ACTIVATION OF ULK1 AND S6K IN THE PDK1$^{K465E/K465E}$ BRAIN

Autophagy is considered as a vital catabolic process in cells which is highly responsible for degradation of abnormal or aggregated proteins and dysfunctional organelles. Phosphorylation of the Unc51-like kinase 1 (ULK1) by mTORC1 downstream of the PI3K/Akt pathway effectively inhibit autophagy (Chan *et al*., 2007; Kundu *et al*., 2008; Kim *et al*., 2011). S6K, also a downstream target of mTORC1, exhibits increased phosphorylation levels in cortex and hippocampus of mice models of AD (Caccamo *et al*., 2011).

Akt acts as an upstream kinase for S6K and ULK1 since Akt leads to mTORC1 activation by inhibiting TSC2, which in turns negatively regulates the mTORC1 activator Rheb (Inoki *et al*., 2002). Active mTORC1 phosphorylates S6K on Thr389, thereby creating a docking site for PDK1 and allowing the phosphorylation of the S6K Thr229 residue in the activation loop by PDK1. The phosphorylation of S6K at Thr389 was reduced in the BDNF-stimulated PDK1$^{K465E/K465E}$ cortical neurons, although the defects were only detected at 5 min after treatment (Zurashvili *et al*., 2013). Therefore, ULK1 and S6K phosphorylation were expected to be affected by the genotype and age-related modulation of mTORC1 activation in the PDK1$^{K465E/K465E}$ mice. Nevertheless, I found that whatever cortex or hippocampus the phosphorylation of ULK at Ser757 was not affected in the PDK1$^{K465E/K465E}$ mutant mice at any age analyzed (Fig. 17), Similarly, phosphorylation of S6K at Thr389 was unchanged in the mutant mice compared to the control (Fig. 17). As mentioned, S6K is a docking site-dependent, PDK1 substrate whose phosphorylation was found transiently reduced upon acute BDNF treatment of PDK1$^{K465E/K465E}$ mutant cortical cells (Zurashvili *et al*., 2013). These results demonstrate that the PDK1 K465E mutation is not affecting the phosphorylation of ULK1 and S6K in the brain, at least in tissues older than 4 months.

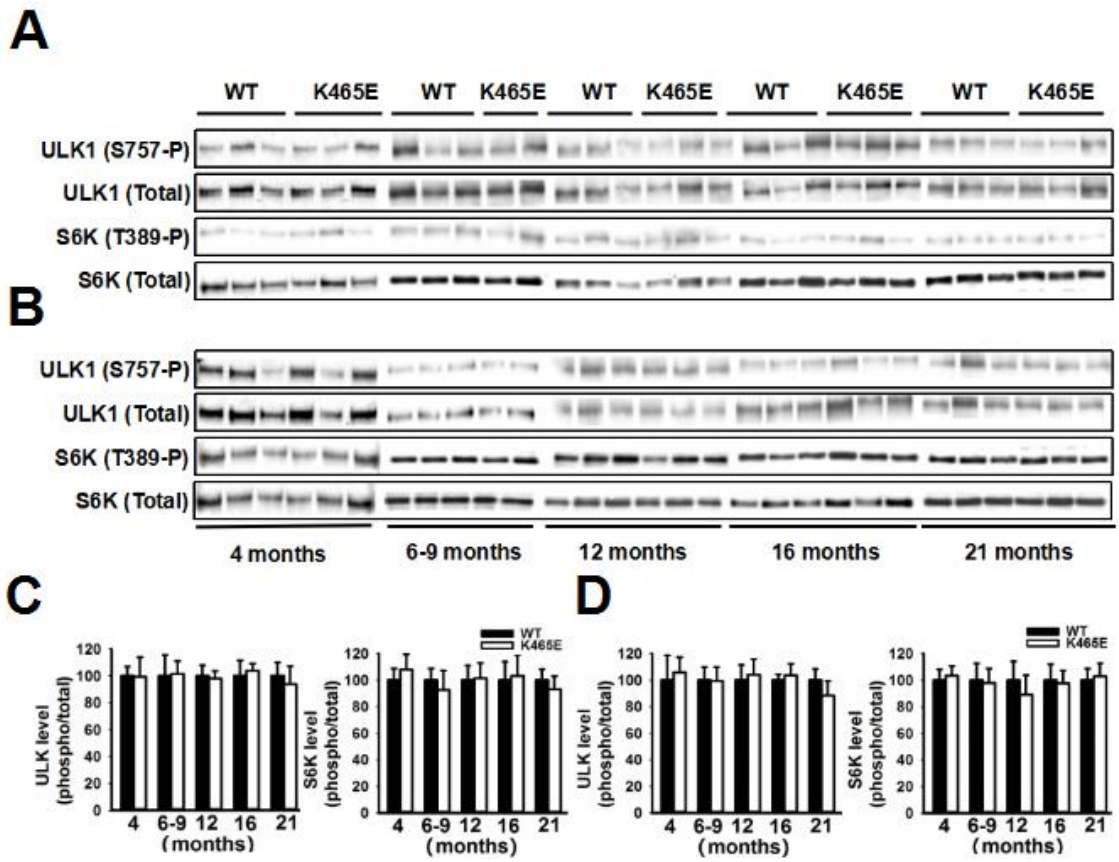


FIGURE 17. Preserved phosphorylation of ULK1 and S6K in the PDK1$^{K465E/K465E}$ brain. (A) Cortical and (B) hippocampal tissues from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) mice at the indicated age in months were subjected to immunoblot analysis with the indicated phospho and total antibodies. Each lane corresponds to a sample derived from a different mouse. (C) Cortical and (D) hippocampal band densitometry quantification of the ratio between phosphorylated and total protein levels is shown at the bottom. Values correspond to the mean ± SEM for at least three different mice per genotype and are presented as percentage of the controls for each dataset.

3. TACE AND sTNFR1 REGULATION IN THE PDK1$^{K465E/K465E}$ MICE

TACE α-secretase activity cleaves amyloid precursor protein (APP) and shed a soluble, non-amyloidogenic fragment, APPsα (Allionson *et al*., 2003), which competitively antagonize the amyloidogenic Aβ generation *via* β-secretase. In Alzheimer's disease (AD) patients, TACE α-secretase activity is reduced and low amounts of sAPPα are observed in the cerebrospinal fluid (CSF) (Sennvik *et al*., 2000). PDK1 activity is increased in samples from Alzheimer Disease patients; moreover, pharmacological inhibition of PDK1 activity with the BX912 compound increased TACE-mediated α-secretase activity and reduced AD pathology and memory impairment in AD models mice (Pietri *et al.*, 2013); these functions

rely on TACE localization at the plasma membrane (Gooz, 2010). Age-related changes in susceptibility to TNF-α were mediated through the TNF receptor 1 (TNFR1), which is also cleaved by TACE (Janelsins *et al*., 2008).

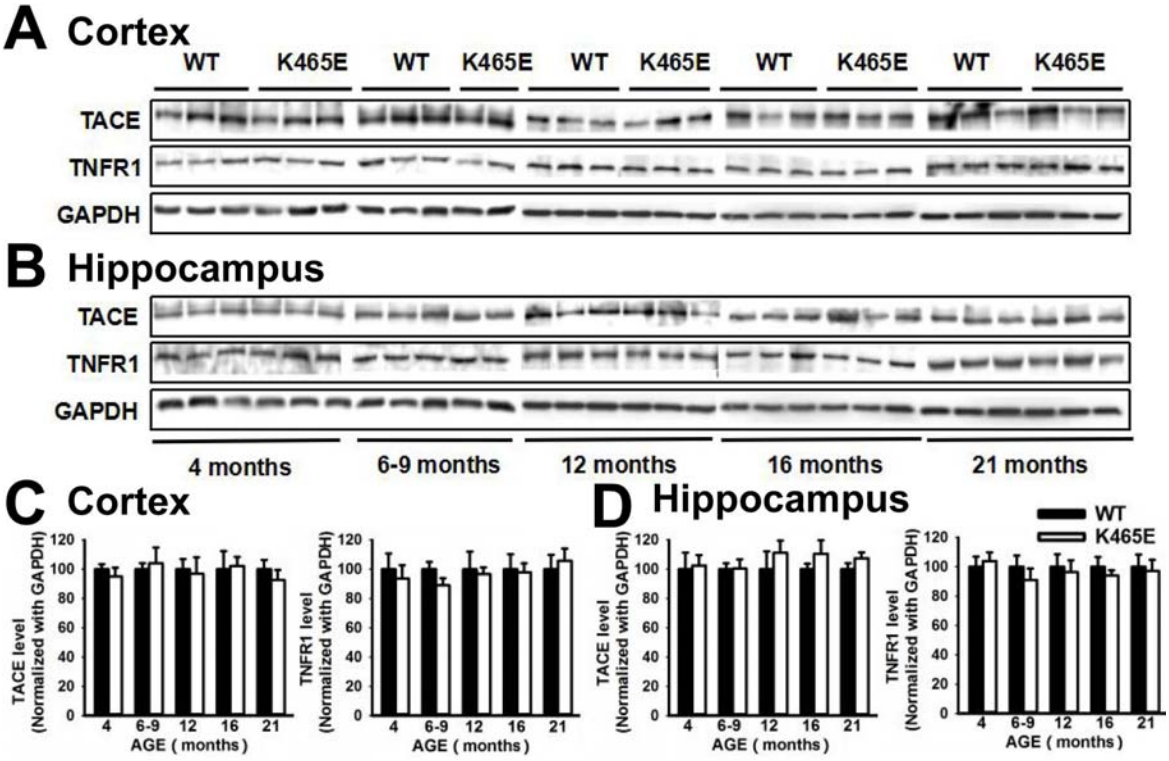


FIGURE 18. Normal expression of TACE and TNFR1 in the PDK1$^{K465E/K465E}$ brain. Cortical (A) and hippocampal (B) tissues from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) mice at the indicated age in months were subjected to immunoblot analysis with the indicated antibodies. Each lane corresponds to a sample derived from a different mouse. Cortical (C) and hippocampal (D) Band densitometry quantification of the ratio between TACE or TNFR1 and GAPDH levels is shown at the bottom, where values correspond to the mean ± SEM for at least three different mice per genotype and are presented as percentage of the controls for each dataset.

3.1. NORMAL EXPRESSION OF TOTAL TACE AND TNFR1 PROTEIN IN THE PDK1$^{K465E/K465E}$ MICE BRAIN

In order to investigate whether TACE activity was affected by the PDK1-PtdIns(3,4,5)P_3 interaction, I measured TACE and TNFR1 protein expression in the cortex and the hippocampus of PDK1$^{+/+}$ and PDK1$^{K465E/K465E}$ mice at different ages. As shown in Figure 18, there was no statistically significant difference in total TACE and TNFR1 expression

levels when compared wild type and mutant mice cortex and hippocampus samples at any age analyzed.

3.2. INCREASED TACE ACTIVITY AND sTNFR1 LEVELS IN THE PDK1$^{K465E/K465E}$ MICE BRAIN

In order to address whether TACE activity was affected by the PDK1 PH-domain mutation, I measured TACE α-secretase activity in the cortex of PDK1$^{K465E/K465E}$ mice and PDK1$^{+/+}$ mice at different age. Interestingly, as the mouse grew older, TACE α-secretase activity was more and more increased in the PDK1$^{K465E/K465E}$ mice brain when compared to the age-matched controls, , and it was significantly higher in the 16 and 21 months old mutant mice than in the control subjects (Fig. 19). I next used TNFR1 shedding as a readout of TACE activity, and correspondingly found the levels of sTNFR1 decreased with the age in the cortex of the control mice, whereas the PDK1$^{K465E/K465E}$ mice seemed resistant to this effect, since the concentration of sTNFR1 was markedely higher in the cortex of the16 and 21 months old mutant mice compared to the matched controls (Fig. 19). These results indicate that the selective deficiency in Akt activation potentiates the TACE α-secretase activity in the mutant mice cortex. Some studies showed that in the brain of AD mice and in CSF of AD patients TACE activity was significantly reduced (Yao *et al*., 2015), whereas in AD patients the concentration of sTNFR1 was 90% reduced in the CSF compared to age-matched normal subjects (Pietri *et al.*, 2013). As described in the introduction, age acts as the main risk factor for AD; since in the aged 16 and 21 months old mutant mice both TACE activity and sTNFR1 levels are increased, this rasise the possibility that the defect of Akt activity could be benefitial against the pathogenesis of AD.

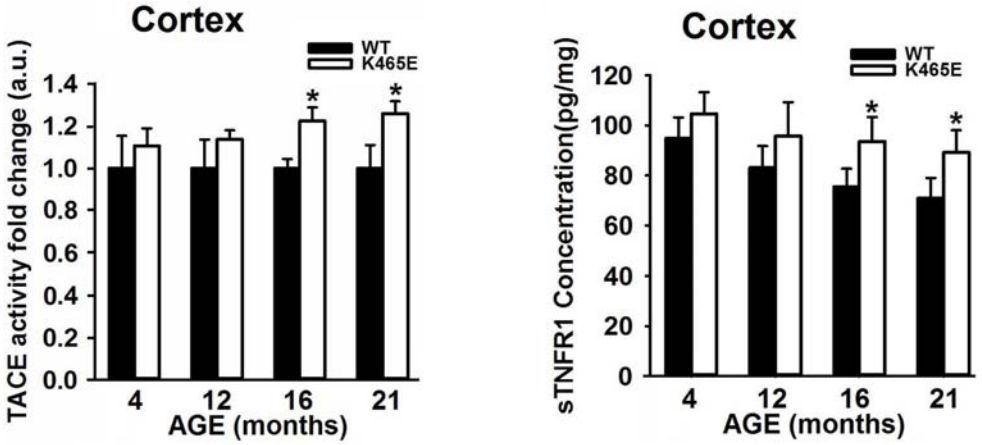


FIGURE 19. Levels of TACE α-secretase activity and sTNFR1 are increased in the PDK1$^{K465E/K465E}$ brain. The concentration of sTNFR1 and TACE α-secretase activity were measured at cortical tissues from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) mice at the indicated age in months. The data are represented as the mean ± SEM for at least three different mice per genotype. * $p < 0.05$ compared with wild types as obtained by the Student' s *t* test.

3.3. DECREASED LEVELS OF SERUM sTNFR1 IN THE PDK1$^{K465E/K465E}$ OLD MICE

Blood-based biomarkers could provide a cost- and time-effective way compared to CSF or brainas the first step in a multi-stage screening and diagnostic process, which is common in medical practice. So it was necessary to validate serum sTNFR1 as a potential biomarker. As shown in Figure 20, there were not significant differences between wild type and mutant mice younger than 12 months. In the 16 and 21 months old aged mice from both genotypes the concentration of sTNFR1 gradually increased, though the sTNFR1 levels were drastically lower in the PDK1$^{K465E/K465E}$ than PDK1$^{+/+}$ old mice, which was opposite to the brain results. Actually, sTNFR1 levels were significantly higher in the AD patients' plasma than controls (Faric *et al.*, 2014). These contradictory results may be caused by the trafficking of sTNFR1 through the blood brain barrier.

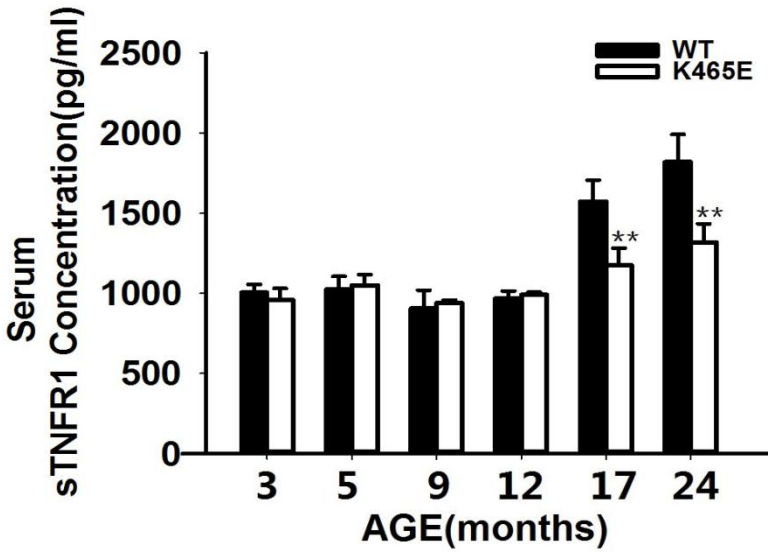


FIGURE 20. Reduction of the levels of serum sTNFR1 in the PDK1$^{K465E/K465E}$ brain. The concentration of serum sTNFR1 was measured from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) mice at the indicated age in months. The data are represented as the mean ± SEM for at least three different mice per genotype. ** $p < 0.005$ compared with wild types as obtained by the Student' s *t* test.

3.4. DYNAMIC CHANGES OF TACE AND TNFR1 IN THE OLD PDK1$^{K465E/K465E}$ BRAIN

Cell surface TNFR1 staining is associated with reduction in the concentration of sTNFR1, and TACE trafficking and internalization is induced by phosphorylation of the C-terminal cytoplasmic tail (Pietri *et al.*, 2013). Based on this theory and on the above described results, it was necessary to further define the dynamic changes of TACE and TNFR1 in the PDK1$^{K465E/K465E}$ old mice brain.

Firstly, I analyzed TACE and TNFR1 expression on brain membrane fractions. I used TrkB as membrane marker and total ERK as cytosolic marker. As shown in Figure 21A, membrane TACE protein levels were higher in the PDK1$^{K465E/K465E}$ brain than in the PDK1$^{+/+}$ controls, whereas cytosolic TACE levels were reduced in the PDK1$^{K465E/K465E}$ brain compared with the PDK1$^{+/+}$ controls. Conversely, TNFR1 protein levels were significantly lower in the membrane and higher in the cytosol of the PDK1$^{K465E/K465E}$ brain samples compared to the PDK1$^{+/+}$ controls. This is in good agreement with the increased TACE activity and sTNFR1 levels observed in the brain of the PDK1$^{K465E/K465E}$ mice.

