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Neuropeptide Y is analgesic in rats after plantar incision

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ABSTRACT

Previous work has demonstrated that neuropeptide tyrosine (NPY), Y_1 receptor and Y_2 receptor are critical in modulation of pain after nerve injury. We hypothesized that NPY was important for nociception after surgical incision. As a model of postoperative pain, rats underwent a plantar incision in one hindpaw. Western blots were used to quantify changes in protein expression of NPY, Y_1 receptor and Y_2 receptor after incision in skin, muscle, and dorsal root ganglion (DRG). Pain-related behaviors were tested after incision in rats treated with intrathecal NPY, Y_1 receptor antagonist (BIBO3304 – Chemical Name: N-[(1R)-1-[[[[4-[[(Aminocarbonyl)amino]methyl]phenyl]methyl]amino]carbonyl]-4-[(aminoiminomethyl)amino]butyl]-α-phenyl-benzeneacetamide ditrifluoroacetate), Y_2 receptor antagonist (BIIE0246 – Chemical Name: N-[(1S)-4-[(Aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino]carbonyl]-butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5H-dibenz[b,e]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentane-acetamide), combined NPY+antagonists, morphine, or vehicle. Pain behaviors were tested after incision in rats treated with locally applied intraplantar injections of NPY, Y_1 receptor and Y_2 receptor antagonists or vehicle. NPY protein expression was significantly downregulated in muscle for two days after incision. In contrast, Y_1 receptor and Y_2 receptor protein expression was upregulated in both skin and muscle. A single intrathecal injection of NPY reduced cumulative guarding pain scores, as did morphine. The intrathecal administration of Y_2 receptor antagonist also reduced pain scores; findings that were not observed when drugs were administered locally. Intrathecal Y_2 receptor antagonists and NPY improved mechanical threshold and heat withdrawal latency 2 h after incision. Intrathecal administration of NPY and/or central blockade of Y_2 receptor attenuated pain behaviors early after incision (postoperative day (POD) 1–2). Y_1 receptor antagonist administration blocked the anti-hyperalgesic effect of NPY. Together these data suggest a role for spinal NPY in postoperative pain.

1. Introduction

Pain is an important parameter in patient recovery and satisfaction after surgery. For many patients, pain due to surgical incision can be severe despite treatment with conventional use of opioids, non-steroidal anti-inflammatory drugs (NSAIDs) and local anesthetics (Apfelbaum et al., 2003). Research into post-operative pain mechanisms has provided some insight into the potential factors involved in the initiation and maintenance of pain after surgery. One factor showing significant regulation in expression after plantar incision is neuropeptide Y (NPY) (Spofford and Brennan, 2012).

NPY is a 36 amino acid peptide, which is widely distributed in the central and peripheral nervous systems. Peripherally, NPY is abundant in the sympathetic nervous system, where it is stored and released with norepinephrine. NPY exerts its biological action via five G-protein coupled receptors (Y_1 receptor–Y_5 receptor) that have been characterized based on their physiological effects. The physiological mechanisms affected by each of these receptors has been partially explored in the neurovascular system (Hodges et al., 2009; Lin et al., 2004) as well as pain from diabetes and intradermal capsaicin (Franco-Cereceda and Liska, 1998; Gibbs et al., 2007). However, there are no reports about the role of NPY in plantar incision. Previous work by others has suggested NPY may sensitize primary sensory afferents. The Y_1 receptor and Y_2 receptor are the likely receptors involved in the transmission and modulation of pain after spared nerve injury (Intondi et al., 2008). However, the role of each of these receptors is controversial with some reports suggesting these receptors can have a pronociceptive, antinociceptive, or mixed effects (Brumovsky et al., 2007; Smith et al., 2007).

In order to elucidate the role of NPY in post-incision pain processing in the peripheral nervous system, we examined the role of NPY in a well-established plantar incision pain model (Brennan et al., 1996). We hypothesize that NPY and its receptors, Y_1 receptor and Y_2 receptor, are important for nociception after surgical incision.

2. Materials and methods

2.1. Animals

This study was approved by the Institutional Animal Care and Use Committee at the University of Iowa and all experiments

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adhered to the ethical guidelines for investigations of experimental pain in conscious animals. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA), 250 to 350 g, were used for all experiments. Rats were housed in groups of 2 on a 12-h light and 12-h dark schedule with food and water made available ad libitum.

2.2. Plantar incision

Plantar incision was made as previously described (Brennan et al., 1996). Briefly, rats were anesthetized with isoflurane (1.5–3%). The right hindpaw was prepared with Povidone iodine and draped. A 1-cm longitudinal incision was made 0.5 cm from the heel on the right hindpaw through the skin, fascia, and the plantar flexor digitorum brevis muscle. The flexor digitorum brevis muscle was raised and stretched. The wound was closed with 2 mattress sutures with 5-0 nylon on an FS-1 needle. Sham surgeries consisted of all procedures except incision. All rats were allowed to recover from anesthesia before being returned to a new, clean cage for testing.

2.3. Western blot analysis

Incised and sham-operated rat hindpaw plantar skin, and flexor digitorum brevis muscle were frozen in liquid nitrogen and stored at −80 °C. Frozen plantar muscle and skin tissue was crushed in liquid nitrogen using a pestle and mortar and transferred to ice-cold 0.2 M RIPA lysis buffer [100 mM Tris–HCl , pH 7.4, 300 mM NaCl, 0.5% deoxycholic acid, 2% NP-40, 2 mM EDTA] (Millipore, Billerica, MA, USA) containing protease and phosphatase inhibitor cocktails (Sigma–Aldrich, St. Louis, MO, USA) for 45 min before being homogenized on ice. Lumbar level 4 and 5 dorsal root ganglion (DRG) from incised and sham-operated rats were pooled and frozen in liquid nitrogen with subsequent storage at −80 °C. DRG were processed in the same manner as skin and muscle except they underwent disruption with a 60-sonic dismembrator (Fisher Scientific, Pittsburg, PA, USA) for 60 s. Lysates were centrifuged at 10,000 g for 20 min at 4 °C and protein concentration of the supernatant was quantified using the BCA protein assay (Pierce, Rockford, IL, USA). All protein lysates were denatured by boiling in reducing sample buffer [1.25% 0.5 M Tris–HCl , pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 1.5% 2-b mercaptoethanol, 0.0025% bromophenol blue] for 5 min at 99 °C. Thirty μg of total protein for each sample was electrophoresed (130 V for 1.5 h) on 4–20 % SDSPAGE gels (Bio-Rad, Hercules, CA, USA). Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (0.45 mm Immobilon-P; Millipore) by semidry electrophoresis (15 V for 1 h). For immunodetection, membranes were incubated in blocking buffer 3% (w/v) BSA and 0.01% (v/v) Tween 20 in 0.1 M Tris-buffered saline (TTBS) (20 mM Tris, 500 mM NaCl, pH 7.5) for 1 h at room temperature followed by washing with 0.1 M TTBS. Membranes were then incubated overnight with rabbit anti-NPY (FL-97) (1:200) or goat anti-Y_1 receptor (A-17) (1:200) or goat anti-Y_2 receptor (L-17) (1:200) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) diluted in blocking buffer 4 °C while being gently rocked. Membranes were washed 3 times in 0.1 M TTBS and incubated in IR Dye 680CW conjugated donkey anti-goat or donkey anti-rabbit IgG secondary antibody (1:5000; LI-COR Biosciences, Lincoln, NE, USA) in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 2 h at room temperature. Where appropriate, after incubating the membranes in Restore™ stripping buffer (Pierce, Rockford, IL, USA), membranes were incubated in blocking buffer and reprobed for the housekeeping gene, actin, overnight at 4 °C using mouse anti-actin (1:1000; Millipore) diluted in blocking buffer. Membranes were washed in 0.1 M TTBS and incubated in IR Dye 800CW conjugated donkey anti-mouse IgG secondary antibody (1:5000; LI-COR Biosciences) in Odyssey blocking buffer for 2 h at room temperature. All membranes were imaged using the Odyssey infrared imaging system (LI-COR Biosciences) at scan intensity and resolution of 1 and 84 mm, respectively. NPY, Y_1 receptor and Y_2 receptor protein expression levels were determined using integrated intensities quantified using Image J 1.41o (NIH, Bethesda, MD, USA). An unloaded gel lane integrated density value was subtracted from the integrated densities from NPY, Y_1 receptor, Y_2 receptor, and actin bands. These values were then used to normalize expression of NPY, Y_1 receptor, and Y_2 receptor to the internal loading control, actin. Each time point studied consisted of 4 rats that underwent incision and 2 rats that underwent sham surgery. Data from sham rats pooled after preliminary analysis showed no change in NPY, Y_1 receptor, and Y_2 receptor protein bands or levels in the tissues across all time points.

2.4. ELISA

Incised and sham-operated rat right hindpaw L4 and L5 DRG were pooled and frozen in liquid nitrogen and stored at −80 °C. Frozen DRG was pounded in liquid nitrogen using a pestle and mortar and transferred to ice-cold 1× DPBS (Invitrogen, Grand Island, NY, USA) containing protease and phosphatase inhibitor cocktails (Roche, Indianapolis, IN, USA) for 45 min with occasional vortexing before being homogenized on ice. Lysates were centrifuged at 10,000 g for 15 min at 4 °C and protein concentration of the supernatant was quantified using the BCA protein assay (Pierce, Rockford, IL, USA). The NPY levels of DRG were quantified using NPY EIA kit (Phoenix Pharmaceuticals, Belmont, CA, USA). The standards and protein lysates were pipetted into the wells precoated with NPY-specific rat antibody. An enzyme-linked polyclonal antibody specific for rat NPY was added to the wells and incubated for 2 h. Any unbound antibody–enzyme reagent was removed by washing. Following the wash HRP substrate solution was added to the wells. The enzyme reaction was stopped using 2 N HCl. The microplates were read at 450 nm.

2.5. Behavioral studies

We measured withdrawal threshold to mechanical stimuli, withdrawal latency to radiant heat, and nonevoked guarding pain behavior. Following 3 days of acclimation to the testing environment and personnel, baseline measurements for all 3 behavioral tests were made. All personnel were blinded to the treatment group. For guarding behavior, a cumulative pain score was used to assess nonevoked pain behavior as described previously (Brennan et al., 1996). Briefly, unrestrained rats were placed onto a plastic mesh surface (8×8-mm^2 grid) under a plastic chamber ($21 \times 27 \times 15$ cm^3) for 15 min before testing. Ipsilateral and contralateral hindpaws were assessed for each animal for 1 min repeating every 5 min for 1 h (30 min for intrathecal drug administration). Following each 1 min observation period of both hindpaws, a score was assigned to each paw; a 0, 1, or 2 score was given accordingly. A 0 score was recorded when the hindpaw touched the mesh causing skin to blanch, a score of 1 was recorded when the hindpaw was partially off the mesh surface, and a score of 2 was recorded when the hindpaw was completely off the mesh surface. The sum of the 12 scores (0–24) recorded for each paw was obtained. The difference between the scores from the incised paw and nonincised paw was the cumulative pain score.

For withdrawal threshold to mechanical stimuli, unrestrained rats were individually placed onto a plastic mesh surface (12×12-mm^2 grid) under a plastic chamber ($21 \times 27 \times 15$ cm^3) for approximately 30 min of acclimation. Using openings created by the mesh surface,