UNIVERZITA KARLOVA V PRAZE

Farmaceutická fakulta v Hradci Králové

Katedra biologických a lékařských věd

LOKALIZACE JNK3 MAP-KINÁZY V MOZKU MYŠÍ POSTIŽENÝCH ALZHEIMEROVOU CHOROBOU

Diplomová práce

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Hradec Králové 2015 Lucie Kohoutová

CHARLES UNIVERSITY IN PRAGUE

Faculty of Pharmacy in Hradec Králové

Department of Biological and Medical Sciences

JNK3 MAP-KINASE LOCALIZATION IN THE BRAIN OF MICE WITH ALZHEIMER´S DISEASE

Diploma thesis

Supervisor: PharmDr. Jana Rathouská, Ph.D.

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13.4.2015 Lucie Kohoutová

Acknowledgement

This work was supported by scholarship grant from European Agency for International Students Exchange ERASMUS. Special thanks belong to the stuff of the Department of Pharmacology University of Navarra. They were always ready to help me with anything and made me feel like home even I was in a foreign country.

Abstrakt

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Student: Lucie Kohoutová

Školitel: PharmDr. Jana Rathouská, Ph.D.

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Název práce: Lokalizace JNK3 MAP-kinázy v mozku myší postižených Alzheimerovou chorobou

Cíl práce: Přesná patogeneze alzheimerovy choroby je doposud nejasná. Objevem c-Jun N-terminálních kináz (JNKs), majích mnoho funkcí zahrnujících regulaci genové exprese, zánětlivých procesů, buněčné proliferace a apoptotické buněčné smrti, se otevřely dveře k hlubšímu poznání mechanismů Alzheimerovy choroby (ACH). JNK3 je forma těchto kináz jedinečná pro mozek, hrající důležitou roli ve vývoji neurodegenerativních procesů, jelikož je spouštečem neuronální apoptozy. Jeho přesná lokalizace v mozku je doposud neznámá a její objasnění by mohlo pomoci najít cestu k novým možnostem léčby ACH. Pomocí knock-outu bylo dokázáno, že utišení jeho exprese vede přímo ke snížení hladin beta amyloidu (Aβ).

Metody: Při studiu exprese aktivní formy JNK3 proteinu byli použity transgenní myší modely s ACH typu Tg2576 ve srovnání s kontrolou bez ACH. Lokalizace této aktivní formy JNK3 proteinu byla prováděna na stejných transgenních modelech za použití imunohistochemie s detekcí protilátkami astroglií (GFAP), mikroglií (Cd11b), neuronů (NeuN), beta amyloidu (6E10) a tau proteinu (PHF11).

Výsledky: Fosforylovaná forma JNK3 proteinu (pJNK3), představující aktivovanou formu, byla prokazatelně zvýšená v mozku trangenních myší Tg2576 ve srovnání s kontrolou. Pomocí imunohistochemie jsme zjistili asociaci aktivní fosforylované formy JNK3 na periferii amyloidních plaků. Protein pJNK3 byl také částečně kolokalizován s nadměrně fosforylovaným tau proteinem uvnitř plaků. Nebyla pozorována žádná kolokalizace JNK3 s markery astrocytů ani neuronů. Předpokládaná asociace s

mikrogliemy nebyla potvrzena, ponevadž stanovení i přes inovaci převzaté metodiky nefungovalo.

Závěry: Vysoká aktivita JNK3 doprovázejícího amyloidní plaky stejně jako zvýšené hladiny tohoto proteinu vyskytující se při ACH nasvědčují tomu, že JNK3 hraje roli v neurodegeneraci související s ACH. Kvůli absenci jeho kolokalizace s jakýmkoliv typem mozkových buněk, otázka, jaké buňky jsou zodpovědny za sekreci této kinázy objevující se při zánětu, zůstává otevřená, stejně jako cesta k objevu nových metod léčby ACH pomocí utišení exprese JNK3 proteinu.

Abstract

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Title of diploma thesis: JNK3 MAP-kinase localization in the brain of mice with Alzheimer´s disease

Background: The pathogenesis of Alzheimer´s disease (AD) is incompletely understood. Discovery of c-Jun N-terminal kinases (JNKs), involved in regulation of gene expression, inflammation, cell proliferation and apoptosis, may contribute to better understanding of AD pathogenesis. JNK3 is a brain-specific JNK isoform involved in apoptosis of mammalian neurons. Precise localization of JNK3 in the brain still remains unclear. JNK3 silencing achieved by gene knockout had directly decreased beta amyloid (Aβ) levels in previous AD models in mice. We performed an immunohistological study to localize JNK3 gene in the mice brain in order to better focus future gene targeting therapy.

Methods: A transgenic mice model of AD (Tg2576) has been used to study the expression of JNK3 by western blotting compared to the wild type. In the same brains localization of pJNK3 in astroglia (GFAP), microglia (Ox42), neurons (NeuN), senile plaques (Aβ) or pTau, using immunohistochemistry staining has also been checked.

Results: Phosphorylated JNK3 levels (pJNK3), representing the activated form of JNK3 protein, were significantly increased in Tg2576 transgenic mice brains compared to normal controls. By immunohistochemistry we observed, that pJNK3 is associated with amyloid plaques on their periphery. pJNK was also partially associated with hyperphosphorylated tau in these plaques. There was no co-localization of pJNK3 with

GFAP or NeuN. Expected association with microglia wasn’t proved, because the stainings with Ox42 antibody didn´t work.

Conclusions: The existence of high JNK3 activity together with amyloid plaques, as well as increased levels of this protein during AD strongly suggests that JNK3 plays a role in Alzheimer-related neurodegeneration. Due to the lack of co-localization with any specific brain cell type, the question as to which cell type is responsible for the release of this inflammatory kinase still remains open, as does the path to discovering new AD treatment methods which silence the JNK3 gene.

Table of contents

1 Abreviations

Aβ	beta amyloid
AD	Alzheimer´s disease
AICD	amyloid precursor protein intracellular domain
AP-1	activator protein-1
APO-E-4	apolipoprotein E, alela 4
APP	amyloid precursor protein
BACE1	beta site amyloid precursor protein cleaving enzyme
BSA	bovine serum albumin
CcO	cytochrome-c oxidase
CD11b(Ox42)	cluster of differentiation molecule 11B
CDK5p35	cyklin-dependent kinase p35
c-JUN	transcription factor of JUN gene
c-FOS	transcription factor of FOS gene
ER	endoplasmatic reticulum
FAD	familiar alzheimer´s disease
GFAP	glial fibrillary acidic protein
GSK3	glycogen synthase kinase 3
hAPP	human amyloid precursor protein
IL-1	interleukin 1
IL-6	interleukin 6
IRS2	insulin receptor substrate 2
IFNγ	interferon gamma
JNK	c-Jun N-terminal kinase
LDL	low density lipoprotein
MAPKs	mitogen-activated protein kinases
NeuN	hexaribonucleotide Binding Protein-3, nuclear antigen

NFAT	nuclear factor of activated T-cells
NFTs	neurofibrilar tangles
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate receptor
OD	optical density
PBS	phosphate buffered saline
PHFs	paired helical filaments
pJNK	phosporylated form of JNK
PPAR	peroxisome proliferator-activated receptor
pTAU	phosphorylated form of tau
RAGE	receptor for advanced glycosylation
SAD	sporadic alzheimer´s disease
SEM	standart error of the mean
Tg	trangenic form
TNFα	tumor necrosis factor
Wt	wild type
XIAP	x-linked inhibitors of apoptosis

2 Introduction

The c-Jun N-terminal protein kinases (JNKs), members of MAP-kinases (MAPKs) were found to be crucial for regulation of brain cell differentiation and cell death program [1]. Briefly, JNKs are agents capable to activate cJun and cFos genes, which initiate apoptosis processing and promotes activation of a number of different pathways connected with intracellular stress [2]. More precisely, in response to cell stress and Aβ production, occurs a translational block in brains affected by AD. Due to this translational block, protein and lipid synthesis is generally decreased in AD brains. In response to that aggression, glucose uptake, β-oxidation and glycolysis must be increased to restore homeostasis to maintain ATP production. Consequently c-Jun pathway is activated by JNK3 in the neurons, which culminate in cell death via apoptosis [3].

Unfortunately these adaptive processes are insufficient to maintain normal brain function and structure resulting in a decreased brain volume (mostly cortical atrophy) and dementia. Alzheimer`s symptoms begin to be manifested.

The c-Jun pathway represents cellular ´´first emergency´´, following maintaining stress stimuli. In response, variable pathways connecting with protection and answer to chronic stressing are activated. This event describes profound mechanism of AD control effects of JNKs, which presents them to be strong prone to shape first answer responding to stress. This basic fact proves JNKs to have strong influence on AD processing. Accordingly, the c-Jun terminal pathway seems to take the most important influence on AD control. In summary JNK3, the less described type of JNKs, induce activation of c-Jun gene mediated neuronal apoptosis.