Phosphorylation of the C-terminal cytoplasmic tail of TACE alters TACE trafficking and promotes its internalization, and one of its phosphorylatable residues, Thr735, is increased in 3x-Tg mice adult neurons, whilst PDK1 inhibitors decrease TACE phosphorylation levels (Pietri *et al.*, 2013). Since there are not suitable antibodies to test specifically TACE phosphorylation according to the literature, I used immunoprecipitation and immunoblotting with antibodies detecting phospho-threonines to study TACE phosphorylation. I found that the phosphorylation of TACE at Threonines was reduced in the PDK1$^{K465E/K465E}$ old brain compared to PDK1$^{+/+}$ controls (Fig. 21B). This result was consistent with Akt promoting TACE trafficking and internalization, which is attenuated in the PDK1 PH-domain mutant mice.

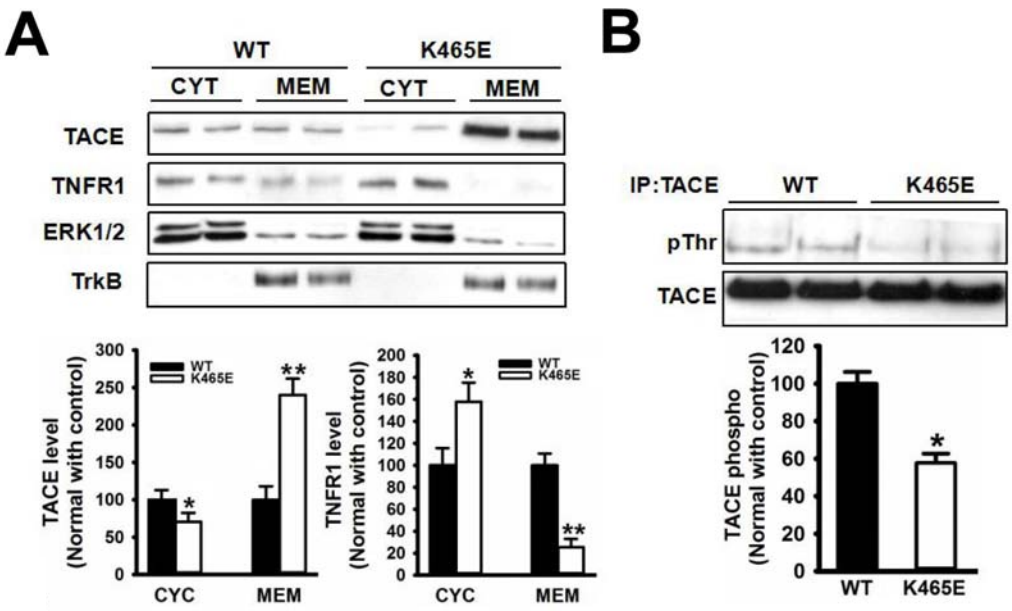


FIGURE 21. Dynamic changes of TACE and TNFR1 induced by TACE phosphorylation in the PDK1$^{K465E/K465E}$ old brain. (A) Brain tissues subcellular fractionation from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) 21-months old mice were immunoblotted with the indicated antibodies. The ERK1/2 and TrkB are shown as membrane (MEM) and cytosolic (CYC) markers respectively. (B) Immunoprecipitation of TACE and immunoblotting for phosphorylation level on threonine residues (pThr) using a pan-pThr antibody in from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) 21-months old mice. Representative Western blots of three independent experiments are shown, where the band densitometry quantification is shown at the bottom. The data are represented as the mean ± SEM for at least three different mice per genotype. * $p < 0.05$ and ** $p < 0.005$ compared with wild types as obtained by the Student' s *t* test.

Secondly, in order to further certify dynamic changes of TACE and TNFR1 in the brain tissue, I used immunofluorescent labeling of TNFR1 and TACE to analyze its expression in the cortex. As shown in Figure 22, TACE staining was increased in the cortex of the PDK1$^{K465E/K465E}$ old brain compared to control litermates; quantification of the intensity confirmed that TACE staining intensity was 50% higher in the mutant mice, which was consistent with the results obtained on membrane fractions. At the same time, TNFR1 accumulation was reduced in the old PDK1$^{K465E/K465E}$ mice cortex compared to wild type controls. These data suggest that the TACE α-secretase activity in the old PDK1$^{K465E/K465E}$ mice brain is increased due to deficient Akt-mediated internalization, which promotes TNFR1 shedding and production of high levels of sTNFR1 capable to elicit resistance to TNFα toxicity.

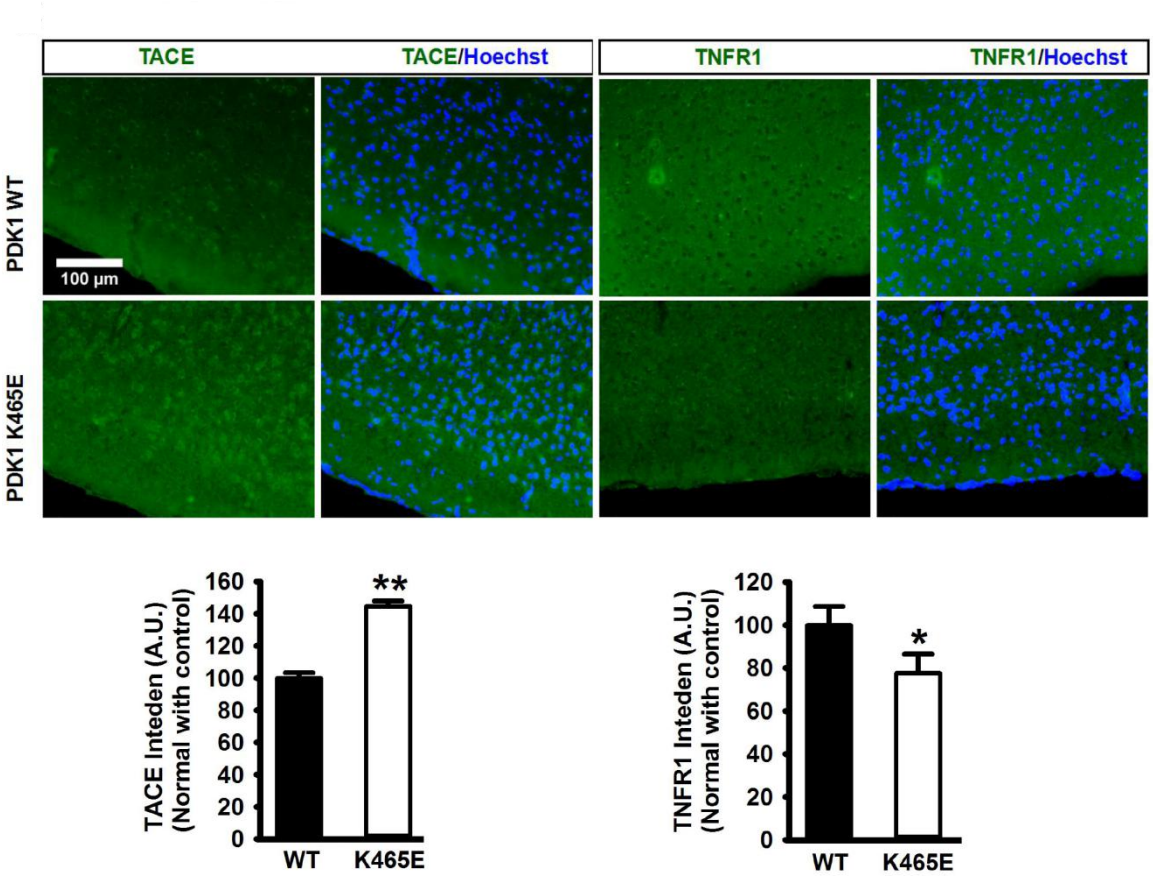


FIGURE 22. Immunostaining of TACE and TNFR1 in the PDK1$^{K465E/K465E}$ old brain. Epifluorescence micrographs of cortex from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) 16-months old mice brain stained with TACE, TNFR1, and the nuclear Hoechst 333342 dye. The intensity of the staining was quantified and expressed as the mean ± SEM for at least three independent sections per mice obtained from three different mice per genotype. Bar, 100 µm. * $p < 0.05$ compared with wild types as obtained by the Student' s *t* test.

Altogether, these data strongly suggest that the Akt branch of the PDK1 pathway seems to be responsible for aging and Aβ peptide-induced TACE activity downregulation. This may be caused by phosphorylation of the C-terminal cytoplasmic tail of TACE, thereby altering its trafficking and promoting the internalization, since the sheddase activity of TACE depends on its localization at the plasma membrane. PDK1 inhibition in the AD model mice increases TACE-mediated α-secretase processing and reduces Aβ production. Similarly, in the $PDK1^{K465E/K465E}$ mutant mice with restricted phosphorylation of Akt at the PDK1 site, TACE activity was shown to be induced by aging.

4. PDK1/AKT SIGNALING PATHWAY IS INCREASED IN ALZHEIMER'S DISEASES MODEL MICE

PDK1 is the master regulator of at least 23 other AGC kinases whose downstream signaling has often been implicated in various diseases. PDK1 and its substrate Akt are both serine/threonine kinases; PDK1 phosphorylates Akt at the T-loop which is crucial for its activitation. This critically depends on the translocation of both proteins to the plasma membrane, perhaps on a PDK1-Akt heterodimer complex (Calleja *et al*., 2007). PDK1 is constitutively active and it is responsible for regulating its phosphorylation and activation. PDK1 activity is increased in human AD whole brains and in primary cultures derived from adult AD model mice (Pietri *et al.*, 2013), which relied on Src-mediated signaling and triggering the phosphorylation and membrane depletion of TACE. Furthermore, PDK1 requires phosphorylation of Ser-241 on its activation loop for catalytic activation, and some evidences suggest that PDK1 catalyzes autophosphorylation of this site (Scheid *et al*., 2005). These notions prompted me to examine whether PDK1 phosphorylation would increase its activity in AD models of disease.

4.1. AGE-DEPENDENT INCREASED OF THE PDK1/AKT SIGNALLING PATHWAY IN THE APP/PS1 MICE

APP/PS1 double transgenic mice coexpress the KM670/671NL "Swedish" mutated version of human APP695 and the L166P mutated form of human PS1 under the regulatory control of the murine Thy-1 minigene promoter, which is a neuron-specific restricting the

transgene expression to the postnatal brain. This model is promptly developing Aβ deposition and a robust neuroinflammatory response toward Aβ plaques, along with synaptic integrity loss and memory impaiments (Radde *et al.*, 2006). Cortical lysates derived from 6 months and 12 months APP/PS1 double transgenic mice maintained on a C57BL/6J genetic background were analyzed. Surprisingly, PDK1 phosphorylation at Ser241 was weakly reduced in APP/PS1 6-months old mice cortex compared to age-matched wild type controls; phosphorylation of Akt at both Thr308, the PDK1 site, and Ser473, the mTORC2 site, were also weakly decreased in APP/PS1 mice (Fig. 23A). Accordingly, the phosphorylation of PRAS40 at Thr246 was also inhibited by the APP and PS1 mutations. In contrast, there were not changes in the phosphorylation of GSK3β at Ser9, FOXO1 at Ser256, and the total TACE and TNFR1 protein levels were also comparable between wild and APP/PS1 mutant mice. With the development of Aβ plaques in the 12-months old mice, although the APP/PS1 mutant mice exhibited only slightly elevated PDK1 phosphorylation at Ser241 compared to wild type mice (Fig. 23B), Akt phosphorylation at Thr308 and Ser473, as well as phosphorylation of the PRAS40, GSK3β and FOXO1 substrates were markedly increased in the APP/PS1 mice, whereas TACE and TNFR1 remained unchanged. Actually, autophosphorylation of PDK1 at Ser241 is critical but not sufficient to induce PDK1 full activity (Gao *et al*., 2006). This process is depending on the cell type and signaling pathways converging on PDK1; additional phosphorylations on Ser, Thr and Tyr residues located within the kinase domain, the pleckstrin-homology (PH) domain and in the linker between the kinase and the PH domains have already been shown to enhance PDK1 activity in a more or less cooperative manner (Li *et al*., 2010). For example, in the presence of insulin, PDK1 can autophosphorylates itself on Ser241 and, at the same time, PDK1 is phosphorylated on Tyr-9 and Tyr-373 with the help of Src and heat shock protein 90 (Hsp90), which further increase PDK1 catalytic activity (Li *et al*., 2010). Also, the discrepancy between the PDK1and Akt activation levels observed in the APP/PS1 mutant mice could be attributed to the inherent amplification capacity of this signaling pathway. To sum up, these results reveal that the accelerated accumulation of Aβ deposition in the APP/PS1 mutant old mice caused PDK1 hyperactivation *via*

autophosphorylation at Ser241 within its activation loop, which resulted in the activation of the downstream Akt signaling pathway.

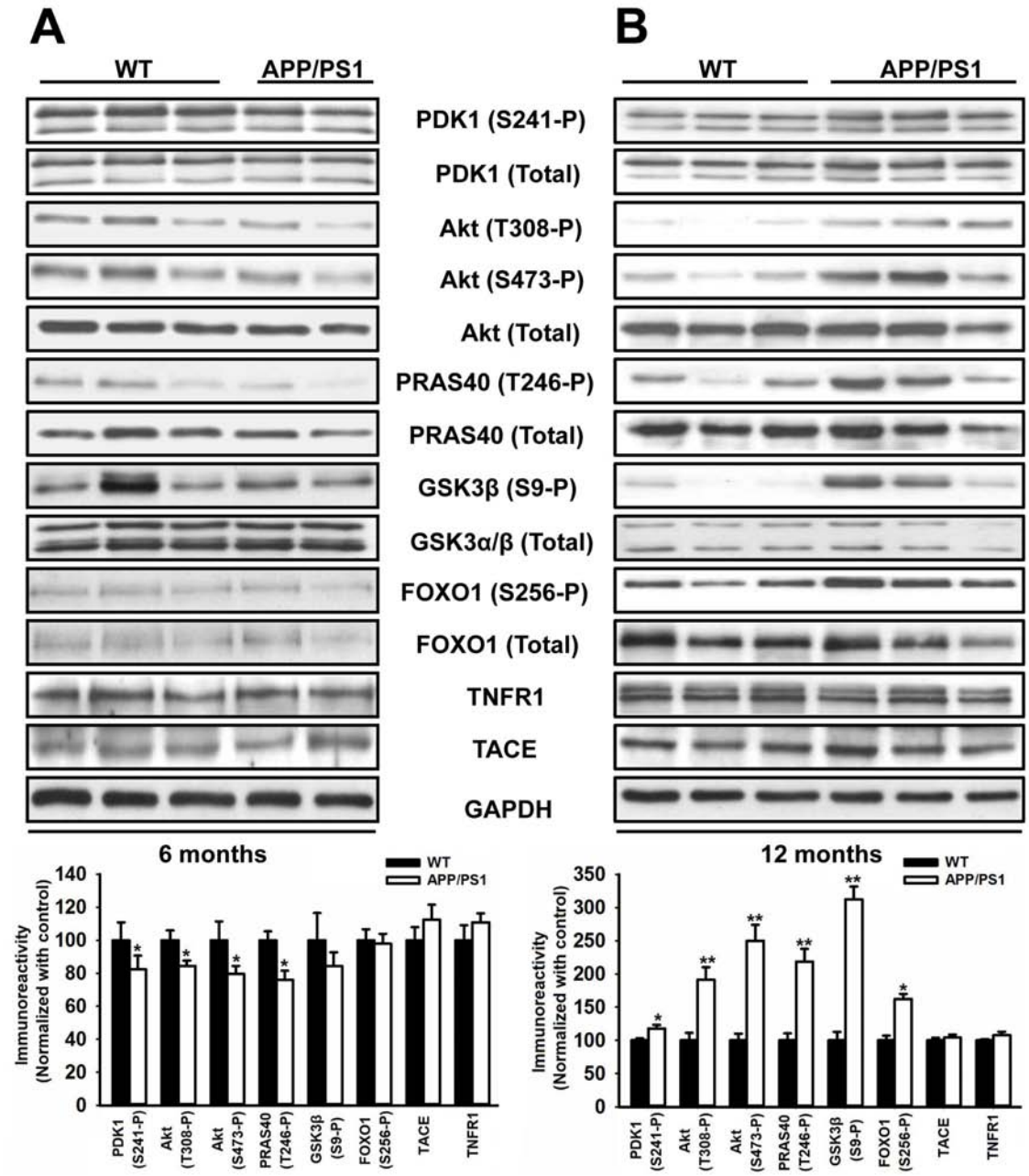


FIGURE 23. PDK1/Akt phosphorylation and activation are increased in the APP/PS1 mice brain. Cortical tissues from wild type (WT) and APP/PS1 double transgenic mice (APP/PS1) at 6 months (A) and 12 months (B) of age were subjected to immunoblot analysis with the indicated phospho and total antibodies. Each lane corresponds to a sample derived from a different mouse. Band densitometry quantification of the ratio between phosphorylated and total protein levels is shown at the bottom. The data are represented as the mean ± SEM for at least three different mice per genotype. * $p < 0.05$ or ** $p < 0.005$ compared with wild types as obtained by the Student' s *t* test.

