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Current Alzheimer Research, 2020, 17, 224-237

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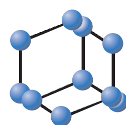

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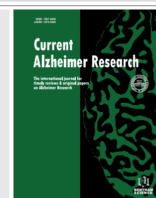
Dangerous Liaisons: Tau Interaction with Muscarinic Receptors



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ARTICLE HISTORY

Received: April 05, 2019
Revised: February 05, 2020
Accepted: March 25, 2020

DOI:
10.2174/1567205017666200424134311

CrossMark

Abstract: The molecular processes underlying neurodegenerative diseases (such as Alzheimer's Disease - AD) remain poorly understood. There is also an imperative need for disease-modifying therapies in AD since the present treatments, acetylcholinesterase inhibitors and NMDA antagonists, do not halt its progression. AD and other dementias present unique pathological features such as that of microtubule associated protein tau metabolic regulation. Tau has numerous binding partners, including signaling molecules, cytoskeletal elements and lipids, which suggests that it is a multifunctional protein. AD has also been associated with severe loss of cholinergic markers in the brain and such loss may be due to the toxic interaction of tau with cholinergic muscarinic receptors. By using specific antagonists of muscarinic receptors it was found *in vitro* that extracellular tau binds to M1 and M3 receptors and which the increase of intracellular calcium found in neuronal cells upon tau-binding. However, so far, the significance of tau signaling through muscarinic receptor *in vivo* in tauopathic models remains uncertain. The data reviewed in the present paper highlight the significant effect of M1 receptor/tau interaction in exacerbating tauopathy related pathological features and suggest that selective M1 agonists may serve as a prototype for future therapeutic development toward modification of currently intractable neurodegenerative diseases, such as tauopathies.

Keywords: Tau, tauopathies, neurodegenerative diseases, muscarinic receptors, cholinergic system, review.

1. INTRODUCTION

The main physiological function of the tau protein is to promote tubulin polymerization and stabilization of microtubules that form a cytoskeletal network in neurons. In physiological condition, tau is located mainly in the distal parts of axon [1]. The abnormal transformation of tau protein in the brain leads to impaired axonal transport and disordered signaling between neurons [2]. Tauopathies are dementias and movement disorders and their common feature is an accumulation of abnormal filamentous deposits inside neurons of various brain structures in the form of PHF (Paired Helical Filaments) or NFT (Neurofibrillar Tangles), composed of aggregates of highly phosphorylated tau protein [3]. The severity of clinical symptoms in the course of these diseases, including Alzheimer's Disease (AD), correlates with the amount of abnormal tau protein deposited in the central nervous system [4].

Numerous studies indicate that neurons which contain toxic forms of tau degenerate and release tau deposits into the extracellular space and that pathological tau, mainly in the form of oligomers then spread between neurons through synaptic connections. One of the first studies indicating that degenerative changes in AD may spread trans-synaptically, was conducted by Saper *et al*. [5].

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Further studies have confirmed these reports and revealed that pathogenic proteins, including beta-amyloid (Aβ), α-synuclein and tau can spread in the nervous system like prion proteins, *i.e.* they can be secreted into the extracellular matrix, and being taken by other cells can inoculate intracellular aggregates in these cells [6-8].

Tau can be secreted from cells actively by exocytosis or passively by secretion and taken up by neighboring cells by endocytosis or by interaction with membrane lipids. Transfection of new cells can also occur through Tunneling Nanotubes (TNTs), which form connections between different populations of cells in the brain [9]. There are many indications that extracellular tau can induce cell death, which in turn causes the release of next pool of intracellular tau into the extracellular space, and this new amount of extracellular tau can interact with healthy neurons and thus promote their degeneration. As noted earlier, extracellular tau secretion may be independent of cell death [10-12]. Tau has been shown to be physiologically released into the extracellular space by neurons both in *in vitro* and *in vivo* studies in mice [13].

Once tau is already in the extracellular space, it can function as a signaling protein. Tau protein can form complexes with enzymes and act as a direct activator or inhibitor of them. It also has the ability to form complexes with many other proteins and elements in the cell and to interact with signaling pathways that determine cell survival. It is suggested that during the initial step of pathological or even

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physiological tau spreading, small tau oligomers specifically interact with neuron specific receptors, although we cannot exclude the existence of other unspecific endocytosis pathways of tau internalization [14-18].

The oldest historical hypothesis on the etiology of AD assumed impaired signal transduction in the cholinergic system, which is responsible for attention processes, learning and memory storage. Recently, it has been hypothesized that impairment of the cholinergic system may be associated with the occurrence of abnormal forms of tau protein in the intercellular space. It has been shown that tau binds to muscarinic M1 and M3 receptors [19]. Moreover, the presence of pathological forms of tau protein in the brain causes a deficiency of the G-protein-coupled receptor kinase 5 (GRK5), this deficit induces a disturbance of presynaptic M2 receptors signaling. Interaction between tau and muscarinic receptors induces a massive influx of calcium ions into the cell [20, 21]. Increased calcium concentration could activate Gq/G11 proteins/phospholipase C (PLC) pathway [22] which, in turn, could modify tau, making it more toxic. Both excessive calcium level and increased tau toxicity lead to neuronal death [23, 24].

M1 receptors dysfunction is involved in several pathological changes observed in AD-parenchymal and cerebrovascular amyloid deposition [25, 26], neurofibrillary tangles [27, 28], neuroinflammation [29] and cognitive decline observed in 3xTgAD mice with the deletion of the M1 receptor gene [30]. In addition, excessive tau phosphorylation and the occurrence of amyloid deposits in AD mice lacking M1 have been found to be associated with high glycogen synthase 3 beta (GSK-3β) and protein kinase C (PKC) activity [31]. These findings were confirmed by the data showing that the deletion of gene for M1 receptor increased the presence of pathological Aβ in $APP_{Swe/Ind}$ mice. Finally, disabling the gene for the M1 receptor increased the pro-inflammatory activation of astrocytes and microglia in response to the deposition of Aβ plaques [32].