Recent studies has shown that JNK3 MAP-kinase plays an important role in inflammation that occurs in AD brains. Moreover, JNK3 knock-out results in a decrease of Aβ levels [3]. However, none of these studies answer the question concerning the exact localization and brain cell type responsible of the expression and activation of this kinase. JNK3 is being distinguished to others JNKs members by being the main isoform expressed in the brain, although it also can be found in lower levels in pancreas and testis [4]. JNK3 has a notorious effect in essential functions such as cell development and apoptosis but also in circadian rhythm [5]. However, JNK3 specific localization and function in the brain still remains unclear, therefore, the main aim of the present work is to find the specific cell type where JNK3 is expressed. Thus, the elucidation of JNK3

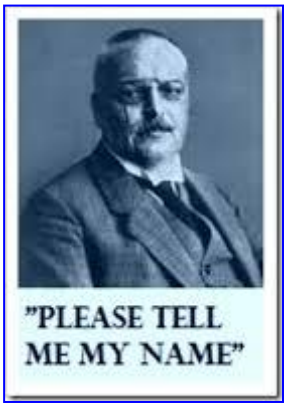
localization could be useful for the generation of new drugs directed to a specific brain cell type for the treatment of AD

.

3 Theoretical part

3.1 Alzheimer´s disease

Alzheimer´s disease was described as neuronal loss and degeneration by the german doctor Alois Alzheimer [Fig. 1] in 1906. Neuronal degeneration occurs mainly in the hippocampus and the cerebral cortex followed by a deterioration of cognitive functions, such as learning and memory. Other processes including chronic inflammation, decrease in number of synapses, oxidative stress or central insulin resistance have been also observed during the course of disease progression [6]. Nowadays it is listed as the third most frequent cause of death after cardiovascular diseases and tumors [7] and it accounts up to the 60% of all dementia cases proved by autopsy in economically advanced countries. In the year 2006 the World Health Organization estimated that 28 millions of people suffer AD in the world. Alzheimer's Association estimated that in 2012 there were 38 million cases of AD worldwide and that every four seconds a new patient was diagnosed with AD. AD prevalence increases with age and after 65 years its frequency doubles every 5 years. Recent studies estimate, that the prevalence rate would quadruple in 2050. The incidence of disease convey, that after 65 years of age one of twenty persons will become AD sufferer, and after 85 every one of four, that is five fold increase [8], [9].



Two diverse forms of this disease are differentiated: Familiar Alzheimer's Disease (FAD), which comprise 5-10% of all AD cases and is a hereditary form of AD and the Sporadic form (SAD) with about 90-95% occurrence. Sporadic form is associated with pathological aging, whereas FAD is caused by known familiarly transmitted genetic mutations. There exist main three stages: mild cognitive impairment, moderate and severe AD, separated from each other by amount of cognitive impairment.

Figure 1 Alois Alzheimer

Copy from: http://sprocketsinside.blogspot.cz/2012/02/dr-alzheimers-magic-bullet.html (13/1/2015)

3.1.1 Pathogenesis of AD

The AD is typically expressed by impairment of neurons and synapses mostly in hippocampus, amygdala and cerebral cortex. The cause of degenerative processes is incompletely understood. It induces reduction of cerebral volume and manifests as loss of ability to learn and impairment of memory and cognitive functions. The main marker responsible for pathological processing is Aβ located into senile plaques [Fig.2], which is physiological product of degradation of APP (beta amyloid precursor protein). There are normally produced soluble monomers of Aβ consisting of 40 amino acids.

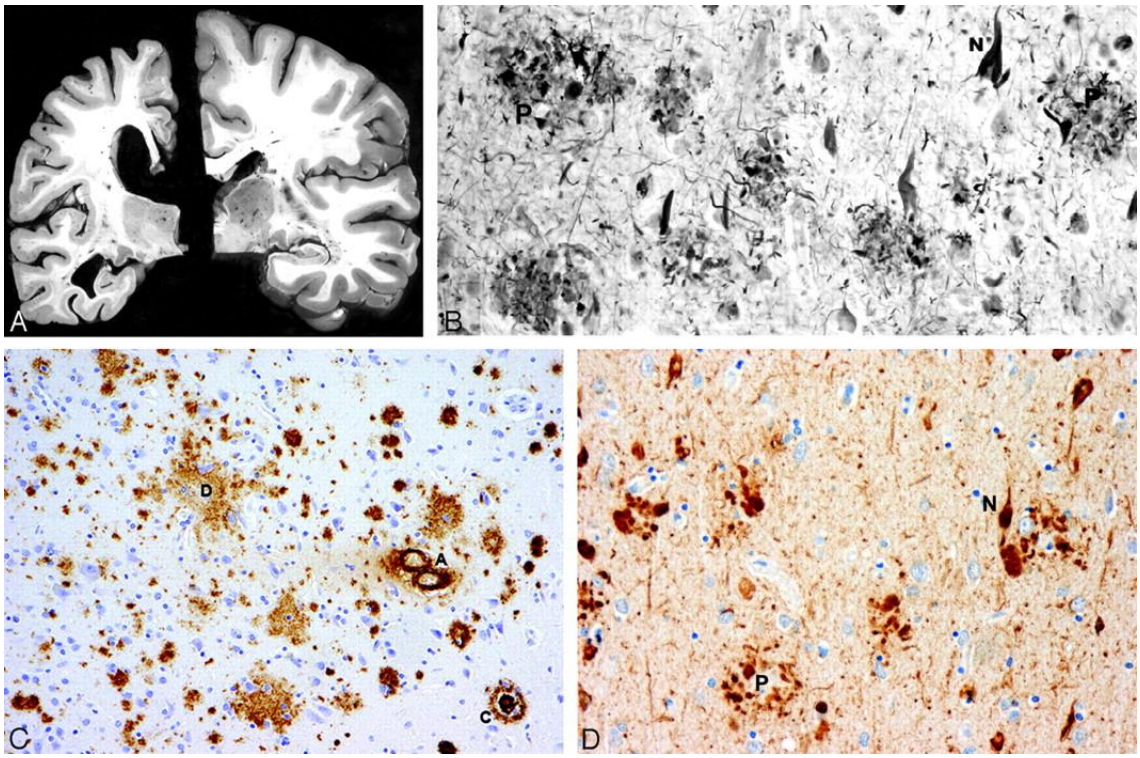


Figure 2 Alzheimer´s disease features

A *On the left - brain of a 70-year-old patient with AD and,*
On the right - a healthy aged control brain. The AD brain shows marked atrophy

B *Neurofibrillary tangles (N) and neuritic plaques (P) in the hippocampus*

C *β-amyloidosis in the frontal lobe: a diffuse plaque (D), a cored plaque (C), and cerebral amyloid angiopathy (A)*

D *Neurofibrillary tangles (N) and neuritic plaques (P) in the frontal lobe*

Copy from: Wipold FJ, Cairnsd N, Voa K et al. Plaques and Tangles. *Neuropathology for the Neuroradiologist*. 2008, 29, 18-22

Pathological nonsoluble Aβ is composed of 42 amino acids and is toxic for cells and more aggregation-prone. These spontaneously aggregate to neurotoxic form of soluble oligomers. The severity of AD is linked with number of oligomers but not to total amount of Aβ protein. The imbalance of production and clearance causes accumulation and aggregation of Aβ. This was described by ``amyloid hypothesis``. The steady-state levels of Aβ can be influenced by degradation enzymes like nephrilysin and insulin-degrading enzyme, thus overexpression of those could prevent plaque formation. Decrease of Aβ oligomers level is foremost target of AD treatment research by various way described thereinafter.

Other pathological features are neurofibrillary tangles NFTs of hyperphosphorylated tau-protein, part of microtubule-associated proteins MAPKs. Tau is hydrophilic cationic protein with molecular weight about 45-65kDa encoded by one gene on chromosome number 17. Normal function of tau is stabilization of microtubules by interacting with actin. Tau promotes axonal growth. Its post-translational modifications mediated by protein kinases GSK3 and CDK5p35 induce production of the most neurotoxic oligomers. Tau will loose its function when hyperphosphorylated into paired helical structure called PHFs. Aβ accumulation precedes and promotes Tau aggregation [10]. Aging, oxidative stress, impaired function of ER and impaired clearance involves accumulation of Aβ, thus increasing tau aggregation. [Fig. 3] The level of total tau and phosphor-tau amino acids are predictive of the Alzheimer´s disease stage.

The modification of platelet tau as well as platelet APP modifications is used like biomarkers of AD. Correlation was found between modifications of platelet tau and level of cognitive impairment [11].

