Neuroendocrinology Letters Nos.1/2, Feb-Apr, Vol.24, 2003
 ISSN 0172–780X **www.nel.edu**

ORIGINAL ARTICLE

Effects of cold stress on morphine-induced nitric oxide production and μ-opiate receptor gene expression in *Mytilus edulis* pedal ganglia

Kirk Mantione, Raymond Hong, Richard Im, Jeong Ho Nam, Maxime Simon, Patrick Cadet & George B. Stefano

Neuroscience Research Institute, State University of New York, College at Old Westbury, Old Westbury, NY 11568-0210,USA and the Long Island Conservatory, Science Program, 1125 Willis Ave, Albertson, NY 11507, USA.

Correspondence to: Dr. G.B. Stefano, Neuroscience Research Institute
State University of New York
College at Old Westbury, Old Westbury
NY 11568-0210, USA
FAX: +1 516-876-2727
PHONE: +1 516-876-2732
E-MAIL: gstefano@sunynri.org

Submitted: *November 18, 2002*
Accepted: *November 22, 2002*

Key words: **nitric oxide; morphine; cold stress; mu opiate receptor; invertebrate neural tissues**

Neuroendocrinology Letters 2003; ***24****(1/2):68–72 pii: NEL241203A10* *Copyright © Neuroendocrinology Letters* ***www.nel.edu***

Abstract

OBJECTIVES: Subjecting the marine bivalve *Mytilus edulis* to an immediate temperature change has been shown to rapidly alter the animals' ganglionic monoamine levels, as well as its ciliary activity. Recently, we extended this observation to include the organism's ganglionic mu opiate receptor and morphine levels. In the past, we demonstrated that *M. edulis* ganglionic mu receptors exposed to morphine was coupled to the immediate release nitric oxide (NO). In this study, we measured morphine-induced NO release in *M. edulis* subjected to acute cold stress.

METHODS: NO release was monitored with an NO-selective microprobe. Temporal changes in mu opiate receptor expression were also examined over 24 hours.

RESULTS: In this study, we demonstrate that after 12h cold exposure (4°C from 24°C), the estimated relative μ-opiate receptor (MOR) gene expression in *M. edulis* pedal ganglia, measured by real-time PCR, did not differ significantly from the control group (1.23 ± 0.25, $p > 0.05$). However, the measured *M. edulis* pedal ganglia MOR expression demonstrated that ganglia significantly (0.77 ± 0.05, $p < 0.001$) down regulated their mu opiate receptor mRNA expression after 24h exposure to the cold water. The mean value for control animal (24°C, n=14) morphine-stimulated NO release was 36.7 ± 9.8 nM. Morphine additions to cold-treated tissues (4°C, n=7) produced an average of 6.7 ± 4.9 nM NO, which was a statistically significant difference between 25°C and 4°C animals ($p = 0.025$).

CONCLUSION: The study further demonstrates that mu opiate receptor expression is coupled to NO release.

Abbreviations:
NO – nitric oxide;
PCR – polymerase chain reaction;
PSU – practical salinity units;
RT – reverse transcription;
dNTP's – 2'-deoxynucleoside 5'-triphosphate;
L-NAME – Nω-nitro-L-arginine methyl ester

Introduction

Through homeostasis, living organisms maintain their survival in the face of both externally and internally generated "stimuli." This balance is constantly challenged [1,2]; and, therefore, the ability to overcome these normal perturbations is essential to survival and longevity. In this regard, subjecting invertebrate animals, i.e., the marine bivalve *Mytilus edulis*, to an immediate temperature change has been shown to rapidly alter the animals' ganglionic monoamine levels as well as functions, such as ciliary activity, which require these specific signal molecules [3,4,5]. Additionally, opiate processes respond to various types of stressors in both vertebrates and invertebrates [6,7,8,9,10,11,12].