Explaining the role of muscarinic receptors in the development of AD may help to modify “Pro-cholinergic” therapies by including compounds which will alter the functional state of these receptors or their affinity to tau, and in this way may prevent or to slow down the progression of tauopathies.

2. TAU: ITS MODIFICATION AND DISTRIBUTION IN BRAIN TISSUE

Tau, called a Microtubule-Associated Protein (MAP), in its native non-aggregated form binds microtubules and is responsible for microtubule assembly, stabilization and spacing [1]. The human tau gene (MAPT) is located on chromosome 17. It contains 16 exons which may be alternatively spliced to give rise to 12 different isoforms of tau. These 12 isoforms are differentially distributed and expressed during various stages of development [33]. The tau gene is coded for by 16 potential exons, exons 1, 4, 5, 7, 9, 11, 12 and 13 being constitutive exons, and spliced together in the order which they occur in the RNA (apart from exon 1 - the promotor exon, which is transcribed but not translated). Whilst exons 2, 3 and 10 may appear in any order. Tau isoforms arise from alternative splicing of exons 2, 3 and 10, which regulate how many microtubule binding domains and n-terminal inserts appear in the protein [34]. Expression of exon 10 determines four repeat microtubule-binding sequences (4R) in tau molecule, while the alternatively spliced isoforms deprived of exon 10 contain three of these sequences (3R) [35].

Tau is a protein located mainly in axons, where it is connected with microtubules [36], but modified forms of tau can be also found in cytosol of neuronal somata and in dendrites [37, 38] or even in dendritic spines [39]. Moreover, tau has also been identified in the plasma membrane [40, 41] and in the nucleus [42].

Apart from tau function as microtubule-associated protein it may play other roles including axonal transport modulation. Tau interaction with the motor proteins, dynein and kinesin, with which tau competes for binding to microtubules slows down the anterograde and retrograde transport along with the tubulin network [43]. Tau can be also responsible for axonal elongation and maturation [44]. In dendrites tau seems to be involved in the regulation of synaptic plasticity [45]. In the nucleus, tau is associated with ribosomes and the nucleolus organizing region and binds to chromatin. DNA-related tau supports DNA repair processes [42, 46]. Furthermore, tau regulates neuronal activity, neurogenesis, iron export and Long-Term Depression (LTD) [47].

Regulation of tau physiological function occurs mainly through post-translational modifications such as phosphorylation, acetylation, glycosylation, glycation, deamination, isomerization, nitration, methylation, ubiquitination, sumoylation and truncation [48]. However, these modifications may also contribute to conformational changes of the tau molecule that could potentially lead to propagation of tau pathology (Fig. **1**). Predominant post-translational changes are introduced by phosphorylation at many amino acid residues. During AD, a large pool of tau protein becomes abnormally phosphorylated resulting in the loss of microtubule (MT) stability and induction of conformational changes that allow the protein to oligomerize and aggregate into PHFs and NFTs [49]. Phosphorylation on the Thr231 residue by GSK-3β kinase is known to prevent tau from binding to microtubules [49] and to relieve the inhibitory activity of the N-terminus over the C-terminus of tau. This allows kinases such as GSK-3β to access and subsequently phosphorylate tau at other epitopes [50].

Monomeric soluble tau may adopt the so-called "Paper Clip" conformation, in which the C-terminal end is located over the domain of the Microtubule Binding Region (MTBR) and the N-terminus bends to lie near the C-terminal [51, 52]. It is believed that the opening of this paper clip conformation is the first necessary stage of tau oligomerization and that the oligomers formed can be stabilized by post-translational modifications such as phosphorylation [53]. Opening the paper clip conformation and exposing the N-terminus of the tau molecule has additional consequences. A domain capable of activating Protein Phosphatase 1 (PP1) present in the axons has been found between the residues Ala2 and Tyr18 of the N-terminus. Activation of PP1 leads to GSK-3 dephosphorylation, which results in the activation of this kinase and consequently, phosphorylation of kinesin light chains. As a result, kinesin is detached from its cargo