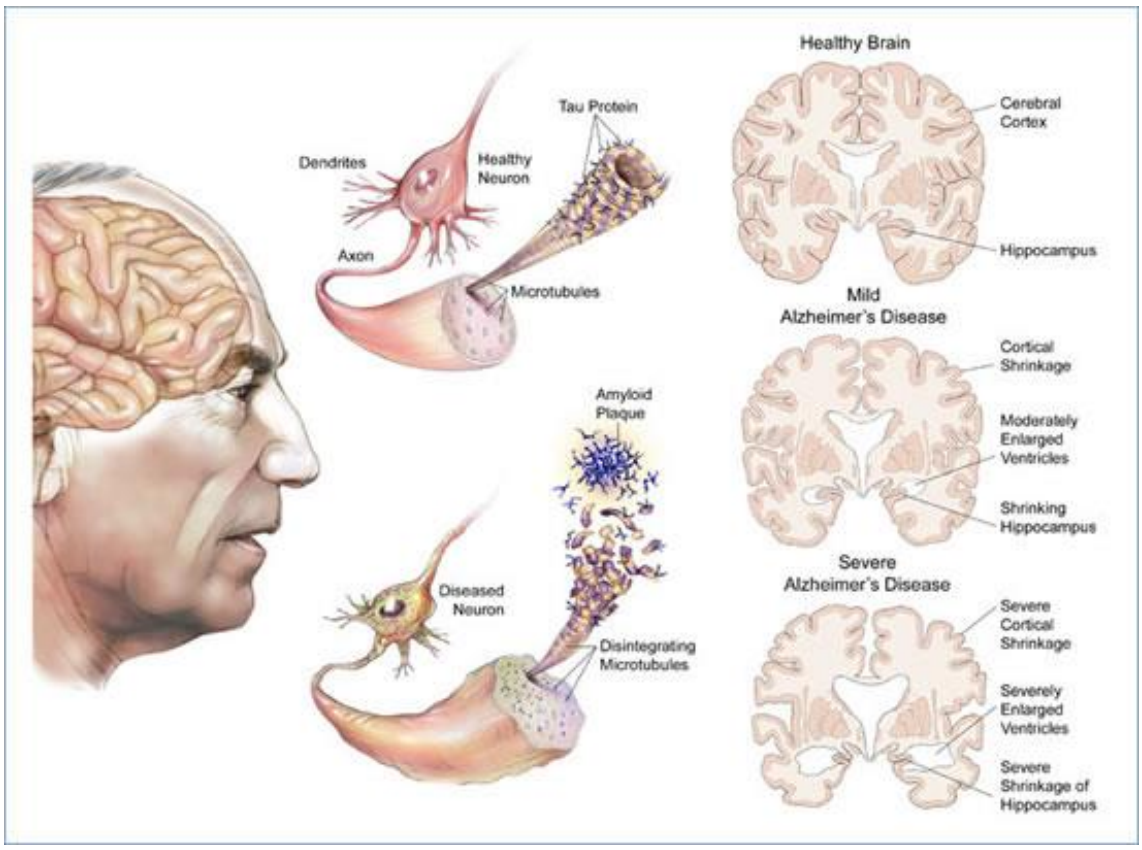


Figure 3 Comparison of normal and Alzheimer's brain

Left inset: Damage of microtubules by hyperphosphorylated tau protein

Right inset: Stages of Alzheimer's: Mild, Moderate & Health

Copy from: http://podcats.in/last-stages-of-alzheimers (13/2/2015)

3.1.2 Beta amyloid

Aβ is a product of proteolysis of APP, transmembrane neuronal protein. Its physiological function remains elusive [Fig.4]. Under physiological conditions it is cleaved by alpha and gamma-secretase to non-amyloidogenic soluble products including alpha APPs, peptide p83, p3 and c-terminal domain. These have a protective and supportive function in neuronal plasticity and formation of synapses.

Under pathological condition amyloidogenic and nonsoluble aggregation-prone molecules of Aβ and other products (betaAPPs, C99 and APP intracellular domain – AICD) are produced. This amyloidogenic process is initiated by beta-secretase beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1). BACE1 could be activated for example by cholesterol and other endogenous molecules. The retained C99 is substrate for gamma-secretase, generating Aβ. There exist different forms of Aβ varying in

number of amino acids. The most prevalent 42 aminoacids form foster aggregation of Aβ into senile plaques, which are the most characteristic features of AD.

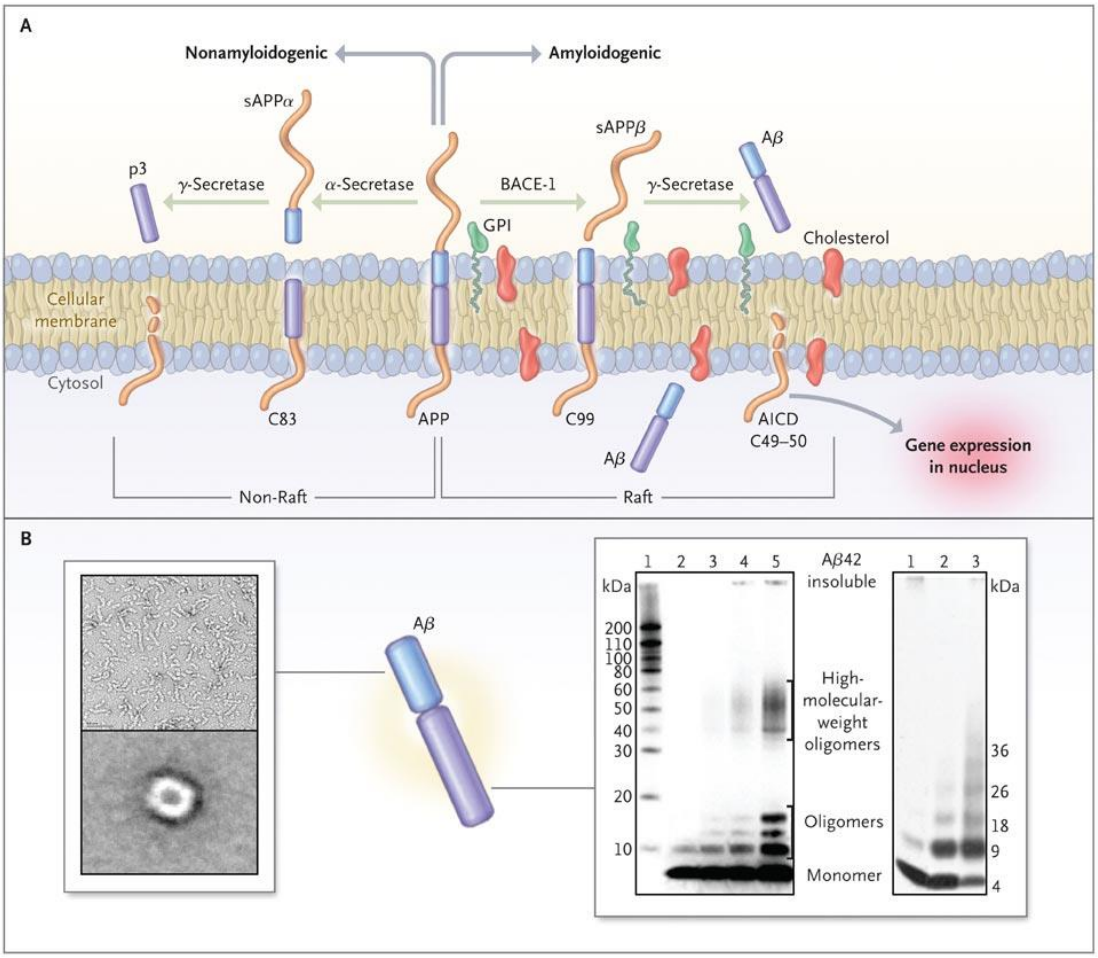


Figure 4 Amyloidogenic and non-amyloidogenic proteolysis of APP.

A *APP cleaved by BACE1- amyloidogenic pathway.*

Alpha-secretase cleavage of APP without pathological condition

B *Beta-amyloid detection, left- photomicrograph, right-immunoblot*

Copy from: Querfurth HW, LaFerla FM.N. Alzheimer´s disease. *N Engl J Med*. 2010,362(4):329-44.

3.1.3 Etiology

Causes are clear in Familiar AD (FAD) and include genetic mutations specific to chromosome 21, which encodes APP, chromosome 14 encoding Presenilin1 [subunit of gamma-secretase] and chromosome 1 encoding Presenilin 2 [subunit of gamma-secretase]. There also plays role a polymorphism in APO-E like well-known risk factor, concretely the haplotype Apo-E-4 increase three times the risk of AD. There is also higher risk of AD in cases of Down syndrome [3-5 times higher risk, above 35 years increases the risk at 25% - every fourth with Down syndrome suffers AD].

In SAD form the main risk factors are age, hyperlipidemia, obesity, diabetes mellitus type 2, high blood pressure, saturated fatty acids intake, smoking and other factors [12].

3.1.4 Mitochondrial dysfunction causing oxidative stress

Physiologically mitochondria are expected to suffer a lot of oxidative stress and have high evidence of mutations. Aβ is mitochondrial poison and impairs key mitochondrial enzymes like cytochrome c oxidase (CcO). ATP production, oxygen consumption and mitochondrial membrane potential are impaired and causes increase of superoxide radicals and generate oxidative stress. Aβ attacks directly the electron transport, Krebs cycle enzymes and damages mitochondrial DNA by fragmentation. It also generate oxygen and nitrogen reactive species, which promote oxidative stress and drives apoptotic damage by activation of JNKs. Damage is mediated by receptor for advanced glycosylation (RAGE), which binds and mediates its toxicity by influx. The metal ion catalyzed hydroxyl-radical formation is driven by reactive radicals and damage several molecular targets by peroxidation of membranes, increase of membrane permeability to calcium and impairs glucose transport leading to energy imbalance. Elevated levels of divalent metal ions like iron, cooper, zinc or aluminium promotes aggregation of tau. Unfortunately attempted treatments by antioxidants all failed [13].

