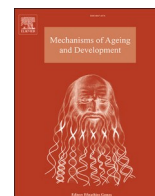




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Nepriylsin expression and functions in development, ageing and disease

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ARTICLE INFO

Keywords:

Ageing
AICD
Alzheimer's disease
A β peptide
amyloid-degrading enzyme
CALLA
cancer
CD10
cognitive behaviour
cortex
diabetes
embryogenesis
endopeptidase 24.11
enkephalinase
epigenetic regulation
HDACs
hippocampus
memory
hypertension
nepriylsin
NEP inhibitors olfactory bulbs
prenatal hypoxia
striatum

ABSTRACT

Nepriylsin (NEP) is an integral membrane-bound metallopeptidase with a wide spectrum of substrates and physiological functions. It plays an important role in proteolytic processes in the kidney, cardiovascular regulation, immune response, cell proliferation, foetal development etc. It is an important neuropeptidase and amyloid-degrading enzyme which makes NEP a therapeutic target in Alzheimer's disease (AD). Moreover, it plays a preventive role in development of cancer, obesity and type-2 diabetes. Recently a role of NEP in COVID-19 pathogenesis has also been suggested. Despite intensive research into NEP structure and functions in different organisms, changes in its expression and regulation during brain development and ageing, especially in age-related pathologies, is still not fully understood. This prevents development of pharmacological treatments from various diseases in which NEP is implicated although recently a dual-acting drug sacubitril-valsartan (LCZ696) combining a NEP inhibitor and angiotensin receptor blocker has been approved for treatment of heart failure. Also, various natural compounds capable of upregulating NEP expression, including green tea (EGCG), have been proposed as a preventive medicine in prostate cancer and AD. This review summarizes the existing literature and our own research on the expression and activity of NEP in normal brain development, ageing and under pathological conditions.

1. Introduction

1.1. Structure and general properties

Nepriylsin (NEP, EC 3.4.24.11, neutral endopeptidase, CD10 and CALLA) is a type II integral membrane protein residing in the plasma membrane with the major part of it, including the active site, located in the extracellular space. As such it is considered as an ectoenzyme providing proteolytic cleavage of various substrates outside cells. It is exclusively a zinc-dependent enzyme with the Zn-binding domain in the extracellular C-terminal part of the molecule (Fulcher and Kenny, 1983). It exists predominantly as a noncovalently associated homodimer

although in some species it was shown to exist as a monomer and a recombinant form of NEP is soluble in aqueous medium (Liu et al., 2010).

Originally NEP was discovered in rat kidney brush border membranes and later purified from rabbit kidney where it constitutes approximately 4% of all proteins of the renal brush border membrane (Kerr and Kenny, 1974). NEP was also shown to be an important brain enzyme cleaving enkephalins and, hence, named enkephalinase. However, after further discovery of a wide range of its substrates NEP was given a more general name - endopeptidase-24.11 based on its enzyme classification number (EC3.4.24.11) (Matsas et al., 1983). In the current literature it is mostly referred to as nepriylsin, neutral endopeptidase or

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¹ With deepest regret we inform the readers that Dr Igor A Zhuravin has passed away on September 16th, 2020.

<https://doi.org/10.1016/j.mad.2020.111363>

Received 7 May 2020; Received in revised form 17 September 2020; Accepted 20 September 2020

Available online 26 September 2020

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NEP. Importantly, NEP was also identified as a leukocyte cell surface antigen, the common acute lymphoblastic leukemia antigen (CALLA or CD10) (Letarte et al., 1988).

NEP has a pH optimum of 6.0 and is inhibited by zinc-chelating reagents (Kerr and Kenny, 1974). A variety of synthetic inhibitors designed to date are widely used for studying NEP properties and also in the clinics. The most widely used NEP inhibitors are thiorphan ([DL-3-mercapto-2-benzylpropanoyl]-glycine) with a K_i in the nanomolar range (Roques et al., 1982) and phosphoramidon, although the latter is less specific and inhibits a range of metalloendopeptidases (Komiyama et al., 1975). Comparative analysis suggested that NEP from the kidney and brain have similar sensitivity both to thiorphan and phosphoramidon (Fulcher et al., 1982).

NEP was shown to be heavily glycosylated with five or six N-linked glycosylation sites depending on the species (Fulcher et al., 1983; Lafrance et al., 1994). It can also be sialylated and hyposialylation of the enzyme was found to lead to myopathy (Broccolini et al., 2008). The cytoplasmic domain of NEP can be phosphorylated by casein kinase II (CKII) and its interaction with tyrosine-phosphorylated Lyn kinase blocks focal adhesion kinase signaling (Ganju et al., 1996). Although the C-terminus of NEP contains a putative prenylation CAAX motif this posttranslational modification has not been detected in an *in vivo* assay (Santos et al., 2002). However, NEP myristoylation at the N-terminal site (Gly²) affects the membrane targeting of the enzyme (Zheng et al., 2010). There are other NEP posttranslational modifications which regulate its binding to various target molecules. For example, phosphorylation of Ser⁶ in the NEP molecule by casein kinase 2 inhibits its interaction with PTEN (phosphatase and tensin homologue deleted on chromosome 10) which acts as a regulator of the cell cycle (Siepmann et al., 2010).

The human NEP gene is located on chromosome 3 spanning more than 80 kb. It exists in a single copy and is composed of 24 exons. The NEP gene is highly conserved among mammalian species (D'Adamo et al., 1989). NEP gene expression is controlled through two distinct promoters whose influence differs between cell types, although both promoters show similar characteristics and activity (Belyaev et al., 2009). Three distinct NEP mRNAs have been identified in human and rat which differ only in their 5' noncoding regions (Li et al., 1995a, 1995b).

Up to now the list of NEP substrates and its functions continues to grow. It includes a range of peptides up to 40-50 amino acids in length, although the efficiency of hydrolysis declines with increasing length of the peptide (Bayes-Genis et al., 2016). The most efficiently hydrolysed substrates, apart from enkephalins are tachykinins such as substance P, bradykinin, endothelins, atrial natriuretic peptide family, somatostatin, adrenomedullin, members of the vasoactive intestinal peptide family, glucagon, thymopentin etc. This makes NEP inhibitors effective in the treatments of pain, inflammation and cardiovascular disease (Roques and Beaumont, 1990). Moreover, the discovery that NEP can cleave amyloid- β peptide (A β) made it one of the major amyloid-degrading enzymes and a target in Alzheimer's disease (AD) pathogenesis (for review see Nalivaeva et al., 2012a).

