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Cholinergic system as pharmacological target in Alzheimer's disease
Cholínergný systém ako cieľ pri liečbe Alzheimerovej choroby

Bachelor´s thesis

Supervisor: Mgr. Jan Jakubík, Ph.D.

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Prehlásenie

Prehlasujem, že som záverečnú prácu spracovala samostatne a že som uviedla všetky použité informačné zdroje a literatúru. Táto práca, ani jej podstatná časť nebola predložená k získaniu iného alebo rovnakého akademického titulu.

V Prahe, dňa 6.5.2016

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder of CNS and very serious type of dementia. AD affects many elderly people and the numbers are increasing with every year. There are two forms of AD: familial (FAD) and sporadic (SAD) form. FAD is an early-onset disease with a genetic cause. SAD is more common, late-onset disease with the age and ε allele of apolipoprotein E as major risk factors. The most crucial symptom is memory disorder, followed by confusion, disorientation, depression and later on, serious psychical and motor-skill problems. These symptoms are as result of neuronal loss, plaques and tangles in the central nervous system (CNS).

As for now, there are no efficient diagnostic or therapeutic approaches to stop the degeneration of brain. Inhibitors of acetylcholinesterase are currently the only approved treatments, that have proven to slow down the progress of AD. Other cholinergic drugs have been developed, but they have shown a lot of side effects, as they are targeting a large scale of receptors. The researchers are trying to find a modulator, that would target only specific receptors in the CNS, to avoid such side effects.

Key words: acetylcholine, Alzheimer´s disease, β-amyloid, cholinergic system, inhibitors of cholinesterase, muscarinic receptors, nicotinic receptors, pharmacology

Abstrakt

Alzheimerova choroba je neurodegeneratíve ochorenie a veľmi vážny typ demencie. Alzheimer postihuje množstvo starších ľudí a tieto čísla rok čo rok narastajú. Existujú dva typy: familiárny a sporadický. Familiárny forma má nástup v rannom veku a príčinou sú genetické faktory. Sporadická forma je častejšia, s neskorším nástupom a hlavnými faktormi sú vek a ε alela apolipoproteínu E. Prvý symptóm je porucha pamäti, ktorý nasleduje zmätok, dezorientácia, depresia a neskôr vážne psychické a motorické poruchy. Tieto symptómy sú ako dôsledok straty neurónov, senilných plakov a neurofibrilárnych klbiek v centrálnej nervovej sústave (CNS).

Zatiaľ neexistujú efektívne diagnostické alebo terapeutické postupy, ktoré by zastavili degeneráciu mozgu. Inhibítory acetylcholínesterázy sú momentálne jediné schválené liečivá, ktoré boli dokázané, že spomaľujú priebeh Alzheimerovej choroby. Ďalšie cholínergné lieky boli vyvíjané, ale ukázalo sa, že spôsobujú mnoho vedľajších efektov. Výskumy sa snažia nájsť taký modulátor, ktorý by sa zameriaval na špecifické receptory v CNS, aby sa takým vedľajším efektom zabránilo.

Kľúčové slová: acetylcholín, Azlheimerova choroba, β-amyloid, cholínergný systém, farmakológia, inhibítor cholínesterázy, muskarínové receptrory, nikotínové receptory

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List of abbreviations:

Aβ - β-amyloid
AC - adenylate cyclase
ACh - acetylcholine
AChE - acetylcholinesterase
AChEI - acetylcholinesterase inhibitor
AD - Alzheimer´s disease
APOE - apolipoprotein E
APP - amyloid precursor protein
Arg - arginine
ATP - adenosine triphosphate
BuChE - butyrylcholinesterase
cAMP - cyclic adenosine monophosphate
CHT1 - choline transporter
CNS - central nervous system
Co-A - coenzyme A
Cys - cysteine
EOAD - early-onset Alzheimer´s disease
FAD - familial Alzheimer´s disease
GDP - guanosin diphosphate
GPCR - G-protein coupled receptor
GTP - guanosine triphosphate
HDL - high-density lipoprotein
ChAT - choline acetyltransferase
LDL - low-density lipoprotein
LOAD - late-onset Alzheimer´s disease
mAChR - muscarinic acetylcholine receptor
MAP - microtubule-associated protein
MCI - mild cognitive impairment
nAChR- nicotinic acetylcholine receptor
NAM - negative allosteric modulator
NFT - neurofibrillary tangle

NS - nervous system

PAM - positive allosteric modulator

PKA - protein kinase A

PLC - phospholipase C

PNS - peripheral nervous system

PS1 - presenilin 1

PS2- presenilin 2

SAM - silet allosteric modulators

SAD - sporadic Alzheimer´s disease

1 Introduction

Alzheimer's disease (AD) is a serious neurodegenerative disorder of elderly people and the most common type of dementia (60-80% of all cases). It affected 46.8 million elderly people worldwide in 2015, according to Alzheimer's Disease International (Tang, Song and Xu, 2016) and the numbers of patients are increasing with every year. In the majority of cases, the most crucial risk factor known so far is aging and over 90% of all cases are first diagnosed after age 65 (Herrup, 2015). The probability of getting AD doubles for every 5 years after that age, with every second person aged 85 years and older being diagnosed. Other possible risk factors are genetics, environment, physical and mental health, ethnicity, alcohol or drug abuse, etc. (Panpalli Ates *et al.*, 2016).

AD is a progressive disease of central nervous system (CNS) with approximately 10 years to live since the initial symptoms are detected. Most typically, the first symptom is short-term memory loss, next are speech problems, mood changes, sleeping and eating disorders, motor skills problems, confusion and depression. In the advanced stages, the symptoms are becoming more compelling and growing into serious memory (short and long-term) and personality disorders, problems with language and orientation (in space as well as time), psychosis, aggression, paranoia and even neuropsychiatric collapse can occur in the last phase of the disease. In the end, patients experience a total loss of speech and motor functions, delusions, apathy and need to be bedridden. AD might result in other kinds of health issues such as seizures, delusional disorders, etc (Born, 2015). The death is generally not caused by the disease itself but by dehydration or infections, such as pneumonia (Gaugler *et al.*, 2016).