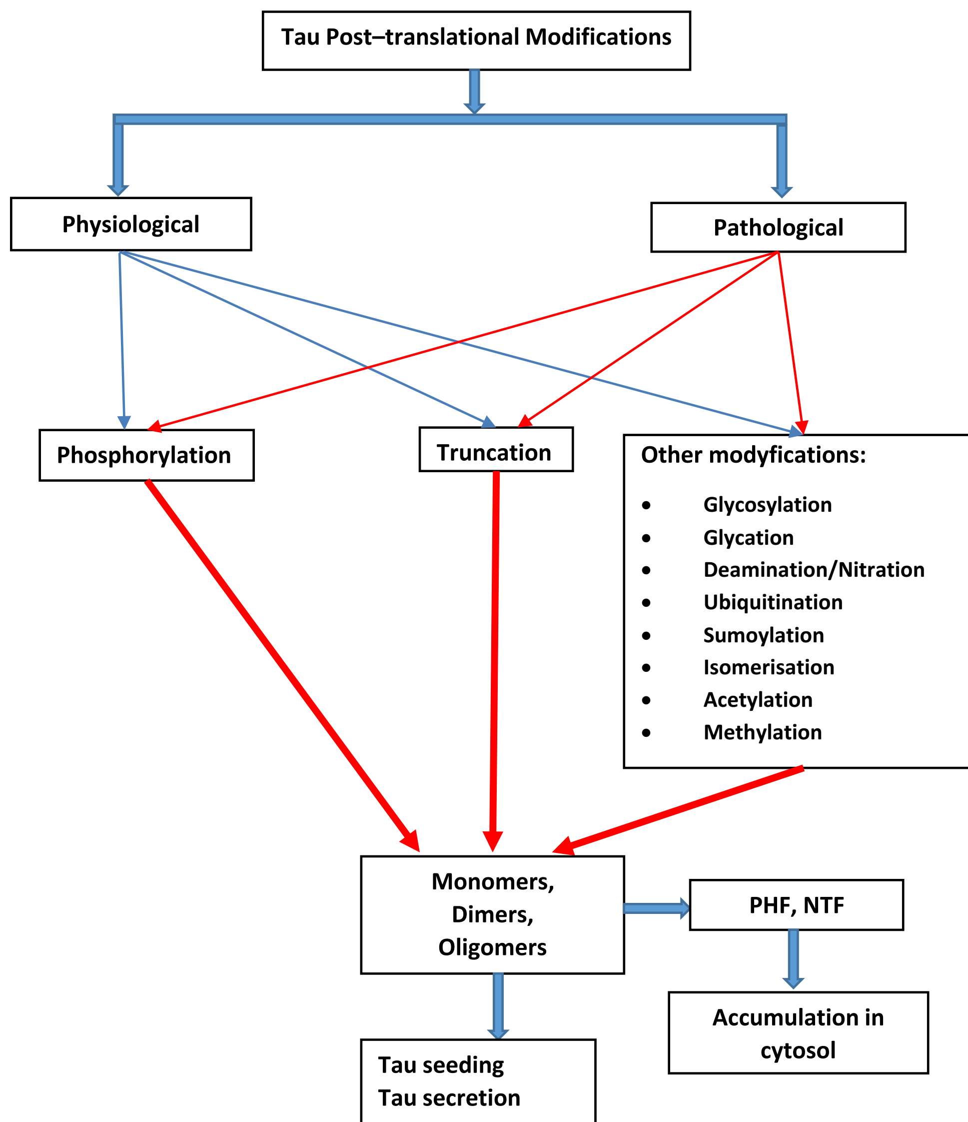

Fig. (1). Schematic presentation of post-translational modifications of tau. When non-physiological (excessive level of a given modification or incorrect modification site) they can lead to formation of toxic tau structures (from monomers to tau aggregates).

and disruption of Fast Axonal Transport (FAT) is observed [54, 55]. In contrast, phosphorylation of the Tyr18 residue abolishes the inhibitory effect of the phosphatase activating domain on FAT [56]. Thus, depending on the modification, Tyr18 may also be a part of the compensatory mechanism reducing toxicity associated with the exposure of the N-terminus of tau oligomers. Furthermore, it was demonstrated that only tau oligomers but not monomeric tau selectively impair FAT [55].

Similarly to the N-terminus, also the C-terminus of tau plays an important role in the formation of tau oligomers or higher-order prefibrillar structures [52, 53]. Ser396 and Ser404 pseudophosphorylation in the AD2 PHF1 tau epitope has been shown to move away the C-terminal from the MTBR region, and to release a paper clip configuration. However, it was found that the combined pseudophosphorylation of epitopes in the N- and C-termini strengthened the structure of the paper clip. As a result, the N-terminus approached the microtubule-biding domain region, forming a conformation-dependent MC1/Alz50 epitope [52]. This conformation promotes tau aggregation and formation of pathological PHF structures observed in AD [57].

An increase in tau phosphorylation reduces its affinity for microtubules and leads to a disruption of the cytoskeleton integrity in neurons [58]. Tau not associated with microtubules can move from the axons to the somato-dendritic compartment, which can cause a disorder of intracellular transport, synaptic conductivity and signaling between neurons [59, 60]. Excessive tau phosphorylation can change its own degradation through autophagy or through the ubiquitin-proteasome complex and increase tau level in cytosol. Tau phosphorylation may increase its aggregation, both observed in AD [61]. Increased tau phosphorylation can also change its interaction with other proteins. *For example*, the phosphorylated form of tau (but not the dephosphorylated form) can interact with the c-Jun N-terminal kinase-interacting

protein 1 (JIP1). Because JIP1 is responsible for cargo binding to kinesin motors, the pathological Tau/JIP1 interaction may interfere with the formation of physiologically active kinesin complex and impair axonal transport [62].

Another important event in the cascade of processes conditioning tau pathology is abnormal acetylation, which causes changes in the conformation of the tau protein molecule and exposes specific sites undergoing then pathological phosphorylation, which in physiological conditions are inaccessible to kinases. Several lysine residues are acetylated by the P300 acetyltransferase and cyclic Adenosine Monophosphate (cAMP) response element binding (CREB) protein. It should be emphasized, however, that acetylation of tau could inhibit its degradation on one side and might suppress tau phosphorylation and aggregation on the other side [63, 64]. Increased acetylation of tau has been found in AD and other tauopathies. Specifically, acetylation at Lys174 has been identified in AD brains and it seems that it may contribute to slowing down tau turnover and may thus be critical for tau-induced toxicity [63].

Because Ubiquitin (Ub) was abundantly located in the senile plaques of AD patients, where it exists in free forms and as protein conjugates, a possible relationship between tau pathology and ubiquitination was considered [65, 66]. Mass spectrometry and immunological data showed that the tau residues Lys48, Lys11 and Lys63 link polyUb chains [67-70]. Tau extracted from PHFs present in the brains of AD co-immunoprecipitated with various proteasome subunits, probably indicating inefficient degradation of the pathological protein [71]. Binding of unprocessed substrates such as tau aggregates reduces the activity of the proteasome complex [72-74]. If the tau level in the cell increases abnormally, the tau should be actively ubiquitinated by Hsp70-interacting C-terminal protein (CHIP) and eliminated by 26S proteasomes. However, when the amount of tau accumulated in the cytosol exceeds the proteasomal capacity, ubiquitinated tau can stimulate the cycle of reciprocal aggregation [75, 76]. Newly formed forms of PHF can interact directly with various proteasome subunits to further inhibit the physiological function of the proteasome, which precludes degradation of damaged tau [76]. Tau ubiquitination observed after exaggerated phosphorylation promotes the aggregation process and prevents its Ub-dependent degradation in proteasomes, especially in people with AD who have reduced proteasome activity.

N-glycosylation similarly to non-enzymatic modifications, such as deamination, is suspected to be involved in tau aggregation, as they may modify conformational structure of tau and decrease its affinity for cytoskeleton [77-79]. On the contrary, other transformations like the O-GlcNAcylation of tau may prevent its phosphorylation [80] and suppress its aggregation [81]. Sumoylation, another tau post-translational modification, may affect tau distribution directly or indirectly in the proteasome [82, 83]. Tau truncation is also a feature of tauopathies and appears to play a significant role in its pathology. Truncated tau oligomers have no tendency to aggregate and may stimulate neurodegeneration as non-aggregated forms that are known to be toxic to neurons [47].