3.1.5 Synaptic failure

Aging causes synaptic loss [14]. Loss of synaptic neuronal connections was demonstrated as impairment of long-term potentiation, as about 25 percent loss of synaptophysin-presynaptic vesicle protein and as endocytosis of NMDA receptor [15]. These processes correlate with dementia. Severe synaptic loss is noticeable in hippocampus, frontal and prefrontal cortex. This process is aggravated by Aβ. The Aβ binds to the nicotinic acetylcholine receptors and blocks them, setting up the persisting long-term potentiation. Moreover the activation of nicotinic acetylcholine or muscarinic receptor limits tau phosphorylation and favours genesis of non-amyloidogenic form of Aβ [16].

3.1.6 Insulin-signaling pathway

Several studies have demonstrated the existence of high fasting insulin levels as well as peripheral insulin resistance in advanced AD patients [17]. These findings correlate with the increased risk of AD in diabetes mellitus type 2 affected patients, which is surrounded by glucose intolerance. Brains of persons suffering from insulin resistance are more vulnerable to oxidative stress and impairment of synaptic plasticity. It represents the mechanism of life span influence by insulin. Moreover the high serum glucose leads to damage of hippocampal structures, where up-regulation of tau phosphorylation kinase GSK3 beta leads to reduction of insulin-degrading enzyme increasing aggregate-prone Aβ [18].

The beneficial effect of PPAR agonists for cognitive functions has been observed in transgenic mice model [19].PPAR agonists have no effect on FAD with APO E-4, although it was shown that APO E-4 improves the glucose utilization. The low glucose utilization is linked with impairment of key mitochondrial enzymes glucose oxidase. Altered glucose metabolism can be detected decades before clinical demonstration of AD symptoms [20].

3.1.7 Vascular effects

Ischemic diseases occur in 60-90% of AD patients, whereas one third of all vascular dementia cases are associated with some pathological features of AD [21]. The vascular injury and parenchymal inflammation drives the cycle of protein aggregation and oxidative changes in brain resulting in cognitive decline. Disruption of brain blood barrier, large-vessel atheroma, lobar hemorrhage, cerebral blood flow reduction, capillary abnormalities and cerebral tissue necrosis may result from arteriosclerotic vascular disease. Perivascular channels impairment impedes the Aβ clearance. Aβ deposits aggravate vasoconstriction and have cytotoxic effect on endothelial and smooth-muscle cells [22]. Increased LDL and RAGE expression and deregulated Aβ transport across the brain-blood barrier was observed. This is what ´´neurovascular uncoupling´´ hypothesis describes to be one of the key processing in AD [23].

3.1.8 Calcium levels changes

In most neurodegenerative diseases the regulation of calcium levels is impaired. Elevated levels of calcium observed in AD leads to Aβ aggregation and the high Aβ levels causes the increase of membrane permeability for calcium and other ions. Therefore, the cycle of Aβ accumulation perpetuates.

The presenilin mutation also modulates calcium homeostasis. The sustained activation of glutaminergic receptors, a common feature in AD, increases cytosolic calcium, resulting from calcium release in Endoplasmic Reticulum. This process causes increased calcium uptake and degeneration of neurons. Decreasing activation of glutamatergic receptor NMDA by its antagonist Memantine now represents first line treatment for AD [24].

3.1.9 Axonal transport deficit

Other relevant effect in AD is the reduction in axonal transport of critical proteins needed for synaptic functions. In response to tau-caused damage of micro tubular continuity, kinases cannot drive vesicles and mitochondria to the synaptic terminals. Impaired transport causes accumulation of several agents: APP, vesicles, kinases, Aβ and indicates inflammation and neurodegeneration. Dysfunctional

microtubules were observed in all stages of AD. The future treatment investigated for microtubular stabilization is a novel drug Paclitaxel [25].

3.1.10 Neuroinflammation by AD

AD brain is also affected by chronic inflammation. Activated microglia plays contradictory role in inflammation occurring by AD. On one side it helps to decrease Aβ levels by fagocytosis, on the other side microglia produces inflammatory cytokines - IL1, IL6, TNF alpha and opsonisants, which were proved to trigger neuronal damage [26]. Microglia also expresses receptor for advanced glycation end products, which mediate toxic and inflammatory effects of Aβ and enable influx of Aβ to neurons [27].

Activated microglia is usually seen in areas associated with Aβ and the activation depends on the load of Aβ. Low Aβ concentration activates more phagocytic function, while high amount of Aβ shift the balance to production of inflammatory products and the phagocytic ability is reduced. Therefore, the beneficial or detrimental function of microglia likely depends on activation state of microglia [28].

The astrocytes have multiple functions connected to neuroinflammatory response. They were proved to generate low amount of Aβ, in addiction to neurons. The reactive cytokines (interleukins, TNFα, IFNγ) are also produced by astrocytes. They have been demonstrated to induce generation of Aβ in human astrocytes and neoplastic astrocytoma cells by elevating the expression of secretasis in neurons. IL-1beta is also able to induce production of free nitrogen radicals and can increase tau phosphorylation, which leads to neurotoxicity. Inhibition of IL-1beta can be helpful to preserve cognitive functions and to reduce Aβ levels, but the knock-out leads to neuronal damage [29]. The production of proinflammatory cytokines has been proved to be promoted by saturated fatty acids, lipopolysacharides or Aβ, which affect this way neuroinflammatory cascade and represents one of the main risk factors of AD [30].

Nonsteroidal anti-inflammatory agents have been reported to lower risk of AD and slow the progression of disease [31].

3.1.11 Aberrant cell-cycle

Non-functional cell-cycle has been detected in all AD stages, including mild stage, most frequently in G1-S phase. It could progress to impairment of DNA replication, resulting in tetraploid neurons and activation of mitotic cyclins, but mitoses are absent. Impaired DNA replication causes neuronal death. Survival is in this case undesirable [32].

3.1.12 Cholesterol metabolism by AD

In AD, defects of cholesterol metabolism are usually included, as evidence by APOE mutation. High levels of esterified cholesterol reduce Aβ clearance and promote his aggregation. APOE is the main cholesterol transporter in brain. The allele ε4 increases the risk of AD four times. Statins ameliorate cognitive state in mild AD as reported in one clinical trial, but it was not confirmed by other studies. Statins can decrease inflammation, up-regulate alpha-secretase, producing non-amyloidogenic Aβ, and improve vascular functions. The exact pathway remains elusive, but it is not directly connected with decrease cholesterol levels [33].

3.1.13 Treatment

Only palliative treatment, which moderates symptoms and slowed cognitive impairment, is used. Scientists are not able to stop neuronal degeneration process. Since 1997 ChEIs are used for symptomatic treatment. Recent first-line therapy includes donepezil, rivastigmin and galanthamin, which slow the atrophy in the hippocampus and ameliorate cognitive impairment by enhancing availability of acetylcholine in synapses [34]. ChEIs can delay the progress of disease up to two month in moderate AD cases and up to 7 month in case of severe AD.

Other first-line pharmacotherapy is Memantin, uncompetitive inhibitor of NMDA receptor, The NMDA receptor is necessary for processes of apprenticeship and memory. NMDA receptor is over-activated in AD and we need to antagonize this process. Memantin is usually used only in moderate and severe AD and generally in combination therapy with ChEIs [35].

Under investigation is lot of diverse pharmaceutical targets based on decrease in Aβ levels like amyloid-base immunotherapy and BACE1 inhibitors. Other pharmacons are targeting on inhibition of tau phosporylation, like GSK3beta inhibitors.

Anti-inflammatory drugs are able to low risk of AD by decreasing transcription of BACE1. Agents preventing tau oxidation and aggregation, are under investigation. One known inhibitor of tau aggregation, Methylene blue - Phenothiazin Methylthioninium Chloride, also called Rember, was presented at Chicago 2008 conference as helpful by decreasing of soluble tau in phase 2 trial [36].

Also natural compounds have been found which prevent tau aggregation and improve cognitive functions. The endemic phytocomplex called Shilajit, located in Himalayan Mountains, is able to inhibit Tau aggregation [37].

Active amyloid-based immunotherapy promises improvement by 80% reduction of senile plaques. The CAD106, ACC-001 and Affitope AD02 principled on activation of T- and B-cells are in phase 2 trial [38]. CMI - cognitive motor intervention – psychomotor and cognitive sessions and exercises improved affective status after one year comminuted therapy with ChEIs in 75% of cases compared to control group-treated only by ChEIs resulting only in 47% improvement [39].

