Neuroscience 250 (2013) 622–631

PHOENIXIN: A NOVEL PEPTIDE IN RODENT SENSORY GANGLIA

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Abstract—Phoenixin-14 amide, herein referred to as phoenixin, is a newly identified peptide from the rat brain. Using a previously characterized rabbit polyclonal antiserum against phoenixin, enzyme-immunoassay detected a high level (>4.5 ng/g tissue) of phoenixin-immunoreactivity (irPNX) in the rat spinal cords. Immunohistochemical studies revealed irPNX in networks of cell processes in the superficial dorsal horn, spinal trigeminal tract and nucleus of the solitary tract; and in a population of dorsal root, trigeminal and nodose ganglion cells. The pattern of distribution of irPNX in the superficial layers of the dorsal horn was similar to that of substance P immunoreactivity (irSP). Double-labeling the dorsal root ganglion sections showed that irPNX and irSP express in different populations of ganglion cells. In awake mice, intrathecal injection of phoenixin (1 or 5 μg) did not significantly affect the tail-flick latency as compared to that in animals injected with artificial cerebrospinal fluid (aCSF). Intrathecal administration of phoenixin (0.5, 1.25 or 2.5 μg) significantly reduced the number of writhes elicited by intraperitoneal injection of acetic acid (0.6%, 0.3 ml/30 g) as compared to that in mice injected with aCSF. While not affecting the tail-flick latency, phoenixin antiserum (1:100) injected intrathecally 10 min prior to the intraperitoneal injection of acetic acid significantly increased the number of writhes as compared to mice pre-treated with normal rabbit serum. Intrathecal injection of non-amidated phoenixin (2.5 μg) did not significantly alter the number of writhes evoked by acetic acid. Our result shows that phoenixin is expressed in sensory neurons of the dorsal root, nodose and trigeminal ganglia, the amidated peptide is bioactive, and exogenously administered phoenixin may preferentially suppress visceral as opposed to thermal pain.

Key words: spinal cord, sensory neurons, thermal pain, visceral pain.

INTRODUCTION

Completion of the human genome sequencing project has uncovered more than 700 genes that belong to the G-protein-coupled receptor (GPCR) superfamily. Approximately half of these genes encode sensory receptors; a large number of which are predicted to be the targets of odorants. Of the remaining 360 receptors, the natural ligand has been identified for approximately 210 receptors, leaving 150 so called orphan GPCRs with no known ligands (Wise et al., 2004; Wettschureck and Offermanns, 2005). Since the 80's, a concerted effort has been made to identify endogenous ligands acting on orphan GPCRs and vice versa. A number of experimental and methodological approaches, including a high throughput screening of small molecules and peptide ligands, reverse pharmacology, and the use of bioinformatics to predict candidate ligands, have been developed to streamline the identification process. By utilizing the bioinformatics algorithm from information provided by the Genome Projects, we have identified several previously unrecognized, secreted, highly conserved neuropeptides; one of which is neuronstatin (Samson et al., 2008; Dun et al., 2010). Recent studies suggest that neuronstatin acts on the orphan GPR107 (Yosten et al., 2012).

Utilizing a similar strategy, two novel peptides phoenixin-14 amide, referred to herein as phoenixin, and phoenixin-20 amide were identified and isolated from the rat brain (Yosten et al., 2013). Phoenixin is identical among multiple species including human, rat, mouse, porcine, canine and Xenopus; whereas, phoenixin-20 amide differs in one amino acid between the human and porcine or canine sequence (Yosten et al., 2013). The precursor for phoenixin is an uncharacterized protein C4orf52, 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, which we termed phoenixin-14 amide. An N-terminal extended peptide, phoenixin-20 amide (AGIVQEDVQ PPGLKVWSDPF-amide) is co-expressed with phoenixin

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Abbreviations: ABC, avidin–biotin complex; aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; BCA, bicinchoninic acid protein assay; EIA, enzyme-immunoassay; FITC, fluorescein isothiocyanate; GPCR, G-protein-coupled receptor; irPNX, phoenixin immunoreactivity; irSP, substance P immunoreactivity; nAmb, nucleus ambiguus; PBS, phosphate-buffered saline.; PEPS, pericentral spikes ; PNX, phoenixin-14 amide; SDB-L, styrene-divinylbenzene polymer; DRG, dorsal root ganglia; RP-HPLC, reverse-phase High-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.07.057

in tissue samples such as the heart and hypothalamus (Yosten et al., 2013). Gene expression of *C4orf52* has been shown in several human organs by the organism specific databases "GC04P025864" and "BioGPS gnf1h09115_at". Serial Analysis of Gene Expression (SAGE) for *C4orf52* also indicates that phoenixin precursor gene expression in the spinal cord is higher than that of several other tissues such as the brain, pancreas, spleen and intestine (see http://gene4.weizmann.ac.il/cgi-bin/cardsisp.pl?gene=c4orf52); a finding that is consistent with a comparative analysis of 45 non-central nervous system tissues (Roth et al., 2006).