1.2. Distribution and biological roles

NEP is a widely distributed enzyme and thiorphan- and phosphoramidon-sensitive activities with the appropriate specificity have been identified in diverse species including the nematode worm, *Caenorhabditis elegans*, and the fly, *Drosophila melanogaster* (Turner et al., 2001; Bland et al., 2008). NEP cDNA cloning suggests that rat, rabbit and human enzymes contain 742, 750 and 742 amino acid residues, respectively (Malfroy et al., 1987; Devault et al., 1987; Malfroy et al., 1988). The similarity of rat NEP to the human enzyme makes it a good model for studying its biology and functions. Three NEP cDNAs isolated in rats have different organ and brain cell distribution suggesting that NEP expression has a specific cell-type dependent mechanism (Li et al., 1995a, 1995b).

Apart from already mentioned NEP expressing tissues it is also abundant in lungs where it plays an important role in lung development and functions via hydrolysing bombesin-like peptides (Sunday et al., 1992) and NEP levels are indicative of lung dysfunctions (Wick et al., 2011). NEP in the lung may play a role in the airways by modulating responses to inflammatory neuropeptides, for example substance P, which constricts the airway smooth muscle (Borson, 1991).

In vivo NEP labelling by ³H-HACBO-Gly in rats has also revealed its abundance in liver, adipose tissue in the neck region, flat bones of the skull, the mandibula, the vertebrae, the long bones of the limbs, articular cartilages and synoviae, while lower labelling was found in the intestine, the glomeruli and submaxillary glands (Sales et al., 1991). NEP is also expressed in the carotid body where it participates in oxygen level sensing (Kumar et al., 1990) and reticular cells of the lymph nodes (Bowes and Kenny, 1986). NEP activity is also identical to skin fibroblast elastase and thus may play a role in skin ageing (Morisaki et al., 2010). NEP may participate in bone metabolism both through its presence on the surface of human osteoblasts and by its ability to metabolize osteogenic peptides (Ruchon et al., 2000).

Like many other metallopeptidases, NEP is also present in the reproductive system. In the testis, a new soluble form of NEP has been identified which plays an important role in sperm formation and the processes related to fertility (Ghaddar et al., 2000). In the ovary NEP regulates follicle maturation, ovulation and ovarian blood flow (Zappulla and DesGroseillers, 2001). NEP is also expressed in placenta where it was shown to hydrolyse oxytocin but not vasopressin (Johnson et al., 1984) and in foetal membranes suggesting its role in the materno-foetal interface (Imai et al., 1994).

NEP is expressed in stem cells and has been used as a marker of stem cells in many tissues (for review see Maguer-Satta et al., 2011). It is also implicated in the differentiation of immature cells in bone marrow (Torlakovic et al., 2005).

In blood plasma soluble circulating NEP was shown not only to degrade natriuretic peptides but also was suggested as a marker and target in therapy of heart failure (Bayes-Genis et al., 2016). There are clinical data suggesting that the heart is a major source of soluble circulating NEP (Arrigo et al., 2018). NEP was also found in animal and human cerebrospinal and amniotic fluids (Spillantini et al., 1990).

In the brain NEP is present in much lower abundance than in the kidney and lungs but its expression was confirmed for such brain structures as the gray matter, caudate-putamen, striatum, globus pallidus, olfactory tubercle, nucleus interpeduncularis, substantia nigra, pia mater and the ependymal lining of the central canal and the spinal cord (dorsal root ganglia and nerve roots) (Matsas et al., 1986) as well as in the choroid plexus (Bourne and Kenny, 1990). In the human, NEP is distributed rather heterogeneously among brain regions with the highest levels being found in the globus pallidus and pars reticulata of the substantia nigra (Llorens et al., 1982). NEP is primarily located on neuronal cells, especially in the striatonigral pathway and in much lower amounts in the hippocampus (Barnes et al., 1988). The enzyme has also been found on Schwann cells in the peripheral nervous system (Barnes et al., 1995). In mouse brain immunocytochemical analysis has revealed NEP presence in the stratum pyramidale and stratum lacunosum-moleculare of the CA1-3 fields of the hippocampus and in the molecular layer of the dentate gyrus (Fukami et al., 2002). Confocal double immunofluorescence analyses confirmed NEP localization along axons and at the synapses suggesting that after synthesis in the soma, NEP is axonally transported to the synapses. More recent studies have demonstrated that in human brain NEP is enriched in GABAergic neurons and that NEP mRNA can be detected mainly in the parvalbumin-expressing interneurons where NEP protein is localized in the perisomatic synapses (Pacheco-Quinto et al., 2016). These data confirmed NEP protein expression in the GABAergic and metabotropic glutamate 2/3 receptor-positive neurons but not in catecholaminergic or cholinergic neurons (Fukami et al., 2002).

The ability of NEP to catabolise A β at multiple sites was first

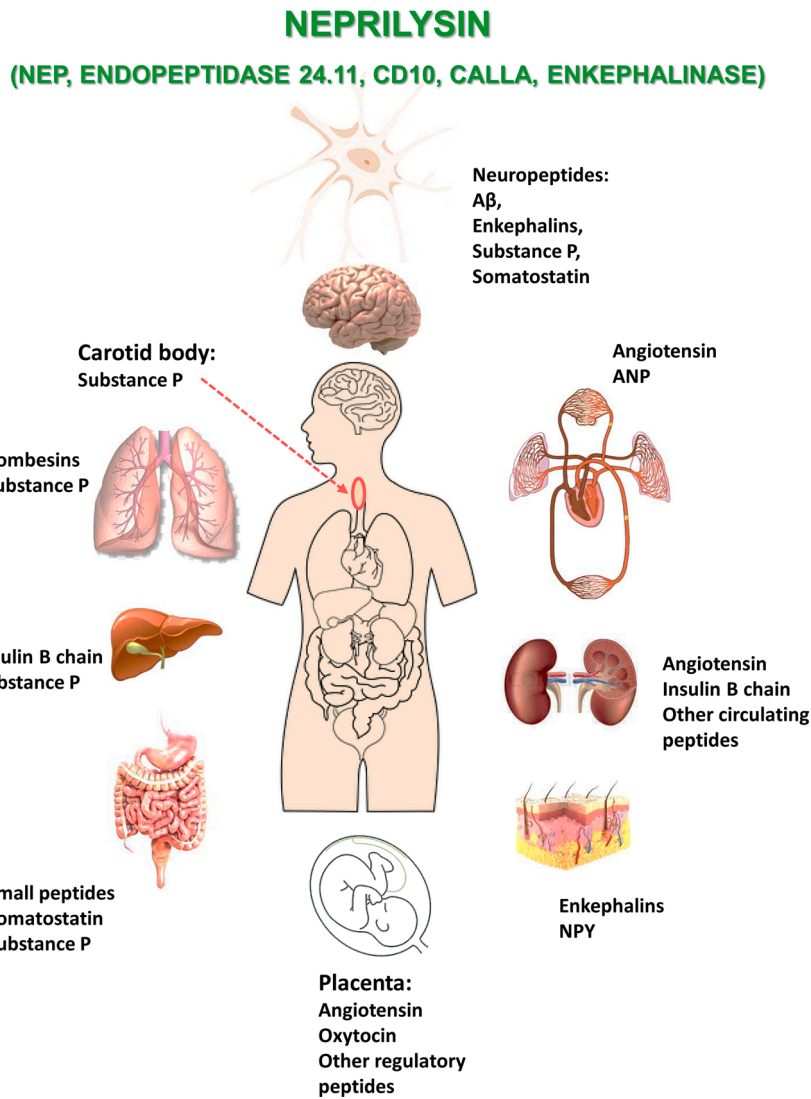


Fig. 1. NEP distribution in various organs and allocation of its main substrates.

demonstrated *in vitro* (Howell et al., 1995) which was later confirmed *in vivo* with intracerebrally administered A β into the rat brain parenchyma (Iwata et al., 2000). Further, the correlation between the levels of A β peptide in the brain and NEP activity was demonstrated both in NEP knockout mice (Iwata et al., 2001), in rats chronically administered with the NEP inhibitor thiorphan (Zou et al., 2006) and in AD brain (Carpentier et al., 2002). This made NEP a very important target in AD studies.