Curcumin supplements, which are able to stop Aβ aggregation, are in second phase:clinical trial [40]. Also antioxidants such as flavonoids can inhibit Aβ generation and aggregation by inhibiting of BACE1. Specifically myricetin, quercetin, kaempherol and morin were proved to be useful [41].

3.2 JNKs and metabolic stress

3.2.1 JNK isoforms and functions

C-Jun N-terminal kinases are members of MAPKs, the family of mitogen activated proteins and play role in apoptosis of mammalian neurons, development of brain and the integrity of skeleton. They are activated in response to various factors like inflammatory cytokines, environmental stresses, heat shock, ionizing radiation, and DNA damage or protein synthesis inhibition. JNKs control expression of genes mediating cell proliferation, differentiation or apoptosis by phosphorylation of transcription factors like c-Jun, p53, ATF-2 and NFAT [42].

Three diverse forms of JNKs are known: JNK1, JNK2 and JNK3. Despite some structural homology of all three isoforms, they have distinct function. JNK1 and JNK2 is expressed in microglia, JNK3 [Fig.5] is prevalent in neurons, testis and pancreatic islet [43]. Proved by knock-out, JNKs have a lot of diverse functions and play special role in cell differentiation and survival. Knock-out of both JNK 1 and 2 caused lethal transformation. Single JNK1 knocked-out mice were more vulnerable to ischemia, atrophy and tauopathy. JNK2 knocked-out mice had ameliorated atherosclerosis process. Furthermore the JNK1-/- mice were surprisingly protected against Diabetes mellitus type 2 and JNK2 -/- mice against hypercholesterolemia induced endothelial dysfunction [44].

JNK1 and JNK2 also cause apoptosis in embryonic neural tube, but also protects against it in forebrain cortex, where they involve survival [45]. Other interesting function was discovered by JNK3 inhibition, which lengthened the period of cell rhythm. JNK plays a role also in the control of the cell circadian rhythm, but the precise mechanism remains elusive [46], [47].

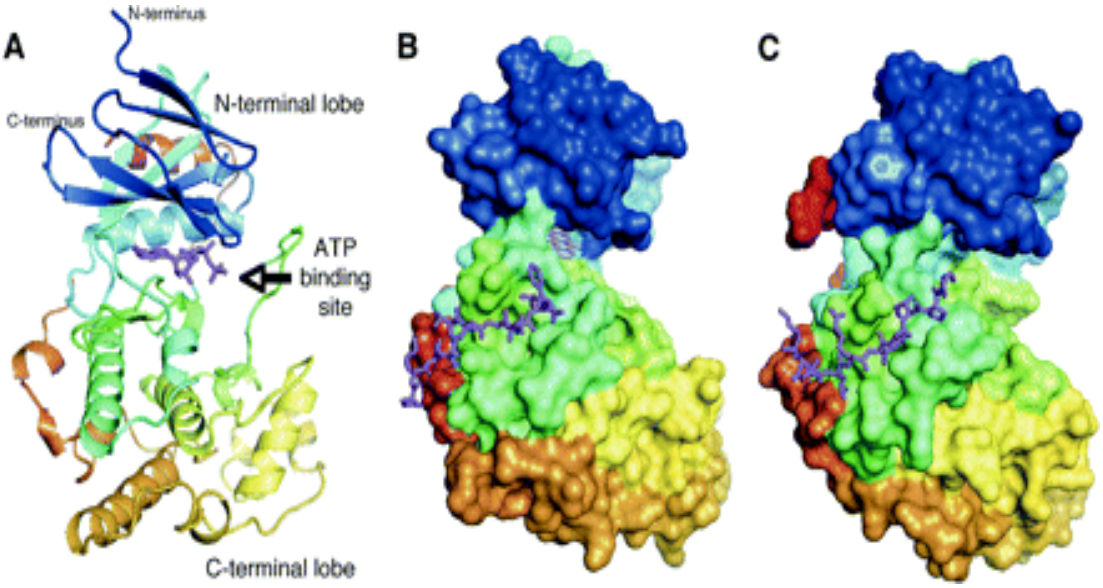


Figure 5 JNK3 structure

A *Crystal structure of JNK3*

B *Crystal structure of JNK1 (shown in surface representation) in complex with the peptide corresponding to residues 153 to 163 of the substrate and scaffold protein JIP1*

C *Crystal structure of the complex of p38 MAPK*

Copy from: http://mmbr.asm.org/content/70/4/1061/F2.expansion.html (13/2/2015)

3.2.2 Mechanism of JNK-mediated apoptosis

As illustrated [Fig.6] JNK pathway can be activated by several stimuli connected with stress and disturbance of homeostasis. Physiologically, JNK pathway plays indispensable role during embryonic and early postnatal development of nervous system by establishment of apoptosis. Surprisingly, about 50 % of neurons are expected to die during neurogenesis by apoptosis to ensure accurate and precise development of nervous system [48].

This finding underlines also potentially noxious function of JNK pathway, represented by promotion of neurodegenerative diseases. This event is strongly connected to the fact, that JNKs are part of apoptosis amplifying cascade. Members of

Jun and the Fos family are first genes to be activated in response to stress or nerve growth factor (NGF) withdrawal. They are called immediate early response genes. Likewise AP-1, which consists of diverse transcriptional factors like c-Jun, Jun B, c-Fos etc., enables binding specific DNA sequence, called AP-1 site. This process likely controls apoptosis. JNK3 is necessary for c-Jun activation .JNK3 mediated phosphorylation of c-Jun is the way, how to activate stress responsible apoptosis [Fig. 6.], [49].

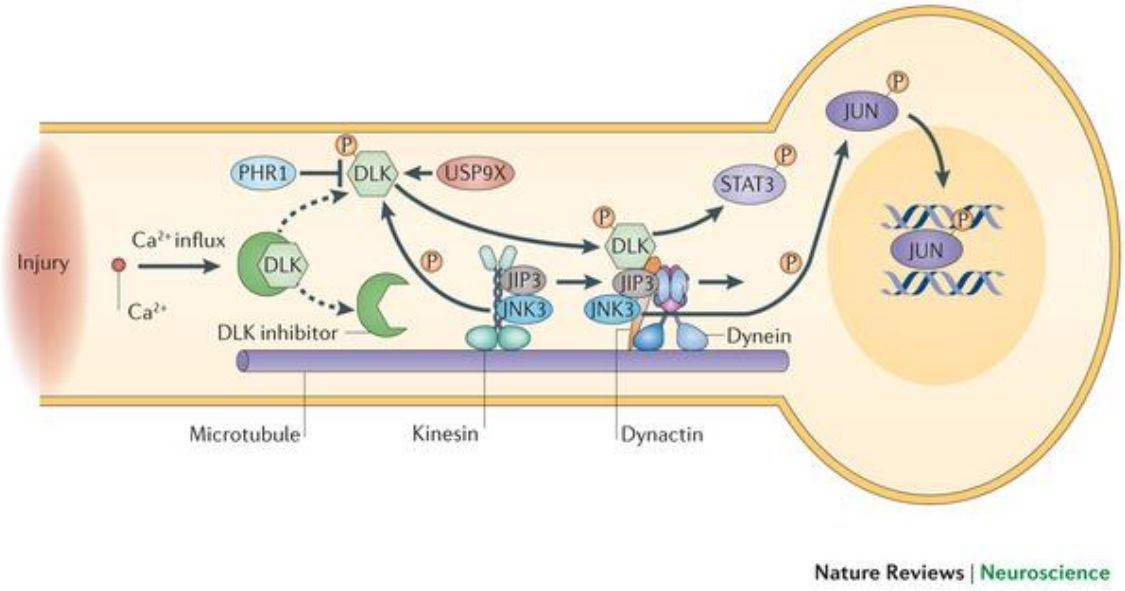


Figure 6 Signaling via the c-Jun N-terminal kinase signaling pathway

Copy from: Rishal I, Fainzilber M. Axon–soma communication in neuronal injury. *Nature Reviews Neuroscience*, 2014, 15(1):32–42

3.2.3 JNK3 like promoter of AD

Aβ was observed to promote neuronal apoptosis by activating the stress cascade. Translational block induced by Aβ leads to ER stress, which activates Jun signaling pathway by unfolded protein response control system, which is supposed to restore homeostasis. It was proved that JNK activation is linked with increase of Aβ endocytosis and also perpetuates the Aβ production. In JNK3 knocked-out mice Aβ levels were markedly reduced and cognition was improved. The deletion of JNK3 in FAD mice caused 87% decrease in Aβ levels in 6 month [50]. This suggests that Aβ

accumulates in a JNK3-dependent manner and thus AD is under tight control of JNK3. These findings set JNK3 to be an important target of research in AD treatment.

