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Targeted high-resolution quadrupole-Orbitrap mass spectrometry analyses reveal a significant reduction of tachykinin and opioid neuropeptides level in PC1 and PC2 mutant mouse spinal cords

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ABSTRACT

Tachykinin and opioid neuropeptides play a fundamental role in pain transmission, modulation and inhibition. The proteolysis control of endogenous tachykinin and opioid neuropeptides has a significant impact on pain perception. The role of proprotein convertases (PCs) in the proteolysis of proneuropeptides was previously established but very few studies have shown the direct impact of PCs on the regulation of specific tachykinin and opioid peptides in the central nervous system. There is an increasing interest in the therapeutic targeting of PCs for the treatment of pain but it is imperative to assess the impact of PCs on the pronociceptive and the endogenous opioid systems. The objective of this study was to determine the relative concentration of targeted neuropeptides in the spinal cord of WT, PC1$^{-/+}$ and PC2$^{-/+}$ animals to establish the impact of a restricted PCs activity on the regulation of specific neuropeptides. The analysis of tachykinin and opioid neuropeptides were performed on a HPLC-MS/MS (High-Resolution Quadrupole-Orbitrap Mass Spectrometer). The results revealed a significant decrease of Dyn A ($p < 0.01$), Leu-Enk ($p < 0.001$), Met-Enk ($p < 0.001$), Tach$_{58-71}$ ($p < 0.05$), SP ($p < 0.01$) and NKA ($p < 0.001$) concentrations in both, PC1$^{-/+}$ and PC2$^{-/+}$ animals. Therefore, the modulation of PCs activity has an important impact on specific pronociceptive peptides (SP and NKA), but the results also shown that endogenous opioid system is hindered and consequently it will affect significantly the pain modulatory pathways. These observations may have insightful impact on future analgesic drug developments and therapeutic strategies.

1. Introduction

It has been widely demonstrated that dibasic residue cleavage site motifs and single basic residue are essential for the processing of proneuropeptide to active neuropeptide [Eisenmann, 2005; Hook et al., 2008; Funkelstein et al., 2010]. Mainly, these reactions are catalyzed by subtilisin-like proprotein convertases and cysteine cathepsin proteases [Miller et al., 2003; Hook and Bandeira, 2015]. Proprotein convertases (PCs) are particularly expressed in the central nervous system (CNS) and it has been widely demonstrated that several neuropeptides are synthesized by the actions of PCs and other specific endopeptidases [Harrison and Geppetti, 2001; Hook et al., 2008]. Comprehensive studies demonstrated that PC1 and PC2 recognize motives composed of either a pair of basic amino acids (KR, RK, RR and KK), or a single residue (R or K) [Zheng et al., 1994; Cui et al., 1998; Jin et al., 2005]. The protease activities of these enzymes are therefore central in the processing of several proneuropeptides but redundant function could counterbalance the down regulation of either one.

Neuropeptides are either neurotransmitters or neuromodulators at various levels in the central nervous system and play an essential role in pain transmission [Levine et al., 1993; Seybold, 2009]. Various members of the tachykinin family (e.g. Substance P, Neurokinin A) are mostly pro-nociceptive neuropeptides and are recognized to play a fundamental role in central sensitization leading to hyperalgesia and allodynia [Lecci et al., 2000]. The tachykinin precursor 1 (Tac1) gene encodes the protachykinin-1 protein containing the sequence of four tachykinin peptides, including Substance P (SP) and Neurokinin A (NKA) [Howard et al., 2006]. The role of PCs in the protachykinin-1 protein C-terminal processing was recently demonstrated in vitro [Saidi et al., 2015]. Specifically, PC1 and PC2 may be important in the biosynthesis of SP and NKA by cleaving at specific sites illustrated in Fig. 1. Opioid peptides (i.e. enkephalins and dynorphins) have potent