Recently, we have demonstrated that the endogenous opiate alkaloid morphine, due to an increase in its levels after a latency period, represents the terminating component of a successful stress response, and that its actions are generally down regulating in both invertebrates and vertebrates (see [13]). Furthermore, this down regulating action occurs because of the coupling of the mu opiate receptor subtype to constitutive nitric oxide (NO) release, see [13]. Thus, it was of interest to determine if *M. edulis*' endogenous neural opiate signaling processes would respond to rapid temperature changes [13,14]. Since the mu opiate receptor gene is expressed at low levels, we apply a novel quantitative method of real-time PCR to accurately measure its change in expression [6,15].

In a recent study, we presented molecular evidence on the effect of rapid temperature changes on μ-opiate receptor expression and morphine levels [6]. In cold stressed organisms, ganglionic μ-opiate receptor expression decreases, whereas opiate alkaloid levels increase. In the present study, we examine the relationship between mu opiate receptor expression and NO release as a result of acute cold stress. This study provides further evidence that μ-type opiate receptors in mollusk ganglia appear to be down regulated in physiological response to cold stress as well as the NO release. This, in turn, strengthens the coupling mechanism of how endogenous morphine exerts its actions.

Materials and Methods

M. edulis, collected from the local waters of Long Island Sound, were maintained in laboratory conditions for at least 14 days prior to use in the experiments. The mussels were kept in artificial seawater (Instant Ocean, Aquarium Systems, Mentor, Ohio) at a salinity of 30 PSU. Control animals were maintained at ambient temperature (20–25 °C), and experimental animals were kept at 4 °C for 24h. All experimental animals, including the controls, were prepared in batches of 20 in 2 L glass containers.

Nitric oxide assay

Ten pedal ganglia dissected from *M. edulis* were bathed in 1 mL sterile phosphate buffered saline. Experiments to demonstrate changes in μ-receptor expression used morphine at a final concentration of 10^{-6} M. NO release was monitored with an NO-selective microprobe manufactured by World Precision Instruments (Sarasota, FL). The redox current was detected by a current-voltage converter circuit and continuously recorded. The dimensions of the probe (30 uM diameter, 0.5 mm length) permitted the use of a micromanipulator (Zeiss-Eppendorff). The sensor was positioned approximately 100 uM above the respective tissue surface. Calibration of the electrochemical sensor was performed by use of different concentrations of a nitrosothiol donor S-nitroso-N-acetyl-DL-penicillamine (SNAP), as previously described by [16]. The NO detection system was calibrated daily. The probe is allowed to equilibrate for 10 min the incubation medium, which is free of tissue, before being transferred to vials containing the ganglia for another 5 min. Manipulation and handling of the ganglia was performed only with glass instruments. Each experiment was repeated 7 times; and the mean NO values were graphed to represent the actual NO release. The data collected were evaluated by the student *t-test*. Data acquisition was by the computer interfaced DUO-18 software (World Precision Instruments, Sarasota, FL). The experimental values were then transferred to Sigma-Plot and -Stat (Jandel, CA) for graphic representation and evaluation.

Isolation of Total RNA

M. edulis pedal ganglia were collected and placed in 1.5 ml tubes. Total RNA was isolated from about 20 ganglia with the RNeasy Protect Mini Kit (Qiagen, Stanford, CA). Ganglia were re-suspended in 600 ul buffer RLT and homogenized for 2 min using a rotor-stator homogenizer. The lysate was centrifuged at 10000g for 3 min and only the supernatant was used in subsequent steps. One volume (600 ul) of 70% ethanol was mixed with the supernatant. The sample was applied to an RNeasy mini spin column sitting in a 2 ml collection tube, and centrifuged for 15 sec at 10,000 rpm. For washing, 700 ul of buffer RW1 was added to the column and then centrifuged as previously mentioned. The column was then transferred into a new 2 ml collection tube, and then washed with 500 ul of buffer RPE. After centrifugation, the column was washed again with 500 ul of buffer RPE and centrifuged for 2 min at maximum speed to dry the RNeasy membrane. The column was transferred into a 1.5 ml collection tube, and RNA was eluted with 50 ul of RNase-free water for 1 min at 10,000 rpm. RNA was analyzed on a 1% denaturing agarose gel and concentrations were determined spectrophotometrically.