4.2. AGE-DEPENDENT INCREASED OF THE PDK1/AKT SIGNALLING PATHWAY IN THE 3xTg-AD MICE

I next used the 3xTg-AD mice AD model mice to certificate this notion. 3xTg-AD is a triple-transgenic model harboring $PS1_{M146V}$, APP_{Swe} and tau_{P301L} mutant transgenes. These model mice progressively develop plaques and tangles as well as synaptic dysfunction including LTP deficits, in an age-related manner (Oddo *et al.*, 2003). To further assess the impact of PDK1 phosphorylation on its activity, different age cortex and hippocampus tissues were collected. Western blot and quantification results show that in contrast to the APP/PS1 mice, the six-month-old 3xTg-AD mice have no detectable alterations neither in the cortex nor in the hippocampus in PDK1 Ser241 phosphorylation levels when compared to matched controls (Fig. 24). By contrast, I found that the levels of Akt phosphorylation at Thr308, Ser473, PRAS40 at Thr246, GSK3β at Ser9 and FOXO1 at Ser256 were weakly increased in the 3xTg-AD mice at this age when compared with age-matched wild types. With gradual accumulation Aβ and Tau pathology in the 12 months old brain (Oddo *et al.*, 2008), the ratio of PDK1 phosphorylation at Ser241 over total PDK1 was slightly elevated in the cortex and hippocampus of the 3xTg-AD mice (Fig. 24). As a consequence, phosphorylation of Akt, PRAS40, GSK3β and FOXO1 were significantly increased in the 12-month-old 3xTg-AD mice. By contrast, total TACE and TNFR1 protein levels were unchanged at both ages analyzed. It has been showed that mTORC1 activity is significantly increased in the 3xTg-AD mice at 6 and 12 months of age when compared with age-and gender-matched controls. PRAS40, which inhibits mTORC1 by directly binding to it, was highly phosphorylated at Thr246 in the 3xTg-AD mice, which prevented mTORC1 binding an inhibition (Caccamo *et al*., 2011). These data suggest that PDK1 autophosphorylation at Ser241 is responsible for PDK1 enzymatic activity in Alzheimer's disease model mice, which acts through the Akt pathway via phosphorylation and inhibition of PRAS40 to activate mTORC1.

Altogether, these results strongly suggest that the Akt branch of the PDK1 pathway seems to be affected by PDK1 hyperactivity in Aβ mediated AD pathology, although the

phosphorylation of PDK1 at Ser241 is not the only determining factor for Akt hyperactivation.

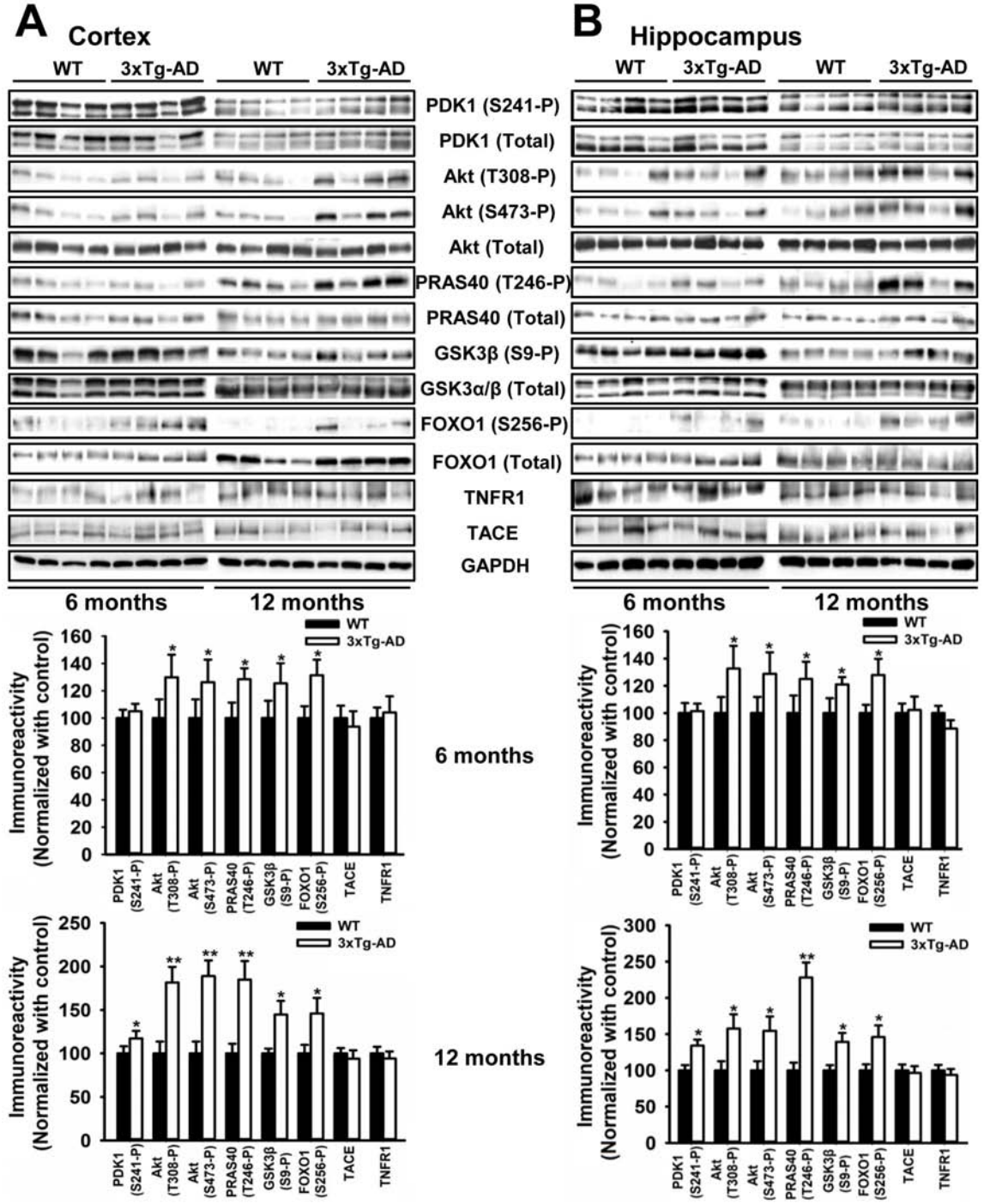


FIGURE 24. PDK1/Akt phosphorylation is increased in the 3xTg-AD model mice brain. Cortical (A) and Hippocampal (B) tissues from wild type (WT) and 3xTg-AD triple-transgenic model mice (3xTg-AD) at 6 months and 12 months of age were subjected to immunoblot analysis with the indicated phospho and total antibodies. Each lane corresponds to a sample derived from a different mouse. Band densitometry quantification of the ratio between phosphorylated and total protein levels is shown at the bottom. The data are represented as the mean ± SEM for at least three different mice per genotype * $p < 0.05$ or ** $p < 0.005$ compared with wild types as obtained by the Student's *t* test.

5. PDK1 LYSINE ACETYLATION IS INCREASED IN THE PDK1$^{K465E/K465E}$ MICE

Akt activation involves its recruitment and binding to plasma membrane; in order to be phosphorylated by PDK1 at Thr308, the PH domain of Akt needs to bind to PtdIns(3,4,5)P_3 (Vinodkumar *et al.*, 2014). This binding induces a conformational change that converts Akt onto a substrate that can be readily phosphorylated by PDK1 at Thr308 within the activation loop, while mTORC2 phosphorylates Ser473 at the hydrophobic motif rendering Akt fully active. As described before, the phosphorylation of Akt at the Thr308 residue was partially age-dependently inhibited in the PDK1$^{K465E/K465E}$ brain. Pharmacological inhibition of Akt S473 phosphorylation totally inhibited the BDNF-induced phosphorylation of Akt at Thr308 in the PDK1$^{K465E/K465E}$ mutant neurons at high doses, which means that in those cells the activation of Akt most likely arises from the ability of the PDK1 PIF-pocket to recognize and bind to the Akt hydrophobic motif (Zhou *et al*., 2014). In order to certify this model, PDK1 was pulled down with a peptide resembling the substrate docking site of S6K able to interact with the catalytic domain of PDK1 independently of the PH domain. PDK1 binding to the peptide was enhanced in the PDK1$^{K465E/K465E}$ mice brain samples compared to the wild type from 4 to 21 months of age (Fig. 25). Since PDK1 can be acetylated at lysine residues within the PH domain to prevent it membrane localization and activation (Nagalingam *et al.*, 2012), I asked whether lysine acetylation of PDK1 was altered in the PDK1$^{K465E/K465E}$ mutant mice. Surprisingly, PDK1 acetylation was increased in the PDK1$^{K465E/K465E}$ brain lysates respect to the control samples, and quantification of the immunoblot signals showed an age-dependent decreasing trend until the 21-month-old mice. It is well known that growth factor stimulus deacetylates and activates Akt (Nagalingam *et al.*, 2012). The PDK1$^{K465E/K465E}$ mice brain possess markedly increased BDNF levels at 21 month (Fig.16), which may have contributed to the lowered PDK1 acetylation levels.

Some researchers show that prion infection increases the pool of PDK1 protein, which acts on the PDK1/TACE signaling (Alleaume-Butaux *et al*., 2015). I found that TACE failed to bind PDK1 in pulldown experiments, suggesting that TACE do not directly associates with PDK1 in the cell membrane (Fig. 25). These data indicates that a synergy between

acetylation-mediated regulation of PDK1 and PDK1-PtdIns(3,4,5)P3 binding accounts for Akt activation in the PDK1$^{K465E/K465E}$ mutant mice brain.

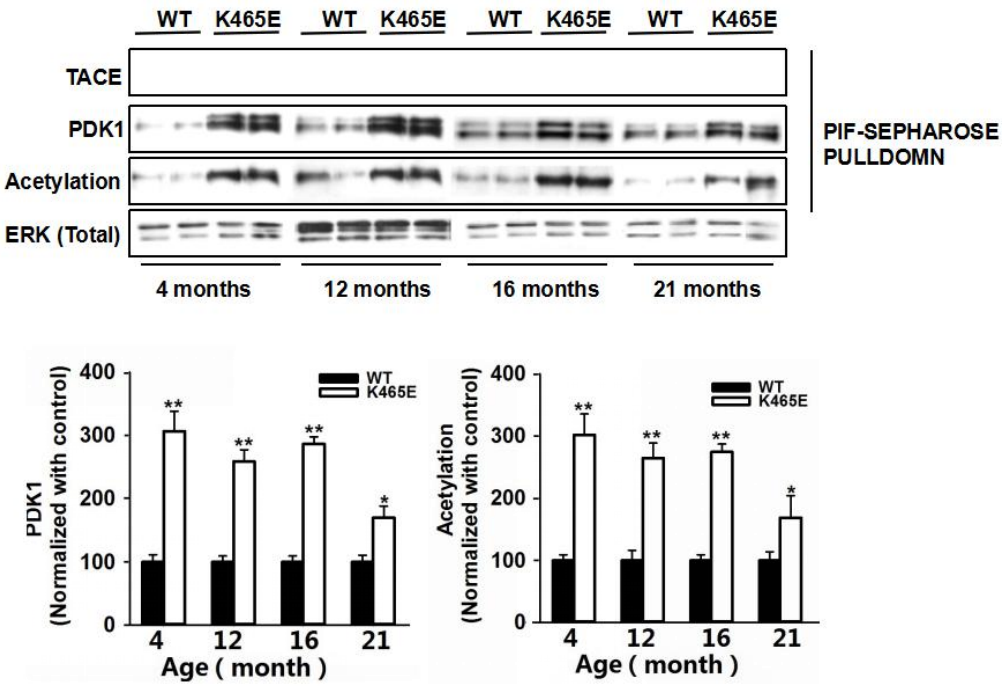


FIGURE 25. Enhanced docking-site binding of PDK1 in the PDK1$^{K465E/K465E}$ brain. PDK1 was affinity purified on PIF-sepharose from PDK1$^{+/+}$ wild type (WT) and PDK1K$^{465E/K465E}$ mutant (K465E) mice of the depicted ages and subjected to immunoblot analysis with the indicated antibodies. The acetylated and total PDK1 levels were normalized by the total PDK1 protein levels derived from the brain tissue lysate immunoblot and expressed as percentage of the controls for each dataseet. Immunoblots with the ERK1/2 antibody are also shown as a loading control. * $p < 0.05$ and ** $p < 0.005$ compared with wild types as obtained by the Student' s *t* test.

6. PDK1 MUTANTION AFFECTS THE UPR SYSTEM IN THE PDK1$^{K465E/K465E}$ MICE

The endoplasmic reticulum (ER) is responsible for processing of proteins that are destined to be secreted, enclosed in a vesicle, or incorporated in the plasma membrane. Nascent peptides that enter the ER undergo a series of highly regulated processing steps to reach maturation as they transit the ER. Alterations in the intracellular environment that induce ER stress are thought to interrupt this process, and result in unfolding of proteins in the ER. Accumulation of unfolded proteins concurrently activates three transmembrane stress

sensors, IRE1, ATF6 and PERK, there are referred to as the UPR (Thomas *et al*., 2015). In AD patients, amyloid plaques and neurofibrillary tangles can disrupt many of the cellular processes controlled by the ER, causing stress that contributes and exacerbates neuronal cell death (Hetz *et al*., 2014). The cell main weapon against ER stress is induction of the UPR. Because age is the primary risk factor for AD, and some studies showed that intraneuronal amyloid-β accumulation increases both in ageing and in AD. I analyzed several UPR markers in the brain of the PDK1$^{K465E/K465E}$ mutant mice at different ages, whilst neuronal survival and apoptotic rates were evaluated under some drugs treatment.

6.1. UPR ACTIVATION IS INCREASED IN THE 3xTg-AD MICE

Aβ oligomers or fibrils can trigger ER stress in different experimental models, which has been shown to lead to the phosphorylation of eIF2α, PERK and other initiators of UPR activation (Katayama *et al*., 2004). Phosphorylation of eIF2α is elevated in the brain of AD patients and model mice, whereas inhibition of PERK prevents deficits in protein synthesis, synaptic plasticity and spatial memory in APP/PS1 mice (Ma *et al.,* 2013). In parallel with this notion, I further check out UPR in another classic AD model mice. Phosphorylation of PERK at Thr980 and its substrate eIF2α at Ser51 were increased in 3xTg-AD mice compared to wild-type littermates, whereas other elements including BIP, IRE1α, PDI and CHOP did not show observable differences between wild and mutant group (Fig. 26). In normal physiological situation, during ER stress, BIP holds PERK and IRE1 in an inactive state until it binds unfolded proteins and dissociates from PERK and IRE1 until the unfolded protein backlog is removed (Thomas *et al*., 2015). Phosphorylated PERK then phosphorylate eIF2α, which usually is considered to be protective. In response to a wide range of cellular stressors, like Aβ, eIF2α is phosphorylated by PERK, leading to inhibition of general mRNA translation, increased ATF4 and CHOP mRNA levels, which in turn has been shown to lead to PERK and eIF2α hyperphosphorylation (Ma *et al.,* 2013). This situation may be inducing a positive feedback mechanism where protein aggregation may exacerbate the conditions that promote the production of the misfolding protein.

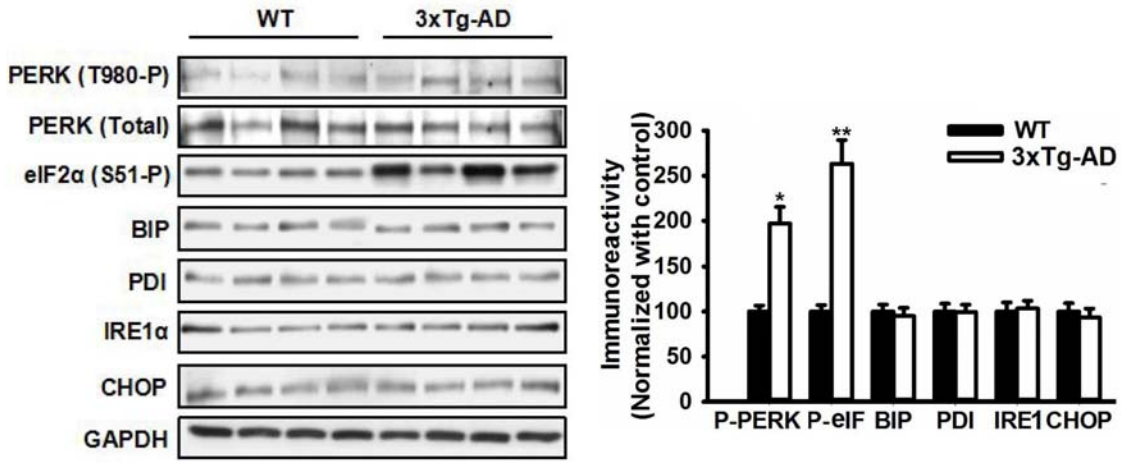


FIGURE 26. UPR activation is increased in the 3xTg-AD model mice brain. Cortical tissues from wild type (WT) and 3xTg-AD triple-transgenic model mice (3xTg-AD) of 12 months of age were subjected to immunoblot analysis with the indicated antibodies. Each lane corresponds to a sample derived from a different mouse. Band densitometry quantification of the ratio between indicated markers and control is shown at the right expressed as a percentage of the controls for each data point. * $p < 0.05$ or ** $p < 0.005$ compared with wild types as obtained by the Student' s *t* test.

6.2. UPR IS DECREASED IN OLD PDK1$^{K465E/K465E}$ MICE BRAIN

As mentioned before, the serum concentration of sTNFR1, which is an inflammation marker in AD, was found elevated in the 16 and 21 months old PDK1$^{K465E/K465E}$ mutant mice brain compared to the matched controls (Pietri *et al.*, 2013). This is due to the fact that the PDK1$^{K465E/K465E}$ mutant mice, with restricted phosphorylation of Akt at the PDK1 site, showed attenuated TACE activity reduction induced by aging.