Unfortunately, the cause of the disease is unclear in most of the cases, which leads to problems with diagnosis and treatment. Pharmacological biomarkers, cerebral or functional imaging can be used to visualize the atrophy of certain parts of the brain, but they are not indicate. As the symptoms vary and the pathological features are not indefinable, the only conclusive diagnosis of AD at the present time is post-mortem autopsy. The most accurate diagnosis of living patients is by excluding the other types of dementia (e.g. vascular dementia).

There is currently no way to effectively prevent, treat or slow the progression of AD. Some drugs, which are targeting the cholinergic system (acetylcholinesterase inhibitors, such as donepezil) have proven effective to ease the clinical symptoms, but are

followed by many side effects like nausea, dizziness or cardiac arrhythmia (Burns *et al*., 1999; Hazlewood *et al*., 2011). Research for the efficient cure is still in progress, like approaches concerning the reduction of Aβ formation or to promote clearance of Aβ from the CNS.

AD is becoming an enormous public health problem. With increasing life expectancy, the number of patients, who are being diagnosed and treated is growing. The patients are dependent and taking care of them is very difficult by their relatives. Since they are disoriented, forgetful and confused. They might also be dangerous to a community as well as to themselves, therefore they either need to be institutionalized or have a caregiver.

2 Pathology of Alzheimer's disease

The main histological features of the disease are amyloid plaques and neurofibrillary tangles. The accumulation of plaques and tangles induces the loss of cholinergic neurons and synapses in the brain, mostly in areas associated with cognition and memory. Other pathological hallmarks are granulovacuolar degeneration, loss of synapses and decreases in cell density in distinct regions of the brain (Neve, McPhie and Chen, 2000). Typically, the first part of the brain to be affected is hippocampus, the center of short-term memory. Usually amygdala, that is responsible for processing memory and linking memory to emotions is next as the other parts of the limbic system. Because these parts of the brain are affected first, the memory and behavioral problems are primary symptoms. As the disease progresses, degenerated areas are spreading from center of the brain to frontal and temporal lobe and eventually, that resolves in cerebral atrophy (Cavedo *et al.*, 2014)(Herrup, 2015).

The β-amyloid protein (Aβ) is derived from amyloid precursor protein (APP), which is an integral membrane protein of yet unknown function. The N terminus of Aβ is located in the transmembrane domain of APP, 28 amino acids from the transmembrane spanning alpha-helix, and its C terminus is within this alpha-helix (Goedert and Spillantini, 2006). Normally, APP is cleaved by α-secretase to sAPPα on the outside of the membrane, which can be dissolved and to C83 at the intracellular terminus. Alternatively, APP is cleaved by β-secretase, that leads to amyloidogenic process and creates extracellular sAPPβ, which resolves in releasing non-soluble β-amyloid and intracellular C99 fragments. Subsequently, C83 and C99 are processed by the γ-secretase complex that

cleaves at the C-terminal site of the Aβ domain and releases Aβ from the C99 fragment (Ferrer, 2012; Hou *et al.*, 2016). These Aβ peptides are 40-42 amino acids long and are accumulating in the extracellular space, where they form $A\beta_{40}$ or $A\beta_{42}$ plaques. $A\beta_{42}$ is believed to be more harmful than $A\beta_{40}$, because of its hydrophobic character, but plaques contain both of them. Some studies show that Aβ plaques are present also in the intracellular matrix of neurons (Takahashi, Nagao and Gouras, 2017). The plaques induce neuronal inflammation due to inefficient clearance of Aβ by microglia or disruption of calcium homeostasis and even neuronal apoptosis (Kern and Behl, 2009; Heneka *et al.*, 2015).

Neurofibrillary tangle (NFT) is a microscopic lesion consist of an abnormal accumulation of a hyperphosphorylated form of protein tau (τ). τ is cytoskeletal protein from a family of proteins associated with microtubules (MAP) (Himmelstein *et al.*, 2012). It is responsible for assembly, organization and dynamics of microtubules and it participates in cell transport. For their stability and function, τ needs to be phosphorylated (Jouanne, Rault and Voisin-Chiret, 2017). This process is regulated by protein kinases and specific phosphatases, deregulation of either of them causes hyperphosphorylation. Increased concentration of hyperphospho-τ, resolves in disassembly of microtubules and disruption of cell signaling. τ is three to four times more phosphorylated in the brains of AD patients than it is in healthy brains (Spillantini and Goedert, 2013).

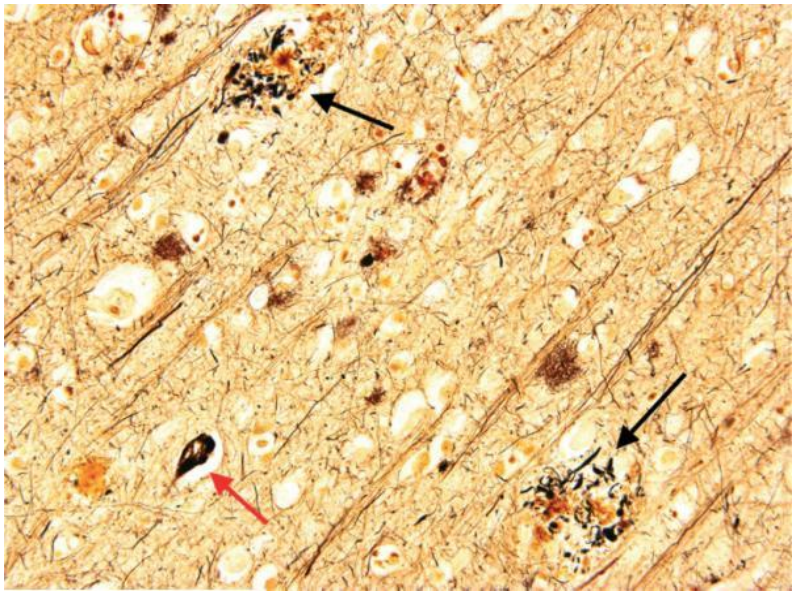

Fig. 1: Histology of the temporal cortex of a patient with AD. Modified Bielschowski stain; original magnification, 100x. Aβ plaques (black arrows) and neurofibrillary tangles (red arrow) are shown (adapted and edited from Perl, 2010).