3.2.4 JNKs and insulin-signaling pathway

It was observed regarding to diabetes risk factor that deletion of JNK3 involved defects in IRS2 expression and increased apoptosis of beta cells of pancreas. JNK1 and JNK2 knock-out has opposite effects. JNKs are therefore supposed to influence insulin resistance by modulation of AKT pathway by increasing its activity leading to pro-survival effects on beta-cells. Silencing of JNK3 decreases the AKT pathway activity. Inhibition of JNK1 or JNK2 activate AKT pathway. JNK3 seems to affect insulin-signaling pathway by influencing transcription in nucleus, whereas JNK1 and JNK2 are acting in cytoplasm. JNK3 is expected to be protective in development of pancreas beta-cells damage, thus prevents development of diabetes [51].

4 Objectives

In consequence to recent works showing decrease of beta amyloid by silencing JNK3 gene in brain we have focused on JNK3 localization in brain. The aim of the present work was to locate JNK3 expression, a strong potent activator of apoptosis in brain and inflammation marker observed in AD, in various types of brain cells. We were supposed to observe if JNK3 is localized with neurons, microglias or astrocytes to elucidade AD pathogenesis. Immunohistochemistry was used, detected by cell markers of following cells: NeuN(neurons), GFAP(astrocytes), Ox42(microglias). Then we also assaid colocalization of JNK3 with tau and beta amyloid markers: PHF1 and 6E10.

5 Experimental part: Methods and material

5.1 Animals

Fourteen month old transgenic mice type Tg2576, bearing the mutation of APP gene were used. Three female transgenic mice (Tg2576) over-expressing human amyloid precursor protein (hAPP) carrying the Swedish familial mutation (K670N/M671L) [52] under the genetic mixed hybrid background C57BL/6/SJL and three wild-type littermates (WT) with the same genetic background were used. At the age of 3 months, the mice were housed in standard laboratory cages (43×27×15 cm) in groups of 4. All mice were kept in the same room under controlled temperature (21±1 C) and humidity (55±5%) on a 12-h light–dark cycle. Cage bottoms, water bottles and sipper tubes were cleaned twice per week, wire lids were cleaned monthly, and food/water were provided ad libitum. All the animals were handled under the same conditions and by the same staff throughout the duration of experiments in order to avoid potential influence on cognition.

The Tg2576 mice develop parenchymal Aβ plaques by 11-13 month and show oxidative lipid damage without neurofibrillary tangles. Cognitive function has been changed in this model. Impaired learning, working memory and fear conditioning have been reported at less than six month, although other studies have reported progressive impairment later at about 12^{th} month of age [53]. Dendritic spine loss has been reported by 4.5 months in the CA1 region of hippocampus. Change in synaptic plasticity has been noted by five month [54].

Experimental procedures were conducted in accordance with the European and Spanish regulations (2003/65/EC; 1201/2005) and approved by the Ethical Committee of University of Navarra (068-11).

5.2 Western blotting

Cytosolic extract preparations from the hippocampus of mice were homogenized in a cold lysis buffer with protease inhibitors (0.2M NaCl, 0.1M Hepes, 10% glycerol, 200mM NaF, 2mM Na4P2O7, 5mM EDTA, 1mM EGTA, 2mM DTT, 0.5mM PMSF, 1mM Na3VO4, 1mM benzamidine, 10μg/ml leupeptin, 400U/ml aprotinin). Samples (20μg) were separated by electrophoresis on a sodium dodecyl sulphate–polyacrylamide gel. Membranes were probed overnight at 4°C with the corresponding primary

antibodies, anti-pSAPK/JNK Thr^{183}/Tyr^{185} (Cell Signaling Technology, USA) or anti-SAPK/JNK (Cell Signaling Technology, USA). Immunopositive bands were visualized using an enhanced chemiluminescense Western blotting-detection reagent (ECL; UK). The optical density (OD) of reactive bands visible on X-ray film was determined densitometrically. β-Actin was used as internal control. Results were expressed as percentage of OD values of control (non-transgenic saline) mice.

5.3 Immunohistochemistry

Left hemispheres from 4 mice per group were fixed by immersion in 4% paraformaldehyde in 0.1M PBS (pH 7.4) for 24 h followed by 10% sucrose solution. Brains were cut into series of 20μm slides. To save free-floating sections more than 7 days were put in etylenglycol, glycerol, PBS and distilled water (proportion 1,5:1,5:1:1). In case of NeuN staining tissues were pre-treated with Citrate Buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) in a water bath at 90ºC for 20 minutes. Tissues for amyloid plaque staining were incubated with 70% Formic Acid for 10 minutes. Tissues were blocked with 0.5% of normal donkey serum in PBS with 0.3% Triton X-100 and 0.1% BSA for 2hours prior to incubation with primary antibody p-JNK (Thr 183/185,1:500), NeuN (1:500), GFAP (1:500), Cd11b (1:500), Aβ (1:500) over-night at 4°C. Then sections were incubated with secondary antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2h in blocking solution. Sections were rinsed in PBS and mounted. 3 sections per mouse were processed and captured into images with Zeiss LSM 510 Meta Confocal Microscope.

5.4 Statistical Analysis

Western blotting results are expressed as mean ± standard error of the mean (SEM). Data were evaluated by Student's *t* test and the level of significance was set at $p<0.05$. All analyses were performed using SPSS 15.0 packages of Windows.

6 Results

6.1 pJNK levels in Alzheimer's disease

As depicted [Fig. 7] there was a significant increase in levels of phosphorylated JNK (pJNK) [$p<0.05$] in the hippocampus of Alzheimer's Tg2576 group compared to wild type. Consistent with a post-transcriptional regulation of this enzyme, total JNK protein levels, normalized using actin, remained unaltered.

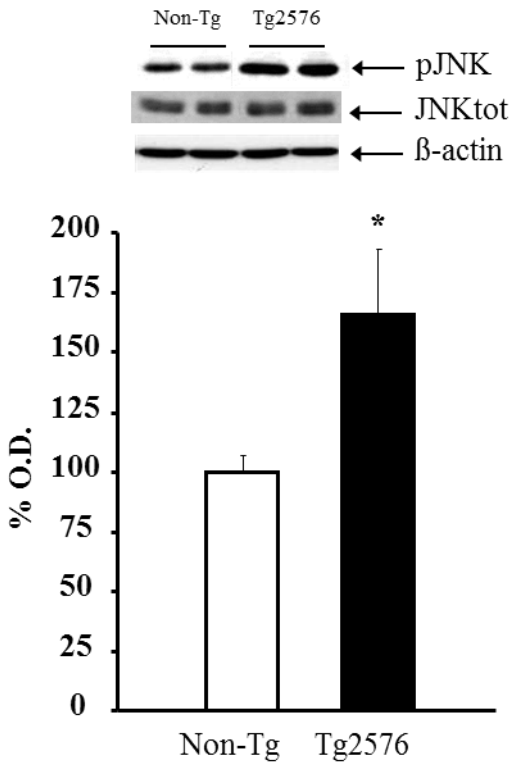


Figure 7 Stress activated protein kinase

JNK levels in the hippocampus of Tg2576 and wild type mice.

Data shows phosphorylated JNK levels

6.2 pJNK localization in neurons

We next determined where active JNK is localized in neurons in Tg2576 brains using active JNK or *p*-JNK and NeuN antibodies in immunohistochemistry. As we expected, in wild type mouse no activated JNK (pJNK) could be observed. That means there is no inflammatory process in those brains. NeuN antibody binds to neuronal cell nucleus. No co-localization between NeuN and pJNK was observed. Indeed, in Tg2576 a NeuN missing area where pJNK is expressed was observed, consistent with a loss of neurons. Therefore, we concluded that NeuN and pJNK were not co-localized. But moreover JNK mediated neuronal degeneration have occurred [Fig.8].