Table I. Primers and Taqman Probe for MOR sequence used in Real Time-Polymerase chain reactions.

Primer / probe	Sequence
Forward primer	5'-ATGCCAGTGCTCATCATTAC-3'
Reverse primer	5'-GATCCTTCGAAGATTCCTGTCCT-3'
Taqman Probe*	5' -CGCCTCAAGAGTGTCCGCATGCT- 3'

*The Taqman probe was constructed with the 5'-reporter dye, 6-carboxyfluorescein (FAM), and a 3'-quencher dye, 6-carboxy-tetramethyl-rhodoamine (TAMRA).

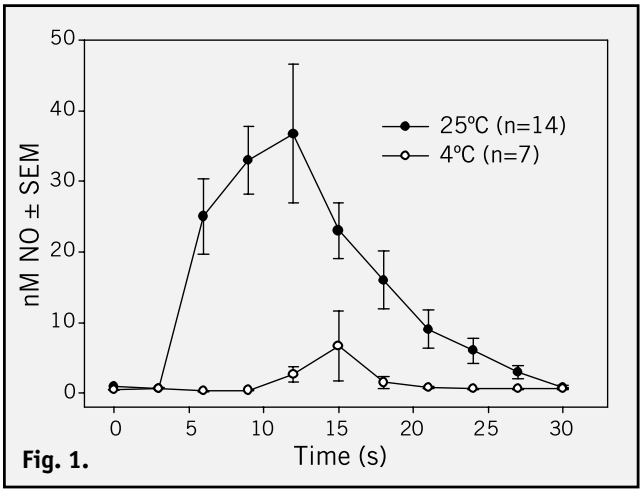

Fig. 1.

Fig. 1. Morphine (10^{-6} M)-stimulated nitric oxide release in *Mytilus edulis* pedal ganglia. Open symbols represent animals kept at 4°C for 24h, closed symbols represent animals kept at ambient temperature. Error bars represent standard error of the mean nitric oxide release at the specified time interval. A *t-test* revealed a significant difference between peak nitric oxide releases (p=0.025).

Reverse Transcription – Polymerase Chain Reaction of Total RNA

First strand cDNA synthesis was performed using random hexamers (GIBCO, BRL, Gaithesburg, MD). 1 ug of total RNA, isolated from the ganglia, were denatured at 95 °C and reverse transcribed at 40 °C for 1 hr using Superscript II Rnase H-RT (GIBCO BRL, MD). Ten microliters of the RT product was added to the PCR mix. Primers and probes that are specific to the μ-opiate receptor gene (Table1), as well as the internal control gene, β-actin, were designed by the software Primer Express (Applied Biosystems). The 2X universal master mix (Applied Biosystems) containing the PCR buffer, $MgCl_2$, dNTP's, and the thermal stable AmpliTaq Gold DNA polymerase was used in the PCR reactions. In addition, 200 uM reverse and forward primers, 100 uM Taqman probe, 10 uL of RT product, and Rnase/DNase-free water were added to the master mix to a final volume of 50 uL. The PCR reaction mixture was then transferred to a MicroAmp optical 96-well reaction plate and incubated at 95 °C for 10 min to activate the Amplitaq Gold DNA polymerase, and was then run for 40 cycles at 95 °C for 30s and 60 °C for 1 min on the Applied Biosystems GeneAmp 5700 sequence Detection System. The PCR result was analyzed with the GeneAmp 5700 SDS software (Applied Biosystems). In order to determine the relative copy number of the target gene transcript, control cDNA, generated from SHY cell total RNA, was used to produce a standard curve. Relative gene expression was calculated using the method of Yoshikawa et al [15].

Results

Nitric oxide release

The mean value for control animal (n=14) morphine (10^{-6} M)-stimulated NO release was 36.7 ± 9.8 nM (Figure1). This process has been shown to be both naloxone and L-NAME antagonizable, see [16,17]. Morphine additions to cold-treated tissues (n=7) produced an average of 6.7 ± 4.9 nM NO (Figure 1). A *t-test* was performed and demonstrated a statistically significant difference between 25 °C and 4 °C animals (p=0.025).