In cytoplasma, the detached hyperphospho-τ aggregates in tangles and creates disulfide bridges. Then it self-assemblies into oligomers and elongates to form a stable NFTs, that block the transport of nutrients and other essential molecules inside neurons (Gaugler *et al.*, 2016). That resolves in neuronal death.

Plaques and tangles are very difficult to see with the common morphological staining used by pathologists. Typical methods to visualize them are fluorochrome dye thioflavin S, silver histochemical staining, modified Bielschowsky staining (aqueous solution of silver nitrate, AgNO3), Gallyas technique and immunohistochemical approaches (Perl, 2010; Šimić *et al.*, 2017)

The formation and pathology of Aβ or NFT are still not completely clear or understood. Both can be only found post-mortem in histological samples and are not strictly associated with AD. Aβ plaques are also found at different kinds of neurodegenerative diseases, like Parkinson´s or Huntington´s disease. Likewise, the NFTs might be markers of other diseases, known as tauopathies. Either Aβ plaques or NFFs as well as loss of neuronal mass may be found in non-affected elders as consequence of ageing.

2.1 History and classification

Alzheimer's disease was first described by German psychiatrist Alois Alzheimer, on 3rd of November 1906. He was observing 51 years old female patient from Frankfurt called Auguste Deter, who was admitted in spring 1901 with series of unusual psychiatric symptoms as well as aggressive behavior (Burns *et al.*, 2002). She suffered from memory loss, confusion, disorientation, sleeping problems and her symptoms were getting more serious as the disease was progressing. While he was studying her, Alzheimer noted, that she has a problem with talking or reading, as she was unable to find right words or pronounce them. She had also problems with recognizing common objects and could not remember how to use them. (Maurer, Volk and Gerbaldo, 1997)Later on, he left Frankfurt, but did not lose his interest about Auguste D. and studied her medical reports. In her final year, she was not able to communicate and became apathic. After her death in spring in 1906, her brain was sent to Alzheimer, who was now in charge of the anatomical laboratory of the Royal Psychiatric Clinic in Munich. He was able to examine the histological changes in her brain, which caused those peculiar symptoms He was the first to report and describe features today known as plaques and neurofibrillary tangles, which he stated as the most probable cause. He presented his discovery at the 37th Meeting of

South-West German Psychiatrists in fall 1906 but got just a little interest and support from other doctors and scientists. Nevertheless, he kept studying patients with similar symptoms and between 1907-1908, he reported three more cases with same pathological features. In 1910 his colleague and good friend doctor Emil Kraepelin published his textbook *Psychiatrie,* where he included the report about Auguste Deter and called this peculiar condition Alzheimer´s disease. A year later Alzheimer published his report of another patient with the same condition (Dahm, 2006). His name was Johann F. and when he died (three years after admission), his autopsy revealed only plaques and no tangles, Alzheimer, therefore stated "plaques only cause of AD" theory (Hippius and Neundörfer, 2003). Alzheimer devoted the rest of his career to studying similar cases and died in 1915, long before his findings made an impact in the world.

2.2 Familial form of AD

The familial form of Alzheimer´s disease (FAD) is autosomal-dominant hereditary early-onset AD (EOAD) and affects people aged 30-65 years. FAD seems to have more rapid development than the sporadic form and it´s caused by specific genetic mutations. There are at least four genes responsible for AD: APP gene on chromosome 21 (more than 30 pathogenic mutations have been described), presenilin-1 gene (PS1) on chromosome 14 (associated with more than 170 mutations), presenilin-2 gene (PS2) on chromosome 1 (only 18 potentially pathogenic mutations have subsequently been reported, making this the least common genetic cause of AD) (Kar *et al.*, no date; El Kadmiri *et al.*, 2014) and ε4 allele of apolipoprotein E (APOE) on chromosome 19, which significant mostly in late-onset AD (Bird, 2009). These genetic findings explain the cause of FAD sufficiently, but they are rare and cause less than 5% of all cases of AD (Kanekiyo, Xu and Bu, 2014; Tellechea *et al.*, 2017).

Because of the high prevalence of AD in people with Down syndrome (trisomy 21), it was believed that gene responsible for causing AD is on chromosome 21 (Wu *et al.*, 2017). People with Down syndrome have an extra copy of APP gene. This means more APP protein may be processed to Aβ plaques, which increases the chance of getting AD, usually around the age of 40 (Annus *et al.*, 2017; Coppus *et al.*, 2012). In 1987 a gene locus for APP on chromosome 21 was found and later on, first APP gene mutation was reported and associated with Aβ plaques Several missense mutations or duplications of this gene and they all cause amyloidogenic processing of APP, therefore induce plaques forming (Veugelen *et al.*, 2016).

Some cases of FAD were reported to have no connection with chromosome 21, thus it was stated that the cause is heterogenic (George-Hyslop *et al.*,1990). In 1992 locus on chromosome 14 was found and 3 years after that PS1 mutations were identified as another cause of FAD. Later on, also PS2 missense mutations were reported. PS1 and PS2 encode proteins, which are essential for γ-secretase (Wolfe and Yankner, 2016). There might be almost 200 mutations in these two genes (most of them in PS1), usually, substitutions, deletions or insertions and each is defective in γ-secretase activity that leads to disruption of APP cleaving.

2.3 Sporadic form of AD

The large majority of the cases (95-97%) is non-hereditary sporadic (SAD) late-onset (LOAD) AD. It affects people above age 65 years and has more typical clinical symptoms. The cause is not defined yet, although it is most probably a combination of genetic, environmental and other risk factors. Commonly it seems that increasing age and possession of APOE ε4 allele has the most serious impact here (Michaelson, 2014).