Abnormal modifications of tau protein lead to a series of CNS disorders known collectively as tauopathies, including AD, Down syndrome dementia, progressive supranuclear palsy, cortico-basal degeneration, Pick's disease, certain forms of frontotemporal lobar degeneration, and chronic traumatic encephalopathy.

Tau is mainly an intracellular protein. However, the presence of tau protein has also been found in extracellular space. In addition, it has been shown that extracellular tau not only comes from degrading neurons, but it can also be actively secreted into the extracellular matrix [84]. Extracellular tau is toxic [20, 85]. *In vivo*, tau was found in the brain of mice with tauopathy before neurodegeneration occurred, which proves that it is secreted in the active process and not only passively after cell death [86-88].

To investigate whether the addition of extracellular tau could modify intracellular tau at proline-directed sites, human neuroblastoma SH-SY5Y cells were treated with truncated tau3RC [85], which was shown before to be toxic to those cells [86]. After 48 h incubation of the cells with tau3RC, Díaz-Hernández *et al.,* [85] observed a decrease in endogenous intracellular unphosphorylated tau recognized by Tau-1 antibody. This antibody recognizes an epitope comprising 3 nonphosphorylateded serines in the endogenous tau, that are followed prolines, namely Ser198, Ser199 and Ser202. Thus, this suggests that in the presence of additional pool of tau (tau3RC) intracellular tau becomes highly phosphorylated at the sequence recognized by Tau-1. Cells treated with tau also changed morphologically, they seemed to be more aggregated. Furthermore, using two antibodies, Br133 and Tau1 recognizing only endogenous tau but not truncated tau3RC added to the medium, endogenous tau was detected in the extracellular matrix of cells upon incubation with tau3RC. This indicated that extracellular tau induces the release of intracellular tau into the extracellular space, where it is then dephosphorylated. In addition, the level of extracellular tau phosphorylated at Ser396 and Ser404 (Western blot staining with PHF-1 antibody) and tau phosphorylated at Ser198, Ser199 and Ser202 (Western blot staining with AT-8 antibody) declined as compared to vehicle treatment, therefore confirming that tau undergoes dephosphorylation in the extracellular matrix probably by the cell membrane phosphatases activity.

The physiological function of tau secreted to the extracellular space is still not recognized. Bright *et al.,* [89] suggested that it may play a role in enhancing neuronal activity. Extracellular tau is also subject to pathological propagation. However, the level of tau that can exert harmful effects is much higher than the physiological one [90]. Tau does not contain a signal sequence to regulate its translocation to the endoplasmic reticulum, thus it cannot be secreted by the conventional secretory pathway. Similarly to other proteins lacking a signal peptide sequence, tau is being secreted by route of so called unconventional protein secretion, which may involve vesicular or non-vesicular pathways.

The non-vesicular pathway may be connected with interaction between tau amino-terminal projection domain [40] and the plasma membrane [91]. Nevertheless, up to date there is no evidence that tau can be released through the non-vesicular secretion pathway [90].

With regard to the vesicular pathway, some possible unconventional tau secretory mechanisms have been proposed. One of the possible ways to secrete tau is the exosome-dependent pathway. Two types of extracellular vesicles have been recognized based on their biogenesis: Exosomes and ectosomes [92]. Exosomes are 40-100 nm diameter membranous vesicles of endocytic origin that are released by a variety of cell types into the extracellular space. Exosomes are created from the intraluminal vesicles involved in endocytosis of cytosolic molecules. After internalization, the content of intraluminal vesicles is recycled to the cytosol or future exosomes are accumulated in the form of multivesicular bodies, which bind to the plasma membrane. Upon fusion of multivesicular bodies with the plasma membrane exosomes are released into the extracellular space together with their content [93]. Ectosomes are large vesicles (50-1000 nm in diameter) that are directly released to the extracellular space from cells by plasma membrane budding [94]. This process is stimulated by the activation of many factors, such as intracellular calcium, inflammatory agents and oxidative stress. Dujardin *et al.,* [95] documented that tau is extracellularly secreted rather in ectosomes than in exosomes. Various cellular mechanisms may influence vesicular tau releasing. They probably include some chaperone complexes [96] and certain Rab GTPases such as Rab7a [97] and Rab1a [98].

Another mechanism of tau releasing proposes the formation of thin TNTs to mediate tau secretion and spreading [99, 100]. These structures have been proven to mediate neuron-to-neuron transfer of pathological tau oligomers or small aggregates and, therefore, have been considered a possible, very fast way of tau spreading [101, 102]. The presence of different tau species and isoforms, as it was documented for mutated [103], abnormally phosphorylated and truncated forms of tau [104], has a positive impact on tau propagation to the extracellular space. It was suggested that also lysosomal dysfunction and starvation could increase tau secretion [98, 105]. Moreover, increased neuronal activity may cause enhanced tau releasing. In return, this extracellular tau influences neuronal activity, which suggests the existence of a positive feedback loop between tau protein and neuronal activity [90]. However, it should be noted that tau released *via* the vesicle-mediated way is a rather small fraction of tau present in the extracellular space [39] and the majority of which comes from degenerating and disintegrating neurons.

3. MUSCARINIC RECEPTORS DISTRIBUTION AND FUNCTION

Muscarinic acetylcholine receptors (mAChRs) are G-protein-coupled, seven transmembrane domain receptors. Five subtypes of mAChRs (M1-M5) have been identified using pharmacological methods. All mAChRs subtypes are expressed in the CNS, while M2 and M3 receptors are also present in peripheral tissues [106, 107]. M1, M3 and M5 receptors preferentially couple with the $G_{q/11}$ family of G proteins, activate PLC and increase intracellular calcium level, whereas M2 and M4 receptors activate $G_{i/o}$ protein, inhibit adenylate cyclase and reduce cAMP level [86, 108, 109].

mAChRs play a crucial role in a wide variety of CNS functions (Table **1**) and many Positive Allosteric Modulators (PAMs) can change their signaling. Selective PAMs of muscarinic receptors or antagonists may have therapeutic potential and be used to treat neurological disorders in which cholinergic signaling is involved (*e.g.* AD, Parkinson's Disease (PD), schizophrenia, epilepsy, sleep disorders, neuropathic pain) [110-113].

