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Review

THE NEUROPEPTIDE KYOTORPHIN AS A POSSIBLE BIOMARKER AND NEUROPROTECTIVE AGENT IN ALZHEIMER'S DISEASE

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Summary

Alzheimer's disease (AD) is an age-related neurodegenerative disorder clinically characterized by memory impairment, disorientation, cognitive deficits, and behavioral disturbances. The neuropathological features are amyloid plaques containing aggregated amyloid-beta (Aβ) peptide, neurofibrillary tangles composed of the hyperphosphorylated form of the microtubule protein tau (HP-tau), and loss of neurons and synapses in the brain.

There are no effective strategies for the prevention or treatment of the disease, leading to an increased need for AD biomarkers to improve early detection, accurate diagnosis, and accelerate drug development in this field. Recently, increasing attention has been dedicated to neuropeptides in searching for new drug targets in the treatment of nervous system disorders. Available data suggest that many neuropeptides may be associated with the pathophysiology and potential therapy of AD because of their wide distribution in brain areas responsible for learning and memory processes and their predominately neuroprotective actions. This short review aimed to briefly describe the neuropathology of AD and summarize the data related to one of its recently proposed biomarker - kyotorphin (KTP) neuropeptide. Our previous experiments showed moderate and selective protective effects of KTP against the late consequences of the intracerebroventricular streptozotocin-induced AD model.

Keywords: Alzheimer's disease, neuropeptides, kyotorphin

Types and etiology of Alzheimer's disease

The most common degenerative disorder of the central nervous system in the elderly is Alzheimer's disease (AD), which was described first in 1907 by German psychiatrist and neurologist Alois Alzheimer [1]. Alzheimer's Association reports that approximately 60– 80% of dementia cases are related to AD [2].

Even though the effects of the *disease* are similar, there are two main *types of AD:* early-onset (EOAD) or familial (fAD) and late-onset (LOAD) or sporadic (sAD) [3]. The familial form of AD is rare (less than 1%) and has an early onset before 65 years [4]. It is primarily related to mutations in the gene for amyloid precursor protein (APP), which was found on chromosome 21 [5]. Other gene mutations that are crucial for the development of the fAD are in PSEN1

(chromosome 14) [6] and PSEN2 (chromosome 1) [7] that code the essential components of the gamma-secretase complex which take part in cleaving APP. The etiology of the more common sporadic version is highly complex and involves genetic, epigenetic, metabolic, environmental, viral, among other factors. However, the risks regarding the disease are not well understood [8].

Many hypotheses attempt to explain the pathogenesis of AD, including the amyloid cascade [9], hyperphosphorylation of tau [10], oxidative stress, neurotransmitters, to mention a few [11]. However, most of the pathogenic mechanisms, which are recently proposed, originated from two leading hypotheses: the amyloid cascade hypothesis and the tau hyperphosphorylation hypothesis.

The amyloid cascade hypothesis was mainly based on genetic evidence and was proposed first in 1991 by Hardy and Allsop [12]. According to this hypothesis, the deposition of the Aβ peptide drives the formation of a neurofibrillary tangle and leads to various toxic events, which finally causes AD [13].

Aβ is a peptide produced in different cell types, including neuronal cells, by cleavage of APP from enzymes known as secretases. Studies have shown that under the hydrolysis by α-, β-, γ, and η-secretases, C-terminal fragments are produced from APP through three pathways [14]. Two of them are non-pathological processes that occur under physiological conditions through the involvement of α-, γ- and η- secretases. The generated products participate in the physiological processes of proliferation, maturation, and synaptogenesis of neuronal cells.

The third pathway in which APP is cleaved first by β-secretase, then by γ-secretase, to several different variants, the predominant species being Aβ38, Aβ40 and Aβ42 comprising 38, 40 and 42 amino acids respectively, is amyloidogenic pathological pathway [15]. Aβ42 is more prone to form oligomers and fibrils and is the most common species in the plaques found in the parenchyma of the brain [16], whereas Aβ40 is more common in the amyloid deposits found in blood vessels [17].