APOE is 299 amino acids long lipoprotein, mainly responsible for transporting cholesterol and other lipids through APOE receptors (Poirier, 2005; Hauser and Ryan, 2013). It is synthesized mostly in liver and CNS. In the brain, it is one of the most important and prevalent protein and it is secreted mainly by astrocytes or microglia. APOE´s major role in CNS is redistribution of lipids for neuronal repairs or remyelination. APOE also regulates neuronal receptors and synaptic formation and plasticity (Yu, Tan and Hardy, 2014).

APOE has three polymorphic alleles, located on chromosome 19: APOE ε2, APOE ε3 (the most common one) and APOE ε4, differing by two amino acids - cysteine (Cys) and arginine (Arg), at positions 112 and 158 (Panza *et al.*, 2012). These differences give each APOE isoform slightly distinct biological functions, for example in the regulation of lipid transport or neuronal signaling (Liu *et al*., 2013). Also, APO ε2 and APO ε3 prefer high-density lipoproteins (HDL), whereas APOE ε4 low-density lipoproteins (LDL) (Kanekiyo, Xu and Bu, 2014). The most important significant difference is that APOE ε4 with Arg residues on both positions increases a risk of getting AD, while APO ε2 (with two Cys) can decrease this risk, or even have the protective effect. Possession of one ε4 allele doubles frequency of AD compared to noncarriers and two ε4 alleles quadruplicate it (Daniell, 2012). APOE ε4 has a negative influence on lipid homeostasis in the brain, that

leads to the reduced signaling efficiency, formation of Aβ plaques and neurofibrillary tangles, disruption in remodeling of synapses, etc. (Poirier *et al.*, 1995; Michaelson, 2014).

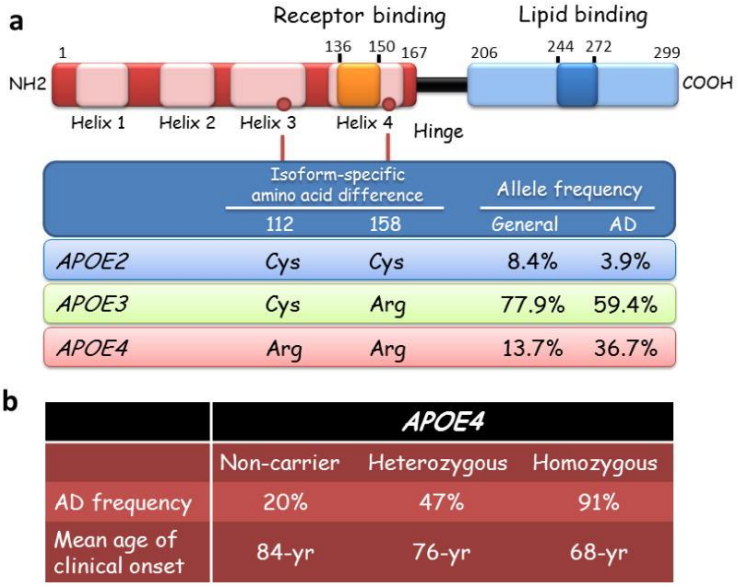

Fig. 2: (a) The ApoE2, E3, and E4 isoforms, which are encoded by the ε2, ε3 and ε4 alleles of the *APOE* gene, respectively, differ from one another at amino acid residues 112 and/or 158 (red circles). ApoE has two structural domains: the N-terminal domain, which contains the receptor-binding region and the C-terminal domain, which contains the lipid-binding region. The two domains are joined by a hinge region. (b) *APOE* ε4 increases the risk of AD and lowers the age of disease onset in a gene-dose-dependent manner (adapted and edited from Liu *et al*., 2013).

3 Physiology of cholinergic system

The cholinergic system is neurotransmission system, that consists of neuronal pathways, which use acetylcholine (ACh) as their neurotransmitter. The receptors stimulated by ACh are called cholinergic receptors and they are present in both central and peripheral nervous system (NS). The main agonists, except of ACh, are muscarine and nicotine, therefore, there are two types of cholinergic receptors: muscarinic (mAChR, activated by muscarine) or nicotinic (nAChR, activated by nicotine). Some chemical compounds can block the receptors for ACh, thus inhibit the biological responses. These compounds are called antagonists and the most common are tubocurarine (for nAChRs) or atropine (for mAChRs). As there are more subtypes of both nicotinic and muscarinic receptors, there are also many subtype-specific antagonists. The cholinergic system of the mammalian forebrain plays a critical role in modulating neuronal activity throughout the cortex, hippocampus, and basal

ganglia, therefore it is essential in higher nervous activities (Allaway and Machold, 2017). Thus, degeneration of the cholinergic system induces a memory defect by reducing the occurrence of information gathering mode (Ikonen, 2001).

3.1 Physiological functions mediated by acetylcholine

ACh is an organic naurotransmiter and it is synthesized from choline and acetyl coenzyme A (acetyl Co-A) in a simple reaction catalyzed by choline acetyltransferase (ChAT). The larger amount of choline used for synthesis comes from the breakdown of ACh or phosphatidylcholine, acetyl Co-A comes from pyruvate. ChAT is a transferase enzyme, highly concentrated in cholinergic neurons, both central and peripheral, mostly in nerve terminals, where most of the synthesis takes place.