M1 is the most abundant subtype of mAChRs in CNS and makes up 50-60% of the total [106, 113]. Main areas of M1 presence are cerebral cortex, hippocampus, thalamus and corpus striatum. Different experimental approaches have shown that mice lacking M1 demonstrate impairments of cognitive functions such as learning and memory [114, 115]. Bradley *et al.,* [9] showed a significant deficit of hippocampal-dependent learning and memory in contextual fear conditioning in M1 knockout (KO) mice whereas the pain threshold and locomotor activity were not altered. At the same time, they showed a positive effect of PAMs of M1 receptors in alleviating cognitive deficits in neurodegenerative disease using a mouse model of prion disease (Tg37 hemizygous mice with an accumulation of misfolded insoluble PrPSc in the hippocampus and cortex). The authors also verified if prolonged daily dosing with one of the tested PAMs could have an impact on prion disease progression. They showed that indeed continued dosing of tested PAMs significantly reduced the onset of clinical symptoms of prion disease, thereby extending the lifespan of prion-diseased mice.

Neuropathology of Aβ and tau proteins and their implication in M1 receptor circuit impairment play a key role in the development of AD. It was shown that activation of M1

Table 1. Localization and function of mAChRs in the brain.

mAChRs	Expression in the Brain	Function
M1	Mainly in cerebral cortex, hippocampus, thalamus and corpus striatum (pyramidal cells, small fraction appear to be on axons and terminals)	Synaptic plasticity, learning and memory, neuronal differentiation, neuronal excitability
M2	Throughout brain, mainly in hippocampus and neocortex	Inhibition of dopaminergic terminals
M3	High in hypothalamus, lower in hippocampus	Food intake, body growth
M4	Mainly in corpus striatum	Important role in psychosis, inhibition of D1 receptor
M5	Mainly in substantia nigra pars compacta, ventral tegmental area	Rewarding effect of abusive drugs

mAChRs promotes the production of the soluble Alpha-Amyloid Precursor Protein (sAPPα) identified to have neurotrophic properties, reduces Aβ production, ameliorates tau pathology possibly by activating PKC and inhibiting GSK-3β, and decreases beta-secretase 1 (BACE1) enzymatic activity [26, 116-119]. On the other hand, loss of M1 mAChRs in transgenic mouse models of AD caused an increase in plaque and tangle levels and activation of tau kinase GSK3β.

Moreover, using transgenic mouse models of AD it was found that the loss of M1 receptors caused activation of astrocytes and microglia, as demonstrated by the pronounced Glial Fibrillary Acidic Protein (GFAP), CD45 and ionized calcium-binding adapter molecule 1 (Iba-1) immunoreactivity and significant up-regulation of Interleukin 1 Beta (IL-1β) and Tumor Necrosis Factor-α (TNF-α). More importantly, the enhanced activation of glial cells positively correlated with the higher Aβ level [30, 120]. Additionally, M1 deletion in these AD mice accelerated their cognitive decline, which progressed with age as compared to non-modified AD animals. Furthermore, the M1 receptor deletion in control and AD animals resulted in a decreased PKA-CREB (Protein Kinase A-cAMP response element Binding Protein) signaling. The M1 knockout in AD mice also led to a dysregulation of transcriptional factor c-Fos and a decline in synaptic proteins levels, mainly postsynaptic density protein 95 (PSD-95) and synaptophysin. The results suggest a role of M1 mAChRs in pathways associated with learning and memory impairment and synaptotoxicity in AD [30].

M2 muscarinic receptors act as autoreceptors *via* a Gi protein signaling pathway, which causes a decrease in cAMP. Thus, M2 receptor are capable of regulating inhibitory pathways in the cells. They appear to serve as autoreceptors. M2 mAChRs are expressed throughout the brain and are mainly responsible for inhibition of dopaminergic terminals which can be used as an approach for the treatment of schizophrenia. Antagonists of presynaptic M2 mAChRs are used as an alternative approach towards increasing cholinergic transmission during AD progression [27, 121].

M3 mAChRs is widely presented in the CNS, mainly in the hippocampus, but with a lower level of occurrence than M1. Due to the fact that M3 mAChRs are expressed peripherally it is not surprising that they are involved in regulation of many processes in the body such as food intake, promoting body growth, proper insulin secretion and glucose homeostasis, regulation and maintenance of cardiac function and mediation of cholinergic vasodilation in small arteries [122-126].

M4 mAChRs are highly expressed in the cortex, striatum and hippocampus, which are key areas of cognitive, neuropsychiatric and motor control. Recent data suggest that modulation of M4 receptors may be used as a potential tool for the development of antipsychotic drugs in schizophrenia and other neuropsychiatric disorders efficacy *via* influence on dopaminergic signalling. It has been shown that the clinically preferred M1/M4 agonist Xanomeline has a positive effect on cognitive and psychotic symptoms (*e.g*., hallucinations, illusions) in the AD [127-129] and may have therapeutic effectiveness in the treatment of schizophrenia. This antipsychotic-like effect of Xanomeline was confirmed in several animal models [130, 131].

M4 knockout mice showed enhanced locomotor activity in the open field test and displayed abnormal social behavior in social interaction test. However, they did not show significant impairment in motor coordination/learning, neuromuscular strength or nociception [132]. It has been also shown that activation of M4 receptors by allosteric modulators can reduce striatal glutamatergic transmission and motor impairments in mice model of Huntington's Disease (HD) [133]. M4 receptors are abundantly expressed in the striatum and are co-expressed with dopamine D1 receptors on Striatal Spiny Projection Neurons (SPNs), suggesting that M4 mAChRs are responsible for the imbalance between cholinergic and dopaminergic projection which is an important pathological factor during development of PD [134, 135]. Moehle *et al.,* [136] showed that M4 PAMs directly inhibit D1 signalling in the *Substantia Nigra Pars Reticulata* (SNr). Based on this observation, and the fact that M4 PAMs locally inhibit dopamine release in the striatum, it is possible that M4 PAMs can selectively reduce dopaminergic signalling in the basal ganglia without causing impairment of cognitive function or other undesirable side effects of dopamine receptor antagonists [137].