However, amyloid plaques and neurofibrillary tangles, which compose the two basic AD pathology features, are increased in the AD brain. The PDK1$^{K465E/K465E}$ mutant mice could potentially be protected from AD pathology by inhibition of the ability of PDK1 to interact with PtdIns(3,4,5)P_3. Autophagy, as a vital catabolic process in cells, is highly responsible for degradation of abnormal and aggregated proteins and dysfunction organelles, but phosphorylation of S6K and ULK, markers of autophagy, are not affected by mutation of

the PDK1 PH-domain. I aimed to determine whether PDK1 could modulate the UPR in the PDK1$^{K465E/K465E}$ mice. I used cortex protein extracts derived from PDK1$^{+/+}$ and PDK1$^{K465E/K465E}$ mice at different ages to analyze the levels of several UPR markers. As shown in figure 27, quantification of the western blot analysis showed that phosphorylation of PERK at Thr980 and its substrate eIF2α at Ser51 were not statistically different between wild type and mutant mice before 12 months of age; BIP, PDI and IRE1 were also not affected by the PDK1 K465E mutation. In the 16 and 21-months old PDK1$^{K465E/K465E}$ mutant mice brain, phosphorylation of PERK at Thr980 was significantly decreased compared with the same age wild types, and phosphorylation of the PERK substrate eIF2α at Ser51 was also reduced in the mutant old mice compared with controls. By contrast, other UPR markers, such as BIP, PDI and IRE1, did not change between PDK1$^{+/+}$ and PDK1$^{K465E/K465E}$ mice. The same sampling depicted in figure 27 was analyzed for TACE activity and sTNFR1, and the results followed a similar trend. While mice are young, ER stress was normal since a balance of unfolded proteins existed allowing the binding of BIP to the apical triggers of the UPR. As mice grow older, more and more amyloid-β is accumulated during the ageing process (Baker-Nigh *et al*., 2015). In response to increased Aβ, ER stress is enhanced and promoted protein refolding, this unbalance caused over-activation PERK, which also leaded to eIF2α hyperphosphorylation. However, in the PDK1$^{K465E/K465E}$ mice, the UPR was extremely decreased in the old mice compared with the same age control, pointing out to Akt as responsible for modulating the UPR.

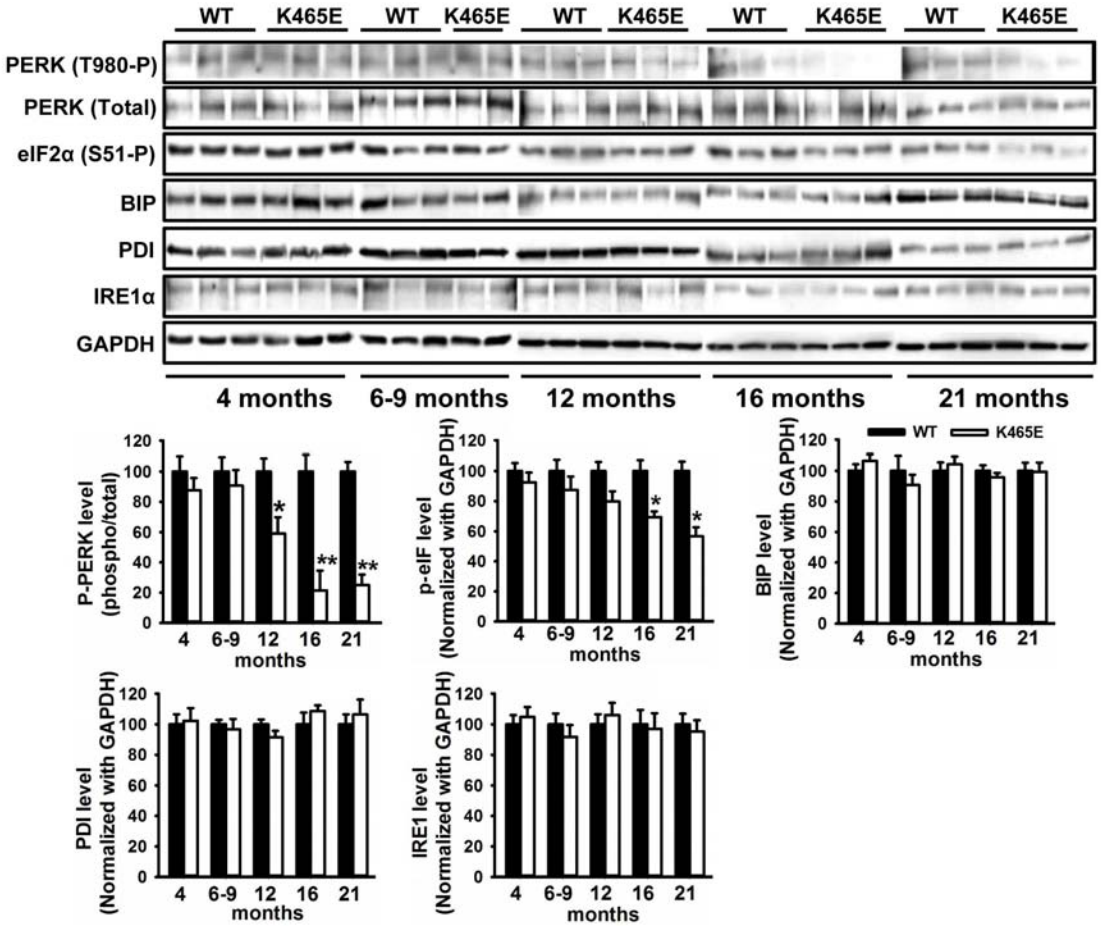


FIGURE 27. UPR activation is reduced in the PDK1$^{K465E/K465E}$ mice brain. Cortical tissue extracts from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) mice of the indicated age in months were subjected to immunoblot analysis with the indicated antibodies. Each lane corresponds to a sample derived from a different mouse. Band densitometry quantification of the ratio between indicated markers and control levels is shown at the bottom. Values are presented as percentage of the controls for each data point. * $p < 0.05$ or ** $p < 0.005$ compared with wild types as obtained by the Student' s *t* test.

6.3. IMPAIRED INTERACTION OF PDK1 WITH PtdIns(3,4,5)P$_3$ ANTAGONIZE NEURONAL CELL DEATH INDUCED BY ER STRESS

Severe ER stress can lead to the activation of apoptotic cell death through an intrinsic/mitochondrial pathway (Smith and Deshmukh, 2007). In order to determine whether mutation of the PDK1 PH-domain can rescue ER stress-induced neuronal cell death, primary cultures of cortical neurons derived from the PDK1$^{+/+}$ and PDK1$^{K465E/K465E}$ mice embryos at E15.5 were treated with tunicamycin (N-glycosylation inhibitor) that can induced neuron death through ER stress (Galehdar *et al*., 2010). Neuronal survival was

determined by the MTT assay and the results confirmed by scoring the percentage of apoptotic nuclei exhibiting chromatin condensation and nuclear fragmentation after Hoechst staining. As shown in Figure 28A, the viability of $PDK1^{+/+}$ and $PDK1^{K465E/K465E}$ cortical neurons was decreased by tunimcamycin in a dose-dependent manner; however, the $PDK1^{K465E/K465E}$ cortical neurons were significantly more viable than the controls at higher doses of tunicamycin of 1 and 3 μg/ml. Consistent with this, the number of apoptotic cells were extremely increased by tunicamycin both in the $PDK1^{+/+}$ and the $PDK1^{K465E/K465E}$ cortical neurons; in the $PDK1^{K465E/K465E}$ mutant neurons, the number of tunicamycin-induced apoptotic cells was attenuated when compared with wild type control, especially at high doses fo the drug. These results suggest that the PDK1 interaction to phosphoinositides likely protects against death induced by tunicamycin. Parallel studies on the UPR signalling pathway revealed phosphorylation of PERK at Thr980 after 6 hours of tunicamycin treatment, which was significantly reduced in the $PDK1^{K465E/K465E}$ cortical neurons compared to $PDK1^{+/+}$ controls, reaching the maximal difference upon 12 hours of tunicamycin treatment (Fig. 28B). Phosphorylation eIF2α at Ser51 was also extremely reduced in the $PDK1^{K465E/K465E}$ mice neurons from 6 hours and sustained for up to 24 hours. By contrast, BIP was detected after 12 hour of stimulation at similar levels in both wild type and mutant neurons, whereas PDI and IRE1 levels were not affected by the PDK1 PH domain mutation. Altogether, these results suggest that the impaired interaction of PDK1 to phosphoinositides protected neurons against tunicamycin-induced neuronal cell death by attenuating the UPR.

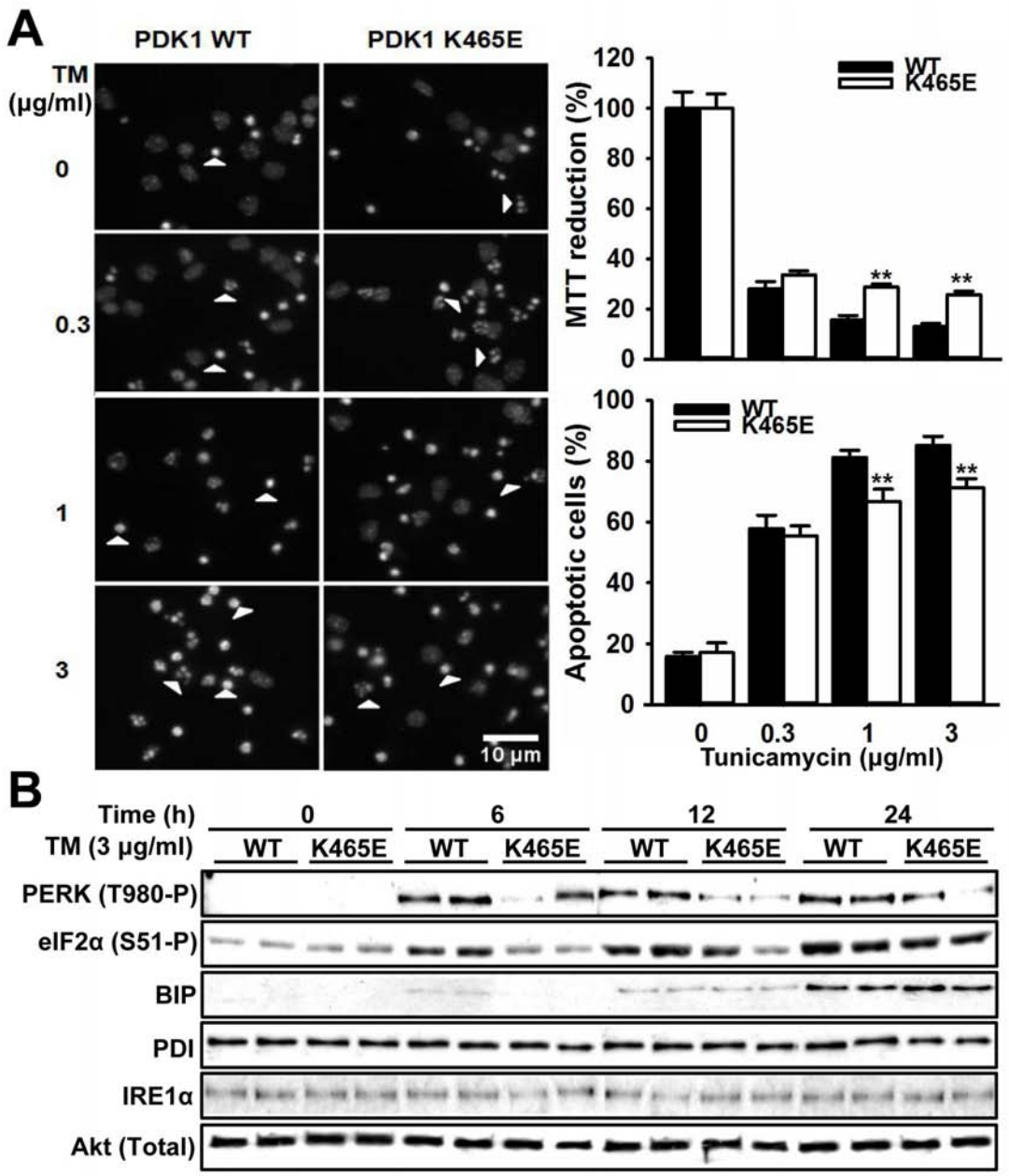


FIGURE 28. Neuronal survival responses are increased in the PDK1$^{K465E/K465E}$ mice. (A) Cortical cells from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) E15,5 embryos were grown for 6 DIV and then treated with the indicated doses of tunicamycin (TM) in complete medium for 48h. Left panel, representative micrographs of Hoechst-stained cortical cultures, where arrowheads indicate apoptotic nuclei; scale bar 10 μm; right upper panel, the cell viability was determined with the MTT reduction assay and is expressed as a percentage of the untreated cells; data represents the mean ± SEM for at least five independent embryos per genotype from two separate experiments, with each sample assayed in triplicate. Right bottom panel, the percentage of apoptotic cells was obtained by scoring the number of nuclei exhibiting chromatin fragmentation divided by the total. (B) Cortical cells from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) E15,5 embryos were cultured for 6 DIV and then either left unstimulated or stimulated with 3 μg/ml tunicamycin for the indicated time points (hours). Lysates were immunoblotted with the indicated UPR marker antibodies, where Akt total levels are also shown as a loading control. * $p < 0.05$ or ** $p < 0.005$ compared with wild types as obtained by the Student' s *t* test.

7. CONTRIBUTION OF THE PDK1-PHOSPHOINSITIDE INTERACTION TO AMYLOID-BETA CAUSED NEURONAL DISFUNCTION

The biochemical analysis on the PDK1$^{K465E/K465E}$ mice demonstrated that the reduced Akt activation observed in the young mice was almost recovered to normal levels when the mice grew older, presumably due to the increased levels of BDNF synthesis that compensate for the defects in the Akt signaling pathway. This raised the question of whether Aβ plays a role in the Akt signaling pathway in the PDK1$^{K465E/K465E}$ neurons. Recent studies showed that PDK1 inhibition in AD model mice stably increases TACE-mediated α-secretase processing and reduces Aβ production (Pietri *et al.*, 2013). In the PDK1$^{K465E/K465E}$ mutant mice, the restricted phosphorylation of Akt at the PDK1 site was also shown to result in increased TACE activity induced by aging. On the other hand, the UPR was significantly decreased in the old PDK1$^{K465E/K465E}$ mice compared with the controls of the same age. Age is the primary risk factor for AD, and some studies present that intraneuronal amyloid-β accumulation increase both during aging and in AD. In order to explore whether Aβ plays an important role in controlling TACE activity and the UPR response in the PDK1$^{K465E/K465E}$ mice, I took advantage of the primary cortical and hippocampal neuronal cultures to evaluate the impact of Aβ treatment on the survival responses of these mutant cells.

7.1. DEFECTIVE AKT ACTIVATION RESTRICTS Aβ-INDUCED ER STRESS AND NEURONAL CELL DEATH UNDER IN THE PDK1$^{K465E/K465E}$ MICE

Pathogenic neurotoxic amyloid-β peptides in AD provoke overactivation of PDK1 in diseased neurons (Pietri *et al.*, 2013). The phosphorylation of PDK1 at Ser-241 in the activation T-loop was found increased in an age-dependent manner in AD model mice. Deficient activation of Akt by BDNF is time and dose-dependent in the PDK1$^{K465E/K465E}$ neurons (Zurashvili *et al.*, 2013). To define the importance of Aβ in the activation of the Akt signaling pathway, primary cultures of cortical neurons were stimulated with the peptides for the indicated times. As shown in Figure 29A, the phosphorylation of Akt at Thr308 in the absence of Aβ was significantly reduced in the PDK1$^{K465E/K465E}$ mutant neurons compared to the controls. Aβ treatment induces an increase in the levels of Akt

phosphorylation at Thr308, reaching the maximum after 6 hours and sustained up to 24 hours of treatment both in wild and mutant neurons. However, compared with the $PDK1^{+/+}$ cells, the reduced phosphorylation of Akt at Thr308 in the $PDK1^{K465E/K465E}$ cortical neurons was attenuated in a time-dependence manner. Similarly, in the brain tissues the differences in the intensity of the Akt signaling were reduced and almost recovered to normal levels in the older $PDK1^{K465E/K465E}$ mice. Corresponding to the activation, the phosphorylation of PRAS40 at Thr246 and TSC2 at Thr1462 were significantly reduced in the $PDK1^{K465E/K465E}$ mutant neurons upon 0 and 6 hours of treatment and then slightly recovered compared to the $PDK1^{+/+}$ controls as stimulation times proceed longer. These western blot data demonstrate that mutation of the phosphoinositide binding site in PDK1 is attenuating Akt activation in a time-dependent manner upon Aβ stimulation. Some studies suggested that Aβ can directly induce ER stress (LaFerla *et al*., 2007). As mentioned before, tunicamicyn-induced UPR was clearly decreased in the old $PDK1^{K465E/K465E}$ mice compared with the same age controls. I analyzed markers of the UPR upon treatment with Aβ at the indicated times. As shown in Figure 30B, the phosphorylation of PERK at Thr980 was first detected at 12 hours to the same levels in $PDK1^{+/+}$ and $PDK1^{K465E/K465E}$ neurons. By contrast, after 24 hours the mutant cortical neurons showed significantly lowered levels of phosphorylation of PERK compared to wild type controls. Phosphorylation of eIF2α at Ser51 was observed along all the time-course treatment in the two genotypes, which was reduced in the $PDK1^{K465E/K465E}$ embryonic cortical cells from 12 hours to 24 hours of treatment. However, BIP was not detected until 24 hours of stimulation, with similar levels in both wild type and mutant neurons. PDI and IRE1 were not affected by the PDK1 PH domain mutation.

Recent studies have reported that Aβ caused neuronal cell death, PDK1 signalling pathway being involved in this process (Manterola *et al*., 2013). Akt is widely believed to mediate the survival-promoting effects of growth factors in various cell types (Dudek *et al*., 1997). However, BDNF can rescue trophic factor deprivation-induced cell death in the $PDK1^{K465E/K465E}$ mice, despite the PDK1-phosphoinositide interaction is important for Akt activation (Zurashvili *et al*., 2013). Moreover, the rate of cortical neuron survival in the presence of tunicamycin is even higher in the mutant than in the wild type cells. To further

investigate the role of the Akt kinase in mediating the death effects of Aβ, embryonic cortical neurons were cultured for 6 DIV and then exposed to soluble Aβ peptides for 24 hours (Fig. 29C).

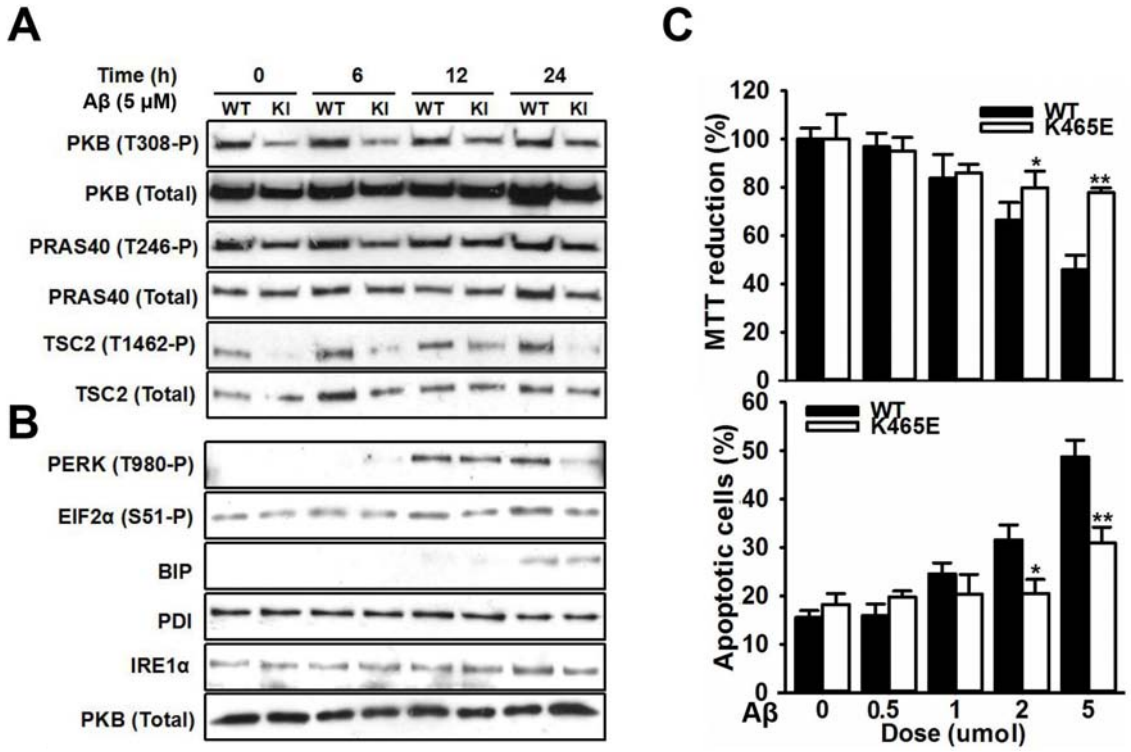


FIGURE 29. The PDK1$^{K465E/K465E}$ neurons are protected against Aβ-induced neurotoxicity. (A) and (B) Primary cultures of cortical neurons from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (KI) E15,5 embryos were grown for 6 DIV and then either left unstimulated or stimulated with 5 μmol of amyloid-β peptides (Aβ) for the indicated time points (hours). Lysates were immunoblotted with the indicated phospho and total antibodies; Akt total protein levels are also shown as a loading control. A representative Western blot of three independent experiments is shown. (C) Cortical cells from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (KK65E) E15,5 embryos were grown for 6 DIV and then treated with different dose of amyloid-β peptides (Aβ) in completely medium for 48 h. The MTT reduction and the percentage of apoptotic nuclei were determined and represented as the mean ± SEM for at least five independent embryos per genotype from two separate experiments, with each sample assayed in triplicate. * $p < 0.05$ or ** $p < 0.005$ compared with wild types as obtained by the Student' s *t* test.

The neurotoxic effects of Aβ were found to be concentration-dependent, with the neuronal cell survival rate decreased in a dose-dependent manner with increasing levels of the peptide. Interestingly, the PDK1$^{K465E/K465E}$ mutant cells were less sensitive than the PDK1$^{+/+}$ wild type neurons at highest concentrations of Aβ from 2 to 5 μM. The percentage of apoptotic value was in line with the MTT reduction, where Aβ-induced number of

apoptotic cells was attenuated in the mutant neurons when compared with wild type controls, especially when treated with high doses of Aβ. These results suggest that defective Akt activation reported in the PDK1$^{K465E/K465E}$ mutant neurons can reduce neuronal cell death caused by Aβ, and that the UPR plays an important role in the control of neuronal survival.

7.2. DYNAMIC CHANGES OF TACE AND TNFR1 IN THE PDK1$^{K465E/K465E}$ NEURONS

As described before, TACE α-secretase activity was higher in the PDK1$^{K465E/K465E}$ aged mice than in the matched PDK1$^{+/+}$ controls. Since the concentration of CSF soluble Aβ increased with aging,o assess whether Aβ peptides affect TACE activity *in vitro*, primary cultures of cortical neurons were grown in the presence of soluble Aβ peptides. As shown in Figure 30, TACE α-secretase activity was decreased after Aβ treatment, whereas the deficient Akt activity of the PDK1$^{K465E/K465E}$ neurons antagonized this phenomenon, resulting in TACE α-secretase activity significantly higher in the PDK1$^{K465E/K465E}$ mutant neurons compared to the PDK1$^{+/+}$wild type cells (Fig. 30C). Correspondingly, the levels of secreted sTNFR1 were reduced after treatment with the Aβ peptide in the media of the PDK1$^{+/+}$ wild type neurons, but not the PDK1$^{K465E/K465E}$ mutant neurons; which displayed higher sTNFR1 concentrations (Fig. 30D). In order to further certify these dynamic changes of TACE and TNFR1 in neurons upon treatment with Aβ, I used immunocytochemistry of TNFR1 and TACE to analyze its expression in hippocampal neurons (Fig. 30A). Soluble Aβ peptides triggered increasing cell surface TNFR1 levels in the wild type neurons which was attenuated in the mutant ones (Fig. 30B). Aβ also promoted the translocation of TACE to the cell surface, as judged by the the intensity of TACE immunoreactivity, which was again enhanced in the PDK1$^{K465E/K465E}$ neurons compared to the PDK1$^{+/+}$ cells (Fig. 30B). These date further suggest that deficient Akt activity in the PDK1$^{K465E/K465E}$ neurons avoided the reduction in TACE α-secretase activity elicited with the soluble Aβ peptide treatment, and increased the translocation of TACE back to the cell surface, which was accompanied by reduced amount of TNFR1 at the plasma membrane and increased sTNFR1 levels in the cell culture medium.

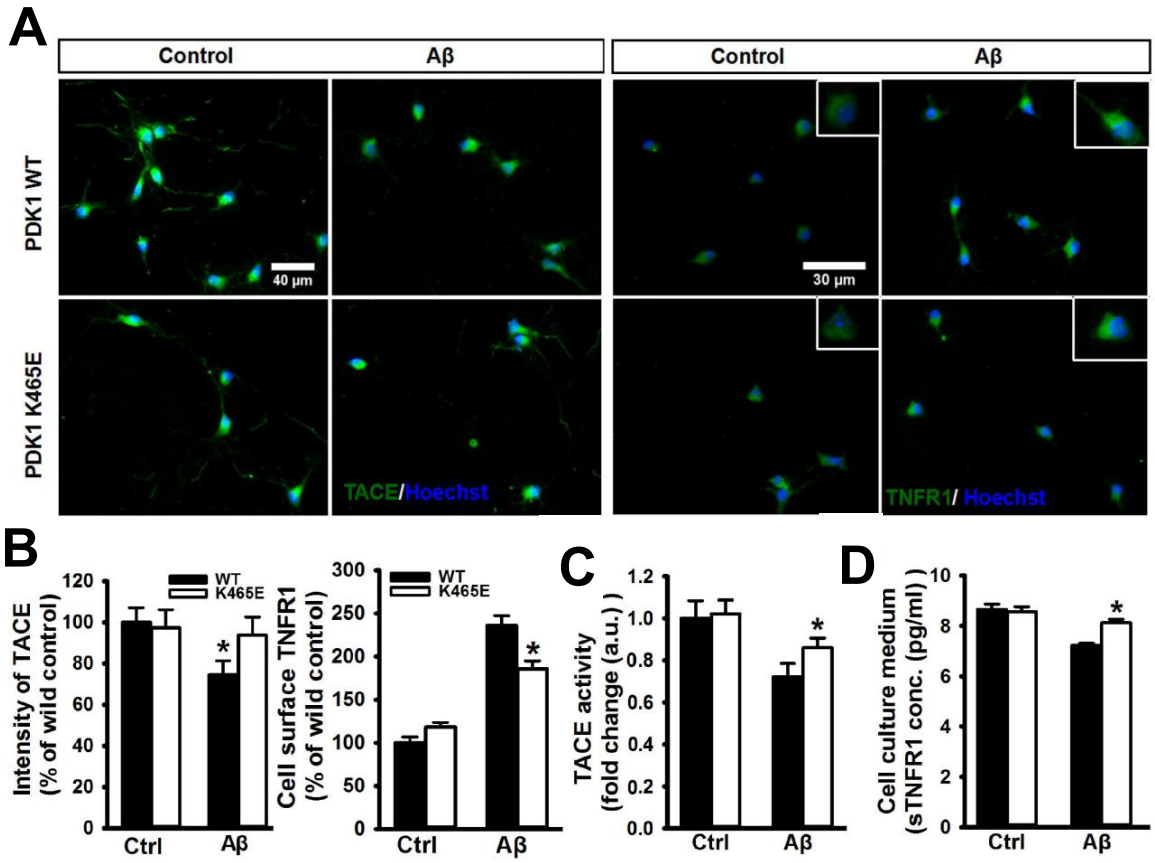


FIGURE 30. Dynamic changes of TACE and TNFR1 in the PDK1$^{K465E/K465E}$ mice neurons in response to Aβ. (A) Representative immunofluorescent labeling of TACE and TNFR1 (green) at the cell surface merged with the nuclei die Hoechst (blue) of PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) E15,5 hippocampal neurons at 6 days *in vitro* (DIV) after treatment or not with 5 µmol of amyloid-β peptides (Aβ) for 24 hours. Scale bar 30 and 40 µm, repectively. (B) Cell surface TACE and TNFR1 intensity of the staining were quantified and expressed as the mean ± SEM for 200 neurons from four different embryos per condition. Cortical cells from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) E15,5 embryos were grown for 6 DIV, then treated or not with 5 µmol Aβ in complete medium for 24 h, and then the TACE α-secretase activity (C) and the cell culture medium sTNFR1 levels (D) measured and expressed as the mean ± SEM from four different embryos per condition. * $p < 0.05$ compared with wild types as obtained by the Student' s *t* test.

7.3. THE PDK1$^{K465E/K465E}$ NEURONS ARE MORE RESISTANT TO TNFα-INDUCED DECREASE OF TACE ACTIVITY

TNFα is produced as a 22 kDa membrane-bound precursor that is cleaved by TACE to release the mature TNFα (Moss *et al*., 1997). TNFα has shown to trigger neuronal cell death by binding to TNFR1 (Gozzelino *et al*., 2008). To identify whether TNFα affects TACE activity, primary cultures of cortical neurons were treated with this cytokine. As shown in Figure 32B, similarly to Aβ, soluble TNFα also reduced TACE α-secretase activity more efficiently in the PDK1$^{+/+}$ than in the PDK1$^{K465E/K465E}$ neurons. As a consequence, the concentration of sTNFR1 released to the media was also higher in the mutant than in the wild type neurons (Fig. 31C). Furtherly, the intensity of TACE immunoreactivity at the neuron surface reduced by the treatment with TNFα in the in the PDK1$^{+/+}$ control neurons but not in the PDK1$^{K465E/K465E}$ mutant neurons; Moreover, the increase in the levels of TNFR1 on the cell surface elicited by TNFα in the wild type neurons was also inhibited by the PDK1 PH domain mutation in the mutant cortical neurons (Fig. 31A). Whilst the neuronal cell survival rate was the same between the PDK1$^{K465E/K465E}$ and PDK1$^{+/+}$ after treatment with TNFα, co-treatment with the protein synthesis inhibitor ActD to avoid the synthesis of antiapoptotic proteins elicited by TNFα revealed again an enhanced resistance of the mutant cells compared to the wild type controls (Fig. 31D).

Altogether, these data further suggest that the Akt branch of the PDK1 pathway seems to be responsible for the opposite effects on the UPR and the TACE activity induced by Aβ, which may result in neuroprotection against TNFα and Aβ-indiced toxicity.

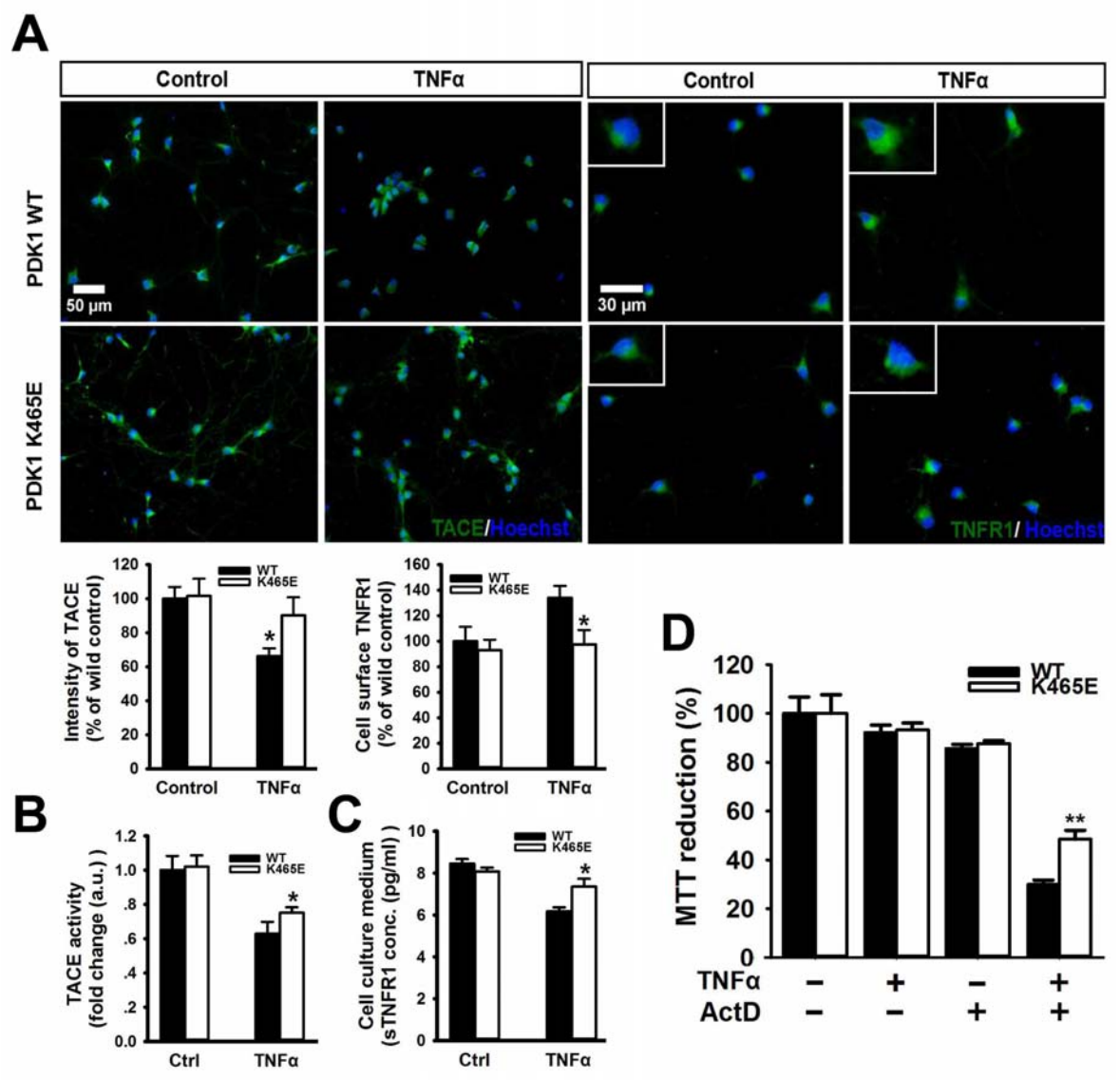


FIGURE 31. Levels of TACE and TNFR1 in the PDK1$^{K465E/K465E}$ mice neurons treated with TNFα. (A) Representative immunofluorescent labeling of TACE or TNFR1 at the cell surface (green) merged with the nuclei Hoechst staining (blue) of PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) E15,5 cortical neurons at 6 days *in vitro* (DIV) treated or not with 100 ng/ml of TNFα for 24 hours. Scale bar 50 µm and 30 µm, repectively. Cell surface TACE and TNFR1 intensity of the staining was quantified and expressed as the mean ± SEM for 200 neurons from four different embryos per condition. Cortical cells from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) E15,5 embryos were grown for 6 DIV and then treated or not with 100 ng/ml TNFα in complete medium for 24 h; TACE α-secretase activity (B) and sTNFR1 on the cell culture medium (C) were measured. (D) Cortical cells from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) E15,5 mice embryos were grown for 6 DIV and then treated with 100 ng/ml TNFα or 1 nM ActD in complete medium for 24h, as indicated. The MTT reduction was determined and represented as the mean ± SEM for at least five independent embryos per genotype from two separate experiments, with each sample assayed in triplicate.* $p <$ 0.05 and ** $p < 0.005$ compared with wild types as obtained by the Student' s *t* test.