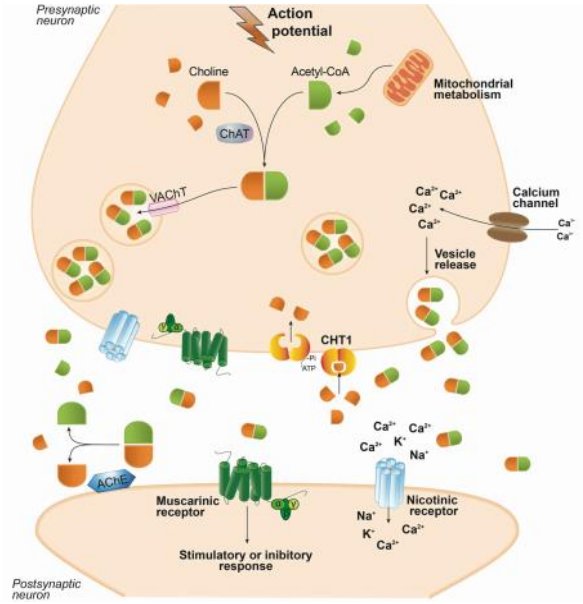

Fig. 3: Schematic representation of biological aspects involving acetylcholine neurotransmission (adapted and edited from Ferreira-vieira *et al.*, 2016)

ACh is stored in vesicles in terminals and released during repolarization via vesicle fusion. After depolarization is ACh is hydrolyzed by the enzyme acetylcholinesterase (AChE), releasing acetate and choline, which is re-uptaken into the presynaptic cholinergic neuron by choline transporter (CHT1). AChE is found mostly in the synaptic cleft and neuromuscular junctions. (Agranoff *et al.*, 1999).

ACh is mainly responsible for neuronal signaling in CNS, mainly present in neurons of the hippocampus, basal forebrain and cerebral cortex. In peripheral NS (PNS), its major role is in the neuromuscular junction. ACh is also important in autonomic NS, as the main

neurotransmitter in parasympathetic neurons and pre-ganglionic naurotransmitter of sympathetic neurons (Ferreira-vieira *et al.*, 2016).

3.2 Nicotinic acetylcholine receptors

The nicotinic receptors are pentameric transmembrane proteins and belong to group of ligand gated ion channels. Binding of ACh to nAChR induces the opening of the pore and the flow of cations through the channel, which causes depolarization of the postsynaptic membrane. The nAChRs consist of five polypeptide subunits surrounding the pore, with selectivity for K^+, Na^+ and Ca^{2+} ions (Lombardo and Maskos, 2015). Different assembly of the subunits creates various subtypes of nAChR.

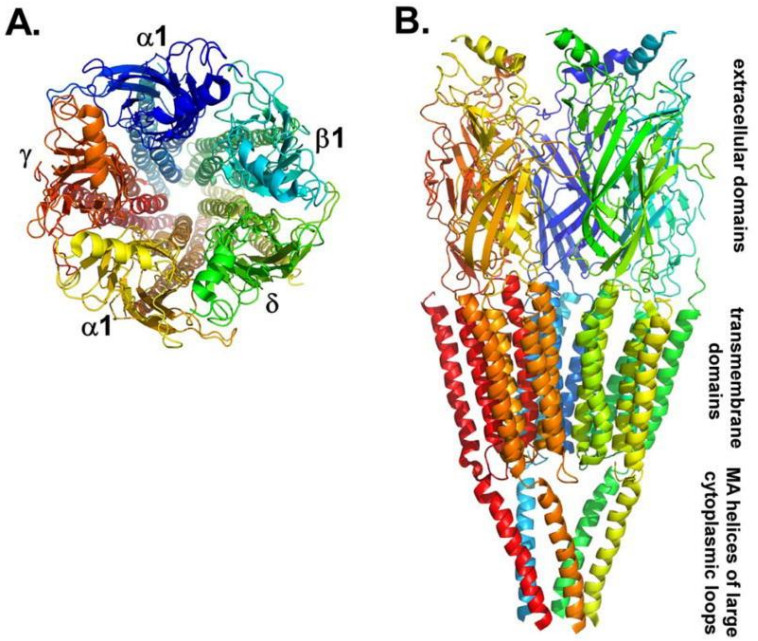

Fig. 4: Ribbon diagram of the pentameric structure of the nAChR from *Torpedo marmorata*. (A) View from the extracellular side of the nAChR and looking along the path of ion travel into a cell. The five subunits are labeled (α1)γ(α1)δ(β1). (B) View in the plane of the transmembrane domains. (These images were created from Protein Data Bank accession 2bg9, adapted and edited from Wells, 2008.)

There are generally two subtypes or nAChR: muscle and neuronal type, which differ in structure and binding sites. The neuronal type is present particularly in NS and has more subtypes, most common are α7 and α4β2. The muscle nAChR are crucial for the neuromuscular junction (Jones, Sudweeks and Yakel, 1999).

The muscle type of nAChRs was one of the first studied ligand gated receptors for neurotransmitters. For the first time, it was isolated from the Pacific electric ray (Torpedo

californica). It has two copies of α and single copies of β, γ, and δ subunits (Agranoff *et al.*, 1999). These subunits are homologous to each other from 35 to 40% (Levin, 2002).

The neuronal nAChR are either heteromeric, consisting of α and β subunits or (e.g. α4β2) or homomeric consisting of five α subunits (e.g. α7). The α4β2 nAChR receptor is widely expressed in CNS and significant roles in attention, learning, memory and also AD. The α4β2 consists of three/two α4 subunits and two/three β2 subunits, thus there are two stoichiometric receptors: ((α4)2(β2)3) and ((α4)3(β2)2) (Carignano, Barila and Spitzmaul, 2016).

The α7 receptor consists of five α7 subunits and it is found in neurons as well as in glial cells. Increased up-regulation of α7 nAChR expression in basal forebrain neurons may signal a compensatory response to maintain baso-cortical cholinergic activity during the onset of AD (Mufson et al. 2009). The evidence of an increase of the α7 corresponds with findings of raised α7 nAChR mRNA and protein expression levels in hippocampal neurons, astrocytes and peripheral blood leukocytes in AD (Teaktong et al., 2003). High expression of α7 nAChR may arise as a compensatory response that is offset by aberrant Aβ-α7 nAChR interactions, leading to cholinergic dysfunction (Mufson et al., 2009). The reported up-regulation in α7 nAChR in early AD might direct baso-cortical cholinergic tone through pre- and postsynaptic processes of cholinergic neurons primary to the degeneration in the later stages of AD. (Dineley, Pandya and Yakel, 2015).

