Medical Hypotheses 131 (2019) 109309

Contents lists available at ScienceDirect

Medical Hypotheses

journal homepage: www.elsevier.com/locate/mehy

Peptidase neurolysin is an endogenous cerebroprotective mechanism in acute neurodegenerative disorders

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ARTICLE INFO

Keywords:
Neuroprotection
Neuropeptide
Neurogenic inflammation
Brain edema
Drug target

ABSTRACT

Stroke and traumatic brain injury (TBI) are significant clinical problems characterized by high rate of mortality and long-lasting disabilities, and an unmet need for new treatments. Current experimental stroke and TBI research are evolving to focus more on understanding the brain's self-protective mechanisms to meet the critical need of developing new therapies for these disorders. In this hypothesis-based manuscript, I provide several lines of evidence that peptidase neurolysin (Nln) is one of the brain's potent, self-protective mechanisms promoting preservation and recovery of the brain after acute injury. Based on published experimental observations and ongoing studies in our laboratory, I posit that Nln is a compensatory and cerebroprotective mechanism in the post-stroke/TBI brain that functions to process a diverse group of extracellular neuropeptides and by that to reduce excitotoxicity, oxidative stress, edema formation, blood brain barrier hyper-permeability, and neuroinflammation. If this hypothesis is correct, Nln could potentially serve as a single therapeutic target to modulate the function of multiple targets, the involved neuropeptide systems, critically involved in various mechanisms of brain injury and cerebroprotection/restoration. Such multi-pathway target would be highly desired for pharmacotherapy of stroke and TBI, because targeting one pathophysiological pathway has proven to be ineffective for such complex disorders.

Introduction

Stroke and traumatic brain injury (TBI) are two major, acute neurodegenerative disorders which constitute the leading causes of death and disability worldwide affecting both old and young individuals [1]. Current experimental stroke and traumatic brain injury research focuses more on understanding the brain's self-protective/repair mechanisms to meet the critical need of developing new stroke therapies [2,3]. The underlying premise of this approach is that the brain has well-developed, complex and highly conserved endogenous mechanisms for self-preservation and repair in adverse conditions and following injury. It is believed that detailed understanding of these mechanisms is important as such knowledge could lead to development of pharmacological or other therapeutic interventions to mimic or engage brain's self-protective/repair mechanisms for successful therapy [2].

While etiologies of stroke and TBI are different, the main secondary brain injury mechanisms are shared in these disorders and involve excitotoxicity, oxidative stress, edema formation, blood brain barrier (BBB) hyperpermeability, and neuroinflammation. All of these brain injury cascades have been studied thoroughly over the last several decades [4,5], however, there has been much less emphasis on the role of neuropeptides in these pathophysiological processes. This is despite the fact that neuropeptides are the most diverse signaling molecules in mammals and primarily come into play when the nervous system is adapting/responding to various challenges, thus representing the "language of the stressed nervous system" [6]. It is well known that the actions of neuropeptides are tightly linked to the function of peptidases, which are hydrolytic enzymes involved in processing of bioactive peptides [7–9]. Altered expression and/or activity of several brain peptidases have been reported in experimental stroke and TBI studies linking the function of peptidases and related neuropeptide systems to the pathophysiology of these disease [10,11]. Here, I summarize the results of our published studies which provide evidence about the role of peptidase neurolysin (Nln) in the post-stroke brain. In addition, based on our unpublished observations and published studies from other laboratories, I make a case that Nln is one of the brain's self-protective mechanisms directed towards preservation and recovery of brain in acute neurodegenerative disorders.

Association of neurolysin with neuronal cell death

Our interest in Nln originates from studies related to the non-AT_1,

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https://doi.org/10.1016/j.mehy.2019.109309
Received 30 May 2019; Received in revised form 6 July 2019; Accepted 12 July 2019

non-AT_2 angiotensin binding site, which was discovered, characterized and identified by us as membrane Nln [12–16]. Nln (EC 3.4.24.16) is a zinc endopeptidase belonging to M3 family which make the most important group of peptidases responsible for hydrolytic processing of bioactive peptides in the extracellular environment [9]. It was by serendipity that in experiments conducted several years ago we observed increased expression of membrane-bound variant of neurolysin (Nln) in primary mouse cortical neurons that were maintained in culture for about 2 weeks without replenishment of the culture medium [17]. This was followed by more systematic set of experiments which revealed upregulation of Nln in primary neurons challenged in four *in vitro* models of cell death (excitotoxicity by NMDA, oxygen-glucose deprivation followed by re-oxygenation, hypoxia by sodium azide, and oxidative stress by hydrogen peroxide), and provided evidence about the potential role of this peptidase in neuronal cell death for the first time. However, these original observations did not answer two critical questions, a) whether upregulation of Nln occurs only *in vitro* or it has *in vivo* relevance, and b) what the function of Nln is in neuronal death.