M5 mAChRs are mainly present in the *Substantia Nigra Pars Compacta* (SNpc) and the Ventral Tegmental Area (VTA). Since the SNpc and the VTA are main structures of the reward system, it has been proposed that M5 receptors may play a role in mediating reinforcing properties of psychostimulants by the modulating dopamine release from midbrain dopaminergic neurons. M5 knockout mice are less sensitive to drug addiction. *For example*, M5 knockout mice show no increased morphine-induced accumbal or striatal dopamine efflux and ~40-50% reduction in morphine-induced locomotion [138-142].

All this information underlines the important role of mAChRs in maintaining homeostasis in the CNS. The possibility of using allosteric modulators that can change the mAChRs conformation and provoke their increased or decreased activity plays a key role in regulating many processes in the human body. This seems to be an attractive future approach in the treatment of neurological diseases.

4. INTERACTION OF TAU AND MUSCARINIC RECEPTORS

4.1. Extracellular Tau is Toxic to Neuronal Cells

The interaction of tau and muscarinic receptor was firstly reported by Gomez-Ramos *et al.,* [20] in their work on the impact of tau protein on neuronal cells. It was observed that the incubation of SH-SY5Y neuroblastoma cells with tau42 (recombinant tau consisting of 2-N terminal inserts and 4 microtubule binding domains) and tau 306-311 (tau lacking residues 306-311) was toxic to these cells, while heparin-induced aggregation of 2R tau (consisting of the first and third microtubule binding domain) reduced the number of dead cells as compared to treatment with non-aggregated 2R tau. Although phosphorylation of tau decreased its toxicity, cell death was still present. In addition, a rise in the toxicity of tau42, tau 306-311, tau 306-311 (peptide consisting of

residues 306-311 of tau) and tau 2R with time was observed. There was also an elevation in the level of phosphorylation of the endogenous tau at the serine 262 upon addition of tau42 to the neuroblastoma cells. Furthermore, a failure of microtubule assembly and an increase in chromatin condensation were observed, which could be linked to calcium homeostasis deregulation [20].

4.2. Tau Deregulates Calcium Homeostasis

Addition of monomeric/non-aggregated tau increased intracellular calcium level, while the effect was not as pronounced upon administration of oligomers. Incubation with PHFs did not cause any change in calcium influx. The mechanism underlying the phenomenon of calcium mobilization was studied with the use of specific antagonists and a blocker, namely atropine for muscarinic receptors, hexafluoroisopropanol for nicotinic receptor (nAChRs) and cadmium for calcium-permeable channels. It seemed that changes in the cytoplasmic calcium were caused by the interaction of tau with muscarinic receptors [20, 86] and not with nicotinic receptors nor calcium-permeable channels [20].

4.3. Muscarinic Receptors Involvement in Tau-Induced Calcium Deregulation

Using a pharmacological approach for specific muscarinic receptor it was shown that calcium influx is inhibited by the M1 and M3 antagonist, but not by the M2 antagonist. These results suggest that tau deregulates calcium homeostasis *via* interaction with M1 and M3 receptors.

To further investigate the interaction, Gomez *et al.,* [86] conducted an experiment on COS-7 cells (African green monkey kidney fibroblast), which physiologically does not express M1 nor M3 receptors, and successfully transfected them with plasmids encoding complementary DNA (cDNA) of the aforementioned muscarinic genes. When tau42 was presented to cells overexpressing M1, M3 separately or in tandem (double M1/M3 transfection) a clear increase in intracellular calcium concentration was visible. It was suggested that the tau3RC peptide (the C-terminal region of tau42, comprising the first, third and fourth microtubule binding repeats) could be involved in the calcium influx, specifically the C-terminal region of tau comprising residues 391-407.

The interaction of tau with M1 and M3 receptors resulting in calcium homeostasis deregulation was confirmed in neuronal cells (neuroblastoma cell line), non-neuronal cells transfected with M1 and M3 receptor constructs, and in primary cell culture [21, 86]. In addition, a co-localization of muscarinic receptor and fluorescently labelled tau42 on the cell surface was observed in COS-7 cells [21]. However, when the cells were pre-incubated with atropine the co-localization did not appear. Furthermore, when cells were firstly incubated with tau and then with an excess of acetylcholine (ACh), the tau-M1 interaction was not interrupted. Since ACh could not replace tau it was inferred that ACh and tau bind to a different site of the receptor. Moreover, a nearly 10 fold higher affinity of tau42 to M1 and M3 than of ACh was measured upon discrete addition of increasing amount of either tau or ACh to transfected cells.

Additionally, a desensitization of M1 and M3 receptors was observed when primary hippocampal cultures were incubated with repetitive ACh stimuli, but the receptors were not desensitized upon tau addition [21]. The cells could respond to successive pulses of tau, but in the case of ACh only the first impulse was affecting the cells. Therefore this suggests that although both tau and ACh are able to activate the mAChRs, they do so in a different manner. Interestingly, the calcium pulses were additive in the primary neuronal culture (COS-7), as opposed to the non-neuronal cell cultures transfected with mAChRs constructs where the calcium levels decreased with time.

Although both ACh and tau induced calcium increase, the only tau evoked neuronal toxicity [20]. This may be due to the aforementioned differences in the kinetic profiles of calcium mobilization and higher affinity of tau than ACh for muscarinic receptors [21]. Moreover, ACh undergoes rapid extracellular hydrolysis by acetylcholinesterase and does not remain in neuronal cell culture for a long period of time, whereas tau is persistent. It is suggested that tauopathies may depend on neurons expressing muscarinic receptors due to their sensitivity to toxic extracellular tau. However the diffusion in the extracellular milieu is yet to be deciphered, degradation of tau seems to remain at a lower level than that of ACh, suggesting that tau may activate mAChRs for a longer period of time [21].

4.4. A Possible Mechanisms for Tau-Muscarinic Receptors Signaling

M1 and M3 receptors are coupled with Gq/G11 proteins leading to activation of PLC. This results in an increase in the level of intracellular calcium and activation of some protein kinases, and these kinases could, in turn, modify tau protein. Recent studies have suggested that G protein-coupled receptor kinase 5 (GRK5) dysfunction augmented tau phosphorylation in APP_{swe} mice. This effect was associated with increased activity of GSK3β and impairment of cholinergic projection [22]. GSK-3β has an important function in abnormal phosphorylation of tau [31, 143-145] and neuronal degeneration [145-148] in AD and plays a well-known role in insulin signalling [143, 149, 150]. On the other hand, GSK-3β was shown to negatively regulate presynaptic glutamate release *via* interfering with the calcium-dependent formation of SNARE complex (Soluble Attachment Protein Receptor) [151]. Additionally, studies on mouse models indicated that GRK5 functional deficiency reduces hippocampal ACh release and leads to cholinergic hypofunction and cognitive decline through selective impairment of presynaptic M2/M4 receptors desensitization [23, 152]. In addition, the GRK5 dysfunction in APP_{swe} mice was able to induce tau abnormal phosphorylation by PKC-mediated activation of GSK-3β signaling pathway [22].

Furthermore, the increased calcium level can also regulate Tissue Non-Specific Alkaline Phosphatase (TNAP) expression. In turn, upregulated TNAP can transform extracellular p-tau into mAChRs agonist (non-phospho tau), which can interact with mAChRs on the neighbouring cells and thus complete the cycle (Fig. **2**). Kellett *et al.,* proposed that neurodegeneration in AD may be caused by TNAP dephosphorylation of tau after its secretion into the extracellular

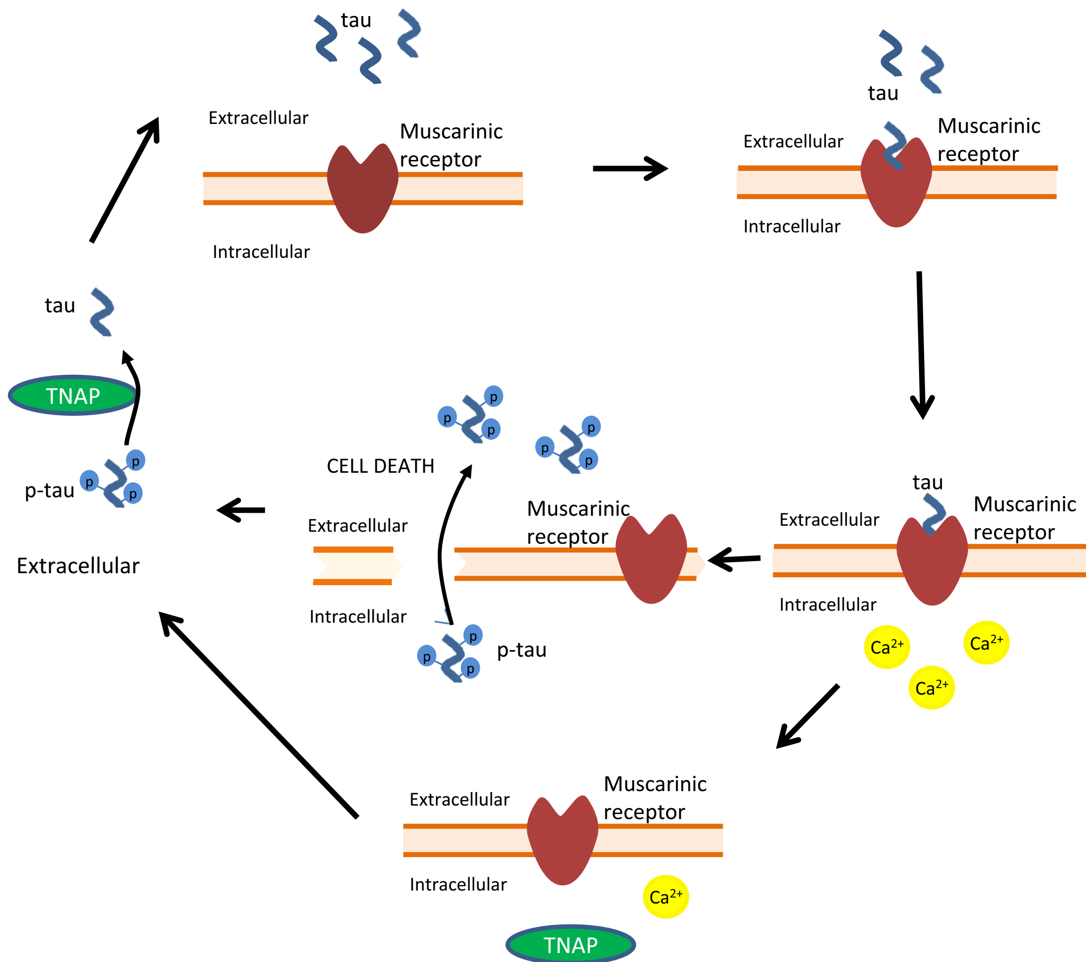

Fig. (2). Tau metabolism in cells. Upon binding of extracellular tau to muscarinic receptor, calcium homeostasis is deregulated. The rise in calcium level results in an increase in TNAP (tissue non-specific alkaline phosphatase) expression and in cell death due to excitotoxicity caused by excessive calcium influx. As a result of this p-tau is released from the damaged cells to the extracellular matrix. Finally, p-tau is extracellularly dephosphorylated by TNAP and the additional pool of unphosphorylated tau can bind to muscarinic receptors on the neighboring cells.

space [153]. TNAP is present on neuronal membranes [154, 155] and plays a role in the neuronal degeneration observed in AD [156]. It was demonstrated that TNAP activity is significantly increased in the brains and plasma of AD patients with both the sporadic and familial forms of the disease [157]. In addition, a significant inverse correlation between plasma TNAP activity and cognitive function in AD has been described [156]. The presence of active TNAP was observed in the membrane fraction of SH-SY5Y cells, where its highest activity was detected [85]. Moreover, TNAP was able to dephosphorylate phosphorylated tau (p-tau). In addition, the pre-treatment with TNAP inhibitor, levamisole could block dephosphorylation of p-tau in the extracellular space. Thus, this indicates that TNAP is a phosphatase responsible for dephosphorylating endogenous intracellular tau after its release into the extracellular matrix. Incubation of cells with unphosphorylated tau resulted in an intracellular calcium increase, while p-tau did not alter the calcium level [85]. Furthermore, when cells were incubated with both p-tau and TNAP, deregulation of the release of calcium from intracellular storages was observed. The addition of non-phosphorylated tau to the cells modulated TNAP gene expression, which may suggest that tau-induced intracellular calcium level increase can regulated expression of the enzyme. Interestingly, an enhanced activity and expression of TNAP was detected in temporal gyrus of AD patients as compared to non-diseased controls. Additionally, a slight decrease in M1 receptor expression was observed in AD with no change in M3 receptor expression [85].

4.5. *In Vivo* Evidence of Tau-Muscarinic Receptors Interaction

The interaction of tau and muscarinic receptors was researched *in vitro*, however almost no data were available on the interaction *in vivo*. This gap was filled by Martinez-Aguila's group [158] with their studies on tear secretion in New Zealand white rabbits. They stated acetylcholine as the main neurotransmitter regulating tear production and therefore an elegant model for studying the interaction of tau with mAChRs. Their data showed that the administration of unphosphorylated human tau isoform (tau42) on a rabbit eye produced a significant increase in the tear production (47±9,12% over basal tear secretion) with no effect when p-

tau was dosed on the rabbit's eye lens as compared to vehicle treatment conducted on the collateral eye.

Previously, it was demonstrated that a tau peptide containing the residues 390-423 of the molecule was sufficient to interact with M1/M3 muscarinic receptors present in neuronal cells and that the interaction of that tau peptide with the cell receptors promoted the same effect as the addition of the whole tau molecule [20]. Therefore, tau peptide comprising the residues 391 to 407 was applied to the rabbit eye and after that the tear secretion was basically the same as with tau42 (50,00±7,14%). In addition, when both carbachol (analogue of the naturally occurring transmitter acetylcholine) and tau were applied, the effects were not additive suggesting that both compounds act *via* the same receptor. Although the maximal efficacy of carbachol was significantly higher than that of tau and tau peptide, tau had higher potency (smaller EC50- smaller dose to reach half of its maximal efficacy). This may suggest that tau and its peptide act as partial agonists of the muscarinic receptor. Furthermore, in studies with mAChRs antagonists a 100% inhibition of tau-induced tear secretion was observed only with M3 antagonist (4-DAMP), whereas M1 and M2 antagonists (pirenzepine and galamine, respectively) were not able to completely block a tau-induced increase in tear secretion. These results show that tau increases tear secretion in a similar way to a muscarinic receptor agonist carbachol and stimulate muscarinic receptors, mainly M1/M3, in a model *in vivo*.

Another function of intracellular tau released upon neuronal death was reported in the study on rat hippocampal synaptosomes [159]. It was demonstrated that monomeric tau at low nanomolar concentrations could affect the high-affinity choline transporter (CHT1) and thus exert a toxic effect on cells. CHT1 is a large transmembrane protein associated with high-affinity choline transport and vulnerable to the damaging effects of Aβ protein applied *in vitro* or *in vivo*. It was supposed that an inhibition of high-affinity choline uptake could occur *via* a direct interaction between the extracellular CHT1 domains (not located in close proximity to the choline recognition site) and of the C-terminally localized microtubule binding repeats of tau. Moreover, the results suggest that residues 25-35 of $A\beta_{1-40}$ can interact with residues 307-325 of tau. Both Aβ and tau seem to be involved in the pathogenesis of AD by acting on high-affinity choline uptake transport, although through different mechanisms. Tau probably exerts its effects through direct interactions of microtubule binding repeats with extracellular portions of the CHT1 protein without influencing the choline recognition site, whereas Aβ acts through lipid rafts in the surrounding membranes [159].

CONCLUSION

Neurodegeneration refers to the progressive loss of specific neuronal populations within the central nervous system. AD is one of neurodegenerative disorder characterized by the pathological modification of the micro-tubule associated protein tau and the aberrant processing of β-amyloid precursor protein. Progression of AD is also correlated with a functional deterioration of cholinergic system and a loss of the basal forebrain cholinergic neurons. The cholinergic hypothesis postulates that selective and progressive deterioration of the cholinergic system is responsible for the cognitive impairments observed in AD. With the growing body of evidence for a correlation between cholinergic deterioration, onset of tauopathy, and onset of cognitive impairment, arguments for a reciprocal interaction between tau and acetylcholine muscarinic receptors seem convincing. Extracellular tau in the unphosphorylated form has been proven to have a neurotoxic effect on surrounding neuronal populations. The cells which may be particularly susceptible to this effect are the basal forebrain cholinergic neurons. Abnormal tau secretion may contribute to synaptic dysfunction of these neurons since extracellular tau can bind to type M1 and M3 muscarinic acetylcholine receptors with higher affinity than acetylcholine. In this way tau could block physiological cholinergic synaptic transmission. Through interaction with muscarinic M1/M3 post-synaptic receptors, extracellular tau is able to reciprocally induce degeneration of the basal forebrain cholinergic neurons. Besides, disruption of synaptic transmission induced by extracellular tau could be due to an excessive increase in the concentration of calcium ions in the cell. Tau binds to M1/M3 receptors as a positive allosteric modulator and prevents their desensitization, leading to prolonged stimulation and an additive effect on intracellular calcium release. Excessive influx of calcium ions leads to excitotoxicity and neuronal death and, consequently, may contribute to the additional release of tau to the extracellular space. Tau has been shown to have increased stability in the extracellular matrix, which allows tau to spread to neighboring neurons before its degradation. This mechanism may explain the propagation of exacerbating brain damage in tauopathies.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

This work was funded by NCN grant 2014/15/B/NZ4/05041 and TauRx Therapeutics/WisTa Laboratories Ltd., Singapore and by statutory funds from the Nencki Institute of Experimental Biology.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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