2. NEP and foetal development

NEP gene knockout is not lethal, and animals appeared developmentally normal (Lu et al., 1995). However, NEP null mice are highly sensitive to endotoxic shock which confirms an important role of NEP in proinflammatory peptide metabolism. Moreover, NEP gene deletion results in an opioid-related increase in thermo-nociceptive threshold in mice suggesting that NEP selectively controls opioid biosynthesis in the hypothalamus and spinal cord (Saria et al., 1997). NEP deficient mice also demonstrate increased consumption of alcohol (Siems et al., 2000). NEP knockout mice also show enhanced aggressive behaviour and altered locomotor activity correlating with compromised NEP enkephalinase activity which cannot be fully compensated by enhanced activities of other enkephalin-degrading enzymes (Fischer et al., 2000).

The importance of NEP for normal foetal development was studied

both in animals and humans. NEP mRNA expression and enzymatic activity were detected in foetal bodies early in embryogenesis with progressive major appearance of NEP in the kidney and lungs, and with transient or enhanced expression observed during development of the heart and the major blood vessels, the intestine, the bones, the genital tubercle and sensory organs (Dutriez et al., 1992). Detailed analysis of cellular and tissue distribution of NEP mRNA and enzyme during rat embryonic development reported its first detection on embryonic day 10 (E10) in the gut lining and then, at E12, in the notochord, otocyst, medial and lateral nasal processes, mesonephros, heart and neuro-epithelium. Later, by E14 and E16, NEP was also detected in a wide range of craniofacial structures, choroid plexus, tongue and perichondrium. Although NEP was detectable in the craniofacial vasculature at E12 and E14, this was no longer apparent at E16. Importantly, distribution of NEP mRNA matched quite closely localization of the protein detected by immunocytochemistry at all investigated stages of rat embryonic development (Spencer-Dene et al., 1994). Injections of NEP inhibitors, phosphoramidon and thiorphan, to rat embryos on E9.5 and E10.5 resulted in asymmetric developmental abnormalities associated with a haematoma and the severity of the effect was dependent on the inhibitor doses (ibid). The developmental profile of NEP in numerous tissues strongly suggests an important regulatory role of NEP in ontogenesis of various organs.

The peak of CD10/NEP transcript levels in human foetus was

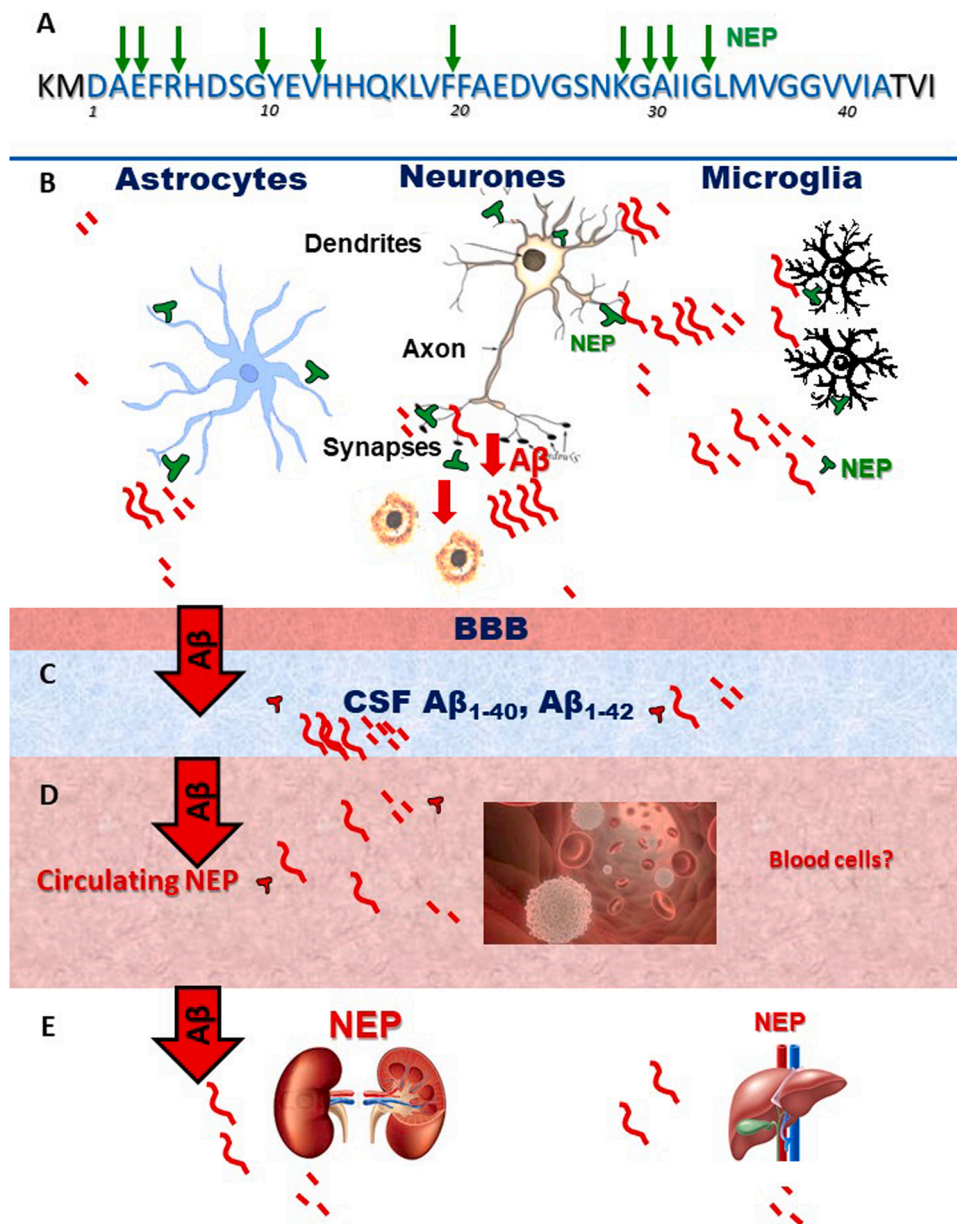


Fig. 2. Sites of NEP action on $A\beta$ and sites of $A\beta$ clearance in the brain and periphery.