In our earlier study, phoenixin was chemically synthesized and an antibody directed against the synthetic peptide was raised in rabbits (Yosten et al., 2013). The antibody was then applied to the development of an enzyme immunoassay (EIA) to quantify the amount of immunoreactive phoenixin (irPNX) in various tissues of the rat. A low level of peptides was detected in peripheral tissues, including the thymus, stomach, and spleen; the tissue with a high level of irPNX was that of the hypothalamus (Yosten et al., 2013). Neuropeptides that are expressed in the brain can, with few exceptions, be expected in the spinal cord and/or peripheral neural tissues. The current study was undertaken to explore the occurrence, distribution and possible function of phoenixin in the rodent spinal cord.

EXPERIMENTAL PROCEDURES

Experimental animals

Adult male ICR mice (Ace Animal Inc., Boyertown, PA, USA), weighing 25–30 g were used in immunohistochemical and behavioral studies; and male Sprague–Dawley rats, weighing 300–325 g (Ace Animal Inc.) were used in EIA and immunohistochemical studies. Experimental protocols were reviewed and approved by the Temple University Institutional Animal Care and Use Committee, in accordance with the NIH Guide for the Care and Use of Laboratory Animals 1996. Animals were housed under a 12/12-h light/dark cycle with free access to food and water. Mice were transported to the behavioral testing room at least 2 h prior to testing. Every effort was made to minimize the distress of the animals and to prevent their suffering.

Isolation and identification of phoenixin from rat spinal cords

Rats (n = 5) were anesthetized with 4% isofurane and decapitated. Spinal cords, with a total wet weight of 0.8 g, were mixed with 0.8 g of sillica beads (0.8 mm), divided into four polypropylene micro-centrifuge tubes, homogenized in 0.8 ml 5% acetic acid twice for each tube, and yielded a total of 4.8 ml of homogenate fraction. After spinning the homogenates at 10,000xg for 20 min, the supernatant was removed and loaded into SDB-L (styrene-divinylbenzene polymer) cartridges (Phenomenex Inc., CA, USA). After washing the cartridges with four volumes of phosphate-buffered saline (PBS), the binding substances were eluted with 60% isopropanol from SDB-L cartridges and lyophilized, reconstituted in PBS. Commercially available Bicinchoninic Acid (BCA) Protein Assay kit (Thermo Scientific) was used to quantify the protein content in each tissue homogenate. To identify the phoenixin peptide, the 60% isopropanol solution was further affinity purified using MagnaBind beads (Pierce/Thermo Scientific) conjugated to anti-phoenixin antiserum. Eluent from magnetic beads was directly applied to MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) for the identification of phoenixin. In the final stage of verification of purified phoenixin, the bioinformatic predicted phoenixin peptide that had been synthesized and the purified phoenixin were processed under the same RP-HPLC (reverse-phase High-performance liquid chromatography) separation conditions. A comparable molecular mass on MALDI-TOF and HPLC profiles of the purified peptide and synthetic phoenixin confirmed the molecular identity of phoenixin.

Enzyme-immunoassay (EIA)

Rats (n = 10) anesthetized with 4% isofurane were decapitated; spinal cords were removed and homogenized in 5% acetic acid buffer. After centrifugation, the large hydrophobic proteins of supernatant were excluded using Strata C18 columns (Phenomenex Co., 411 Madrid Ave. Torrance, California 90501, USA), and eluents were collected and lyophilized. Samples were prepared and analyzed with phoenixin-14 amide or substance P EIA kit according to the manufacturer's instructions (Phoenix Pharmaceuticals, Burlingame, CA, USA).

Immunohistochemistry

Animals anesthetized with 4% isofurane were intracardially perfused with PBS followed by 4% paraformaldehyde in PBS. Spinal cord, medulla oblongata, dorsal root ganglia, nodose ganglia, trigeminal ganglia and superior cervical ganglia were dissected, postfixed for 2 h, and stored in 30% sucrose/PBS solution overnight. Tissues were processed for irPNX or substance P immunoreactivity (irSP) by the avidin–biotin complex (ABC) procedure or the immunofluorescent method (Dun et al., 2010).

For the ABC method, tissues were first treated with 3% H_2O_2 to quench endogenous peroxidase, washed several times, blocked with 10% normal goat serum, and incubated in phoenixin-antiserum [1:1000 dilution; a rabbit polyclonal against phoenixin-14 amide; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA]. The specificity of phoenixin antiserum has been previously characterized (Yosten et al., 2013). After thorough rinsing, sections were incubated in biotinylated anti-rabbit IgG (1:200 dilution; Vector Laboratories, Burlingame, CA, USA) for 2 h and rinsed with PBS and incubated in avidin–biotin complex solution for 1.5 h (1:200 dilution; Vector Laboratories). After several washes in Tris-buffered saline, sections were developed