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PHOENIXIN: A CANDIDATE PRURITOGEN IN THE MOUSE

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Abstract—Phoenixin (PNX) is a 14-amino acid amidated peptide (PNX-14) or an N-terminal extended 20-residue amidated peptide (PNX-20) recently identified in neural and non-neural tissue. Mass spectrometry analysis identified a major peak corresponding to PNX-14, with negligible PNX-20, in mouse spinal cord extracts. Using a previously characterized antiserum that recognized both PNX-14 and PNX-20, PNX-immunoreactivity (irPNX) was detected in a population of dorsal root ganglion (DRG) cells and in cell processes densely distributed to the superficial layers of the dorsal horn; irPNX cell processes were also detected in the skin. The retrograde tracer, Fluorogold, injected subcutaneously (s.c.) to the back of the cervical and thoracic spinal cord of mice, labeled a population of DRG, some of which were also irPNX. PNX-14 (2, 4 and 8 mg/kg) injected s.c.to the nape of the neck provoked dose-dependent repetitive scratching bouts directed to the back of the neck with the hindpaws. The number of scratching bouts varied from 16 to 95 in 30 min, commencing within 5 min post-injection and lasted 10–15 min. Pretreatment of mice at −20 min with nalfurafine (20 μg/kg, s.c.), the kappa opioid receptor agonist, significantly reduced the number of bouts induced by PNX-14 (4 mg/kg) compared with that of saline-pretreated mice. Our results suggest that the peptide, PNX-14, serves as one of the endogenous signal molecules transducing itch sensation in the mouse.

Key words: dorsal root ganglion, itch, kappa opioid receptor, nalfurafine, primary afferent neuron, pruritogen.

INTRODUCTION

Two novel peptides, phoenixin-14 amide (referred to herein as PNX-14) and phoenixin-20 amide (PNX-20), have recently been isolated and identified in the rat brain and spinal cord as well as non-neural tissues including the heart (Lyu et al., 2013; Yosten et al., 2013). PNX-14 is identical among multiple species including human, rat, mouse, porcine and canine; whereas PNX-20 differs in one amino acid between the coding region of human, canine or porcine sequences (Yosten et al., 2013). The precursor of phoenixin is an uncharacterized protein C4orf52 (or *Smim20*), which contains a glycine residue that can undergo C-terminal amidation, and several conserved dibasic residues after glycine, indicative of potential carboxypeptidase cleavage sites (Fricker, 2012). The most abundant peptide generated from C4orf52 is a 14 residue peptide, DVQPPGLKVWSDPF-amide, termed PNX-14. An N-terminal extended peptide, with a sequence of AGIVQEDVQPPGLKVWSDPF-amide, is co-expressed with PNX-14 in tissue samples, such as the heart and hypothalamus, which we called PNX-20 (Yosten et al., 2013).

Insofar as the distribution of PNX-14 or PNX-20 in neural tissue is concerned, immunohistochemical studies show that PNX-immunoreactive (irPNX) cells are expressed in several regions of the rat brain including hypothalamus, brainstem and pituitary (Yosten et al., 2013). Because PNX antiserum, a rabbit polyclonal directed against the peptide PNX-14, cross-reacted 100% with PNX-14 amide or PNX-20 amide (Yosten et al., 2013), the term irPNX referred to irPNX-14 and/or irPNX-20 indiscriminately. In the rat spinal cord, irPNX is expressed in networks of nerve fibers distributed to superficial layers of the dorsal horn, and in small-to-medium dorsal root ganglion cells (Lyu et al., 2013). This pattern of distribution of irPNX in the dorsal horn and DRG is similar to that of several sensory peptides including substance P (SP), calcitonin gene-related peptide (CGRP) and gastrin-releasing peptide (GRP). The latter is reported to be an endogenous ligand, acting on gastrin-releasing peptide receptors (GRPR), which promote itch sensation in the mouse (Sun and Chen, 2007). A similar expression pattern of PNX and GRP in the spinal cord and DRGs raised the intriguing possibility that PNX-14 may serve as one of the heretofore unrecognized signaling molecules that transduces itch sensation (Yosipovitch et al., 2003; Sun and Chen, 2007; Davidson and Giesler, 2010).

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Abbreviations: BN, Bombesin; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion/ganglia; FITC, fluorescein isothiocyanate; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; 5′-GNTI, 5′-guanidinonaltrindole; i.c.v., intracerebroventricular; NPPB, natriuretic polypeptide B; NMB, neuromedin B; norBNI, norbinaltorphimine; PNX, phoenixin; PNX-14, phoenixin-14 amide; PNX-20, phoenixin-20 amide; irPNX, phoenixin immunoreactivity; PBS, phosphate-buffered saline; SP, substance P.

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EXPERIMENTAL PROCEDURES

Experimental animals

Male Swiss-Webster mice (20–25 g) were purchased from Taconic Biosciences (Hudson, NY, USA). Animals were housed under a 12-h light/dark cycle with food and water available *ad libitum*. Experimental procedures were approved by the Temple University Institutional Animal Care and Use Committee, in accordance with the 1996 NIH Guide for the Care and Use of Laboratory Animals. Experiments were designed to keep the number of animals to a minimum and care was taken to minimize pain or suffering.

