JOURNAL OF PROTEOMICS XX (2014) XXX–XXX

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Peptidomic analysis of the neurolysin-knockout mouse brain☆

Leandro M. Castro[a,b], *Diogo M.L.P. Cavalcanti*[a], *Christiane B. Araujo*[a], *Vanessa Rioli*[c], *Marcelo Y. Icimoto*[b], *Fábio C. Gozzo*[d], *Maria Juliano*[b], *Luiz Juliano*[b], *Vitor Oliveira*[b], *Emer S. Ferro*[a,*]

[a]*Department of Pharmacology, Support Center for Research in Proteolysis and Cell Signaling (NAPPS), University of São Paulo, 05508-000 SP, Brazil*
[b]*Department of Biophysics, Federal University of São Paulo, 04039-032 SP, Brazil*
[c]*Butantan Institute, LETA-CETICS, 05503-900 São Paulo, SP, Brazil*
[d]*Chemistry Institute, Campinas State University, 13083-970 Campinas, SP, Brazil*

ARTICLE INFO

Keywords:
Intracellular peptides
Neurolysin
Hemopressin
Peptide metabolism
Endopeptidase
Oligopeptidase

ABSTRACT

A large number of intracellular peptides are constantly produced following protein degradation by the proteasome. A few of these peptides function in cell signaling and regulate protein–protein interactions. Neurolysin (Nln) is a structurally defined and biochemically well-characterized endooligopeptidase, and its subcellular distribution and biological activity in the vertebrate brain have been previously investigated. However, the contribution of Nln to peptide metabolism *in vivo* is poorly understood. In this study, we used quantitative mass spectrometry to investigate the brain peptidome of Nln-knockout mice. An additional *in vitro* digestion assay with recombinant Nln was also performed to confirm the identification of the substrates and/or products of Nln. Altogether, the data presented suggest that Nln is a key enzyme in the *in vivo* degradation of only a few peptides derived from proenkephalin, such as Met-enkephalin and octapeptide. Nln was found to have only a minor contribution to the intracellular peptide metabolism in the entire mouse brain. However, further studies appear necessary to investigate the contribution of Nln to the peptide metabolism in specific areas of the murine brain.

Biological significance
Neurolysin was first identified in the synaptic membranes of the rat brain in the middle 80's by Frederic Checler and colleagues. Neurolysin was well characterized biochemically, and its brain distribution has been confirmed by immunohistochemical methods. The neurolysin contribution to the central and peripheral neurotensin-mediated functions *in vivo* has been delineated through inhibitor-based pharmacological approaches, but its genuine contribution to the physiological inactivation of neuropeptides remains to be firmly established. As a result, the main significance of this work is the first characterization of the brain peptidome of the neurolysin-knockout mouse.
This article is part of a Special Issue entitled: SMP Cancun 2013.

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* *Corresponding author at: Department of Pharmacology, University of São Paulo, Av.* Prof. Lineu Prestes 1524, Room 315, São Paulo, SP 05508-000, Brazil. Tel.: +55 11 3091 7493; fax: +55 11 3091 7322.
E-mail address: emersferro@gmail.com (E.S. Ferro).

http://dx.doi.org/10.1016/j.jprot.2014.03.043
1874-3919/

1. Introduction

Peptidases play many physiological roles through the production, regulation, and degradation of peptides *in vivo*. Neurolysin (EC 3.4.24.16, EP24.16 or Nln) belongs to the M3 metallopeptidase family and contains a His-Glu-Xaa-Xaa-His (HEXXH) zinc-binding motif in its active domain [1]. This peptidase was originally purified and characterized from rat synaptic membranes [2] and later from tissues such as the rat ileum and kidney [3,4] based on its ability to cleave neurotensin (NT) at Pro10-Tyr11 to generate the biologically inactive fragments NT1–10 and NT11–13 [5]. In addition, Nln was able to cleave the bioactive peptides bradykinin, dynorphin A, GnRH, angiotensin I, substance P, and somatostatin *in vitro* [4,6–8]. This enzyme is distributed ubiquitously in mammalian tissues, with high concentrations in the brain, including the ventral midbrain, olfactory bulb and tubercle, cingulate cortex, neostriatum, and globus pallidus [9–11]. Immunocytochemical studies of the rat brain at both the light and electron microscopic levels and the use of a cell biology-based approach using primary cultured neurons and glial cells have shown that Nln is both cytoplasmic and membrane-associated in neurons [10,12–14]. In addition, the Nln contribution to the central and peripheral neurotensin-mediated functions *in vivo* has been delineated [15,16] through inhibitor-based pharmacological approaches, but its genuine contribution to the physiological inactivation of neuropeptides remains to be firmly established. As a result, the identification of substrates of Nln may reveal their specific role in the cellular metabolism.