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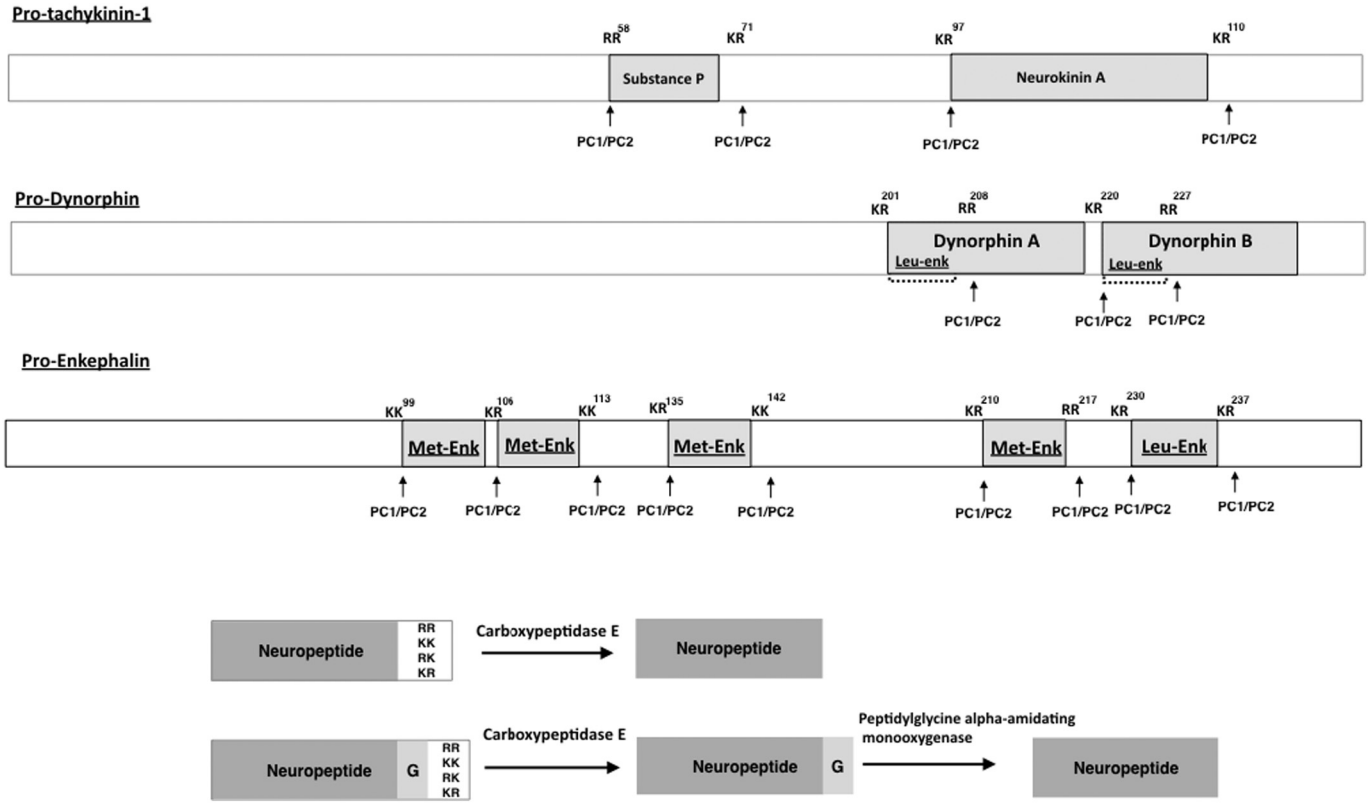

Fig. 1. Prodynorphin, Proenkephalin and Protachykinin-1 processing by proprotein convertases (PC1 and PC2). Dynorphins, enkephalins and tachykinins have several paired and single basic amino acid cleavage sites and based on the sequence, various processing intermediates can be derived leading to specific neuropeptides. Carboxypeptidase E catalyze the sequential removal of basic amino acids and peptidylglycine alpha-amidating monooxygenase will catalyze the C-terminal alpha-amidation of peptides following the elimination of the glycine residue.