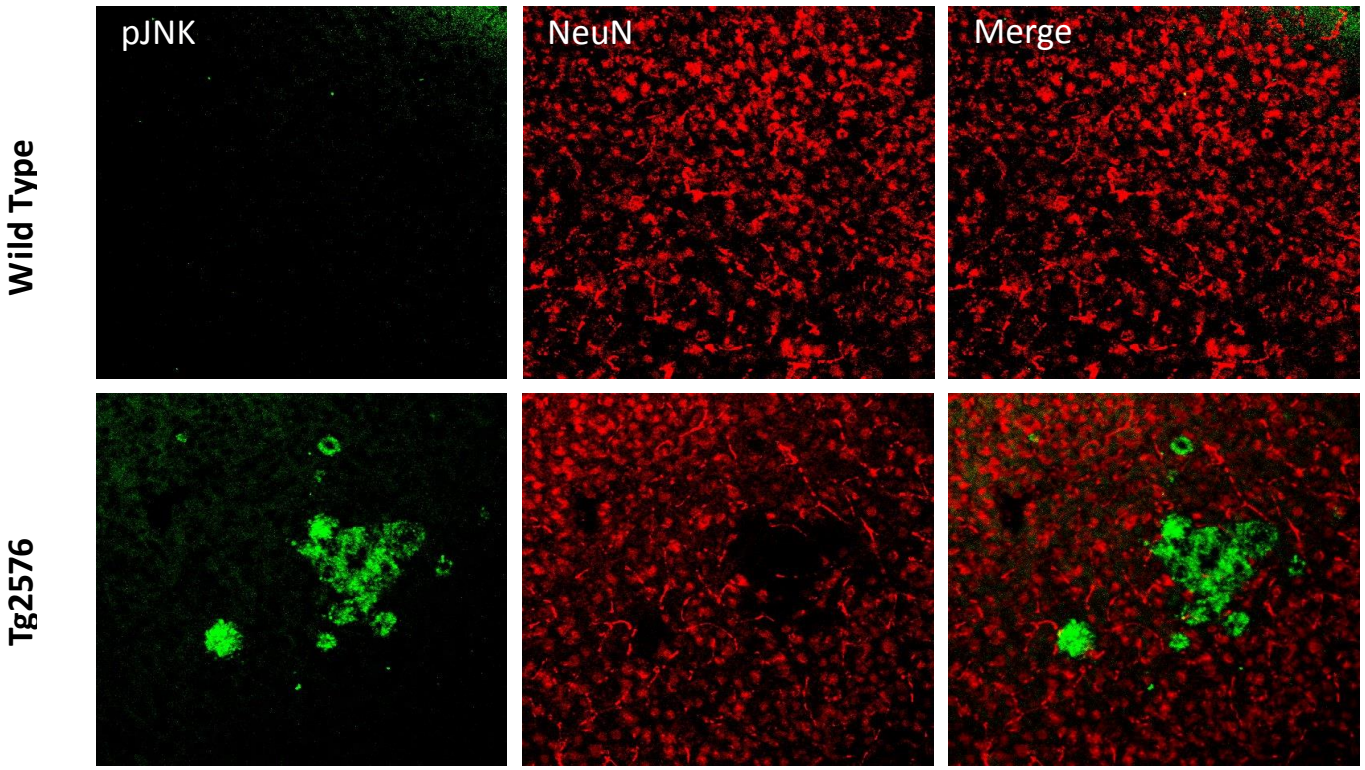


Figure 8 JNK staining in neurons

pJNK is not co-localized with the neuronal marker NeuN.

Upper panels, pJNK and NeuN stainings in wild type mice.

Lower panels, pJNK and NeuN stainings in Tg2576 mice.

6.3 pJNK localization in astrocytes

As described in the previous figure, no activated JNK was observed in wild type mice and in Tg2576 mice brains. There is no co-localization between the astrocyte marker GFAP and pJNK [Fig. 9]. However, astrocytes are accumulated around pJNK which could indicate that astrocytes are attracted to the area where an inflammatory process is occurring.

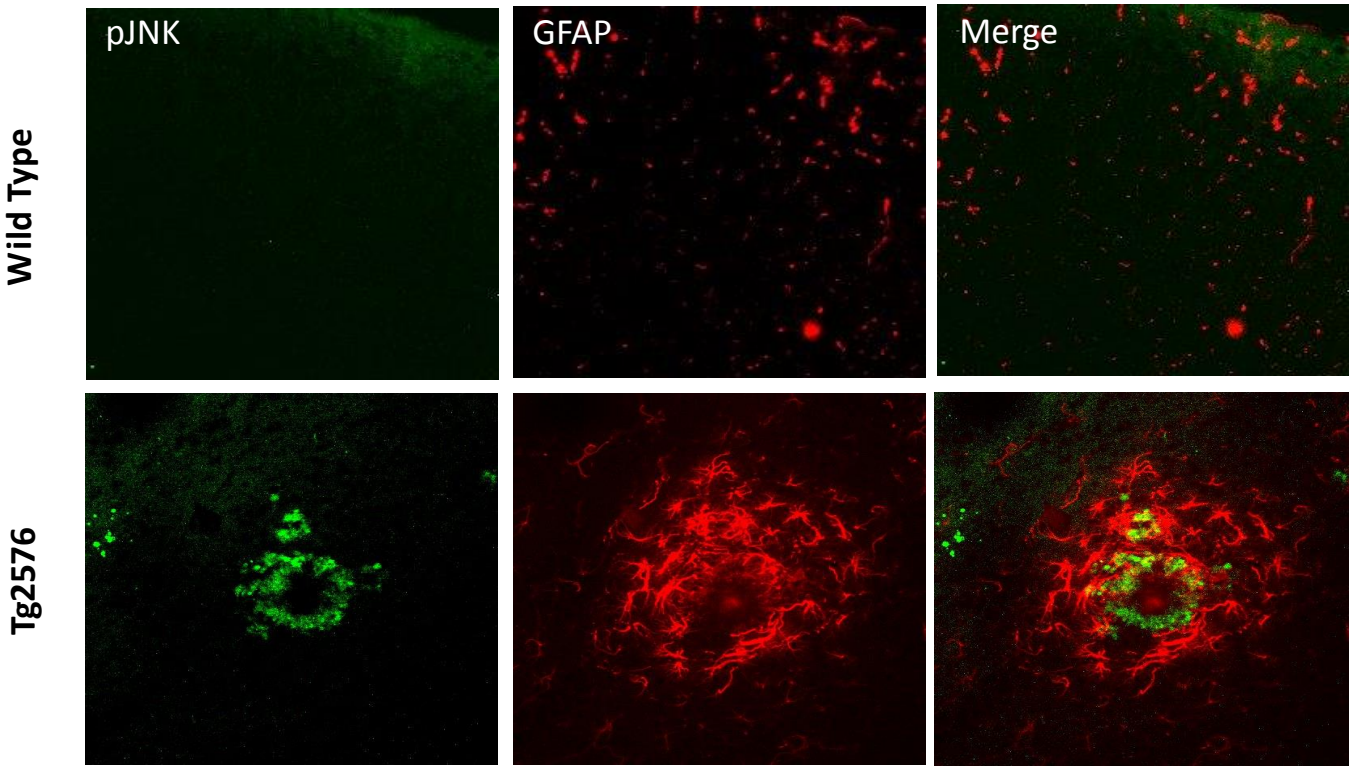


Figure 9 JNK staining in astrocytes

pJNK is not co-localized with the astrocyte marker GFAP.

Upper panels, pJNK and GFAP stainings in wild type mice.

Lower panels, pJNK and GFAP stainings in Tg2576 mice.

6.4 pJNK localization in senile plaques

We next determined where active JNK is localized in senile plaques. p-JNK signals were predominantly detected in plaque structures, co-localizing with 6E10 immunoreactivity, an amyloid beta marker [Fig. 10]. This result indicates that pJNK is accumulated in the periphery of the senile plaques, where dystrophic neurites are localized.

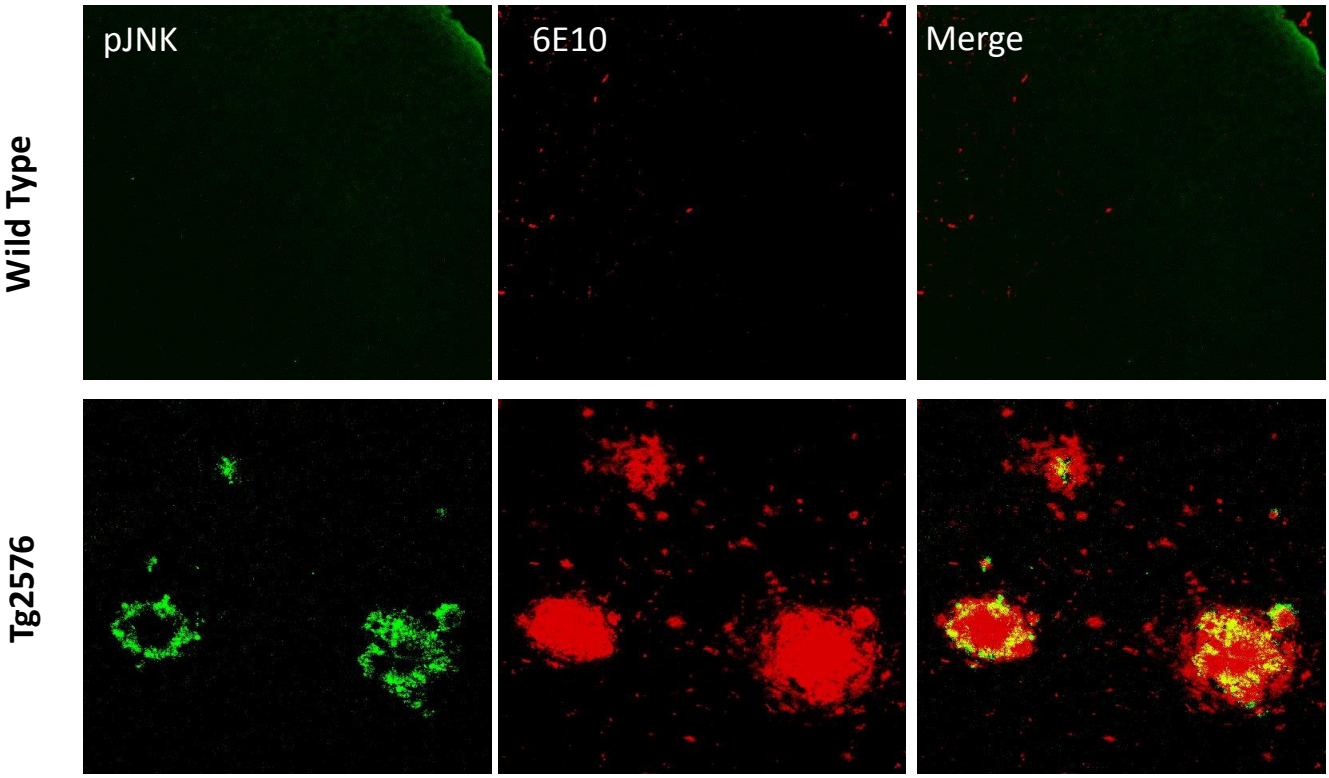


Figure 10 JNK staining in senile plaques

pJNK is co-localized with the amyloid beta marker 6E10.