A – diagram of $A\beta$ sequence and the sites cleaved by NEP.

B- Schematic presentation of brain localization of NEP.

C – E – $A\beta$ which is not cleaved by brain NEP is transported via BBB and the glymphatic system into the CSF, blood and peripheral organs (kidney and liver) where it can be degraded by circulating or resident NEP.

identified at 11-13 weeks of gestation and localized to epithelial cells and mesenchyme of developing airways suggesting that NEP plays an important role in lung development (Sunday et al., 1992). It was later confirmed that NEP regulates foetal lung growth and maturation by mediating endogenous bombesin-like peptides (King et al., 1997). In human kidney NEP (CD10) expression was detected starting from early stages of embryogenesis increasing with maturation of the foetuses which suggests that it plays an important role in renal embryogenesis (Faa et al., 2012) (Figs. 1 and 2).

In fibroblasts NEP activity was found to increase during foetal to adult transition although in fibroblasts from a centenarian donor it was comparable to that in the cells from young donors (Kletsas et al., 1998). Adrenocorticotropin hormone (a regulatory peptide that can be cleaved by NEP) was shown to increase NEP enzymic activity in foetal cells and young adult donors but decrease in adults and old donors suggesting that ageing affects NEP activity in this type of cell. NEP was also detected in growing ligaments of calf foetuses (Johnson et al., 1990).

NEP also plays an important role in early thymic development and maturation of T lymphocytes as shown in both *in vivo* and *in vitro* studies

in mice (Guérin et al., 1997). Although the amino acid sequence of CD10 expressed in lymphocytes is almost identical to NEP, its enzymic activity was never detected biochemically in normal lymphocytes (Leoni and Losa, 1996).

The status of the maternal organism plays an important role in NEP expression and activity in the developing foetuses and offspring. Increased NEP expression in placenta was observed in such conditions as pre-eclampsia suggesting that it may be involved in regulation of peptide activity at the foetal-maternal interface, and as such could be implicated in the developmental changes observed in the growing foetus under such pathological conditions (Li et al., 1995a, 1995b). It was also shown that placenta releases active NEP into the maternal circulation at significantly greater levels in preeclampsia which can also predispose to various complications both in the maternal organism and the foetus including hypertension and heart failure (Gill et al., 2019) (Fig. 3). On the other hand, maternal obesity was found to downregulate NEP expression in foetal-placental endothelium and in the umbilical cord blood which also may alter the balance of vasoactive peptides and affect foetal development and predispose the offspring to various metabolic

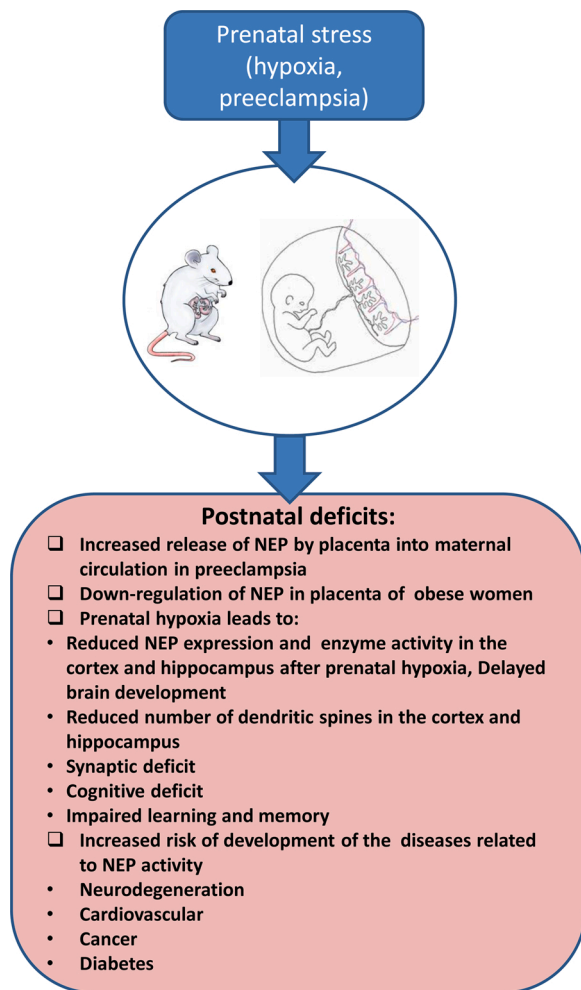


Fig. 3. Role of prenatal stress in development of NEP-related pathologies.

A significant amount of experimental, epidemiological and clinical studies suggest that gestational factors play a crucial role in development of all organ functions in postnatal life. Such adverse factors as prenatal hypoxia, preeclampsia, maternal diet or drugs of abuse have a negative effect on the foetus and increase its vulnerability to later development of various disorders (for review see Nalivaeva et al., 2018). Changes in NEP expression and activity during pregnancy affect the balance of a variety of regulator peptides. Prenatal hypoxia also affects levels of expression of NEP in the brain and blood plasma of the offspring predisposing them to development of neurodegenerative and cardiovascular diseases, cancer and diabetes.

diseases in later life (Weiß et al., 2020).

3. NEP in development of the nervous system

In the central nervous system, the external layer of the olfactory bulbs is the only structure prominently labelled in the foetal rat brain before birth while other structures enriched with NEP in the adult brain, such as the nigrostriatal tract, were shown to become progressively labelled after birth (Dutriez et al., 1992). In murine embryonic brain NEP expression was reported in the nigro-striatal axis, globus pallidus and nucleus accumbens (Dauch et al., 1993). NEP protein levels in rat brain cortex were found to be the highest during the first two weeks after birth declining significantly by the end of the first month of postnatal development. On the contrary, in the striatum, NEP protein levels reached their peak by the end of the first month and stayed at this level practically during all life (Nalivaeva et al., 2004). Several studies from our laboratory have demonstrated that prenatal hypoxia in rats results in a significant reduction of NEP mRNA and protein levels and its enzyme

activity in the cortex and hippocampus of rat offspring which correlates with a reduced number of dendritic spines in these brain structures and cognitive impairment of animals during postnatal development and can predispose to development of neurodegeneration, including AD, in later life (Fig. 3) (Nalivaeva et al., 2018; Zhuravin et al., 2019). On the other hand, NEP levels in postnatal life were shown to be dependent on the environment in which animals have been raised and elevated NEP activity was reported in the brains of mice raised in an "enriched environment" which inversely correlated with the amyloid burden (Lazarov et al., 2005).

Analysis of NEP expression in the peripheral nervous system has demonstrated that from E16 to maturity in rat sciatic nerve NEP expression declined both in the levels of immunoreactive protein and enzyme activity in the Schwann cells suggesting age-dependent down-regulation of its expression in this particular type of cell since in non-myelin-forming cells in the sciatic nerve NEP levels were retained in adulthood (Kioussi et al., 1992). The significant increase in NEP expression by Schwann cells after axonal damage suggests that this enzyme plays an important role in nerve development and regeneration (Kioussi et al., 1995). A loss-of-function NEP mutation in humans is also linked with development of Charcot-Marie-Tooth disease characterised by late-onset axonal neuropathy (Higuchi et al., 2016). These patients had decreased or lack of NEP protein expression in the peripheral nerves correlated with muscle weakness, atrophy, and sensory disturbance in the lower extremities although they did not demonstrate symptoms of dementia.

4. NEP and brain functions

Being an important and promiscuous neuropeptidase, NEP participates in various brain functions moderated by its corresponding substrates. This includes NEP roles in metabolism of sensory and inflammatory neuropeptides such as tachykinins and neurokinins (Turner and Nalivaeva, 2007) and pain perception (Fischer et al., 2002). By terminating the effects of neuropeptides in the basal ganglia NEP also participates in movement regulation (Chen et al., 2020) and loss of NEP expression results in altered locomotor activity (Fischer et al., 2000).

Experiments either with NEP knockout mice or NEP inhibitor injections have demonstrated that NEP plays certain roles in cognitive function. NEP knockout mice demonstrated increased aggressive behaviour in the resident-intruder paradigm (Fischer et al., 2000) as well as considerably weaker conditioned taste aversion which extinguished faster than in the age-matched controls (Madani et al., 2006). Rats continuously injected with thiorphan via an osmo-pump during 4 weeks displayed significant cognitive dysfunction in the ability to discriminate in the object recognition test and spatial memory in the water maze test, but not in other hippocampus-dependent learning and memory tasks (Mouri et al., 2006). Single microinjections of phosphoramidon or thiorphan into rat sensorimotor cortex led to disruption of short-term memory tested in a two-level radial maze test (Dubrovskaya et al., 2010).

Studies performed in 5XFAD mice modelling AD demonstrated that NEP deficiency aggravates the behavioural and neuropathological phenotype demonstrating impairment in spatial working memory (Hüttenrauch et al., 2015). Intracranial injections of thiorphan together with A β 42 to aged rhesus monkeys accelerated development of AD phenotype with significant intraneuronal accumulation of endogenous A β in the basal ganglia, cortex and hippocampus, accompanied by neuronal atrophy and loss (Li et al., 2010). On the contrary, over-expression of the human NEP gene in 5XFAD mice under control of the α CaMKII promoter was shown to ameliorate the AD-like phenotype in aged animals (Devi and Ohno, 2015). It also was reported that, in the absence of the NEP gene, other neuropeptides e.g. glucagon-like peptide 1 and galanin could substitute for NEP activity and support the processes involved in learning in aged NEP-deficient mice (Walther et al., 2009).

5. NEP in the ageing brain

As already mentioned above, NEP expression and activity decrease with age in some peripheral tissues and in brain structures of experimental animals (Kletsas et al., 1998; Nalivaeva et al., 2004; Zhuravin et al., 2019). There are also several studies reporting that in human brain, and especially in the hippocampus, which is vulnerable to AD pathology, NEP steady-state levels diminish as a function of age (Cacamo et al., 2005). Reduced NEP protein levels were reported in the temporal and frontal cortex of normally aged and AD brains (Hellström-Lindhahl et al., 2006) while another study confirmed that NEP immunoreactivity was decreased in AD but not in other pathological ageing (Wang et al., 2005). More detailed analysis showed that NEP mRNA and protein levels, as well as enzyme activity were decreased in AD brain compared to normal controls with no cognitive impairment (Wang et al., 2010). Comparative mRNA analysis in high plaque areas of AD brains also demonstrated reduced NEP expression compared to age-matched controls while in the periphery they were much higher suggesting that in the periphery the capacity of NEP to degrade A β is stronger than in the brain and explaining the lack of A β deposits in the peripheral organs (Yasojima et al., 2001). Although another group reported no differences in NEP mRNA levels in the temporal lobe of AD patients they noted that the changes in the somatostatin system in this brain area might impair NEP regulation causing a deficit in its activity leading to A β accumulation (Gahete et al., 2010). There are also data, demonstrating that NEP protein levels do not significantly change in adult human brain with advanced ageing from 40 to over 60 years old and in AD and that NEP activity is higher in the homogenates from advanced age and AD samples. However, such a discrepancy is most likely to be attributed to the methodological approach used in this study (Miners et al., 2010).

Since amyloid clearance and NEP deficit play an important role in development of late-onset sporadic AD (Farris et al., 2007), more studies of NEP developmental profile and regulation in various brain areas both in non-transgenic animals and humans using state of the art unified techniques are still needed for a better understanding of its role in AD pathogenesis and preventions.

6. Role of NEP in diseases

6.1. Cancer

The identity of NEP (CD10) as the common acute lymphoblastic leukemia antigen CALLA (LeTarte et al., 1988; Le Bien and McCormack, 1989) and its transient expression on the surface of certain hematopoietic cells makes CD10 an important marker for some types of lymphomas (de Jong et al., 2007) and is widely used for diagnosis of certain forms of childhood leukemia (Cutrona et al., 2002). More recently NEP was also reported as a marker for primary central nervous system lymphoma (Yang and Liu, 2017).

Apart from lymphomas, NEP has been implicated in the progression of various types of cancers due to its ability to inactivate such bioactive peptides as endothelin-1 (ET-1), angiotensin-II, and bombesin which participate in neoplastic transformation. The most studied types of cancer in which NEP expression and activity are affected by tumour progression include prostate (Papandreou et al., 1998; Usmani et al., 2000), kidney (Ahn et al., 2013; Erin et al., 2016), lungs (Shipp et al., 1991; Goldstein et al., 2017), breast (Louhichi et al., 2018), endometrium (Pekonen et al., 1995) and cervix (Terauchi et al., 2005). In the majority of cases NEP has tumour suppressive action and its loss contributes to cancer progression (Sumitomo et al., 2005). However, in colorectal cancer, CD10 expression was shown to increase with tumour progression (Jang et al., 2013). NEP overexpression was also reported in melanomas (Velazquez et al., 2007).