8. CHARACTERIZATION OF CYTOKINES AND CHEMOKINES IN THE PDK1$^{K465E/K465E}$ MICE

A chronic inflammatory process has been implicated in the neuropathology of Alzheimer's diseaseinvolving microglia, astrocytes and various inflammatory mediators (cytokines and chemokines). Microglia, the resident immune cells of the brain, is constantly surveying the microenvironment under physiological conditions. In AD, deposition of Aβ peptide initiates a spectrum of cerebral neuroinflammation mediated by activated microglia. Activated microglia may play a potentially detrimental role by eliciting the expression of pro-inflammatory cytokines (Wang *et al*., 2015). Cytokines are heterogeneous protein mixtures of low molecular weight, which may act on the cells that secrete the proteins (autocrine action) or receptor cells (paracrine action)playing a fundamental role in the regulation of inflammatory processes. Chemokines are a superfamily of chemotactic cytokines that are essential for the activation and migration of leukocytes in both physiological and pathological contexts (Lee *et al*., 2009). Experimental and clinical evidence have demonstrated dysregulation of pro-inflammatory cytokines or chemokines in the AD. In addition, several studies suggest that sustained inflammation from the periphery can cause pro-inflammatory cytokines in the central nervous system by crossing the blood brain barrier and thereby contribute to the cognitive decline in AD patients (Holmes *et al*., 2009). As mentioned before, the PDK1$^{K465E/K465E}$ mutant mice showed increased brain TACE activity and lowered sTNFR1 levels, which may be markers for AD. In order to check out whether other inflammatory factors are changed in the PDK1$^{K465E/K465E}$ mutant mice, I measured cytokines and chemokines levels on serum from mutant and control mice at different ages.

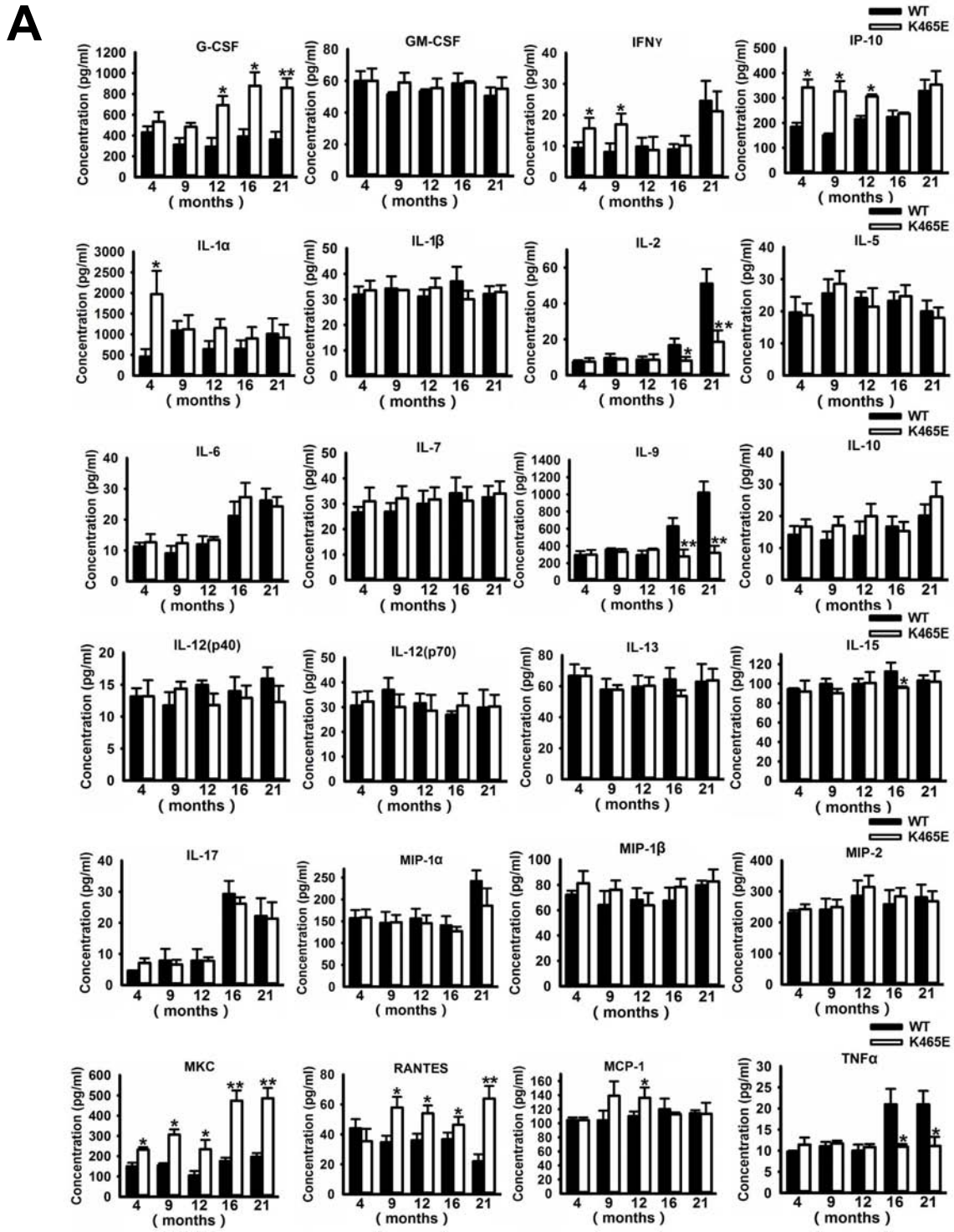
8.1. PERIPHERAL CYTOKINES AND CHEMOKINES IN THE PDK1$^{K465E/K465E}$ MUTANT MICE

The levels of a cytokine panel, consisting on proinflammatory and anti-inflammatory members, were measured in serum samples from PDK1$^{K465E/K465E}$ mutant and PDK1$^{+/+}$ wild type mice at different ages. IL-1α is a member of IL-1 family group, and can act as proinflammatory and anti-inflammatory; IL-1β is another IL-1 family member, belong to

the proinflammatory group (Teixeira *et al*., 2008). IL-2 is pro-inflammatory and plays a dual role in induction/suppression of immune responses via activation of conventional and regulatory T lymphocytes (Kariminik, 2016). IL-5 is an anti-inflammatory cytokine, which belong to the group of Th2 cytokines and may act as immunosuppressor. IL-6, a cytokine belonging to proinflammatory and anti-inflammatory group, mediates immune responses and inflammatory reactions affecting CNS (Qian *et al*., 2006). IL-7 is a pro-inflammatory factor (Bermejo *et al*., 2011). IL-9, IL-10 and IL-13 are potent anti-inflammatory cytokines in the CNS that could reduce inflammation during AD (Stoeck *et al*., 2014). The function of IL-12, which can both promote inflammation and inhibit the pathological process, relies largely on the expression and degradation of the different component proteins. IL-15 is a pro-inflammatory cytokine, and it exacts role in neuro-inflammation has been studied as a possible marker of AD (Yang *et al*., 2015). IL-17 is a pro-inflammatory cytokine produced by Th17 cells. The levels of IL-1β, IL-5, IL-7, IL-10, IL-12, IL-13 and IL-15 did not change between PDK1 $^{K465E/K465E}$ and PDK1$^{+/+}$ mice serum (Fig. 32A), although the serum levels of IL-1α were extremely elevated in the 4 months old PDK1 mutant mice compared with controls. The levels of IL-6 and IL-17 were increased with ageing to the same extent in both the wild type and the mutant mice serum. TNFα and IFNγ are special pro-inflammatory cytokines (Alvarez *et al*., 1996), involved in the neuroinflammatory related to the progression of neurodegeneration. I found significantly elevated levels of IFNγ in the serum of PDK1$^{K465E/K465E}$ adult mice compared with controls (Fig. 32A), while it was recovered to normal levels in the old mice. However, the concentration of TNFα was increased dependently on ageing in the wild type control, but not in the PDK1$^{K465E/K465E}$ mutant, which were extremely reduced compared to the same age controls (Fig. 32A).

G-CSF is a neurotrophic factor increasing neuroplasticity and suppressing apoptosis (Lu and Xiao, 2007; Minnerup *et al*., 2008). GM-CSF induces programmed cell death in the brain tissue of patients with dementia. Serum G-CSF was significantly higher from 12 months up to 21 months in the PDK1$^{K465E/K465E}$ mice compared to the same age wild type control (Fig. 32A). The levels of GM-CSF were not changed in both of the two genotypes analyzed. IP-10 belonging to the CXC chemokine subfamily is constitutively expressed and up-regulated in AD neurons. Serum IP-10 levels were increased with ageing and there

were significant differences before 12 months between PDK1$^{K465E/K465E}$ and PDK1$^{+/+}$ mice. MIP-1 could be involved in the regulation of the inflammation process of AD; MCP-1 is stimulated by Aβ



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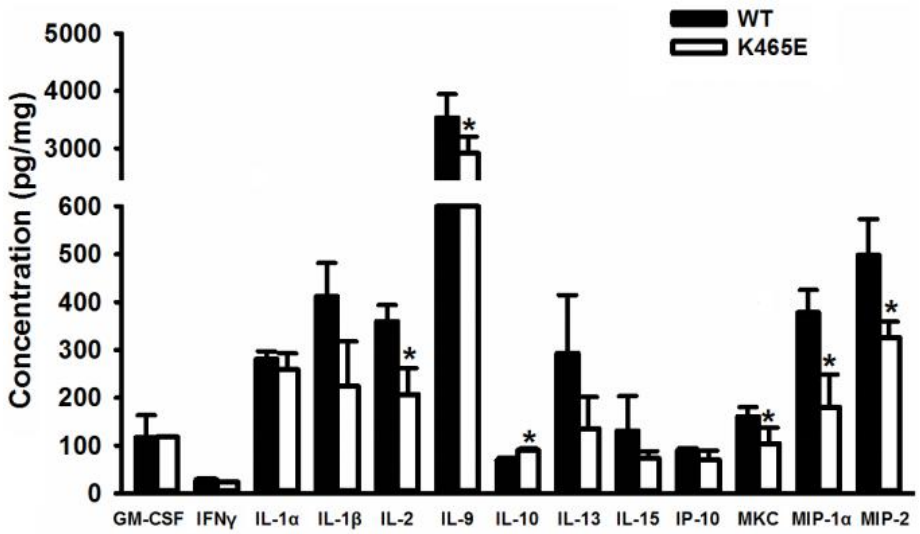

FIGURE 32. Characterization of cytokines and chemokines in the PDK1$^{K465E/K465E}$ mice. The concentration of 24 different cytokines and chemokines were measured by using multiplex methodology on serum (A) or brain (B) samples from PDK1$^{+/+}$ wild type (WT) and PDK1$^{K465E/K465E}$ mutant (K465E) mice at the indicated age in months The data are represented as the mean ± SEM for at least three different mice per genotype. * $p < 0.05$ and ** $p < 0.005$ compared with wild types as obtained by the Student' s t test.

and by inflammatory factors. As shown in Figure 32A, the levels of MIP-1α, MIP-1β, MIP-2 and MCP-1 were not difference between PDK1$^{K465E/K465E}$ and PDK1$^{+/+}$ mice. RANTES plays an important role in neuroprotection (Tripathy *et al*., 2008), the levels of serum RANTES were significantly increased in PDK1$^{K465E/K465E}$ mice compared with PDK1$^{+/+}$ control.

8.2. BRAIN CYTOKINES AND CHEMOKINES IN THE PDK1 $^{K465E/K465E}$ MUTANT MICE

In brain tissues, the data revealed significantly decreased levels of cytokine IL-2 and IL-9, chemokine MKC (Puig *et al*., 2012), MIP-1α and MIP2 in the PDK1$^{K465E/K465E}$ mice compared with PDK1$^{+/+}$ control. In contrast, the levels of IL-10 were higher in the PDK1 mutant mice than in the control. The concentration of IL-1α, IL-1β, IL-5, IL-6, IL-7, IL-12, IL-13 IL-15, IL-17, G-CSF, IFN-γ, MCP-1,IP-10, RANTES and TNF-α was either not

measurable or not significantly changed in the brain of PDK1$^{K465E/K465E}$ and PDK1$^{+/+}$ mice (Fig. 32B).

DISCUSSION

The present study has examined the neuroprotective contribution of the PDK1 signaling network to Alzheimer's Diseases and ageing. The findings derived from the data in this work show that PDK1 activity is increased in Alzheimer's Disease mice models. The Akt branch of the PDK1 pathway seems to be necessary in Aβ mediated AD pathology. Indeed, Akt substrates like GSK3β and FOXO were also responsible for PDK1-mediated pathology in an age-dependent manner. Collectively, this study suggests that PDK1 autophosphorylation at Ser241 is responsible for PDK1 enzymatic activity and Akt hyperactivation in Alzheimer's Disease model mice.

It is also shown that the phosphorylation of Akt at the Thr308 residue, the PDK1 site, was reduced in the $PDK1^{K465E/K465E}$ brain tissues compared with the $PDK1^{+/+}$ wild type brain, whereas the phosphorylation of Akt at Ser473, the mTORC2 site, was not affected by the PDK1 mutation. This is in accordance with the fact that BDNF-induced activation of Akt is partially reduced in the $PDK1^{K465E/K465E}$ mice embryonic cortical neurons (Zurashvili *et al*., 2013).

This study also suggests that the Akt branch of the PDK1 pathway seems to be responsible for age-induced TACE activity downregulation. TACE activity was reduced by the Aβ peptide, which may be caused by phosphorylation of the C-terminal cytoplasmic tail of TACE altering its trafficking and internalization, since the sheddase activity of TACE depends on its localization at the plasma membrane. Indeed, PDK1 inhibition in AD model mice increases TACE-mediated α-secretase processing and reduces Aβ production, whilst in the $PDK1^{K465E/K465E}$ mutant mice, the restricted phosphorylation of Akt at the PDK1 site increased TACE activity and thereby antagonized the effect of aging.

AD is characterized by the presence of amyloid plaques and neurofibrillary tangles, which can disrupt many of the cellular processes controlled by the ER, causing stress that contributes and exacerbates neuronal cell death. In the $PDK1^{K465E/K465E}$ mice, the phosphorylation of PERK at Thr980 and eIF2α at Ser51 were decreased in the old mice compared with the same age $PDK1^{+/+}$ controls, whereas other UPR elements including BIP, IRE1α, PDI and CHOP were unaffected. Since the PDK1 K465E knock-in mutation within

the PH-domain results in inefficient binding of PDK1 to PtdIns(3,4,5)P_3 and compromise the activation of Akt by phosphorylation at Thr308, Akt seems to play an important role in the regulation of the UPR.

1. MUTATION OF THE PDK1 PH-DOMAIN IMPAIRS AKT ACTIVATION IN BRAIN

PDK1 is a serine-threonine protein kinase of 556 amino acids that comprises an N-terminal catalytic domain, a C-terminal PH domain and a nuclear export sequence (Fyffe *et al*., 2013). PDK1 acts as the upstream activator phosphorylating a specific Thr or Ser residue located at the T-loop of the kinase domain of at least 23 members of the AGC family of kinases (Bayascas, 2010). Activation of Akt is dependent on prior activation of PI3K and generation of the second messenger PtdIns(3,4,5)P_3 (Alessi *et al*., 1997a). PDK1 is localized at the plasma membrane due to the interaction of its PH domain with phosphoinositides and exhibits the highest affinity to the PtdIns(3,4,5)P_3. Binding of the Akt PH-domain to PtdIns(3,4,5)P_3 promotes the translocation of Akt to the plasma membrane and induces a conformational change that allow the phosphorylation of Akt at its residue threonine 308 by PDK1 (Currie *et al*., 1999). The PDK1 K465E knock-in mutation was enginered by replacing Lysine 465 by Glutamic acid within the PDK1 PH domain in order to disrupt the conformation of the lipid-binding pocket and abrogate the ability of PDK1 to interact with PtdIns(3,4,5)P_3; as a consequence, it was expected that the Akt phosphorylation at Thr308 in the absence of PDK1-PtdIns(3,4,5)P_3 interaction was going to be completely ruled out (McMannus *et al*., 2004). Although in the PDK1$^{K465E/K465E}$ homozygous mice Akt phosphorylation at Thr308 and its activity was lower fivefold level than in control in insulin-sensitive tissues after injected insulin (Bayascas *et al*., 2008), BDNF-induced activation of Akt and phosphorylation at the Thr308 was partially reduced in the PDK1$^{K465E/K465E}$ mice embryonic cortical neurons by only two-fold than the PDK1$^{+/+}$ neurons (Zurashvili *et al*., 2013). These studies support the notion that binding of PDK1 to phosphoinositides is necessary for optimal activation of Akt.