The nAChRs are preferentially blocked by the competitive antagonist *d*-tubocurarine, but they have other antagonists, such as snake α-toxins (for muscle nAChRs), or neuronal bungarotoxin, trimethaphan and mecamylamine (for neuronal nAChRs) (Agranoff *et al.*, 1999).

3.3 Muscarinic acetylcholine receptors

The muscarinic receptors belong to class I of a group of G-protein coupled receptors (GPCR) complexes with the seven-transmembrane domain. The GPCRs are metabotropic receptors, which means that they signal through second messengers (Fryer, Christopoulos and Nathanson, 2012). The GPCR binds with a ligand, that leads to a change in conformation of GPCR and interaction with G-protein. G-protein is a heterotrimeric protein with α, β, γ subunits and GDP (guanosine diphosphate). The interaction causes exchange of GDP for GTP (guanosine triphosphate), that makes G-protein activated. A conformation change of G-protein follows, as α subunit with GTP dissassociates from β and γ and activates effectors and secondary messengers (Vischer *et al.*, 2011).

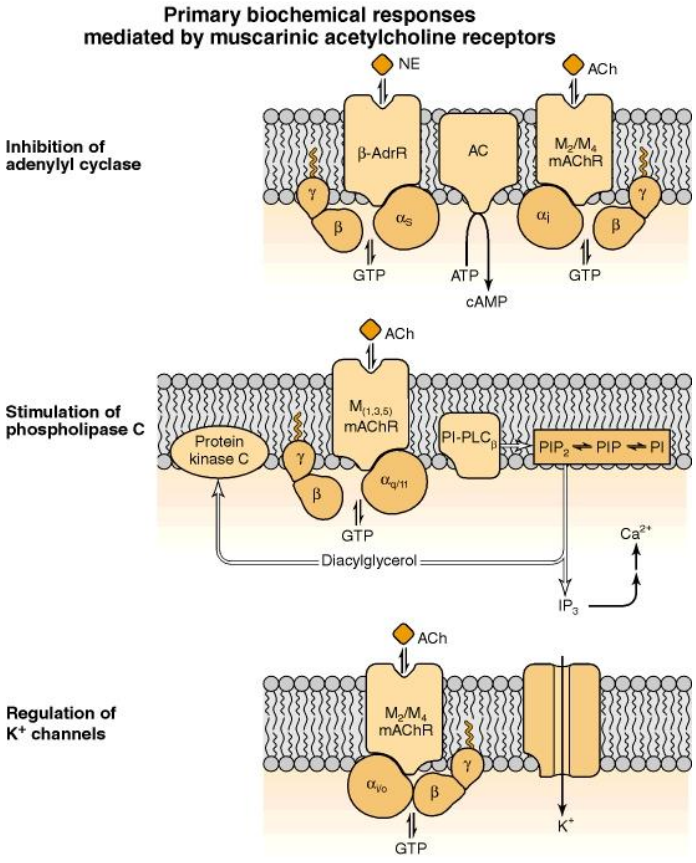

Fig. 5: ACh interacts with mAChR of the subtypes indicated to induce various responses. The M_2 and M_4 interact with the α subunit of G-protein, that inhibits adenylyl cyclase (AC). The M_1, M_3 and M_5 mAChRs interact with G-proteins and the $G\alpha_q$ and α_{11} subunits activate phosphoinositide-specific phospholipase C (PI-PLC). The M_2 and M_4 mAChRs regulate inwardly rectifying K^+ channels through the βγ subunit. Diffusible second messengers formed within the cell include cAMP, inositol trisphosphate (IP_3) and diacylglycerol (DAG) (adapted and edited from Arganoff *et al.*, 1999)

There are five subtypes of mAChR, which are topographically distributed throughout the body: M_1, M_2, M_3, M_4 and M_5.

M1 receptors form a significant part of the total amount of muscarinic receptors in the frontal part of the brain (in the cortex, hippocampus or striatum up to about 50%). On the other hand, the amount of M1 is decreasing in the caudal direction, in the thalamus and hypothalamus there are about one quarter and even less in pons or cerebellum. M1 receptors are found in pyramidal cells of all layers of the cortex, especially in layers II, III and VI. They are found on the neurons and dendrites, which is consistent with the role of M1 as the main muscarinic postsynaptic receptor (Fryer, Christopoulos and Nathanson, 2012).

Also, M2 receptors are very prevalent very in the CNS, where they are main auto-inhibitory receptors. Their density across the brain seems to be approximately constant. In proportion to other muscarinic receptors, most M2 receptors are in the caudal parts of the brain, such as the cerebellum, the medulla oblongata, the pons, but also in the hypothalamus and thalamus (Eglen, 2012).

Compared to the previous two mAChR subtypes, the number of M3 receptors is noticeably lower in the CNS and accounts for approximately 5-10% of all receptors. M3 receptors are scattered throughout the brain but in small and separate neuronal subpopulations. We find them for example in the cortical regions of the limbic system, the striatum, the hippocampus, the front nuclei of the thalamus or the kernels of the pons. (Fryer, Christopoulos and Nathanson, 2012)

M4 is again one of the most abundant mAChR subtypes in CNS. Large numbers are found in the cerebral cortex, and in many of its parts, M4 sites overlap with M1. Many M4 receptors are found in the occipital cortex, which represents the primary visual area. Most of the M4 receptors in the caudate and putamen nucleus, as well as in the globus pallidus. Like M2, M4 also serves as a presynaptic auto-receptor. M2 have function mainly in the hippocampus and cerebral cortex (Fryer, Christopoulos and Nathanson, 2012)

The distribution of M5 is very dense in the outermost parts of the cortex, substantia nigra and ventral tegmental area. In the hippocampus, M5 are found in small separate subpopulations of neurons. Almost no M5 is found in the brain stem. Only few M5 receptors are found in PNS (Fryer, Christopoulos and Nathanson, 2012).