Mechanisms of NEP gene regulation were studied in great detail in prostate cancer (Usmani et al., 2000; Hong et al., 2012) where NEP

contributes to androgen-independent progression of the disease through the loss of ability to metabolise mitogenic peptides, such as endothelin-1. Down-regulation of NEP expression in prostate cancer was shown to be attributed to extensive hypermethylation of its gene promoter region (Usmani et al., 2000). NEP gene transcription in the prostate is up-regulated by androgens (Shen et al., 2000) as well as by estrogen through a hormone-responsive element (Xiao et al., 2009). Although in neuronal cells the NEP gene is regulated by the amyloid-precursor protein (APP) intracellular domain (AICD) (Belyaev et al., 2010) and APP is expressed in prostate, there is no evidence that a similar AICD-dependent mechanism is also involved in NEP gene regulation in this tissue (Hong et al., 2012) although histone acetylation in the region of the NEP gene promoter may facilitate partial re-activation of NEP expression in advanced prostate cancer cells. Recently it was shown that long noncoding RNA (lncRNA) within mRNA sequences of APP is progressively activated in prostate cancer promoting its development by competitive binding to miR218 (Shi et al., 2020) although its involvement in NEP expression has not yet been investigated.

Up-regulation of NEP activity in cancer cell lines was also achieved by treatment with *Epilobium* extracts and polyphenols which also inhibited cell proliferation suggesting that this herbal remedy can be efficient in cancer cells with dysregulated NEP expression and activity (Kiss et al., 2006). Re-expression of NEP via an inducible tetracycline-regulatory gene expression system in mice resulted in inhibition of tumour formation and progression in their prostates (Dai et al., 2001) which opens new avenues in prostate cancer therapy.

6.2. Cardiovascular diseases

Taking into account a very important role of NEP in inactivating the natriuretic peptides *in vivo* and its participation in the renin-angiotensin system (RAS), much effort has been paid to the design of potential NEP inhibitors for the treatment of certain forms of cardiovascular and renal diseases. Moreover, circulating plasma NEP was shown to be a predictor of heart failure (Bayes-Genis et al., 2016, especially in patients on dialysis (Claus et al., 2020). However, the observations that loss of NEP activity can promote accumulation of A β in the brain (Newell et al., 2003) or enhance the activity of mitogenic peptides (Dawson et al., 2004) urged caution for the chronic application of NEP inhibitors in humans (Campbell, 2017). Currently a novel angiotensin receptor/neprilysin inhibitor (ARNI) sacubitril/valsartan has been approved for treatment of hypertension and prevention of heart failure in elderly hypertensive patients (for review see Kario, 2018). While there is no doubt that hypertension should be treated for prevention of cardiovascular diseases as well as of stroke and vascular dementia, the role of NEP inhibitors as antihypertensive agents in AD pathogenesis remains unclear and needs further investigation (Lebouvier et al., 2020).

6.3. Obesity and Diabetes

Recent studies have revealed that NEP is involved in the development of obesity and type 2 diabetes. NEP-deficient mice become obese even under a normocaloric diet at the age of 6-7 months (Becker et al., 2010). In humans, a direct correlation between NEP activity, body mass index (BMI) and insulin resistance was observed in subjects with multiple cardiovascular risk factors. NEP protein production in human adipocytes increased during cell differentiation, and plasma and adipose tissue levels of NEP were increased in obese insulin-resistant mice (Standeven et al., 2010). It was also demonstrated that NEP accelerates adipogenesis through enhancement of insulin-mediated PI3K-Akt activation (Kim et al., 2017). Treatment of obese mice (Berlin fat mice) with green tea significantly reduced body fat and prevented its further accumulation. This correlated with increased peripheral (in the kidney and intestine) NEP expression and downregulation of orexigens (Muenzner et al., 2016). This effect of green tea was not observed in obese NEP-knockout mice. NEP deficient mice are also characterised by

increased islet β -cell mass when fed on a high fat diet which suggests a role for NEP in modulating metabolism of this type of cells (Parrilla et al., 2018). Elevated NEP activity was also observed in the vitreous of patients with diabetic retinopathy which inversely correlated with $A\beta$ levels (Hara et al., 2006). Hyperlipidemia and hyperglycemia associated with type 2 diabetes cause delayed diabetic wound healing and were found to be associated with increased NEP activity in human microvascular endothelial cells which might impair normal response of the tissues to the injury (Muangman et al., 2003). Topical application of thiorphan to the wounds of diabetic mice, on the contrary, improved wound healing (Spenny et al., 2002).

In patients with diabetes NEP levels in urine are increased and could be used as early biomarkers to predict the incidence or progression of chronic kidney disease in individuals with type 2 diabetes (Gutta et al., 2018).

In a monkey model of streptozotocin-induced type 1 diabetes a decrease in NEP mRNA levels were observed in the hippocampus which correlated with increased levels of $A\beta$ in this brain structure suggesting a link between diabetes and AD pathogenesis (Morales-Corraliza et al., 2016).

Accumulating data suggest that NEP plays an important role in glucose metabolism and clinical studies with a dual angiotensin receptor-neprilysin inhibitor (ARNi) demonstrated that such treatment improved insulin sensitivity and glycaemic control in patients with type 2 diabetes (Seferovic et al., 2017). This suggests that NEP inhibitors in combination with angiotensin II receptor blockers might be a useful therapeutic approach for treatment of type 2 diabetes (Esser and Zraika, 2019). However, considering the promiscuous nature of NEP and its involvement in normal homeostasis and development of a range of diseases, this should be done with significant care and preliminary laboratory research.

6.4. Immune system diseases

Involvement of NEP in development of immunodeficiency can be anticipated based on its involvement in development and maturation of haemopoietic cells and pathogenesis of lymphomas. In HIV patients an increased ratio of CD10-positive B cells has been reported suggesting B cell activation and immaturity (Martínez-Maza et al., 1987). Although NEP enzymatic activity was not reported in normal lymphocytes, it was detectable in HIV + cells (Leoni and Losa, 1996). However, in the brain of HIV patients the increase of a viral transactivating transcription factor (Tat) correlated with reduced NEP activity and reported accumulation of $A\beta$ in the brain of HIV-infected patients (Rempel and Pulliam, 2005). Later it was reported that NEP hydrolyses the Tat protein and the peptides derived from it inhibit NEP activity competitively and reversibly leading to increased $A\beta$ levels (Daily et al., 2006).

Although no direct correlation was reported between $A\beta$ 42 and NEP activity in the CSF of HIV patients (Mothapo et al., 2015) the changes in NEP/ $A\beta$ ratio was found to be dependent on HIV subtype suggesting higher $A\beta$ deposit in HIV1-C infected patients than in HIV1-B (de Almeida et al., 2018).

6.5. Alzheimer's disease

As documented above NEP, being the major amyloid-degrading enzyme in the brain (Iwata et al., 2000, 2001), plays a significant role in AD pathogenesis, in particular, of the late onset sporadic form (for review see Nalivaeva et al., 2012a). A recent meta-analysis of NEP levels in AD demonstrated both that NEP expression and activity are decreased in the cortex of elderly AD patients (Zhang et al., 2017). NEP inhibition or gene silencing in animal models recapitulate the pathological hallmarks of AD detected in human brains with accumulation of amyloid peptide and cognitive impairment (Hanson et al., 2011; Li et al., 1995a, 1995b). Interestingly, NEP gene knockout in APP_{Sw} mice modelling AD led to the changes in expression of more than 600 proteins including

some which were not previously reported to be linked to AD but associated with increased $A\beta$ levels (Nilsson et al., 2015). These mice also exhibited enhanced amyloid pathology compared with only APP transgenic ones and showed impaired learning and memory in the Morris water maze test (Mohajeri and Wolfer, 2009).

Numerous studies have demonstrated the efficacy of NEP up-regulation for lowering $A\beta$ levels in the brain, including lentiviral or adenoviral NEP gene transfer (Marr et al., 2003; Carty et al., 2013). By this approach it was shown that NEP gene transfer was able to abolish increased $A\beta$ levels at the presynaptic sites in young transgenic mice (Iwata et al., 2004). Moreover, up-regulation of peripheral NEP in experimental animals (in the skeletal muscles or plasma) could also reduce $A\beta$ levels in the brain and ameliorate AD pathology (Liu et al., 2009; Liu et al., 2010). There are also numerous pharmacological approaches for increasing NEP expression and activity recently reviewed in (Nalivaeva and Turner, 2019). Some of them are based on the endogenous feedback mechanism for upregulating the NEP gene by the APP C-terminal fragment AICD which is formed alongside $A\beta$ in the amyloidogenic APP proteolytic processing in neuronal cells (Pardossi-Piquard et al., 2005; Belyaev et al., 2010). This mechanism involves competitive binding of AICD and histone deacetylases (HDACs) to the promoter region of the NEP gene which, in the cells with low levels of NEP expression, can be activated by HDAC inhibitors (Belyaev et al., 2009). On the other hand, there is convincing evidence that DNA methylation of the NEP promoter is not involved in AD pathogenesis (Nagata et al., 2018).

Our work in cell and animal models has demonstrated that NEP expression and activity is reduced after hypoxia and that this down-regulation is related to increased cleavage of AICD by caspases activated by hypoxia (Kerridge et al., 2015; Kozlova et al., 2015). Intracranial injections to rats, subjected to prenatal hypoxia, of a caspase-3 inhibitor (Z-DEVD-FMK or Ac-DEVD-CHO) at the age of three weeks reduced caspase activity and restored AICD levels and NEP protein expression (Vasilev et al., 2016; Kozlova et al., 2015). Moreover, administration of an HDAC inhibitor, sodium valproate, or the antioxidant EGCG were shown to increase NEP expression and activity in rats subjected to prenatal hypoxia (Nalivaeva et al., 2012b; Zhuravin et al., 2019).

One of the substrates of NEP, somatostatin (Barnes et al., 1995), which plays an important role in the hippocampus facilitating memory processes (Eyre and Bartos, 2019), was suggested to upregulate NEP expression (Saito et al., 2005). Somatostatin levels decline in the brain with ageing (Hayashi et al., 1997) and are consistently reduced in the brain and CSF of AD patients (Burgos-Ramos et al., 2008) which together with reduced NEP activity may trigger and promote $A\beta$ accumulation leading to late-onset AD. Incubation of primary cortical neurons with somatostatin was shown to upregulate NEP activity (Saito et al., 2005). Moreover, *i.c.v.* injection of a selective somatostatin receptor-subtype agonist to 12-month SAMP8 mice not only increased NEP activity in the cortical tissue but also enhanced their learning and memory (Sandoval et al., 2012).

Screening 25 curcumin analogues for their ability to upregulate NEP activity it was shown that only four of them (namely dihydroxylated curcumin, monohydroxylated demethoxycurcumin, and mono- and dihydroxylated bisdemethoxycurcumin) increased NEP activity while curcumin itself did not have this effect (Chen et al., 2016). Although such pharmacological approaches can be beneficial for NEP-dependent prevention of $A\beta$ accumulation and development of late onset AD, treatments have to start rather early even before disease manifestation (El-Amouri et al., 2008). Further works on the role of HDAC modifications in AD pathology demonstrated that HDAC1 SUMOylation might be an intrinsic defence mechanism protecting neuronal cells against $A\beta$ toxicity which opens a new avenue in therapeutic strategy in AD prevention (Tao et al., 2017).

In relation to AD pathology it is important to mention another NEP-like endopeptidase termed neprilysin-2 (NEP2) whose expression in the brain is restricted to developing and differentiating areas (Facchinetti

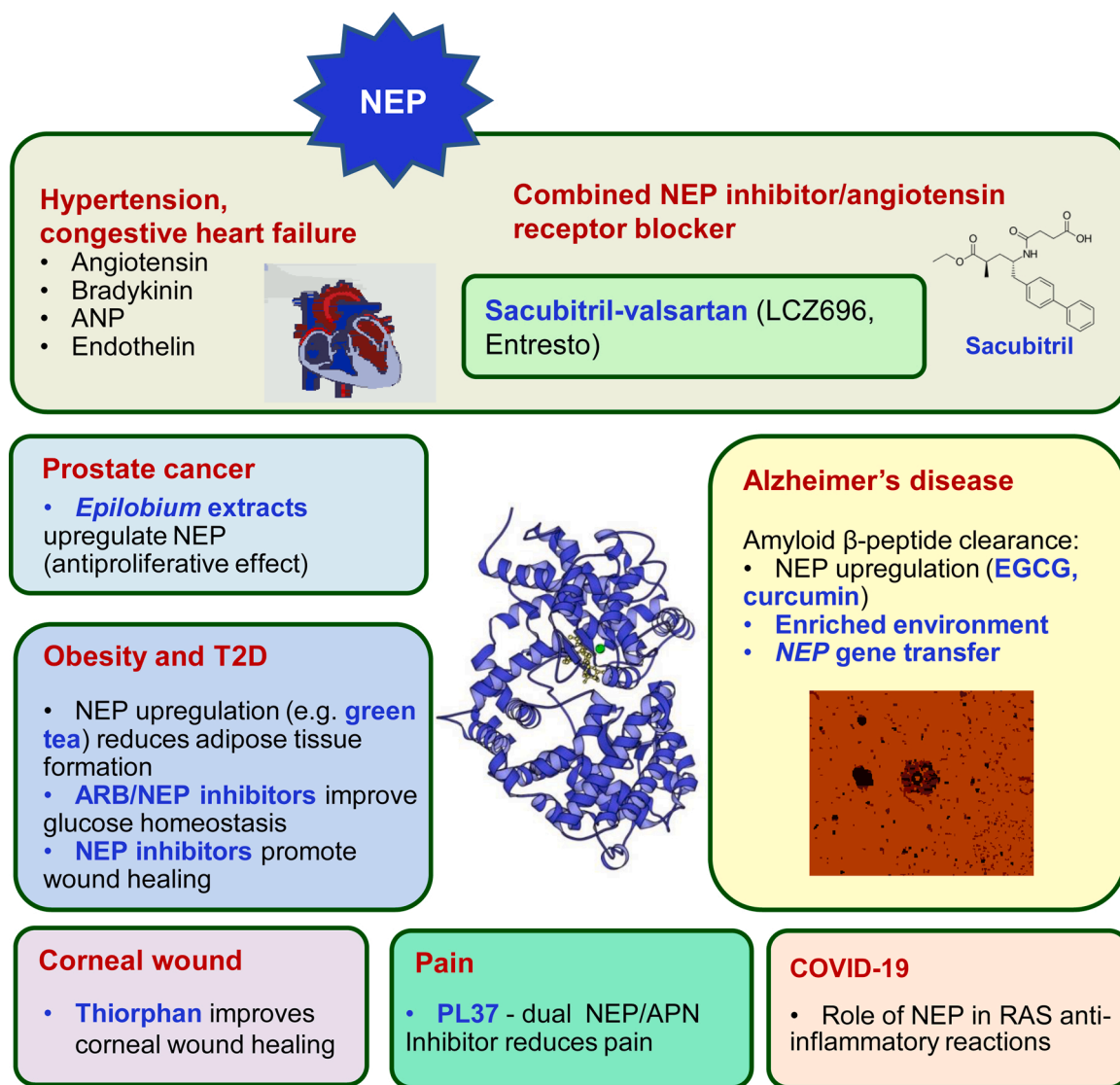


Fig. 4. NEP dysregulation leads to various diseases of old age and creates a platform for designing therapeutic targets. The involvement of NEP in diverse disease conditions, its relevant substrates and inhibitors, and strategies for its upregulation are highlighted, alongside the enzyme 3D structure.

et al., 2003) and which has different subcellular localization and substrate preference from NEP (Whyteside and Turner, 2008) but contributes to Aβ metabolism *in vivo* (Hafez et al., 2011; Huang et al., 2012). It was demonstrated that NEP2 activity is reduced in individuals with mild cognitive impairment and in AD patients compared to non-impaired individuals (Huang et al., 2012). NEP2 was suggested to be even more selective as a therapeutic target than NEP since it has a more restricted substrate specificity (Whyteside and Turner, 2008).

There are reports on a biallelic polymorphism in the 3'UTR of the NEP gene associated with an increased risk for AD in an age-dependent manner adjusting for sex and ApoE status (Clarimón et al., 2003). Also, a single nucleotide polymorphism (SNP) in the NEP gene correlating with development of AD was reported in Finnish patients (Helisalme et al., 2004). However, studies in Swedish patients failed to demonstrate significant differences in the distribution of promoter polymorphisms between AD cases and controls (Lilius et al., 2003) as well as in a cohort from a Southern Chinese community (Fu et al., 2009). Nevertheless, analysis of NEP SNP rs1816558 in two Han Chinese cohorts demonstrated significant association with AD after adjustment for APOEε4 allele while NEP mRNA levels were substantially increased during AD progression (Wang et al., 2016).

Reduced CSF NEP activity levels have been shown to occur in early AD, suggesting that altered CSF NEP activity levels may also be predictive of the progression of dementia (Maetzler et al., 2010). There are also reports on reduced NEP activity in blood plasma in individuals with mild cognitive impairment and AD patients (Zhuravin et al., 2015), which are in line with studies showing that NEP is reduced in CSF in prodromal AD (Maruyama et al. 2005).

6.6. COVID-19

The recent ongoing pandemic caused by the new coronavirus (SARS-CoV2) has raised the question about a possible role of NEP in the pathogenesis of the complications developed following the viral infection. Despite intensive search for potential drug candidates and development of effective vaccines there is still no treatment to prevent the infection or reduce the severity of the disease progression. The most severe complications of SARS-CoV2 infection are observed in the lungs and heart where the viral receptor, angiotensin-converting enzyme-2 (ACE2), a metallopeptidase determining together with its homologues ACE and NEP the effectiveness of the RAS, is expressed (Tipnis et al., 2000; Rice et al., 2006). Taking into account the role of NEP in the lung

RAS and its ability to convert angiotensin I to the protective angiotensin-(1-7) (Rice et al., 2006), it might be protective against the inflammatory reactions and fibrosis caused by viral infections in the lungs (for review see El Tabaa and El Tabaa, 2020; Manolis et al., 2020).

7. NEP as a therapeutic target

As already discussed above, development of NEP inhibitors for controlling blood pressure has been compromised by the facts that chronic NEP inhibition might result in angio-oedema, bronchial reactivity, inflammation, cancer, and could predispose to polyneuropathy and AD (for review see Campbell, 2017). Despite these concerns, in recent years interest in this strategy has been revived leading to the development of sacubitril-valsartan (LCZ696), a combined NEP inhibitor and angiotensin receptor blocker, whose use for the treatment of heart failure was approved in 2015. The dual cardioprotective action of the drug appears to have significant advantages in the management of heart failure (the PARADIGM-HF phase III trial) (Seferovic et al., 2017; Kario, 2018).

NEP inhibition was also suggested for treatment of corneal injury and corneal wound healing (Genova et al., 2018). Another proposed area for NEP inhibitor application was female sexual arousal disorders although no significant progress in this area has been achieved (Angulo, 2010).

NEP inhibitors may also be suitable for pain management because of their effects on nociception (Aykan et al., 2019; Ramírez-Sánchez et al., 2019). Oral administration of a dual enkephalinase (NEP/ANP) inhibitor PL37 was shown to suppress completely osteosarcoma-induced thermal hyperalgesia in mice via activation of opioid receptors (Menéndez et al., 2008). Apart from synthetic NEP inhibitors there are some natural endogenous NEP inhibitors, including the stress-response peptide, sialorphin (Rougeot et al., 2003) and “opiorphins”, which possess strong analgesic properties (Wisner et al., 2006). Some studies have also suggested that opiorphins promote sperm motility which adds another important role to the list of NEP functions and therapeutic avenues (Bosler et al., 2014). Role of NEP in development and modulation of pain, stress response, addiction, sexuality, and food intake has recently been reviewed in (Roques, 2018).

Although some areas of medicine are seeking compounds to inhibit NEP there is clearly an urgent need to upregulate NEP expression and activity for treatment of other groups of diseases such as cancer and AD (Fig. 4). Various pharmacological and epigenetic approaches have been developed to up-regulate NEP expression (for review see Nalivaeva and Turner, 2019). However, for designing strategies to treat each particular type of pathology it is not only necessary to develop specific drugs but also to acquire a deeper knowledge on the specificity of NEP regulation by endogenous and exogenous factors at the levels of its gene expression, mRNA translation, protein post-translational modification and local modulation of its activity. Such knowledge could help us to use this rather unique enzyme for the benefit of human health.

Declaration of Competing Interest

Authors declare no conflict of interest.

Acknowledgements

Supported by RFBR (19-015-00232) and Russian Federation state budget (assignment AAAA-A18-118012290373-7).

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