Introduction

Enkephalins, classified under endorphins, are opioid peptides involved in nociception through opioid receptors [1]. The two major types of enkephalins, leucine-enkephalin (Leu-Enk) and methionine-enkephalin (Met-Enk), and the opioid antagonist naloxone on gonad development in the Eastern lubber grasshopper, Romalea microptera. Injection of either Leu-Enk or naloxone alone significantly increased the testicular index and testicular follicular diameter in males, and the ovarian index, oocyte length, and oocyte diameter in females. In contrast, injection of Met-Enk inhibited all measures of reproductive development in both sexes. Surprisingly, co-injection of naloxone with either enkephalin enhanced the effect associated with administration of the enkephalin alone. This study clearly demonstrates the ability of enkephalins to disrupt insect sexual development and also suggests the existence of conserved enkephaline-dependent regulatory mechanisms in insects and crustaceans.

Abstract

Enkephalins play a major role in reproductive physiology in crustaceans; however, their role in reproductive development in insects is largely unknown. We investigated the effect of exposure to exogenous Leu-Enk, methionine-enkephalin (Met-Enk), and the opioid antagonist naloxone on gonad development in the Eastern lubber grasshopper, Romalea microptera. Injection of either Leu-Enk or naloxone alone significantly increased the testicular index and testicular follicular diameter in males, and the ovarian index, oocyte length, and oocyte diameter in females. In contrast, injection of Met-Enk inhibited all measures of reproductive development in both sexes. Surprisingly, co-injection of naloxone with either enkephalin enhanced the effect associated with administration of the enkephalin alone. This study clearly demonstrates the ability of enkephalins to disrupt insect sexual development and also suggests the existence of conserved enkephaline-dependent regulatory mechanisms in insects and crustaceans.

Citation: Kumar S, Ganji PN, Song H, von Kalm L, Borst DW (2012) Exposure to Exogenous Enkephalins Disrupts Reproductive Development in the Eastern Lubber Grasshopper, Romalea microptera (Insecta: Orthoptera). PLoS ONE 7(11): e51126. doi:10.1371/journal.pone.0051126

Editor: Irina Kerkis, Instituto Butantan, Brazil

Received September 1, 2012; Accepted October 31, 2012; Published November 30, 2012

Copyright: © 2012 Kumar et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This research was supported by a grant from National Science Foundation, NSF-0611447 (DW Borst). The contents are solely the responsibility of the authors and do not necessarily represent the official views of NSF. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: skumar@knights.ucf.edu
platanis after ingestion of naloxone containing food. Recently, naloxone has been found to bind with retinoid X receptors (RXR) [23], which are associated with reproduction [24], suggesting that naloxone may also play a role in reproductive physiology in crustaceans and vertebrates. However, the effect of naloxone on reproduction and its interaction with enkephalins has not been investigated in insects. In this study we hypothesized that the effects of exposure to exogenous enkephalins in insects would be similar to previously reported studies in crustaceans. While the hypothesis is supported for Leu-Enk and Met-Enk, we find unexpectedly that naloxone enhances the effect of both enkephalins in the Eastern lubber grasshopper Romalea microptera (Insecta: Orthoptera), which has been used as a model organism for insect physiology and reproductive endocrinology [25,26,27,28].

Materials and Methods

Grasshoppers were collected from in and around University of Central Florida, Orlando, Florida (No specific permits were required for the collection of grasshoppers and studies) and were reared for two generations in laboratory conditions at 28±2°C in a 16:8 L:D cycle. Food consisted of Romaine lettuce and wheat bran offered ad libitum until the final molt to adult instar. Leu-Enk, Met-Enk, and naloxone were purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA) in a powder form and were dissolved in 1X PBS prior to use.

A total of one hundred and forty adult grasshoppers (third generation, laboratory reared) were used and divided into 14 groups of 10 insects each (N = 10). Two groups were used as control (no treatment) and another two groups were used as concurrent control (injection with 1X PBS). The remaining 10 groups were further divided into five treatment groups (each with two groups or 20 insects): Leu-Enk only, Met-Enk only, naloxone only, Leu-Enk + naloxone, and Met-Enk + naloxone. For each treatment, grasshoppers were injected on the first, fifth, tenth, and fifteenth day from adult emergence at a fixed dose of 10^-3 mol/grasshopper in 10 μL volume per injection. No significant mortality was observed in either the control or experimental groups, and the grasshoppers were sacrificed on the 20th day from adult emergence. Animals were first immobilized with carbon...
dioxide to measure body weight. Then, reproductive organs were dissected into 1X PBS and excessive fat body was removed. Organs were lightly blotted with paper towel and weighed on an electronic balance. Gonadal indices (GI) for oocytes and testicular follicles were measured using the following formula: GI = (weight of gonads/weight of animal) X 100. The linear dimensions of the reproductive structures were measured using an ocular micrometer attached to a Leica MZ6 microscope.

For dose-dependent studies of Leu-Enk, Met-Enk and naloxone, male and female insects were injected with 10⁻¹⁴mol/grasshopper, 10⁻¹³mol/grasshopper, 10⁻¹²mol/grasshopper, and 10⁻¹¹mol/grasshopper in 10 μl volume. Gonadal indices were measured for each dose as described above.

For statistical analyses, one-way ANOVA followed by Tukey-Kramer multiple comparison test was used to compare different treatments.

**Results**

To investigate the role of enkephalins and naloxone in *Romalea* reproductive physiology we first studied the dose-dependent effects of Leu-Enk, Met-Enk, and naloxone when administered singly (Figure 1). For all three compounds, an optimal dose of 10⁻¹¹mol/animal was observed.

For Leu-Enk, ovarian index increased in a dose-dependent manner up to a concentration of 10⁻⁸mol/animal (Figure 1A). A higher dose (10⁻⁷mol/animal) did not yield further development. Similar results were observed for testicular index (Figure 1A) as well as average oocyte diameter, average oocyte length and average testicular follicular diameter (data not shown). As previously documented in crustaceans [11], the opposite result was observed following administration of Met-Enk where ovarian index and testicular index both declined with increasing dose (Figure 1B). Naloxone, an opioid antagonist, had a similar dose-dependent profile to Leu-Enk with an increase in all reproductive indices observed as dose increased (Figure 1C). Similarly to Leu-Enk, the concentration of 10⁻⁸ mol/animal appeared to be the maximally sensitive dose for Met-Enk and naloxone.

To investigate potential interactions between naloxone and Leu-Enk or Met-Enk, we performed experiments with animals injected with two compounds. A similar profile was observed for all reproductive indices measured, where a combination of enkephalin with naloxone served to enhance the effect of administration of the enkephalin alone (Figure 2). For example, a combination of Met-Enk and naloxone significantly reduced the ovarian index when compared to Met-Enk alone (Figure 2A). Naloxone has been previously reported to enhance reproductive indices in crustaceans [21,22,30] and our work extends this finding to insects. Because naloxone is an opioid antagonist, we expected that it would negate the effect of both enkephalins when co-administered. Instead, naloxone acted as a synergistic agonist of both enkephalins in our study, significantly increasing the stimulating effect of Leu-Enk and enhancing the inhibitory effect of Met-Enk. These data are interpretable in a context where naloxone increases receptor sensitivity to enkephalins. Schulz et al. [31] reported that chronic injection of naloxone in guinea pigs resulted in an increased sensitivity to Met-Enk action in muscle mesenteric plexus of ileum. Similarly, Tang and Collins [32] reported the enhancement of analgesic action of morphine following chronic administration of naloxone in rats. The mode of action for naloxone in *R. microptera* is unknown; however naloxone has been reported to stimulate the action of gonad stimulating hormone (GSH) in the red claw crayfish [22]. Further studies are needed to determine the mode of action of naloxone and the mechanism of its interaction with enkephalins in insects.

To date there is no direct evidence for the existence of Leu- and Met- enkephalins in insects or crustaceans. Indirect evidence is based on cross-immunoreactivity with mammalian antibodies [7,16,18], or exogenous application of these compounds [11,12,13]. The effect of exogenous enkephalins on reproductive development has been studied in depth in crustaceans as reviewed by Nagaraju [29], and our findings are consistent with the previous reports in crustaceans. The similarity between our observations and those reported in crustaceans imply the possible existence of a conserved enkephalin response mechanism in arthropods. This suggests that the response to enkephalins may be an ancestral trait that evolved before the divergence of hexapods and decapods. In our opinion it seems unlikely that an artifactual response to exogenous administration of enkephalins would be conserved throughout the Pancrustacea unless the enkephalins were interacting with molecules in another highly conserved neuroendocrine pathway. Clearly, further studies now require a definitive demonstration of the existence of endogenous enkephalins in insects and crustaceans. If endogenous enkephalins are identified, it will be interesting to determine how they interact with juvenile hormone, ecdysone, ecdysteroids, and gonadotropin neurohormones such as allatotropin and allatostatin to regulate insect reproductive physiology.

**Discussion**

In this study we demonstrate that exposure to exogenous enkephalins influences reproductive development in *Romalea microptera*. As previously reported in decapod crustaceans [29], we find an antagonistic interaction between Leu-Enk and Met-Enk in terms of reproductive development, Leu-Enk stimulated gonad development in both male and female grasshoppers, while Met-Enk had the opposite effect. The biological effects of both enkephalins were dose-dependent with a maximum effect observed at 10⁻¹² mol/animal. Our findings are consistent with the observations of Schoofs et al. [10] who reported immunoreactivity to Met-Enk in the gonads of two distantly related insect species, *L. migratoria* and *S. bullata*, and speculated that Met-Enk might play a role in insect reproductive physiology. In contrast, while Schoof et al. [10] observed immunoreactivity against Leu-Enk in the central nervous system of both species, immunoreactivity was not detected in the ovaries. This raises the possibility that enkephalins or enkephalin-like peptides may regulate insect reproduction indirectly via the neuroendocrine system. In support of this hypothesis, Duve and Thorpe [16] reported the presence of enkephalin-like peptides in the brain, corpus cardiacum, and corpus allatum in the blowfly *Calliphora vomitoria*.

Naloxone has been previously reported to enhance reproductive indices in crustaceans [21,22,30] and our work extends this finding to insects. Because naloxone is an opioid antagonist, we expected that it would negate the effect of both enkephalins when co-administered. Instead, naloxone acted as a synergistic agonist of both enkephalins in our study, significantly increasing the stimulating effect of Leu-Enk and enhancing the inhibitory effect of Met-Enk. These data are interpretable in a context where naloxone increases receptor sensitivity to enkephalins. Schulz et al. [31] reported that chronic injection of naloxone in guinea pigs resulted in an increased sensitivity to Met-Enk action in muscle mesenteric plexus of ileum. Similarly, Tang and Collins [32] reported the enhancement of analgesic action of morphine following chronic administration of naloxone in rats. The mode of action for naloxone in *R. microptera* is unknown; however naloxone has been reported to stimulate the action of gonad stimulating hormone (GSH) in the red claw crayfish [22]. Further studies are needed to determine the mode of action of naloxone and the mechanism of its interaction with enkephalins in insects.

**Acknowledgments**

This study is dedicated to the memory of Dr. David Borst who passed away on September 27, 2010. Sandeep Kumar is grateful for his wonderful support and guidance. He was a brilliant teacher and researcher and will always be remembered.

**Author Contributions**

Conceived and designed the experiments: SK GPN. Performed the experiments: SK GPN. Analyzed the data: SK GPN. Contributed reagents/materials/analysis tools: SK DWB HS LV. Wrote the paper: SK. Provided guidance for the research, worked further on data analysis,
Figure 2. Effect of enkephalins alone, naloxone alone or combinations on ovarian index (A), average oocyte diameter (B), average oocyte length (C) of female grasshoppers, and average testicular index (D) and average testicular follicular diameter (E) of male grasshoppers. ‘ns’ indicates statistical non-significance ($p>0.05$) compared to the control and the letters indicate that the effects of various doses were significantly ($p<0.05$) different from each other and the control.

doi:10.1371/journal.pone.0051126.g002
References

1. Michael-Titus A, Dourmap N, Caline H, Costenen J, Schwartz JC (1989) Role of endogenous enkephalin in locomotion and narcosis studied with peptidase inhibitors in two inbred strains of mice (C57BL/6J and DRA/2J). Neuropharmacology 28: 117–122.

2. Simanton R, Snyder SH (1976) Morphine-like peptides in mammalian brain: isolation, structure elucidation, and interactions with the opiate receptor. Proc Natl Acad Sci USA 73: 2319–2323.

3. Hughes J, Smith TW, Kosteritz HW, Forghill LA, Morgan BA, et al. (1975) Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature 256: 577–580.

4. Gubler U, Seeberg P, Hoffman BJ, Gage LP, Uderyenfied S (1980) Molecular cloning establishes proenkephalin as precursor of enkephalin-containing peptides. Nature 295: 206–208.

5. Uderyenfied S, Kilpatrick DL (1983) Biochemistry of the enkephalins and enkephalin-containing peptides. Arch Biochem Biophys 221: 309–323.

6. Salzet M, Stefano GB (1997) Invertebrate proenkephalin: d opioid binding sites in leech ganglia and immunocytochemistry. Brain Res. 768: 224–232.

7. Duve H, Thorpe A (1988) Mapping of enkephalin related peptides in the nervous system of the blowfly, Calliphora vomitoria. Prog Clin Biol Res. 342: 293–299.

8. Kishori B, Reddy PS (2003) Influence of leucine-enkephalin on moulting and oviposition in Leucophaea maderae. J Insect Physiol. 49: 561–567.

9. Froehlich JC (1997) Opioid peptides. Alcohol Health Res World 21: 132–136.

10. Schoofs L, Schoonjans S, Haybrendt R, De Loof A (1993) Methionine-enkephalin immunoreactivity in the gonads and nervous system of two insect species: Locusta migratoria and Sarcophaga bullata. Gen Comp Endocrinol. 89: 1–12.

11. Reddy PS (2000) Involvement of opioid peptides in the regulation of reproduction in the prawn Penaeus indicus. Natuurwetenschappen 87: 535–538.

12. Kishori B, Reddy PS (2003) Influence of leucine-enkephalin on moulting and vitellogenesis in the freshwater crab, Ocypode ceratophthalma. J Insect Physiol. 49: 1201–1208.

13. Sarojini R, Nagabhushanam R, Fingerman M (1995) Evidence for opioid involvement in the regulation of ovarian maturation of the fiddler-crab, Uca Pagulana. Comp Biochem Physiol Part C Pharmacol Toxicol Endocrinol. 107: 137–141.

14. Harrison LM, Kastin AJ, Webers JT, Banks WA, Hurley DL, et al. (1994) The opiate system in invertebrates. Peptides 15: 1309–1328.

15. Thorpe A, Duve H (1990) Morphological, biochemical, and physiological studies on invertebrate enkephalins. Prog Clin Biol Res. 342: 293–299.

16. Duve H, Thorpe A (1988) Mapping of enkephalin related peptides in the nervous system of the blowfly, Calliphora vomitoria, and their co-localization with Cholecystokinin (CCK)-like and Pancreatic Polypeptide (PP)-like peptides. Cell Tissue Res. 298: 395–415.

17. Hansen BL, Hansen GN, Scharrer B (1982) Immunoreactive material resembling vertebrate neuropeptides in the corpus cardiaca and corpus allatum of the insect Locusta migratoria. Cell Tissue Res. 225: 319–329.

18. Duve H, Thorpe A (1990) Distribution and functional significance of Met-Enkephalin-Arg6-Phe7-like and Met-Enkephalin-Arg6-Gly7-Leu8-like peptides in the blowfly Calliphora vomitoria 1. Immunocytochemical mapping of neuronal pathways in the brain. Cell Tissue Res. 258: 147–161.

19. Bruni JF, Vanvugt D, Marshall S, Meites J (1977) Effects of naloxone, morphine and methionine enkephalin on serum prolactin, lutetinizing-hormone, follicle-stimulating hormone, thyroid stimulating hormone and growth hormone. Life Sci. 21: 461–466.

20. Cicero TJ, Adams ML, O’Connor LH, Nock B (1989) In vivo evidence for a direct effect of naloxone on testicular steroidogenesis in the male rat. Endocrinology 125: 957–963.

21. Sarojini R, Nagabhushanam R, Fingerman M (1996) In vivo assessment of opioid agonists and antagonists on ovarian maturation in the red swamp crayfish, Procambarus clarkii. Comp Biochem Physiol Part C Pharmacol Toxicol Endocrinol. 115: 149–153.

22. Cahansky AV, Medesani DA, Rodriguez EM (2008) Induction of ovarian growth in the red claw crayfish, Cherax quadricarinatus, by the enkephalergic antagonist naloxone: in vivo and in vitro studies. Invert Rep Dev. 51: 61–67.

23. Nagaraju GPC, Prasad GLV, Taliaferro-Smith L, Aruna BV, Naik BR, et al. (2010) Computational analysis of the structural basis of ligand binding to the crustacean retinoid X receptor. Comp Biochem Physiol Part D Genomics Proteomics 5: 317–324.

24. Mark M, Ghyselinck NB, Chambon P (2006) Function of retinoid nuclear receptors. Lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. Annu Rev Pharmacol Toxicol. 46: 451–489.

25. Brust DW, Lekow MR, Wagner SJ, Shores K, Hunter J, et al. (2000) Quantification of juvenile hormone III, vitellogenin, and vitellogenin-mRNA during the oviposition cycle of the lubber grasshopper, Romalea microptera. Comp Biochem Physiol Part C Pharmacol Toxicol Endocrinol. 117: 135–141.

26. Hatle JD, Juliano SA, Borst DW (2000) Juvenile hormone is a marker of the onset of reproductive cannalization in lubber grasshoppers. Insect Biochem Mol Biol. 30: 811–819.

27. Sandberg SV, Luong-Skowmandh MH, Whitman DW (2001) Morphology and development of oocyte and follicle resorption bodies in the Lubber grasshopper, Romalea microptera (Beauvior). J Orthoptera Res. 10: 39–51.

28. Judd ET, Hatle JD, Dreyer MD, Wessells EF, Hahn DA (2010) Allocation of nutrients to somatic tissues in young ovariectomized grasshoppers. Integr Comp Biol. 50: 818–828.

29. Nagaraju GPC (2011) Reproductive regulators in decapod crustaceans: an overview. J Exp Biol. 214: 3–16.

30. Sarojini R, Nagabhushanam R, Fingerman M (1997) An in vitro study of the effect of opioid agonists and antagonists on ovarian maturation in the red swamp crayfish, Procambarus clarkii. Comp Biochem Physiol Part C Pharmacol Toxicol Endocrinol. 117: 207–210.

31. Schulz R, Wuster M, Herz A (1979) Supersensitivity to opioids following the chronic blockade of endorphin action by naloxone. Naunyn Schmiedebergs Arch Pharmacol. 306: 93–96.

32. Tang AH, Collins RJ (1978) Enhanced analgesic effects of morphine after chronic administration of naloxone in the rat. Eur J Pharmacol. 47: 473–474.