A new family of M1 partial agonists was developed (AF102B, AF150(S) and AF267B-i) (Mufson *et al.*, 2009) in the context state, that summarizes the principal deficits of AD, chronic AF267B treatment protected cognitive deterioration and reduced Aβ42 and tau impairments in the cortex and hippocampus. The transformation reportedly related with M1 mAChR-mediated activation of the TNFα-converting enzyme ADAM17/TACE, decreased β-secretase steady state levels and inhibition of g lycogen synthase kinase 3β (GSK3β) (Caccamo et al., 2006).

4 Pharmacological targets in AD

The mild cognitive impairment (MCI) is a typical condition affecting people after the age of 65 and seems to be a normal consequence of aging (Sanford, 2012; Ward, 2011). The MCI leads other stages of dementia. The pharmacological goal is to prevent further

impairment or to slow down the progress of MCI. This goal includes finding a way to diagnose AD before the MCI symptoms start to develop (Mufson *et al.*, 2009).

After the loss of cholinergic neurons and decrease of activity of ChAT in patients with AD was discovered, the cholinergic hypothesis was brought forward (Francis *et al.*, 1999) Anatomic reports supported it and linked it with memory and cognitive dysfunctions. This observation meant, that the drugs for treating AD, would need to target cholinergic transmission and increase the level of ACh. The pharmacology of AD emerged from this hypothesis and tried to develop drugs, that would selectively target cholinergic receptors in the brain and enhance the cholinergic activity.

The cholinergic drugs (or cholinomimetics) imitate the effect of ACh and therefore improve the cholinergic transmission. The cholinergic drugs can act directly as muscarinic and nicotinic agonists or indirectly as cholinesterase inhibitors.

The most of the researchers report that physostigmine and oral anticholinesterases have been discovered as a beneficial factor for the AD treatment. Generally, it can be assumed that the basal forebrain system remains somewhat preserved in the course of dementia, in spite of the well-documented decline of the cholinergic biosynthetic complex (ChAT and AChE enzyme loss). Furthermore, research has also identified some specific part of the hippocampal and cortical cholinergic projection system, that seems to have the potential of compensation and neuroplasticity within the early stages of AD (Mufson *et al.*, 2009).

A second major research line of AD is based on in the concept of the loss of basal forebrain cholinergic neurons within the late phase of AD (Davies and Maloney, 1976; Whitehouse et al., 1981). Considerably complexity of baso-cortical cholinergic system is currently emphasized as can be deduced on the base of former experimental lesions in animals (Gilmor et al., 1998) and postmortem human brain studies (Pearson et al., 1983), which have proposed that a huge amount of cholinergic neurons more likely than degenerate are lessened, depleted of phenotypic markers or survive during an atrophic state caused by trauma or pathological process. The findings indicate that basal forebrain neurons might be, despite their dysregulation, viable and thus open to pharmacologic interventions with potential to prevent or reduce degeneration.

Another possible approach to treat AD patients could be targeting CHT1 since choline uptake by cholinergic neurons is the rate-limiting step for ACh production. It has been shown that changes in the expression, trafficking or activity of CHT1 could directly affect choline uptake and, consequently cholinergic neurotransmission.

4.1 Inhibitors of cholinesterase

The inhibitors of cholinesterase inhibit the hydrolyze of ACh, thus increase the synaptic levels of ACh. AChE, but also butyrylcholinesterase (BuChE) are both responsible for ACh degradation, which means that specific targeting drug, with inhibitive effect, might decrease cholinergic loss. The inhibitors of AChE (AChEI) are the most potent and also the only clinically approved drugs for the treatment of AD (Foster *et al.*, 2012). Donepezil is one of the most used AChEI and it has proved to be very efficient to slow down or delay cognitive decline (Burns *et al.*, 1999). Rivastigmine inhibits both AChE and BuChE, and galantamine selectively inhibits AChE and also modulates nicotinic ACh receptors. The physostigmine has been shown to enhance selective attention (Kuo and Rajesh, 2017). The clinical significance of these additional mechanisms of action has yet to be determined. It has been suggested that glutamate-mediated toxicity may play a role in neurodegeneration in AD (Hynd, Scott and Dodd, 2004).

On the other hand, AChEIs have shown several side effects in PNS. The nAChRs are greatly expressed in muscle, where AChEI induce muscle rigidity. AChEI increases level of ACh, which activates mAChRs. This activation leads to slow down a heart rate in M2 and causes urinary incontinence and increased sweating in M3 receptors (Fryer, Christopoulos and Nathanson, 2012; Ferreira-vieira *et al.*, 2016). A wide range of clinical trials concentrates on treating the cholinergic disruption in AD and even has focused on testing the efficacy of cholinesterase inhibitor drugs (Mufson *et al.*, 2008).

4.2 Pharmacology of nicotinic receptors

There are two main types of these receptors, differing in their subunits neuronal type in the vegetative ganglia, and the muscle type on the neuromuscular junction. The opening of the channel occurs when two molecules of acetylcholine are bound to sites at the aδ and aγ Subunits. The channel is open for about one millisecond, with a flow of Na^+ cations, less K^+ and Ca^{2+} (Levin, 2002). The central canal pore consists of five homologous M2 α helixes, each of one subunit. The cations flow here without being stripped of their water molecules. Nicotine receptors interact with many natural and synthetic substances. Depending on their effect on nAChR, they can be classically divided into several groups. However, the boundaries between them may not always be quite clear, as their effect is often more complex. Simplified, however, these substances can be grouped between receptor activating agonists, antagonists that inhibit their activation, or allosteric modulators that otherwise modulate

receptor activity. Allosteric modulation may be positive or negative. In addition, the effect of one substance may vary depending on the subunit composition of the receptor. Therapeutically interesting in particular are substances that selectively bind only to a particular subtype of the receptor. However, such substances have been reported relatively little for nAChR (Linden, 2012).

4.3 Pharmacology of muscarinic receptors

The structure and further signal transmission of mAChR are completely different from nAChR. The mAChR are associated with different types of G-proteins and also differ in cellular responses that ACh makes by receptor binding. M1, M3, M5 transmit a signal to cells, in particular, G-protein, designated as Gq or G11, that activate the phospholipase C (PLC). Other subtypes associate with Gs G-proteins, whose subunit stimulates the activity of adenylate cyclase (AC) and cyclic adenosine monophosphate (cAMP). M2 and M4 are preferably coupled to other types of G-proteins - Gi and Go, that inhibit AC activity (Agranoff *et al.*, 1999). As a result of cAMP depletion, protein kinase A (PKA) mediates phosphorylation of various cellular proteins and transcription factors. M2 and M4 also directly interact with potassium channels in the heart, which affects ion channels such as, suppressing calcium intake through voltage-controlled calcium channels at the nerve terminals and thereby stimulated the release of mediators.

In addition to the above-mentioned heart, different mAChR subtypes also are expressed in many different tissues. One of these is the detrusor - the smooth muscle that is involved in urinating urine. All subtypes of mAChR are present, but M2 and M3 predominate, M3 being three times more. However, it is interesting to note that minor M3 receptors are involved in the contraction of this muscle. The binding of acetylcholine to M3 mAChR is also the source of contraction of all of the smooth muscle (Greenlee *et al.*, 2001; Racké, Juergens and Matthiesen, 2006).

4.3.1 Muscarinic agonists

The muscarinic agonists activate mAChRs, by direct binding to the active site. There are endogenous (neurotransmitters) and exogenous agonists (drugs). The pharmacological approach of finding the M1 receptor-subtype selective agonists appears to be therapeutically very promising but is still in the clinical testing phase. The M1 receptor is the most represented subtype of mAChRs in CNS and plays a significant role in the development of ACh. For the time being, M1 muscarinic agonist (AF267B) has shown to support in vivo and

in vitro tests, a non-amyloidogenic APP cleavage pathway mediated by α-secretase, which instead of toxic Aβ produces soluble APPsα with a neuroprotective character (Linden, 2012). In addition, a decrease in Aβ level in cerebrospinal fluid was observed, and even a beneficial effect on τ protein, that was not hyperphosphorylated.

Other M1 receptor-inducing agents introduced into clinical trials are talsaclidine and xanomeline, which are also shown to have a positive effect on decreasing Aβ and τ protein levels. They also potentiate the effect of AChEIs, thereby enhancing the cognitive function of ACh in patients. Receptor-non-specific test substances have many undesirable effects, therefore they will probably not be used (isrolin, oxotremorin) (Fisher *et al.*, 1998; Greenlee *et al.*, 2001)

The idea for the development of M1 agonists seems promising, but there are difficulties with realizing it, as the agonists shown only modest selectivity for the M1 receptor. (Fisher *et al.*, 1998; Lütjens and Rocher, 2017)

4.3.2 Positive allosteric modulators

Allosteric modulators indirectly induce the effect of natural agonists by binding to other than the orthosteric site, followed by a change of conformation. Monod, Jacob and Wyman have developed an idea of how allosterically regulated enzymes should look. They are able to switch from active to inactive, with both states varying in their spatial conformation. The transition from one state to the other is caused by the binding of an allosteric effector (or several effectors, for example, one acting inhibitory and the other activating).

There are positive (PAM), negative (NAM) or silent (SAM) allosteric modulators, which can enhance, inhibit the function of an agonist or neutrally occupy the allosteric binding site. Modulation of the receptor functions by PAM offers many therapeutic advantages over agonists or antagonists (Lütjens and Rocher, 2017). They can be used to reduce or increase the effect of a standard release endogenous agonist without causing sustained inhibition or activation of the receptor. There are also allosteric modulators, which activate receptors from the allosteric site in absence of ACh.

Above all, there is an attempt to find such PAM that will bind only to a specific subtype, allowing for a specialized modulation of the receptor function. For this reason, it could be substances with fewer side effects that could be safer and more specific (Linden, 2012).

The clinical trial with the M1/M4 orthosteric agonist xanomeline has shown improvement in cognitive functions, however, it has also shown gastrointestinal side effects, therefore PAM could be a better fit. Recently, Merck, Vanderbilt, Takeda and Pfizer have identified M1 PAMs active in rodent models but gastrointestinal, cardiovascular and convulsion findings have been reported with M1 PAM compounds and the safety margin of this class should be well established (Lütjens and Rocher, 2017). Activating of M1 proved to reduce pathology of APP and accumulating of plaques (Fisher, 2008).

The discovery of M1/M4 positive allosteric modulators has also provided a new approach that holds great potential for the treatment of CNS disorders (Ferreira-vieira *et al.*, 2016). The advantage of allosteric modulation is, in particular, the maintenance of natural timing and localization of ACh signalling. Its effect is only to enhance the cholinergic transmission in specific subtypes of mAChR in CNS and no undesirable stimulation or side effects (Ciruela, 2008).

Conclusion

In this thesis, we were trying to summarize informations of possible pharmacological targets in cholinergic transmission, for AD treatment.

The cholinergic system plays an important role in cognitive functions and the loss of cholinergic neurons is one of the hallmarks of AD. There are only a few drugs that have been approved for treating AD patients so far, including the cholinesterase inhibitors. However, none of them treat the cause of disease, but they merely slow down the progression or delay the symptoms.

Therapeutic approaches that are very much needed must consist of an early diagnosis before MCI symptoms occur and efficient treatment to prevent the neurodegeneration. The treatment has to inhibit the Aβ and NFT formation and a decrease of neuronal loss and synapses. Therapies that might have the potential to stop the cholinergic neuronal death, are based on targeting cholinergic system, in either direct or indirect way.

The most promising approaches seem to be PAM, which thanks to the ability to bind to an allosteric site, might be able to target subtype-specific receptors and thus avoid side effects.

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