The development of mass spectrometry techniques for peptidomic studies has provided new insights into the biochemistry and biology of peptidases in the nervous system [17]. This approach has allowed 1) the analysis of hundreds of peptides in a single experiment, 2) a relative quantification of the peptide levels in two or more different samples, 3) the identification of natural substrates of peptidases, and 4) the identification of post-translational modifications [18]. Peptidomic studies of mammalian cells and tissues have identified [19–22], in addition to the known neuropeptides, a large number of intracellular peptides from cytosolic, mitochondrial, or nuclear proteins that are produced from their precursors by selective endo- and exopeptidases [23–25]. The treatment of HEK 293 cells with epoxomicin, an irreversible inhibitor of the proteasome, suggested that the proteasome is the main enzyme responsible for intracellular peptide production [26]. Furthermore, it has been shown that intracellular peptides isolated from rat brain homogenates using an inactive thimet oligopeptidase capture substrate assay [27] can efficiently interfere with G-protein-coupled receptor signal transduction [28]. These data were confirmed in HEK293 cells transfected with thimet oligopeptidase siRNA, which showed modulation in the levels of specific intracellular peptides and isoproterenol signal transduction [29]. In addition, peptides generated from mitochondrial proteins by the matrix-localized protease ClpP are able to signal the stress of protein misfolding and activate nuclear-encoded mitochondrial chaperone genes in *Caenorhabditis elegans* [30]. Taken together, these findings show that intracellular peptides can act as signaling molecules inside of the cell before the complete degradation of amino acids.

The aim of the present study was to analyze changes in the mouse brain peptide profile using Nln knockout mice, which were used for the first time in this study. Moreover, an *in vitro* digestion assay using a recombinant enzyme was performed to confirm and identify the substrates and/or products of Nln. The quantitative mass spectrometry analysis of Nln-knockout mouse showed an intracellular peptide profile similar to the wildtype with significant differences in the quantity of some neuropeptides. These data are in accordance with previously published data [12] that show that in the nervous tissue Nln may be better placed for the degradation of neuropeptides than intracellular peptide metabolism.

2. Material and methods

2.1. Reagents

Acetonitrile, glycine, sodium hydroxide, sodium chloride, and trifluoroacetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide, methanol, hydrochloridric acid, and hydroxylamine were supplied by Merck (Darmstadt, DE, DEU). Anhydrous dibasic sodium phosphate (Na_2HPO_4) and anhydrous monobasic sodium phosphate (NaH_2PO_4) were obtained from Amresco (Solon, OH, USA). The 4-trimethylammoniumbutyryl (TMAB) stable isotopic labeling reagents containing three, six, or twelve atoms of deuterium (D3, D9, and D12-TMAB) or no deuterium (D0-TMAB) were synthesized as described [31] and generously provided by Prof. Lloyd D. Fricker (Department of Molecular Pharmacology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, USA). Fluorescamine was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Animals

The generation and characterization of Nln-knockout mice are described in detail elsewhere (Cavalcanti et al., in preparation). Briefly, the Nln gene-trap knockout mouse strain NPX481 was generated by the C57BL/6 blastocyst micro-injection of genetically modified embryonic stem cells (129ola) obtained from Baygenomics through the International Gene-Trap Consortium (IGTC, http://www.genetrap.org/). The genetically modified Nln allele of the NPX481 strain has the gene-trap vector pGT1dTMpfs inserted between exon 1 and exon 2 (Supplemental Fig. 1A), as evidenced by 5′RACE sequencing of the cDNA from the original NPX481 ES cell line (CC178547) that was used to generate this mouse strain (the details of this ES cell line can be found at http://www.genetrap.org/cgi-bin/annotation.py?cellline=NPX481).

After transmission of the knockout allele from the chimera to the F1 generation, the Nln mice were obtained from heterozygous breeding, and the line was further maintained on the mixed background by breeding $Nln^{+/-}$ with $Nln^{+/-}$animals. To obtain such mice on a pure genetic background, we bred the F1 (129/OlaHsd/C57BL/6 background) heterozygous Nln-deficient animals to the inbred C57BL/6 mouse line for 10 generations before their use in experimental investigations.

The mice were maintained in individual ventilated cages (Tecniplast, DE) under standardized conditions with an artificial

Please cite this article as: Castro LM., et al, Peptidomic analysis of the neurolysin-knockout mouse brain, J Prot (2014), http://dx.doi.org/10.1016/j.jprot.2014.03.043