Upregulation of Nln in mouse brain after stroke

We sought to answer the first question in a mouse model of ischemic stroke which confirmed our *in vitro* observations. Experiments carried out in transient, middle cerebral artery occlusion model of stroke (MCAO, 1 h occlusion followed by 24 h re-perfusion) revealed significant increase (≥ twofold) in quantity and activity of membrane Nln in ischemia-affected parts of the mouse brain [18]. Importantly, this study also documented sustained functional upregulation of Nln in ischemia-affected cerebral cortical membranes for at least 7 days after stroke. Upregulation of Nln did not appear to be transcriptionally or translationally regulated, but rather depended on translocation of cytosolic Nln to the membranes and mitochondria [18]. Collectively these findings confirmed our *in vitro* observations and suggested that Nln may play a role in processes modulating the brain's response to stroke.

Additional *in vivo* evidence linking Nln to cell death

The potential role of Nln in neuronal cell death is additionally supported by published studies which evaluated the expression of Nln in developing rodent brains [17,19]. Expression of membrane Nln was shown to increase gradually in mouse forebrain membranes from E14 to P10, followed by a dramatic drop in P21 animals and similar levels in 9- to 12-week-old animals [17]. It is noteworthy that the pattern of developmental changes in the expression of Nln is very similar to the occurrence of neuronal death in developing brain [20]. The number of dead neurons in rat cerebral cortex is low at birth, which increases from P2, peaking at the end of the first week, and decreases during the second week followed by low numbers at the end of the first month [20]. These *in vivo* observations complement the above discussed data and support the association between neuronal cell death and Nln. Moreover, these results clarify earlier observations in adult rat brain where distribution of Nln was studied by *in vitro* receptor autoradiography [15]. In the latter study, among brain regions with the highest expression of Nln were the olfactory bulb (highest compared with other brain regions), walls of ventricles (throughout brain), and dentate gyrus. Complementary to this, it was shown that the frequency of apoptosis in adult rat brain is up to 100 times higher in olfactory bulb, ventricular wall, and dentate gyrus (in decreasing order) compared with other brain areas [21]. Lastly, another line of evidence comes from a list of functional proteins which have ontogenic and post-stroke expression profile very similar to Nln (i.e. overexpression), and are viewed as key molecular players in response of brain to ischemia and acute injury [22].

It is important to note that in the brain, Nln is expressed not only in neurons but also in astrocytes [23] and likely in endothelial cells of the brain vasculature [24]. Cellular localization (plasma membrane vs. cytosol and mitochondria) of Nln differs in these cell types and changes during brain development [19,23]. Currently, it is unknown which transcription factor(s) control expression of Nln and how the latter is affected in dying astrocytes and endothelial cells.

Nln substrates and their role in acute neurodegeneration

To understand the potential significance of Nln upregulation in the post-stroke brain it is important to recognize the peptide substrates that are hydrolyzed by Nln and their function in the setting of acute brain injury. Endogenous substrates of Nln are neurotensin, bradykinin, substance P, angiotensins I and II, hemopressin, dynorphin A(1–8), and metorphamide [9,18,25]. Nln cleaves and inactivates all of these peptides except angiotensin I, dynorphin A(1–8) and metorphamide, which are converted into angiotensin-(1–7) and enkephalins, respectively. Importantly, all of these peptide systems are directly involved in pathogenesis of stroke and/or TBI [18]. Integral role of bradykinin in these disorders was demonstrated by *in vivo* studies indicating that both B1 and B2 receptors are involved in development of neuroinflammation, brain edema and cell death [26,27]. Pathological role of neurotensin in ischemic brain is supported by *in vitro* studies demonstrating decreased survival of primary neurons after ischemia in the presence of neurotensin, and blockade of its effects by NT1 receptor antagonists [28,29]. This effect involves potentiation of glutamate release and amplification of NMDA-R-mediated glutamate signaling in neurons [28,30]. Substance P is another member of the kinin family with critical role in neurogenic inflammation, oxidative stress, genesis of edema and cell death after stroke and TBI [31,32]. Angiotensin I is an inactive peptide, serving as a precursor for bioactive angiotensins. Cleavage of angiotensin I by Nln results in angiotensin-(1–7), which was shown to have neuroprotective and anti-inflammatory effects in *in vivo* stroke and TBI models [33,34]. Complementary to this, Nln inactivates angiotensin II, which is the most studied peptide in the renin-angiotensin system with mounting evidence supporting its pathological role in stroke and TBI [35,36]. Hemopressin is a potent CB1 cannabinoid receptor antagonist (also acts as inverse agonist) which is inactivated by Nln. Inactivation of hemopressin facilitates CB1 receptor activation, which is known to mediate neuro- and angiogenesis, and survival of new neurons [37]. Dynorphin A(1–8), a kappa-opioid receptor agonist, and metorphamide, a kappa- and mu-opioid receptor agonist, are converted into potent delta-opioid receptor agonists Leu- and Met-enkephalins, respectively, by Nln. The role of delta-opioid receptors in resistance to ischemia, neuroprotection and post-stroke brain repair was demonstrated by our and other groups [38,39]. It is important to note that findings of a small number of studies do not support the cerebrotoxic effects of some Nln substrates in the setting of acute brain injury [27,40,41]. Unfortunately, it would be impossible to discuss all experimental studies on the pivotal function of Nln substrate peptides in pathophysiology of stroke and TBI here, given their vast number and page limitations of this article. Importantly though, a number of recent clinical studies support this preclinical evidence and strongly relate the severity of stroke injury and TBI, and subsequent mortality to elevated serum and/or cerebrospinal levels of bradykinin, substance P and neurotensin [42–45], in some cases documenting 5 to 10-fold increase of these peptides following injury.

Hypothesis and future directions

Considering diversity of endogenous Nln substrates and the well-documented role of these peptidergic systems in pathogenesis of stroke and TBI, I hypothesize that upregulation of Nln following neuronal cell death is a compensatory and self-protective mechanism which functions to preserve expansion of damage and restore the brain after injury. To test this hypothesis, our current studies focus on ischemic stroke using mouse models, where we are applying two independent approaches, inhibition of Nln by a specific inhibitor and brain overexpression by an

AAV vector, to verify functional significance of Nln in the post-stroke brain. The outcome measures in this study include brain infarction and cell death, formation of edema, disruption of the blood brain-barrier (BBB), functional impairment and overall survival. Our preliminary results support the hypothesis and it is believed that cerebroprotective function of Nln is, at least in part, linked to hydrolysis of its extracellular peptide substrates and by that prevention of cellular and vasogenic edema, oxidative stress, preservation of BBB, reduction of neuroinflammation and cell death following stroke. These ongoing studies will be followed by more mechanistic and cell type-specific investigations to reveal the role of different brain cell types and substrate peptides in Nln-mediated cerebroprotection. If this hypothesis is confirmed in stroke settings, it will be critical to evaluate the potential of Nln function in other neurological disorders including TBI and vascular dementia.

It is important to recognize that the ability of Nln to process several neuropeptides suggests that it could potentially serve as a single therapeutic target to modulate the function of multiple targets, the noted neuropeptide systems, critically involved in various mechanisms of brain injury or cerebroprotection/restoration. Such multi-pathway target would be highly desired for pharmacotherapy of stroke and TBI, because in recent years it has been recognized that targeting one pathophysiological pathway is unlikely to be therapeutically effective in such complex disorders. If this idea is confirmed by systematic studies, then brain-penetrating variants of Nln or small molecule activators of Nln, which are being developed in our laboratory, could become a new class of drugs to be used for cerebroprotection and restoration of the brain after acute injuries.

Declaration of Competing Interest

VTK is the senior inventor on a provisional patent, filed by Texas Tech University Systems, focusing on discovery of small molecule activators of Nln.

Acknowledgements

This research was supported by research grants from the American Heart Association (14BGIA20380826) and NIH (1R01NS106879). I apologize that the scope of this manuscript prevented citation of many important studies.

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