analgesic effects in the central nervous system (CNS) and play a fundamental role in endogenous pain inhibition [Machelska, 2007; Wahlert et al., 2013]. They are interacting with μ, κ and δ-opioid receptors expressed widely in the brain and in the spinal cord [Carr and Lovering, 2000; Stanojevic et al., 2008]. Initial endoproteolytic studies of prodynorphin (Pdyn) have shown that PCs play an essential role in Pdyn processing [Day et al., 1998; Berman et al., 2000; Mika et al., 2011; Kastin, 2013; Orduna Ruiz and Beaudry, 2016]. Specifically, these initial studies revealed that the action of PC2 is needed for the formation of Big dynorphin (BDyn), Dyn A and dynorphin B (Dyn B) [Day et al., 1998; Miller et al., 2003]. Moreover, our recent results from in vitro experiments demonstrate that both PC1 and PC2 are involved in the proteolysis regulation of Dyn B and Dyn A with a more important role for PC1 [Orduna Ruiz and Beaudry, 2016]. Interestingly, the proteolysis of Dyn A generated Leu-enkephalin (Leu-Enk), another important opioid peptide mediating potent painkilling effects [Akil et al., 1997]. Proenkephalin (Penk) is the protein precursor of the enkaphalin peptides, mainly Met-enkephalin (Met-Enk) but also Leu-Enk. Upon cleavages mediated by PC1 and PC2, the proteolysis of Penk will generate four copy of Met-Enk and one copy of Leu-Enk [Vats et al., 2009; Gonzalez-Nunez et al., 2013; Takahashi, 2015]. Both enkaphalin peptides are essential for the regulation of nociception by acting on μ and δ-opioid receptors [Carr and Lovering, 2000; Stanojevic et al., 2008; Orduna Ruiz and Beaudry, 2016].

The regulation of endogenous tachykinin and opioid peptide levels by proteolysis of precursor proteins is very important and can help to better understand the cellular and molecular mechanisms of pain. Our hypothesis is PC1 and PC2 mutant mouse will have significant deficit of endogenous tachykinin and opioid peptides therefore affect significantly the pain modulatory pathways. We used a state-of-the-art high-resolution accurate mass spectrometry strategy to quantify targeted neuropeptides in mouse spinal cords.

2. Materials and methods

2.1. Chemicals and reagents

Leu-enkephalin (Leu-Enk), Met-enkephalin (Met-Enk), Dynorphin A (Dyn A), β-Tachykinin$_{58–71}$ (Tach$_{58–71}$), Substance P (SP) and Neurokinin A (NKA) were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). Deuterium labeled analogue peptides were synthesized (CanPeptide, Inc., Pointe-Claire, QC, Canada) and used as internal standards. Acetonitrile and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (NJ, USA). Hexane and formic acid (FA) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Standard solutions were prepared in 0.25% TFA solution as described previously [Beaudry et al., 2009].

2.2. Sample preparation

Spinal cord tissues (n = 6 per genotypes) from male wild type (C57BL/6 J) and male PC2$^{-/+}$ mice (product #002963) were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) and kept frozen at − 80 °C until analysis. Heterozygote animals only were used since PC2$^{-/-}$ exhibit many abnormalities and the survival rate is low. Animal genotyping was performed for each animal by Jackson Laboratory using a standard PCR assay. Only heterozygous and normal animals were selected for this study. All mice were 8 weeks old at time of tissues collection. The animals from both groups were euthanized with an overdose of isoflurane followed by a transection of the cervical spine. A flush of saline was performed within the spinal canal to collect the spinal cord lumbar enlargement. Tissue sample was snap-frozen in cold hexane (60 °C) and stored immediately at − 80 °C pending analyses. The study protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary