American Pain Society
RESEARCH
EDUCATION
TREATMENT
ADVOCACY
PUBLISHED BY ELSEVIER

The Journal of Pain, Vol 14, No 11 (November), 2013: pp 1522-1530
Available online at www.jpain.org and www.sciencedirect.com

Spinal Endomorphin 2 Antinociception and the Mechanisms That Produce It Are Both Sex- and Stage of Estrus Cycle–Dependent in Rats

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Abstract: **Endomorphin 2 (EM2) is the predominant endogenous mu-opioid receptor (MOR) ligand in the spinal cord. Given its endogenous presence, antinociceptive responsiveness to the intrathecal application of EM2 most likely reflects its ability to modulate nociception when released in situ. In order to explore the physiological pliability of sex-dependent differences in spinal MOR-mediated antinociception, we investigated the antinociception produced by intrathecal EM2 in male, proestrus female, and diestrus female rats. Antinociception was reflected by changes in tail flick latency to radiant heat. In females, the spinal EM2 antinociceptive system oscillated between analgesically active and inactive states. During diestrus, when circulating estrogens are low, spinal EM2 antinociceptive responsiveness was minimal. In contrast, during proestrus, when circulating estrogens are high, spinal EM2 antinociception was robust and comparable in magnitude to that manifest by males. Furthermore, in proestrus females, spinal EM2 antinociception required spinal dynorphin and kappa-opioid receptor activation, concomitant with MOR activation. This is required for neither spinal EM2 antinociception in males nor the antinociception elicited in proestrus females by spinal sufentanil or [D-Ala^2,*N*-methyl-Phe^4,Gly-ol^5]-enkephalin, which are prototypic MOR-selective nonpeptide and peptide agonists, respectively. These results reveal that spinal EM2 antinociception and the signaling mechanisms used to produce it fundamentally differ in males and females.**

Perspective: ***The inability to mount spinal EM2 antinociception during defined stages of the estrus (and presumably menstrual) cycle and impaired transition from spinal EM2 analgesically nonresponsive to responsive physiological states could be causally associated with the well-documented greater severity and frequency of chronic intractable pain syndromes in women vs men.***

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Key words: *Endomorphin, estrus cycle, kappa-opioid receptor, dynorphin, antinociception.*

There is compelling evidence that women experience greater frequency and severity of numerous chronic pain syndromes than do men. Sexually dimorphic expression of idiopathic chronic pain syndromes pertains to migraine headaches,[11] irritable bowel syndrome,[26] interstitial cystitis,[59] musculoskeletal pain,[49] temporomandibular joint disorders,[23] and fibromyalgia.[68,69] Stage of menstrual (estrus) cycle is an important determinant of pain sensitivity in women[44,54,57,60] as well as laboratory animals.[13,18,24,63] The mechanism(s) responsible for sexually dimorphic occurrence of chronic pain states, variability in pain sensitivity across the menstrual (estrus) cycle, and possible linkages between the 2 remains unidentified.

Endomorphins (EMs) are widely considered to be the endogenous ligands for the mu-opioid receptor (MOR), the predominant opioid receptor mediating antinociception. EM1 (Tyr-Pro-Trp-Phe-NH2) and EM2 (Tyr-Pro-Phe-Phe-NH2) produce potent MOR-mediated antinociception following intracerebroventricular[53,70] or intrathecal administration,[51] which is abolished by selective MOR blockade.[51] Notably, spinally applied EMs are also potent antiallodynic agents in neuropathic pain.[45,46] Notwithstanding the inability to identify a precursor protein for either EM1 or EM2,[58] 1) the wide distribution of EMs in areas of the central nervous system relevant to pain (spinal cord dorsal horn,

Received June 17, 2013; Revised September 10, 2013; Accepted September 12, 2013.
The study was supported by grant DA027663 to N.-J.L. and A.R.G.
The authors have no conflicts of interest to report.
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1526-5900/$36.00
© 2013 by the American Pain Society
http://dx.doi.org/10.1016/j.jpain.2013.09.002

parabrachial nucleus, periaqueductal gray, medial thalamus, amygdala[33,34,41,64,65]), 2) the expression of EM2 (the predominant spinal EM) in dense core vesicles of primary afferent terminals in the lumbar dorsal horn,[52] 3) colocalization with spinal substance P,[52] and 4) the dramatic decrease in EM2 content in spinal cord ipsilateral to sciatic nerve lesion[52] collectively strongly suggest that EM2 is relevant to endogenous spinal MOR-coupled antinociception, particularly in the spinal cord where it is more potent in producing antinociception than at supraspinal sites.[15,70]