The phosphorylation of Akt at the Thr308 residue, the PDK1 site, was also reduced in the

PDK1$^{K465E/K465E}$ brain tissues compared with the PDK1$^{+/+}$ wild type brain; the phosphorylation of Akt at Ser473, the mTORC2 site, was not affected by the PDK1 mutation. Quantification of the immunoblot signals shows that the maximal difference in Akt activation between PDK1$^{+/+}$ and PDK1$^{K465E/K465E}$ brain was achieved the age of 4 months, but this difference in the levels of phosphorylation of Akt at Thr308 was attenuated below two fold both in the cortex and hippocampus as the mice were ageing, and almost recovered to normal levels at 21 months of age in the hippocampal tissues (Fig. 15B). The findings in this study suggest that the interaction of PDK1 with phosphoinositides in the PDK1$^{K465E/K465E}$ mutant mice brain just avoided partially Akt activation in a tissue specific-manner. This data could be explained if a pool of PDK1 protein exists at the plasma membrane, which is not dependent on the phosphoinositides binding neither on agonist stimulation that could be phosphorylating Akt at the T-loop, since in PDK1$^{K465E/K465E}$ brain PDK1 catalytic activity is totally preserved (Bayascas *et al*., 2008).

Actually, BDNF-mediated Akt activation was time and dose-dependent in the mutant PDK1$^{K465E/K465E}$ primary cortical neurons; the reduction in the phosphorylation of Akt at Thr308 reached the maximal point after 5 min of BDNF stimulation, and it was mostly recovered after 30 min (Zurashvili *et al*., 2013). Indeed, the concentration of cortical and hippocampal BDNF levels is increased in the older PDK1$^{K465E/K465E}$ mice compared with PDK1$^{+/+}$ wild type mice, which can compensate for the defects of the Akt signaling pathway (Fig. 16). BDNF plays a neuroprotective role and the concentration of BDNF was decreased with aging in the cortex of PDK1$^{+/+}$ wild type, while the levels of BDNF were maintained in the PDK1$^{K465E/K465E}$ old mice cortex and hippocampus compared with PDK1$^{+/+}$ wild type brain tissues. As the content of Aβ is increased with aging, and BDNF seems to be sensitive to aging, it might be possible that Aβ activates the production of more BDNF in the PDK1$^{K465E/K465E}$ cortical neurons and brain. I am led to this hypothesis as the phosphorylation of Akt at Thr308 upon Aβ stimulation is more increased in a time-dependent manner in the PDK1$^{K465E/K465E}$ cortical neurons compared with the PDK1$^{+/+}$ wild type neuronal cells (Fig. 29A).

In relation with this finding, Najafov and co-workers proposed that Akt can be activated by either the PtdIns(3,4,5)P_3 binding or PIF-pocket-dependent pathways, this dual mechanism presumably enables Akt to be efficiently activated in response to agonists, causing resistance to anticancer PDK1 inhibitors (Fig. 33). The GSK2334470 PDK1 inhibitor obviously reduced the phosphorylation of Akt at Thr308 in the PDK1$^{K465E/K465E}$ cortical neurons in a dose-dependent manner compared with PDK1$^{+/+}$ wild type controls (data not show). The reduced Akt activation was still observed in the PDK1$^{K465E/K465E}$ cells that are dependent on the binding of the PDK1 PIF-pocket to Akt when phosphorylated at Ser473 (Najafov *et al*., 2012). Recently, the treatment of PDK1$^{K465E/K465E}$ knock-in mice neurons with the mTORC2 inhibitor AZD8055 to reduce phosphorylation of Akt at Ser473 totally inhibited the phosphorylation of Akt at Thr308. The authors proposed an interesting model for the evolution of the Akt activating mechanisms. In ancestral eukaryotes possessing a PDK1 protein lacking PH domain or in the PDK1$^{K465E/K465E}$ mice, the PIF-pocket dependent activation of Akt was sufficient to guarantee essential functions. In evolved animals in which PDK1 possesses a functional PH domain or in PDK1$^{+/+}$ cells, the mutual binding of both PDK1 and Akt to PtdIns(3,4,5)P_3 dramatically increases the rate of Akt activation allowing the adquisition of novel functions (Zhou *et al*., 2014). The current data suggest that the existing phosphorylation of Akt at Thr308 in the PDK1$^{K465E/K465E}$ mice brain in the absence of PDK1-PtdIns(3,4,5)P_3 binding is likely to be dependent on the PDK1 PIF pocket mechanism. This kind of mechanism of phosphorylation of Akt at Thr308 is much more potent in brain than other tissues as BDNF/TrkB signal pathway exists. Indeed, PDK1 strongly binds to the PIF peptide (PDK1 interacting fragment), and this interaction was much more enhanced in the PDK1$^{K465E/K465E}$ mice brain than in the PDK1$^{+/+}$ wild type cells (Fig. 25). This further explain how the phosphorylation of Akt at Thr308 still can be efficiently achieved due to the binding of PDK1 to the phospho-Ser373 docking site in the PDK1$^{K465E/K465E}$ mice brain.

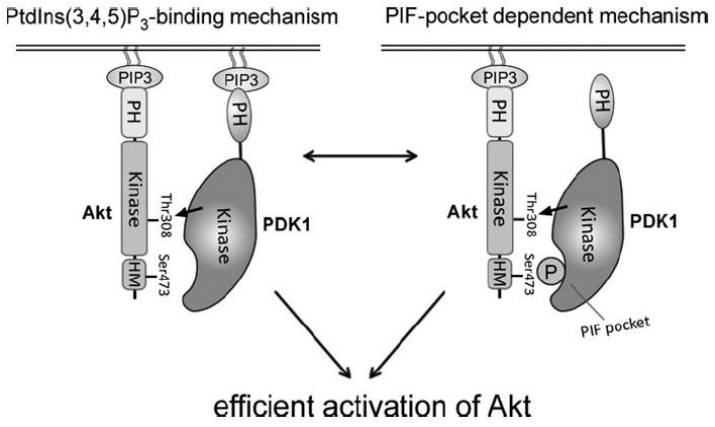

FIGURE 34. Two alternative PtdIns(3,4,5)P_3 binding and PIF pocket-dependent mechanisms enabling Akt to be efficiently activated, which account for the resistence to PDK1 K465E knock-in mutation in neurons. (Najafov *et al*., 2012)

Akt activation involves its recruitment and binding to plasma membrane, in which PDK1 need to phosphorylate Akt at Thr308, this happens only when the PH domain of Akt binds to PtdIns(3,4,5)P_3. Some study show that membrane localization of PDK1 can also be regulated by reversible acetylation; PDK1 is acetylated at lysine residues within the PH domain to prevent its membrane localization as well as activation; SIRT1 deacetylase expression increased the phosphorylation of PDK1 at Ser241 in the membrane fraction (Nagalingam *et al.*, 2012). PDK1 acetylation was found increased in the PDK1$^{K465E/K465E}$ brain lysates compared to the control samples (Fig. 25), since the acetylation of the PH-domain inhibits the ability of PDK1 to bind PtdIns(3,4,5)P_3 in the membrane. Akt activation is dependent on the binding of PDK1 to membrane PtdIns(3,4,5)P_3 and lysine acetylation of the PDK1 PH-domain in the PDK1$^{K465E/K465E}$ mice. Quantification of the immunoblot signals showed that the trend lowered in the 21-month-old mice. Growth factor stimulation deacetylates and activates Akt (Nagalingam *et al.*, 2012), PDK1$^{K465E/K465E}$ mice exhibited markedly enhanced BDNF levels at 21 month brain (Fig. 16). I propose that BDNF may promote SIRT1 deacetylates function and increase membrane localization of PDK1 in the PDK1$^{K465E/K465E}$ old mice brain even in the absence of PtdIns(3,4,5)P_3 binding.

Similar to Akt, the phosphorylation and inhibition of PRAS40 and TSC2 are limited due to the reduction of Akt activation in the PDK1$^{K465E/K465E}$ adult brain, which results in incomplete activation of mTORC1. Akt also acts as an upstream kinase for S6K and ULK1 since Akt activates mTORC1 by inhibiting the TSC2, which negatively regulates the

mTORC1 activator Rheb (Inoki *et al*., 2002). Active mTORC1 phosphorylates S6K on Thr389 on its hydrophobic motif, thereby creating a docking site for PDK1 and allowing the phosphorylation of the S6K at the Thr229 residue in the activation loop by PDK1. However, the reduced PRAS40 and TSC2 activation did not lead to impaired phosphorylation of S6K and ULK1 in the $PDK1^{K465E/K465E}$ adult brain (Fig. 17). The deficient Akt activity leads to decreased S6K phosphorylation within the T-loop, but this effect was transient and only detected 5 min after stimulation with BDNF in the $PDK1^{K465E/K465E}$ neuronal cells (Zurashvili *et al*., 2013). These findings demonstrate that the non PH-domain dependent substrate of PDK1 S6K is not affected by the PDK1 PH domain mutation, at least in adult brain tissues.

2. IMPACT OF THE PDK1 K465E KNOCK-IN MUTANT ON TACE ACTIVITY AND sTNFR1

In Alzheimer's disease (AD) patients, TACE α-secretase activity is reduced and lower amounts of sAPPα are found in the cerebrospinal fluid (CSF) (Sennvik *et al*., 2000); moreover, pharmacological inhibition of PDK1 activity with the BX912 compound increased TACE-mediated α-secretase activity and reduced AD pathology and memory impairment in AD models mice (Pietri *et al.*, 2013). One of the main targets of this study was to test the hypothesis that inhibiting the binding of PDK1 to phosphoinositides and thereby the Akt signaling pathway is useful against AD.

There was no statistically notable difference in total TACE and TNFR1 protein levels between $PDK1^{+/+}$ and $PDK1^{K465E/K465E}$ mice at any age in cortex and hippocampus (Fig. 18). Furthermore, the PDK1 inhibitor did not affect total TACE expression in the cortical neuronal cells (Fig. 22B); TACE and TNFR1 expression were also significantly unchanged in the two AD mice models tested (Fig. 23; 24).

Some study showed that in the brain of AD mice and in CSF of AD patients TACE activity was significantly reduced (Yao *et al*., 2015); moreovern AD patients the concentration of

sTNFR1 was 90% decreased in the CSF compared to age-matched normal subjects (Pietri *et al.*, 2013). In accordance with these observations, I found that the TACE α-secretase activity and the levels of sTNFR1 were decreasing with ageing in the $PDK1^{+/+}$ control mice, and they were significantly higher at 16 and 21 months of age in the $PDK1^{K465E/K465E}$ mice brain when compared to age-matched controls (Fig. 19). Also, TACE α-secretase activity and the concentration of sTNFR1 were not extremely difference between the $PDK1^{K465E/K465E}$ mutant and $PDK1^{+/+}$ wild type mice before 12 months of age. Using TNFR1 shedding as readout of TACE activity, remarkably, the TACE α-secretase activity and the concentration of sTNFR1 were significantly higher in the $PDK1^{K465E/K465E}$ neuronal cells treated with Aβ or TNFα compared with the $PDK1^{+/+}$ wild type cells (Fig. 30C; 31A). Thus, these evidences show that the inhibition of the PDK1-PtdIns(3,4,5)P_3 binding impedes the reduction of TACE activity induced by Aβ or other inflammatory factors.

Because blood-based biomarkers could provide a cost- and time-effective diagnosis compared to CSF and brain, it was necessary to test serum sTNFR1. There was not significantly different between wild type and mutant samples before 12 months of age; at 16 and 21 months, the concentration of sTNFR1 exhibited a tendency to increase in the two genotypes analyzed, but the sTNFR1 levels were drastically lower in the $PDK1^{K465E/K465E}$ than in the $PDK1^{+/+}$ old mice (Fig. 20), which was opposite to the brain results. Some studies show that sTNFR1 levels are significantly higher in the AD patients' serum or plasma than controls (Faric *et al.*, 2014; Diniz *et al.*, 2010). Accumulating evidence suggests that inflammatory mechanisms are an early event and prime mover in the development of AD; TNFα is one of the major inflammatory cytokines produced by activated astrocytes and microglia (Zhao *et al.*, 2003). In addition, peripheral inflammation and TNFα increased expression correlate with poorer cognitive performance (Rafnsson *et al.*, 2007). So the serum sTNFR1 levels rely on the TNFα induction by the inflammatory activity in the periphery, since the levels of TNFα in serum tend to be increased (Buchhave *et al.*, 2010); moreover, the brain TNFα promotes inflammatory responses into periphery via blood-brain barrier, leading to increased levels of serum sTNFR1. Interestingly, TACE activity is significantly higher in both MCI and AD subjects compared with healthy control,

which is associations with CSF T-tau and Aβ42 levels (Sun *et al*., 2014). Some results identified sTNFR1 from a set of 25 serum inflammatory markers as a singular marker with best discriminatory performance between AD patients and healthy controls (Laske *et al*., 2013). Altogether, considering that the expression of TNFRs and their conversion into soluble forms is a complex multistage process that not solely depends on TNFα activity, but also other inflammatory mechanismsthis work data suggest that sTNFR1 could be considered as independent inflammatory marker. This study shows that levels of brain TACE activity and sTNFR1 were increased, as well as the concentration of serum sTNFR1 was reduced, in the $PDK1^{K465E/K465E}$ mutant mice; these processes were therefore dependent on the association of PDK1 with $PtdIns(3,4,5)P_3$ and Akt activation.

Cell surface TNFR1 staining was associated with reduction in the concentration of sTNFR1; TACE is primarily known for its role in mediating the shedding of cell surface sTNFR1. PDK1 activity is increased in the AD old mice neurons and the amount of TNFR1 at the cell surface is increased with the age, which is dependent on TACE trafficking induced by phosphorylation at residue Thr735. PDK1 inhibition with BX912 or silencing with PDK1 siRNA in 3xTg-AD neurons increased the concentration of sTNFR1 (Pietri *et al.*, 2013). In agreement with this previous observation, TACE staining was increased and TNFR1 accumulation was reduced in the cortex of $PDK1^{K465E/K465E}$ old mice brain compared to control litermates (Fig. 22). Using the fraction membrane of brain tissues to analyse TACE and TNFR1 localization, TACE presence was increased and unshedded TNFR1 protein was reduced in the membrane of $PDK1^{K465E/K465E}$ old mice compared to $PDK1^{+/+}$ wild type mice brain. Moreover, the phosphorylation of TACE at Thr735 was reduced in the $PDK1^{K465E/K465E}$ old mice brain (Fig. 21A). These data altogether suggest that the PDK1 PH-domain mutant could be attenuating the Aβ peptide-induced phosphorylation of TACE and therefore ther TACE recycling out from the plasma membrane. In order to further certify that Aβ and TNFα affect the dynamic changes of TACE and TNFR1, it was shown that cell surface TACE immunostaining intensity was higher and TNFR1 was lower in the PDK1 PH-domain mutant neurons than in wild type

ones after treatment with Aβ or TNFα (Fig 30 and 31). As described before, these changes were associated with TACE α-secretase activity.

In the PDK1 PH domain, Lys465 is the central residue conforming the phosphoinositide-binding pocket, and its mutation to Glu prevents the association of PDK1 with PtdIns(3,4,5)P_3, which attenuates Akt phosphorylation. As mentioned before, in cortical and hippocampal mutant tissues, Akt phosphorylation is recovered with the age presumably due to increased BDNF. On the other hand, TACE activity increases appear after 16 months in the PDK1$^{K465E/K465E}$ mice. Moreover, PDK1 inhibition with BX912 or silencing with siRNA increased the TACE membrane levels and the concentration of sTNFR1 in 3xTg-AD mice (Pietri *et al.*, 2013). Taken together all the above findings, they raise the possibility of Akt playing a major role in TACE activity induced by Aβ and TNFα, and therefore I postulate that inhibition of Akt resulting from PDK1 inhibition with BX912 or silencing with siRNA might have protected mice against Alzheimer's disease, whereas inhibition of the docking-site dependent PDK1 substrates might be responsible for the toxicity of the BX912 treatment reported by Pietri et al (Pietri el al 2013).

3. PDK1/AKT SIGNAL PATHWAY IS INCREASED IN ALZHEIMER'S DISEASE MODEL MICE

PDK1 is the master regulator of at least 23 other serine/threonine kinasesof the AGC family whose downstream signaling has often been implicated in various diseases. PDK1 phosphorylates Akt on the T-loop that is crucial for its activity. The activation of Akt by PDK1 has been shown to critically depend on the translocation of both proteins to the plasma membrane, perhaps on a PDK1-Akt heterodimer complex (Calleja *et al*., 2007). PDK1 is constitutively active and it is responsible for regulating its own phosphorylation and activation. PDK1 activity is increased in human AD whole brains and in primary cultures derived from adult AD model mice (Pietri *et al.*, 2013), which relied on Src-mediated signaling and triggered in turn the phosphorylation and membrane depletion of TACE. Furthermore, PDK1 requires phosphorylation of Ser241 of its activation loop for

catalytic activity, and some evidences suggest that PDK1 catalyzes autophosphorylation of this site (Scheid *et al*., 2005). One of the main goals of this study was to test the hypothesis that the consequences of PDK1 hyperactivation in AD are mediated by the PDK1-Akt signalling branch. Two classic Alzheimer's disease model mice were used to investigate several PDK1 signalling markers.