Upper panels, pJNK and 6E10 stainings in wild type mice.

Lower panels, pJNK and 6E10 stainings in Tg2576 mice.

6.5 pJNK localization with pTau

As depicted [Fig.11], pTau immunoreactivity (marked with PHF1 antibody) was found mainly in the center of the senile plaque and was only partially co-localized with the pJNK that accumulates around that plaque.

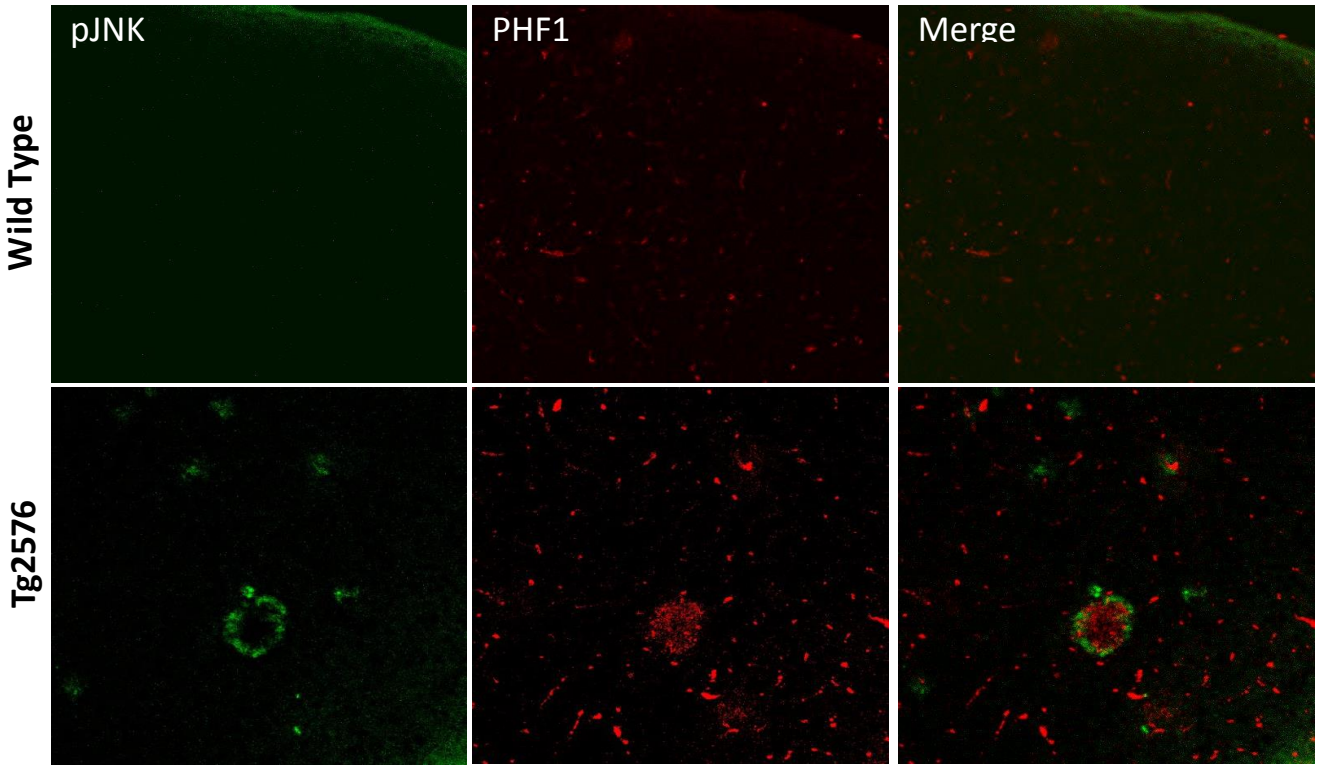


Figure 11 JNK staining in Tau

pJNK is partially co-localized with the pTau marker PHF1.

Upper panels, pJNK and PHF1 stainings in wild type mice.

Lower panel, pJNK and PHF1 stainings in Tg2576 mice.

6.6 JNK staining in microglia

The microglial staining using CD11b antibody, following previously described protocols failed to work. Thus the co-localization between pJNK and microglia could not be probed. Microglia is attracted by inflammation around the senile plaque similarly to astrocytes, therefore, a partial co-localization with pJNK was expected.

7 Discussion

One of four hypotheses of neurodegeneration underlies the key role of inflammation in AD development [55]. JNK3 was found to be strong potent activator of apoptosis induced by Aβ promoted stress, essential in pre- and postnatal neuronal growth [56]. In addition, JNK3 serve like regulator of cell cycle rhythm [55].

It is proved that inflammation occurs in all - mild to advanced - stages of AD compared to non-alzheimeric brain. Those healthy brains show totally none sign of inflammation. Moreover, it was showed in JNK3 knock-out mice, that the silencing of this gene caused rapid decrease of Aβ (87% in 6 month), which according to recent studies caused postnatal neuronal death in AD [56]. In connection to a known fact about JNK3 tight relation to basic mechanism of pathology in AD, that is the inflammation, main objective of present work was to target specific cell type potentially responsible of its expression.

Our findings surprisingly did not demonstrate localization of JNK in any kind of brain cell, but only co-localization with senile plaques was clearly observed. It remains elusive, if increased activated JNK3 is typical for AD or occurs in all types of neurodegenerative diseases. Few studies about this topic have been published, mostly about JNK3 basic functions [57].

We hypothesized that pJNK is associated or released by microglia or astrocytes, like recent few of papers published [58] but our results failed to show that. By using the immunohistochemistry we localized the pJNK directly around the plaques of Aβ, not associated with any kind of specific cells. Based in the lack of co-localization with any cell type we also hypothesize that pJNK3 are secreted.

Our staining has found JNKs in areas where death neurons – dystrophic neurites are. Around these places were accumulated activated microglias. It corresponds with the fact that JNK is presented like inflammation marker. Microglias are attracted by JNKs to the place of chronic inflammation in death neurons [Fig.7].

This finding helped us to complete the information about this key enzyme involved in AD to focus on better future treatment of AD. According to all found facts and our staining results we hypothesized, that JNK3 influence on apoptosis is part of cyclic process of Aβ accumulation. The whole process starts with pathological aging in brain. Aβ levels are increased and cause translational block. Synthesis of proteins and lipids in brain is starving. Other sources of energy must be increased to maintain

homeostasis. Glucose uptake, β-oxidation and glycolysis are over-activated. Brain cells get stressed because of this abnormal metabolism. JNK3 is activated by stress and drives Aβ production and its endocytosis. Moreover, neuronal apoptosis is activated by JNK pathways. Aβ accumulates and apoptosis cycle starts again.

This information drove us to try to find out if there exist some studies about JNK inhibitors. JNK inhibition is studied also in treatment of other diseases. Histone deacetylase inhibitor, Panobistat, inhibiting JNK activation, was observed to increace leukemia animal model survival [59].

Other potential agent called nuclear-factor-kappaB was found to over-activate XIAP (x-linked inhibitor of apoptosis) gene expression, which decrease JNK activation NFkappaB inhibition is studied to avoid hepatocellular carcinoma [60].

There was also observed effect of JNK1 and JNK2 RNA silencing on breast carcinoma. The cell growth was decreased after decreasing of JNKs expression up to 70 % [61].

It could be seen, that JNK inhibitors have widespread future in therapy of various diseases. But in connection to AD we haven't found any recent papers and studies about JNK3 silencing, although they are evidently potent to be more investigated.

This brings up more questions about possibilities of treatment of AD. In conclusion, the further research should be focused on improving the current poor ways of AD treatment where JNK3 down regulation or neutralization could be one of the main targets.

8 Conclusion

According to recent studies, several facts about JNK3 function, but nothing is understood about its carriers. JNK3 is a crucial activator of neuronal apoptosis induced by Aβ promoted stress. We failed to locate it in astrocyte, microglia or neurons of our AD mouse model. In transgenic AD mice pJNK has been particularly co-localized with the periphery of Aβ plaques, in the area where dystrophic neuritis are present. Moreover, JNK3 was observed extracellulary, which could suggest that it may have been either secreted there or was released upon death of carrier cells. Recent studies have shown connection between silencing of JNK3 gene expression and decrease of Aβ levels. This supports idea to target it for AD treatment.

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