Physiologic, naturally occurring fluctuations in the functional state of the endogenous spinal EM2/MOR analgesic system during the menstrual cycle in women (estrus cycle in laboratory animals) could contribute to cyclic variations in their pain sensitivity, which over the long run could be a risk factor for developing chronic pain. Because EM2 not only is endogenously expressed in the spinal cord but also is the predominant spinal EM,[33] it is reasonable to assume that antinociceptive responsiveness to its exogenous application reflects its ability to modulate nociception when released endogenously. Therefore, sex-dependent and stage of estrus cycle–dependent differences in the ability of intrathecally applied EM2 to elicit antinociception would likely reflect variations in the effectiveness of the endogenous spinal EM2 system to modulate pain.

Accordingly, we compared antinociceptive responsiveness to spinally applied EM2 among male rats, diestrus female rats, and proestrus female rats. Results revealed not only a dramatic dependence of the magnitude of EM2 antinociception on stage of estrus cycle but also a sexual dimorphism in the mechanisms utilized by spinal EM2 to elicit antinociception. The observed absence of antinociceptive responsiveness to intrathecal EM2 during diestrus, the longest stage of the estrus cycle, could potentially amplify the consequences in women of nociceptive events that occur during analogous stages of the menstrual cycle, thus contributing to the greater risk of women than men for developing more severe and frequent chronic pain.

Methods

Animals

Experiments employed Sprague Dawley rats (225–300 g; Charles River, Kingston, NY), which were maintained in an approved controlled environment on a 12-hour light–dark cycle. Food and water were available ad libitum. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of SUNY Downstate Medical Center.

Determination of Stage of Estrous Cycle

Stage of cycle was determined using histology of vaginal smears. Predominance of small leukocytes was indicative of diestrous; a predominance of large round nucleated cells was indicative of proestrous. Disruptions of the estrous cycle that could result from surgery did not confound data interpretation because vaginal smear histology and not predictions that assumed regularity of cycling was used to define diestrous and proestrous cycles.

Intrathecal Administration of Drugs

Each drug was administered in 5 to 10 μL vehicle (water, saline, or 3% dimethyl sulfoxide in saline) over a 60-second period to the subarachnoid space of the lumbar spinal cord via a permanent indwelling intrathecal cannula. Complete delivery was ensured by flushing the cannula with an additional 10 μL of saline. Thereafter, responsiveness to nociceptive stimuli was determined at various intervals and compared with pre-drug values. Sufentanil and [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]-enkephalin (DAMGO) were used as nonpeptide and peptide, respectively, prototypic MOR-selective agonists. Intrathecal kappa-opioid receptor (KOR) blocker nor-binaltorphimine (norBNI)[43] was applied 18 hours before intrathecal EM2[9,27] in order to ensure that reported interactions between norBNI and spinal MOR[16] did not confound interpretation of results. Neither vehicle (water, saline, or 3% dimethyl sulfoxide saline), norBNI, nor anti-dynorphin antibody had detectable effects on tail flick latency (TFL).

Assessment of Nociceptive Responses

TFL to radiant heat was quantified using a Tail Flick Analgesia Meter (IITC, Woodland Hills, CA). Intensity of the radiant heat was adjusted such that baseline values were 3.0 to 4.5 seconds. A cutoff of 10 seconds latency prevented any untoward consequences to the tail.

Statistical Analysis

Repeated measures analysis of variance (ANOVA) was used to analyze effect of drug treatment at multiple time points after intrathecal administration within each group. Subsequent Dunnett's Multiple Comparison Test was used to locate the time point(s) that showed significant effect ($P < .05$). Two-way ANOVA was used to analyze the sex, group, or dose by time effects among groups. Subsequent Bonferroni posttests were used to locate differences between groups.

Results

Antinociceptive Responsiveness to Intrathecal EM2 Is Sexually Dimorphic

As expected, in males, intrathecal EM2 produced a robust and dose-dependent antinociception. A 2-way ANOVA (dose × time) revealed a significant dose effect of EM2 [$F_{(2,60)} = 35.50$, $P < .0001$]. Surprisingly, however, in females, intrathecal EM2 failed to produce significant antinociception, even at a dose of 90 nmol. The apparent sexually dimorphic antinociceptive response to spinal EM2 was validated by a 2-way ANOVA (sex × time), which revealed a significant sex effect [$F_{(1,40)} = 36.22$, $P < .0001$, n = 6 for each group; Fig 1].

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