The phosphorylation of PDK1 at Ser241 was increased with aging both in the APP/PS1 and the 3xTg-AD mice cortex and hippocampus, and the phosphorylation of its substrate Akt at Thr308, was also increased similarly to PDK1 (Fig. 23 and 24). Also, these data suggest that the increased PDK1 activity arise from PDK1 antophosphorylation at Ser241, at least in old AD mice. Actually, autophosphorylation of PDK1 Ser241 is critical but not sufficient to induce PDK1 full activity (Gao *et al*., 2006). This process is depending on the cell type and signaling pathways converging on PDK1, and additional phosphorylations on Ser, Thr and Tyr residues located in the kinase domain, the pleckstrin-homology (PH) domain and in the linker between the kinase and PH domains have already been shown to enhance PDK1 activity in a more or less cooperative manner (Li *et al*., 2010). For example, in the presence of insulin, PDK1 can autophosphorylates itself on Ser241 and, at the same time, PDK1 is phosphorylated on Tyr-9 and Tyr-373 with the help of Src and heat shock protein 90 (Hsp90), which further increase PDK1 catalytic activity (Li *et al*., 2010). AKT isoforms are among the different PDK1-activated kinases the only ones possessing PH domains, a PtdIns(3,4,5)P_3 binding domain that is also present in the PDK1 protein. The binding of the Akt PH domain to PtdIns(3,4,5)P_3 becomes rate-limiting for the translocation of Akt to the plasma membrane and co-localization with PDK1, where PDK1 can then activate Akt by phosphorylating the Thr308 T-loop site (Bayascas, 2010). Furthermore, phosphorylation of Akt at Ser473, the mTORC2 site, was also obviously increased in old AD mice. As detailed in the introduction, mTORC2, which is insensitive to rapamycin, controls cellular shape by modulating actin function and contains mTOR, rictor, Mlst8, and Sin1 (Wullschleger *et al*., 2006). Recently, a study has described that Aβ induces mTORC1 hyperactivity in 3xTg-AD mice (Caccamo *et al*., 2011). Whether Aβ could induce also mTORC2 hyperactivity in 3xTg-AD mice and thereby potentiate the phosphorylation of Akt at Ser473 is an open question. PRAS40 is a mTORC1 regulatory

protein which inhibits mTOR by directly binding to it. When PRAS40 is phosphorylated at Thr-246, it detaches from mTORC1, thereby facilitating mTORC1 activity (Sancak *et al*., 2007). mTORC1 hyperactivity is mediated by PRAS40 in AD (Caccamo *et al*., 2011). Indeed, PRAS40 phosphorylation at Thr-246 was increased both in APP/PS1 and 3xTg-AD old mice (Fig. 23 and 24). Thus, taken together, these data indicate that Aβ accumulation increases the activity of the PI3K/PDK1/Akt pathway, leading to PRAS40 phosphorylation and mTORC1 hyperactivity.

GSK3 is critical for the normal function of the central nervous system, where it regulates a variety of neuronal responses including protein synthesis. Tau is a cytosolic protein associated with microtubule dynamics; GSK3β induce tau hyperphosphorylation leading to the NFTs formation in AD. As expected, the phosphorylation of GSK3β at Ser9 was markedly increased in APP/PS1 and 3xTg-AD old mice (Fig. 23 and 24).

FOX family of transcription factors are inhibited by phosphorylation downstream of the PI3K signaling pathway. Overexpression of a constitutively active of FOXO promotes apoptosis in a number of cell types (Brunet *et al*., 1999). The phosphorylation of FOXO at Ser256 was also increased in both AD model mice (Fig. 23 and 24), which might protect cells against apoptosis and thereby suggest that other Akt substrates are also involve in Aβ-induced toxicity rather than FOXOs.

Altogether, these data strongly suggest that the Akt branch of the PDK1 pathway is also hyperactivated in Aβ-mediated AD pathology.

4. CONTRIBUTION OF THE PDK1-PHOSPHOINOSITIDE INTERACTION TO THE UPR SYSTEM

Another main objective of this study was to test the hypothesis that the inhibition of PDK1 binding to phosphoinositides leading to reduction of Akt activation had consequences for the UPR system. Firstly, using cortex derived from $PDK1^{+/+}$ and $PDK1^{K465E/K465E}$ mice at different ages to analyze different UPR markers, it was observed that phosphorylation of PERK at Thr980 and eIF2α at Ser51 were reduced in the old $PDK1^{K465E/K465E}$ mutant mice compared with the $PDK1^{+/+}$ wild type, while other markers were unchanged. Secondly, by inducing ER stress on primary cultures on cortical neurons, I found the number of

apoptotic cells induced by both tunicamycin and Aβ treatment attenuated in the PDK1$^{K465E/K465E}$ neurons compared with the PDK1$^{+/+}$ wild type controls, especially when they were used at highest doses. Thirdly, parallel studies on the UPR signalling pathway revealed that phosphorylation of PERK at Thr980 and eIF2α at Ser51 induced by tunicamycin and Aβ were significantly reduced in the PDK1$^{K465E/K465E}$ cortical neurons compared to PDK1$^{+/+}$ ones.

The UPR is a protective cellular response induced during periods of endoplasmic reticulum (ER) stress, which reduces unfolded protein load and return protein-folding homeostasis. Alterations in the intracellular environment that induce ER stress are thought to interrupt these processing steps, resulting in unfolded proteins in the ER (Thomas *et al*., 2015). AD is characterized by the presence of amyloid plaques and neurofibrillary tangles; many of the cellular processes controlled by the ER can be disrupted by the disease pathology, causing stress that contributes and exacerbates the neuronal cell death (Hetz *et al*., 2014). The cell main weapon against ER stress is the induction of the UPR. The levels of phosphorylation of both PERK at Thr980 and eIF2α at Ser51 were markedly increased in the cortex of the 12-months old 3xTg-AD mice, whereas the protein levels of other components of the UPR analyzed such as BIP, PDI, IRE1α or CHOP remained unchanged (Fig. 26). Phosphorylation of eIF2α is elevated in the brain of AD patients and model mice, and inhibitions of PERK prevent deficits in protein synthesis, synaptic plasticity and spatial memory in APP/PS1 mice (Ma *et al.,* 2013). In AD, in response to some cellular stressors, especial Aβ, PERK phosphorylates eIF2α leading to inhibition of general mRNA translation and the decrease of the rate of general protein synthesis allows cells to conserve energy resources; at the same time. It enhances the translation of selected mRNAs coding stress-related proteins, thereby reconfiguring gene expression to manage the stress condition (Paschen *et al*., 2007). PERK-eIF2α phosphorylation state is important in maintaining long-lasting forms of synaptic plasticity and long-term memory (Wek *et al*., 2007).

The phosphorylation of PERK and eIF2α were not statistically different between PDK1$^{K465E/K465E}$ and PDK1$^{+/+}$ cortex tissues before 12 months, but from 16 months the phosphorylation state begun to change (Fig. 27). A study showed that PERK was a

substrate of Akt, thus implicating a regulation of eIF2α phosphorylation by Akt signaling in response to stress (Mounir *et al*., 2011). In the PDK1$^{K465E/K465E}$ mice, the inefficient binding of PDK1 to PtdIns(3,4,5)P_3 leading to reduced phosphorylation of Akt at Thr308 and hypoactivation of the downstream pathway seems to play an important role in attenuating the UPR in an age-dependent manner. These observations point out to an Akt-dependent activation of the PERK/eIF2α pathway with aging. When mice are young, ER stress is normal, a balance exists for unfolded and dissociated proteins until the unfolded protein backlog is removed, phosphorylation of PERK and eIF2α is considered to be protective. In older ages, on one hand, more and more amyloid-β is accumulated during the process (Alaina *et al*., 2015). On the other hand, abnormally high levels of reactive oxygen (ROS) are constantly generated caused by the endogenous antioxidant mechanisms (Lin and Beal, 2006). In response to Aβ and high ROS, ER stress is enhanced and protein refolding is promoted, making the dysregulation of protein synthesis more pronounced and long lasting. This unbalance induces over-activation of PERK, which induces eIF2α hyperphosphorylation. However, in the old PDK1$^{K465E/K465E}$ mice the phosphorylation of PERK and eIF2α were attenuated because of the deficient activation of Akt. In agreement with this observations, using cultured primary neurons treated with the ER stressor tunicamycin or with Aβ, the phosphorylation of PERK and eIF2α was reduced in the PDK1$^{K465E/K465E}$ cortical neurons compared to PDK1$^{+/+}$ wild type cells (Fig. 28B and Fig. 29B). The present study clearly demonstrate a reduced phosphorylation of PERK and eIF2α after stress due to the deficient Akt activation determined by the lack of the binding of PDK1 to PtdIns(3,4,5)P_3.

Severe ER stress can lead to the activation of the apoptotic cell death through an intrinsic/mitochondrial pathway (Smith and Deshmukh, 2007). This study provides evidence that the defective Akt activation can reduce neuron death caused by stress like Aβ and tunicamycin. The PDK1$^{K465E/K465E}$ cortical cells exhibited higher viability compared with control at elevated doses of Aβ and tunicamycin (Fig. 28A and Fig. 29C). Furthermore, these results were corroborated when specific cell death nuclear markers characteristics of apoptosis were scored. It was previously shown in ther group that the PDK1$^{K465E/K465E}$ and PDK1$^{+/+}$ cortical cells exhibited normal survival responses to BDNF

after trophic factor deprivation, and suggested that other PDK1 substrates might play a major role in controlling neuronal survival rather than Akt, such as S6K, PKC, RSK and SGK1 (Zurashvili *et al*., 2013). Low doses of Aβ and tunicamycin caused neuronal death to the same extent in the two genotypes analyzed; active PI3K/Akt might have served as a general mediator of cell survival by phosphorylating and inactivating a number of cytoplasmic substrates both of the PDK1$^{K465E/K465E}$ and PDK1$^{+/+}$ cortical cells; with higher doses of Aβ or tunicamycin, ER stress was highly enhanced and cells needed to promote protein refolding, making the dysregulation of protein synthesis more pronounced and longer lasting, which cause higher neuronal death. The PDK1 PH domain mutant cells exhibited reducted sensitivity to the neuronal death induced by ER stressors due to its reduced Akt phosphorylation. As described before, cell death is dependent on the PERK-eIF2α pathway, and the reduction of phosphorylation of PERK and eIF2α allowed to recovery of the cell viability in the PDK1$^{K465E/K465E}$ neurons.

Taken together, all the above findings raise the possibility of the existence of a "positive feedback effect" of Akt on the PERK-eIF2α axis activation under an unbalance of the ER stress, which might be attenuated in the PDK1$^{K465E/K465E}$ mice leading to a reduction in the neuronal cell death response. It is worth mentioning that other markers of UPR seem not to be affected by this function.

5. CHARACTERIZATION OF CYTOKINES AND CHEMOKINES IN THE PDK1$^{K465E/K465E}$ KNOCK-IN MICE

Investigating immune markers in neurodegenerative diseases may be relevant for a better understanding of the role of inflammation in pathological processes related to age-related cognitive impairment. As mentioned before, in the PDK1$^{K465E/K465E}$ mutant mice brain TACE activity and sTNFR1 levels AD markers are increased. In order to investigate the potential relationship between Akt activity and inflammatory factors, serum and brain cytokines and chemokines levels were analysed at different ages, with different results derived from peripheral and central samples.

Several studies report that inflammatory mechanisms may be involved in the development of cognitive decline and pathophysiology of brain (Stoeck *et al*., 2014). In this study, I

observed a significantly increased level of proinflammatory cytokines (IL-2, IL-6, IL-17, TNFα and IFN-γ), as well as anti-inflammatory cytokines (IL-9, IL-10) and some chemokine (IP-10, MIP-1α) in the wild type mice serum with ageing; while others cytokines (IL-1, IL-5, IL-7, IL-12, IL-13 and IL-15) and chemokines (G-CSF, GM-CSF, MIP-1, MIP2, MKC, RANTES and MCP-1) were unchanged (Fig. 32A). Furthermore, the levels of IL-2, IL-9 and TNFα were extremely reduced in the serum of the $PDK1^{K465E/K465E}$ old mice compared with the corresponding wild type controls. IL-2 is pro-inflammatory and plays a dual role in induction/suppression of immune responses via activation of conventional and regulatory T lymphocytes (Kariminik, 2016); TNFα is produced by activated microglial cells, mainly in response to Aβ and oxidative stress (Lee *et al*., 2009). Dysregulation of TNFα production has been implicated in AD; although still controversial, higher serum TNFα concentrations have been also reported in AD patients (Bruunsgaard *et al*., 1999; Alvarez *et al*., 1996). In contrast, it has been report that TNFα concentrations are lower in serum from demented compared with healthy controls (Paganelli *et al*., 2002). Interestingly, the levels of sTNFR1were in-line with TNFα, which was increased in $PDK1^{K465E/K465E}$ old mice serum compared with wild type. Opposite to the $PDK1^{K465E/K465E}$ old mice, IL-9 levels are significantly augmented in AD patients (Dardalhon *et al*., 2008). Higher levels of IL-6, IL-10 and IL-17 have been observed as well in plasma of patients of AD (Qian *et al*., 2006; Bermejo *et al*., Saresella *et al*., 2011), whereas these seem unchanged between of $PDK1^{K465E/K465E}$ and $PDK1^{+/+}$ mice. It was surprising that the levels of IL-1 and IFN-γ were significantly increased in the $PDK1^{K465E/K465E}$ young mice compared with the wild type. IL-1 is secreted by reactive microglia and IFN-γ is produced by phagocytic cells, indicating an increased immune response in the PDK1 PH-domain mutant mice.

Chemokines are essential for the activation and migration of leucocytes, the proliferation and differentiation of progenitor cells as well as the regulation of the infiltration and activation of immune cells (Stuart and Baune, 2014). A significantly increased levels of certain chemokines (IP-10 and MIP-1) in the serum of the old wild type mice was observed, whereas the levels of G-CSF, MKC and RANTES were extremely increased in the $PDK1^{K465E/K465E}$ mice compared with the $PDK1^{+/+}$ wild type mice, suggesting that these

chemokines are relevant to ageing in relation with the PDK1 signalling pathway. G-CSF is a glycoprotein known to promote neuroprotection (Minnerup *et al*., 2008), and the increased G-CSF levels elicited by defective Akt activation may have been protective in the PDK1 mutant mice neurons. It was interesting that the MKC levels were upregulated in the $PDK1^{K465E/K465E}$ mice, indicating that mutation of PDK1 enhances infection (Puig *et al*., 2012; Hinojosa *et al*., 2012). RANTES has been found upregulated in the AD brains and has a neuroprotective role (Tripathy *et al*., 2008), whereas its levels were also significantly increased in the $PDK1^{K465E/K465E}$ mice serum, which look opposite with the brain result.

In brain the expression patterns are different from serum, and even the levels of certain cytokines such as G-CSF and TNFα were too low to be detected in brain. A number of cytokines (including IL-2 and IL-10) and chemokines (MKC, MIP-1 and MIP-2) were downregulated in the $PDK1^{K465E/K465E}$ mice brain. To sum up, this study suggests that there is a potential association between inflammatory markers and Akt activation, but further studies are needed.

CONCLUSIONS

CONCLUSIONS

1-In brain tissues, the phosphorylation of Akt at Thr308, the PDK1 site, is significantly reduced in the PDK1$^{K465E/K465E}$ mutant mice from 4 months and is then sustained for up to 21 months. The phosphorylation of Akt at Ser473, the mTORC2 site, is not affected at any age analyzed by the PDK1 mutation.

2-In cortex and hippocampus of the aged PDK1$^{K465E/K465E}$ mutant mice, increased levels of BDNF synthesis compensate for the defects of the Akt signaling pathway.

3-Phosphorylation of ULK1 and S6K are not affected in the PDK1$^{K465E/K465E}$ mutant brain.

4-The deficient activation of Akt results in increased TACE α-secretase activity, which is translated onto elevated concentration of sTNFR1 in the aged PDK1$^{K465E/K465E}$ knock-in mice cortex.

5-The levels of phosphorylation of TACE at Thr735 are significantly reduced in the PDK1$^{K465E/K465E}$ TACE immunoprecipitates compared to the controls. TACE protein levels are markedly increased in the 60000 g membrane fractions of the PDK1$^{K465E/K465E}$ 21-month old mice brain when compared to their corresponding controls, which is accompanied by a nearly absence of TNFR1 on those membrane fractions.

6- Phosphorylation of PDK1at Ser241, Akt at Thr308 and Ser473, and that of the Akt substrates PRAS40, GSK3β and FOXO1 are markedly increased in the APP/PS1 and the 3xTg-AD transgenic mice models in an age-dependent manner.

7-PDK1 pull down and acetylation are enhanced in the PDK1$^{K465E/K465E}$ mice brain compared to the wild type from 4 to 21 months of age.

8-Phosphorylation of PERK at Thr980 and its substrate eIF2α at Ser51 are decreased in the PDK1$^{K465E/K465E}$ old mice brain and cortical neurons treated with tunicamycin or amyloid β peptide compared to wild-type littermates. By contrast, other UPR markers including BIP, IRE1α, PDI and CHOP are unchanged.

9- The interaction of PDK1 with phosphoinositides is protecting against UPR-induced neuronal cell death and allows the PDK1$^{K465E/K465E}$ neurons to survive better when compared with PDK1$^{+/+}$ controls in response to high doses of tunicamycin or amyloid β.

10- The deficient Akt activition of the PDK1$^{K465E/K465E}$ neurons impedes the reduction of TACE α-secretase activity after treatment with soluble Aβ peptide or TNFα, and inhibits the internalization of TACE out of the cell surface. As a consequence, the PDK1$^{K465E/K465E}$ mutant mice neurons show reduced amounts of TNFR1 at the plasma membrane, and furtherly increased sTNFR1 levels secreted to the cell culture medium.

11- The concentration of inflammatory factors (cytokines and chemokines) derived from the PDK1$^{K465E/K465E}$ and the PDK1$^{+/+}$ mice peripheral and central samples present different but not conclusive profiles when comparing genotypes, ages and the origin of the samples.

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