Special histochemical and immunohistochemical stains reveal that in the hippocampus and basal segment, Aβ is deposited *extracellularly* in the form of neurotoxic amyloid plaques. The constituents of the plaques, also known as senile plaques (SPs), generally include abnormal neuronal processes (neurites) and amyloid deposits in varying proportions. The plaques recruit more Aβ to form aggregates that are insoluble and induce damage in mitochondria [18], destabilize homeostasis, and loss of synaptic function. Related inflammatory reactions and oxidation are induced by activated glial cells, such as astrocytes and microglia. Ultimately, the developed neuronal dysfunction and apoptosis leads to AD [18-20]. Aβ activates an enzyme, tau protein kinase 1, leading to hyperphosphorylation of tau protein [13], a microtubule-associated protein, and prompting the formation of paired helical filaments (PHFs) and neurofibrillary tangles (NFTs), which finally speed up the development of tau pathology [21]. NFTs are deposits found within the perikaryal region of neurons in brain regions affected by AD. It is generally believed that Aβ precedes tau in the pathogenesis, i.e., Aβ causes the tau pathology [22, 23].

However, due to the emergence of new evidence for the pathological role of soluble oligomeric Aβ species, before plaque formation, and the role of Aβ within the cells, some modifications of the amyloid cascade hypothesis have been made recently. In 2011, the "Aβ oligomer pathogenic theory" was officially proposed by Ferreira and Klein. According to this theory, soluble Aβ oligomers are neurologically significant toxins, possibly the most important ones, related to a sequence of pathological changes in AD [24]. The built-up of soluble Aβ oligomers correlates with loss of synapses [25] and memory impairment [26]. Some researchers even propose that the formation of plaques in AD has a protecting role against this toxic influence of Aβ species. Fundamentally, the latest hypothesis states that increased production of Aβ42, due to mutation or improper clearance, leads to its oligomerization and deposition, and ultimately to neuronal or synaptic dysfunction, resulting in dementia, the pathologic events related to AD [27].

The Tau hyperphosphorylation hypothesis arose in 1988 when the tau protein was isolated from brain plaques of AD patients from Claude Wischik and colleagues. They proposed for the first time that perhaps the cause of dementia in

AD was this protein [28] found mainly in the axons of brain neuronal cells combined with microtubules [29]. Under normal conditions, tau function is to maintain the structure of axons and to facilitate cytoplasmic transport [30]. It is responsible for maintaining the structure and function of synapses and the regulation of cell signaling [31]. In the base of the mechanism of its action lies the fact that the peptide has a few phosphorylation sites that negatively regulate the binding of tau to microtubules. Phosphorylation and dephosphorylation of tau may depend on the balance of protein kinase and protein phosphatase activity. In turn, this balance is regulated by brain development. In pathological conditions, the phosphorylation of tau saturated.

Hyperphosphorylated tau (HP-tau) was dissociated from microtubules and aggregated into dense, compact paired helical filaments (PHF), resulting in defective microtubule functioning and ultimately killing the neuron [13, 32]. HP-tau and NFTs are not only seen in the brains of AD patients. They can also be found in other neurodegenerative diseases, collectively known as 'tauopathies' [33]. However, as AD progresses, about 8% of the neuronal loss in the hippocampus is caused by NFTs formation. This fact suggests that the primary cause of cell death is not the HP-tau [34].

Alzheimer's disease affects brain areas and symptoms

Although β-amyloid and tau are involved in AD› etiology, the exact mechanisms liable for the regional specificity of neuronal loss are still unclear. The brain region, which is first affected in AD, is the transentorhinal region, followed by the amygdala and hippocampus [35].

Typically, the impaired short term memory is the initial symptom in AD. In early stages, behavioral and psychological changes occurred, including a gradual inability to handle normal daily activities and inability to recall recently learned facts or to form new memories.

Usually, at this stage of the disease, the patients' spatial orientation, vocabulary, and language skills tend to be noticeably affected, but they can manage with daily routines. These problems are often mistaken for age-related problems or stress-induced indicators. Long term memory and general intelligence are generally not affected.