Identification of phoenixin from mouse spinal cords

Swiss-Webster mice (n = 20) were anesthetized with 4% isoflurane and decapitated. Spinal cords, with a total wet weight of 1.6 g, were mixed with 1.6 g of 0.8-mm silica beads (OPS Diagnostics, Lebanon, NJ, USA), divided into eight polypropylene micro-centrifuge tubes, homogenized in 0.8 ml 5% acetic acid and heated at 95 °C for each tube for 5 min, which yielded a total of 4.8 ml of homogenates. After spinning the homogenates at 10,000*g* for 20 min, the supernatant was transferred to a 15-ml polypropylene tube. Commercially available Bicinchoninic Acid (BCA) Protein Assay kit (Life technologies, Grand Island, NY, USA) was used to quantify the protein content in tissue homogenates. To identify phoenixin peptide, 0.8 mg of tissue homogenates were further affinity purified using MagnaBind beads (Life technologies, Grand Island, NY, USA) which had been conjugated to anti-phoenixin antibody. Briefly, saturating amounts of phoenixin immunoreactive peptides from tissue homogenates were bound to antibody conjugated magnetic beads after 4-h incubation at 4 °C; the latter were washed four times with 1 ml of phosphate-buffered saline (PBS). After the last wash, beads were transferred to new tubes. The captured peptides were eluted from beads with 50 μl of 60% acetonitrile in 1% triflouroacetic acid. Lastly, 3 μl of the eluent from magnetic beads was directly applied to the MALDI-TOF plate of Maxima LNR (Kratos-Shimadzu Co., Kyoto, Japan), after mixing with 1-μl matrix solution of alpha-cyano-4-hydroxycinnamic acid for the identification of phoenixin. In the final stage of verification of purified phoenixin, the bioinformatics predicted phoenixin peptide that had been synthesized, and the purified phoenixin were processed by the same procedures as indicated in the previous publications (Lyu et al., 2013; Yosten et al., 2013). A comparable molecular mass on MALDI-TOF and HPLC profiles of the purified peptide and synthetic phoenixin confirmed the molecular identity of phoenixin.

Immunohistochemistry

Animals anesthetized with 4% isoflurane were intracardially perfused with PBS followed by 4% paraformaldehyde in PBS. Spinal cords and DRG were dissected, and several pieces of 5 × 5-mm skin patches were clipped and removed from the back of the mouse, postfixed for 2 h and stored in 30% sucrose/PBS overnight. Tissues were processed for irPNX by the immunofluorescent method (Lyu et al., 2013).

Tissues were sectioned to 40-μm thick by a cryostat, blocked with normal goat serum (1:100 dilution in PBS, 0.5% bovine serum albumin, 0.4% Triton X-100), rinsed and then incubated in PNX antiserum (1:1000 dilution; a rabbit polyclonal against PNX-14; Phoenix Pharmaceuticals Inc., Burlingame, CA, USA). After thorough rinsing, sections were incubated in biotinylated anti-rabbit IgG (1:100 dilution; Vector Laboratories, Burlingame, CA, USA) for 2 h, rinsed with PBS, and incubated in Texas Red or avidin fluorescein isothiocyanate (FITC, 1:50 dilution) for 5 h. Sections were washed with PBS, mounted on subbed slides, covered with Citifluor mountant medium (Ted Pella Inc., Redding, CA, USA) and coverslipped.

Fluorogold injection

In four mice, the retrograde fluorescent tracer Fluorogold (3% solution, 10 μl, Biotium Inc., Haywood, CA, USA) was injected s.c. to three spots, separated by 5 mm, to the nape of the neck. Three to five days later, animals under 4% isoflurane anesthesia, were intracardially perfused with chilled PBS followed by 4% paraformaldehyde. DRG removed from cervical, thoracic and lumbar segments were processed for irPNX using the fluorescent methods described above. As preliminary results showed that the fluorescence intensity of Fluorogold in DRG sections was less than optimal, an additional step was carried out involving incubation of DRG sections in anti-Fluorogold antibody (Chemicon International, Inc., USA) followed by incubation in anti-rabbit secondary antiserum conjugated to FITC to amplify the signal. Sections were examined under a confocal scanning laser microscope (Leica TCS SP5, Heidelberg, Germany) with excitation wavelengths set to 488 nm for FITC and 561 nm for Texas Red.

Pre-absorption protocol

To assess cross-reactivity between PNX antiserum and PNX-14 or PNX-20 peptide, spinal cord or DRG sections were incubated overnight with PNX antiserum pre-absorbed with either PNX-14 amide or PNX-20 amide (1 μg/ml). Additional control studies were performed in which the spinal cord or DRG sections were incubated overnight with PNX antiserum pre-absorbed with substance P (1 μg/ml), gastrin-releasing peptide (GRP, 1 μg/ml) or calcitonin gene-related peptide (CGRP, 1 μg/ml). Tissues were then processed with the pre-absorbed PNX antiserum in a manner similar to that described for PNX antiserum.

Scratch protocol

The methodology originally introduced by Kuraishi et al. (1995) was employed here and was similar to that described in our earlier studies (Inan et al., 2009; 2011; Zhang et al., 2015). Mice were acclimated individually in rectangular plastic observation boxes for at least 2 h prior