In later stages, neurons of parietal and frontal cortical lobes and neurons in the posterior cingulate gyrus, are destroyed. Relatively spared are neuronal cells in the anterior cingulate, motor, sensorimotor, and occipital cortices [35]. As a result of massive cell loss, shrinkage of the gyri and widening of the sulci can be recognized in the affected areas [36]. Moreover, the specificity of neuronal destruction in these regions is evidenced by the targeting of specific cell types [37-39]. Pyramidal neurons of the neocortex, projecting corticocortically, are preferentially lost in AD. While the CA3 region is spared, pyramidal neurons of the entorhinal cortex and the hippocampal neurons in CA1 and subiculum are destroyed early in the disease process.

Nevertheless, the best correlate of cognitive impairment appears to be a synaptic loss, not cell death per se [26]. Axons, inhibitory interneurons, glia, motor areas, thalamic projections, and primary sensory areas are not set to NFTs formation and subsequent neurodegeneration [40, 41]. With the disease's progress, symptoms like personality changes, problems with language, daily life activities such as dressing and eating, or not being able to remember family members appear. In the later stages of AD, when due to loss of muscle mass, complete loss of speech and mobility are presented, ultimately causing the patient to become bedridden. At this point in the disease, the patient needs and is entirely dependent on the caregiver. Confusion, irritability, delusional symptoms, aggression, and wandering tend to become less common than in the intermediate stages of the disease.

Usually, five to15 years after the onset of the disease, death occurs, most often because of secondary infections unrelated to AD, like pneumonia or others. With 100% accuracy, AD can only be diagnosed *post mortem* after brain autopsy [27].

Improved imaging techniques and brain scans have given researchers further insight into the disease's etiology, although there is still no cure for AD [42]. To date, established treatments are only symptomatic. They do not affect the actual disease process, but they try to counterbalance the disturbance in neurotransmitters and to provide some improvement in cognitive function and memory for 1 or 2 years. For

instance, to patients with mild to moderate AD, cholinesterase inhibitors are given. These drugs increase both the levels and duration of action of acetylcholine in the cholinergic synapses. The antagonist of the NMDA (N-methyl d-aspartate) receptor is applied to the patients with moderate to severe AD to reduce the adverse effects of glutamate in the brain [27].

Neuropeptides as potential biomarkers and possible therapeutic targets in AD

Neuropeptides are peptides, or small proteins (from 3 to about 100 amino acids), which act as messenger hormones, true neurotransmitters or neuromodulators [43]. Their receptors are widely distributed in the CNS and peripheral nervous system. Generally, neuropeptides originate in the cell body of the neurons from precursors, inactive molecules with high molecular weight, whose enzymatic cleavage leads to the formation of one or more neuropeptides with biological effects (Figure 1) [44].

Neuropeptides are slow mediators since they have to diffuse to reach their receptors. However, they induce prolonged physiological response since they are slowly inactivated by enzymatic proteolysis or diffusing away from the synaptic cleft.

The role of neuropeptides in brain functions is prominent. Because they are involved in activities such as social behaviors, learning, and memory, the authors suggest that the AD's pathophysiology is associated with some neuropeptides such as substance P, vasopressin, corticotropin-releasing factor, ghrelin, neuropeptide Y, neurotensin, orexin, and others. [45]. In addition to classical Aβ and tau proteins, several neuropeptides with neuroprotective properties, involved in learning and memory processes have been demonstrated to be altered in both cerebrospinal fluid (CSF) and blood of AD patients. These represent potential biomarkers and possible therapeutic targets [41, 45-48]. The general interpretation is that their reduction is likely to be, at least to a certain degree, a consequence of the degeneration of neuropeptide-generating neurons in AD brains. However, this assumption does not exclude the possibility that the reduction of a specific neuropeptide or neuropeptides may be causal in the pathological cascade of AD development. Consistent with this alternative hypothesis is the finding that the amounts of some neuropeptides decline with healthy aging, without the onset of AD [49, 50].

Kyotorphin neuropeptide

Kyotorphin (KTP) is an analgesic neuropeptide described by Takagi et al. (1979) during the

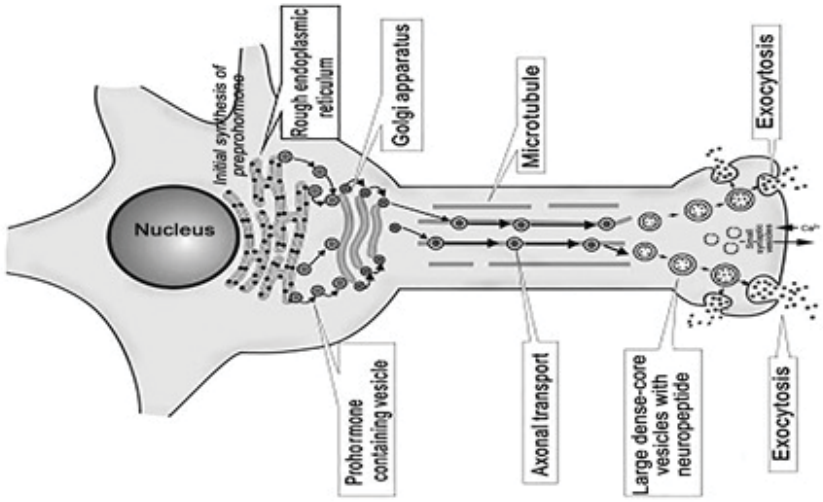

Figure 1. Schematic illustration of neuropeptides synthesis, storage and secretion. Neuropeptides are synthesized in the rough endoplasmic reticulum and Golgi apparatus in the cell body of neurons from biologically inactive pre-pro-peptides and then transported to the axon while undergoing their processing. The bioactive neuropeptides are stored in large dense-core vesicles and released by exocytosis, reaching their cognate receptors also at a considerable distance from the site of release

fractionation of bovine brain extracts [51]. Subsequently, it was isolated from the brains of other vertebrates such as mice, guinea pigs, rabbits, rats, and CSF in humans [52]. Studies have shown that KTP is a dipeptide, whose sequence is L-tyrosyl-L-arginine [53, 54]. It may excite cortical neurons directly or indirectly through modulation of the synaptic transmission.

Synthesized in nerve terminals, KTP is released by depolarization [53]. Two mechanisms for its formation in the brain are known: 1) by a specific enzyme, kyotorphin synthetase dependent of ATP and Mg^{2+}, from its constituent amino acids (Figure 2) [55]; 2) degradation of the precursor proteins by Ca^{2+}-activated protease [56].

Kyotorphin receptors mediate activation of phospholipase C (PLC) in synaptosomal membranes through G_{i1}, followed by an opening of inositol 1,4,5-triphosphate (IP_3)-gated calcium channels, located in the endoplasmic reticulum membrane of nerve terminals, thereby directly leading to a production of the action potential [57, 58].

The uptake of KTP showed properties, alike to those of classical neurotransmitters. However, the mechanism is comparatively specific, as it is not affected by other neurotransmitters, their uptake inhibitors, tyrosine, or arginine [59].

To date, there is no immunohistochemical mapping of kyotorphin-containing neurons in the brain. Regional distribution of KTP in rat brain homogenates was reported only in the manuscript of Ueda et al. [60]. According to it, the highest levels are found in the brainstem, corresponding with areas most sensitive to opioids and analgesia (Table 1).

Surprisingly, it was reported that approximately 50% of the total KTP content was found in the cortex, an area where enkephalin's receptors are low. Therefore neurochemical action of KTP is not only opioid but also non-opioid [60]. Other studies confirm that the physiological effects of KTP fall clearly into two groups: those mediated via opioid peptides and opioid peptide-independent ones. It was proposed that the opioid-like action of the peptide was indirect, and analgesia was produced through

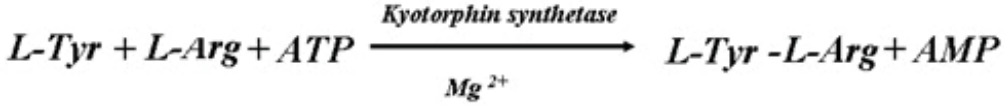

Figure 2. Kyotorphin formation by kyotorphin synthetase from L-Tyr and L-Arg dependent on ATP and Mg2+. L-Arg has rate-limiting or regulatory role in kyotorphin biosynthesis

Table 1. Regional distribution of kyotorphin from the rat brain and spinal cord (according to Ueda et al., 1980 [60])

Region	Tissue weight (mg)	Kyotorphin content	
		[ng/g tissue]	% of the total brain
Brain			
Whole brain	1724.1 ± 10.0	261.9 ± 33.4	100
Cortex	623.6 ± 16.5	367.1 ± 85.9	48.4 ± 8.5
Striatum	159.4 ± 2.0	45.5 ± 8.2	1.7 ± 0.4
Hippocampus	192.3 ± 8.4	61.8 ± 20,3	2.7 ± 0.7
Thalamus	157.3 ± 4.1	119.3 ± 30.6	4.5 ± 1.5
Hypothalamus	36.9 ± 1.4	391.8 ± 47.8	3.5 ± 0.8
Midbrain	101.3 ± 2.6	719.5 ± 113.3	16.9 ± 2.8
Pons + medulla	190.6 ± 8.0	556.5 ± 89.6	25.0 ± 4.5
Cerebellum	262.7 ± 2.0	101.8 ± 25.2	6.4 ± 1.8
Spinal cord			
Dorsal part	188.5 ± 8.5	405.1 ± 71.0	-
Ventral part	172.7 ± 10.2	230.2 ± 37.7	-

the release of met-enkephalin [61]. It has been shown that KTP concentration in human CSF in patients with persistent pain from normal 1.19 ± 0.51 pmol/ml^{-1} decreases to 0.24 ± 0.04 pmol/ml^{-1}. This data suggest that KTP probably acts as a neuromediator and/or a pain modulator in the human brain [52]. According to the literature data, the transport capacity of the peptide to cross the blood-brain barrier (BBB) is limited despite its more potent analgesic action than other endogenous opioids [54].

Nevertheless, some authors have demonstrated that KTP derivatives can penetrate the BBB and exert analgesic effects after systemic administration in several pain models [61, 62]. It was assumed that KTP was transported by the high-affinity and low-capacity type transporter known as H^+-coupled peptide transporter or PEPT2, [63, 64] expressed in astrocytes of the cerebral cortex, thalamus, hippocampus, but primarily in the kidney [65]. In situ hybridization histochemistry has shown that PEPT2 mRNA is expressed in the entire rat brain and is localized in non-neuronal cells [66].

The neuroprotective actions of KTP in the hippocampus and cerebellum have revealed in other animal models studies [67, 68-70]. There is further evidence that this peptide possesses neuroprotective and neuromodulating properties [71, 72], acting as a neuroleptic, and inhibiting a calcium-dependent current in the postsynaptic membrane [71, 73], or showing anticonvulsant activity in animal models of epilepsy [74, 75]. Some studies have reported its inhibitory effect on heart rate, respiration, and body temperature regulation in animals that hibernate [76, 77]. Our previous findings also showed probable an anti-opioid action of KTP since it was capable of decreased stress-induced antinociception [68-70, 72, 78, 79].

KTP as a possible neuroprotective agent in AD

In 2013 Santos et al. revealed the correlation between HP-tau levels in CSF samples of patients with AD and KTP [80]. Their study results were in agreement with previous ones, showing that the progress of the AD correlated with diminished levels of several neuropeptides. [81, 82]. The decreased KTP level in the CSF of AD patients was suggested to result from a hippocampal shrinkage and specific for the disease cortical thinning with an acceleration phase during the early stages [83]. Thus, the loss of cortical mass may consequently decrease the cortical capability for KTP production and diminish the levels of the dipeptide in the CSF. Furthermore, they discovered an inverse correlation between KTP and HP-tau level. As the AD progresses, more neurons are destroyed, KTP production is impaired, and more HP-tau is released.

New clinical data have shown the existence of a link between pain, KTP, and AD. In the same study mentioned above, Santos et al. (2013) pointed that in AD patients with chronic pain, KTP levels decrease due to irreversible structural changes in the CNS areas involved in the transmission and/or modulation of nociceptive information [84]. Furthermore, both components of the pain response, the sensory-discriminative, and the affective-emotional are differentially affected. Because the thalamic nuclei and somatosensory cortex are preserved in AD, the sensory-discriminative component is retained. Simultaneously, the pain tolerance associated with the affective-emotional aspect was amplified due to the neuronal loss in structures involved in such reactions - the prefrontal and limbic system [80, 84-87].

Lately, there has been an increasing interest in the synthesis of peptides that gather both analgesic and anti-inflammatory activities, due to their unique range of molecular properties [88, 89]. Such peptide derivatives are expected to overcome the limitations of conventional analgesic peptides and to increase the potential of developing novel and safer medicinal products. In that field, efforts were focused on developing two designed efficient drugs derived from KTP - amidated (KTP-NH_2) and conjugated to ibuprofen (IbKTP-NH_2). Both were studied concerning their ability to improve behavioral functions, cognitive dysfunction, and post-ischemic neuronal damage caused by chronic cerebral hypoperfusion in female rats. Also, they were evaluated regarding their impact on microcirculation and retained anti-inflammatory activity. The experimental data showed that both new peptide derivatives prevented neuronal damage in the CA1 subfield of the hippocampus and have ameliorated cognitive impairment. Furthermore, the derivate containing ibuprofen proved to be more effective

in recovering cognitive function than amidated KTP [90]. Besides, both KTP derivatives did not cause any damage in microcirculation and efficiently decreased the number of leukocyte rolling induced by lipopolysaccharide [91].

Over the last few years, our team was also focused on experiments to elucidate the role of exogenous intracerebroventricularly administered KTP in neuroprotection during the streptozotocin-induced model (STZ-ICV) of sAD in rats. We studied behavioral, biochemical, and histological changes in some brain areas and blood vessels after 14 days of subchronic treatment with KTP. Three months after streptozotocin administration, rats developed decreased anxiety-like behavior, increased exploratory behavior in the open field test, impaired spatial and working memory. Changes in these basic behavioral parameters were in agreement with other authors [92]. Histological data showed the accumulation of Aβ in the leptomeningeal and cerebral blood vessels and the hippocampus and decreased the number of neurons in the hippocampal CA1 and CA3 subfields [93, 94]. Biochemical data showed that in the prefrontal cortex and the hippocampus, the total protein content was significantly decreased. These data were accompanied by increased levels of carbonylated proteins in the hippocampus, showing the presence of oxidized and impaired proteins. Our observations showed that the early phase of this sAD model was characterized by sporadic motor seizures, which disappeared with the progression of the disease [94].

Our data also showed a moderate protective effect of sub-chronically infused KTP against the impaired anxiety and habituation to a new environment in STZ-induced sAD. The beneficial effects associated with KTP treatment were prevented object recognition memory loss, abolished protein loss in the prefrontal cortex, and decreased neuronal loss in the hippocampal CA3 subfield. The impaired spatial memory, neuronal loss in the CA1 subfield, and the rise of carbonylated proteins in the hippocampus remained unchanged by the dipeptide. These diverse effects of KTP in different brain structures draw attention to its possible selective neuronal protection against STZ-induced structural impairment, related maybe to the selective distribution of its receptors. The comparison between saline-treated and KTP treated STZ groups did not show any significant changes in motor activity, spatial memory, or level of Aβ in studied brain structures. However, there was a tendency for diminishment [93, 94].

Summarizing our data, we can conclude that the sub-chronic intracerebroventricular administration of KTP showed a moderate and selective protective effect on the pathological changes induced by an experimental model of sAD in rats. We proposed that one of the mechanisms in the base of these protective effects of KTP is a nitric oxide-dependent. Due to its L-Arginine residue, it was known that KTP could be used as a substrate for neuronal nitric oxide synthase (nNOS) [95, 96]. Recently, there is increasing evidence that AD may be primarily a vascular disease with neurodegenerative consequences, rather than a neurodegenerative disorder with vascular consequences [97]. Two key factors for the development of AD, aging, and decreased cerebral perfusion, lead to abnormalities of the brain capillary architecture and impair nitric oxide (NO) release to such an extent that it can initiate neurodegenerative changes characteristic of AD [97].

Conclusion

KTP, like many other neuropeptides, might be associated with the pathophysiology of AD. Decreased levels of the dipeptide and its inverse correlation with HP-tau, in the CSF of patients with AD, prove that the dipeptide can be used as a biomarker in early diagnosis of disease. What comes first – Alzheimer's disease because of low KTP levels or low KTP because of loss of neuropeptide-generating neurons in AD brains remains to be clarified. The experimental data about the neuroprotective effects of KTP and its derivatives are encouraging for further study of their potential use as therapeutic agents.

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