Morphine receptor expression

Real time PCR for mu opiate receptor expression yielded threshold cycle numbers between 31 and 26 cycles for all samples amplified (Figure 2). Negative template controls did not amplify (Figure 2). The calibration standards used, bracketed the threshold cycle numbers of the samples, ranging from 36 to 26 cycles (Figure 2, inset). The RNA standards used for calibration ranged from 0.35 to 350 ng total RNA per reverse transcription reaction. The reference gene, β-actin, determined that equal amounts of *M. edulis* total RNA were used for the reverse transcription reactions.

After 12 h cold exposure, the estimated relative μ-opiate receptor (MOR) gene expression, measured by real-time PCR, did not change significantly from the control groups (1.23 ± 0.25, $p > 0.05$). However, the measured *M. edulis* pedal ganglia MOR expression demonstrated that ganglia significantly (0.77 ± 0.05, $p < 0.001$) down regulated their mRNA expression after 24 h exposure to cold water (Figure 3).

Discussion

Rapid exposure of whole mollusks to cold temperatures from maintenance at room temperature alters ganglionic opiate processes. We have provided such treatment results in significantly enhanced levels of ganglionic opiate alkaloids that are time dependent [6]. Subsequently, ganglionic μ opiate receptor expression slightly increases before a decrease to below control levels [6]. We surmise that the decrease in μ expression results from the enhanced levels of the endogenous opiate alkaloids, representing a compensatory response. In the earlier study, we also demonstrated as that both μ opiate receptors and opiate alkaloids are expressed under basal conditions, suggesting their constant use

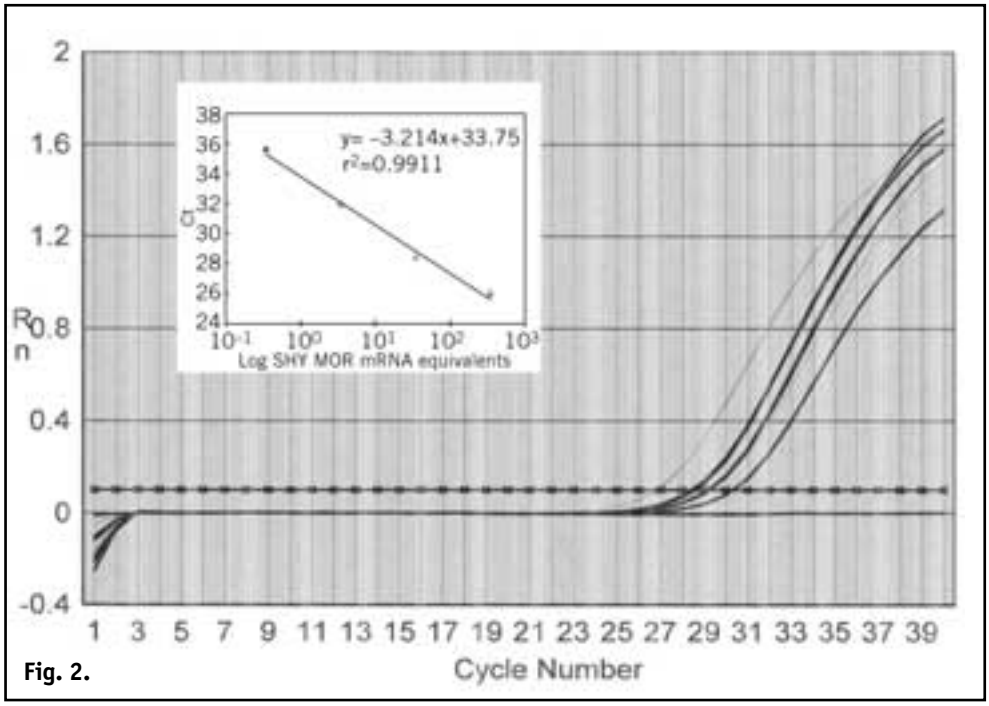

Fig. 2.

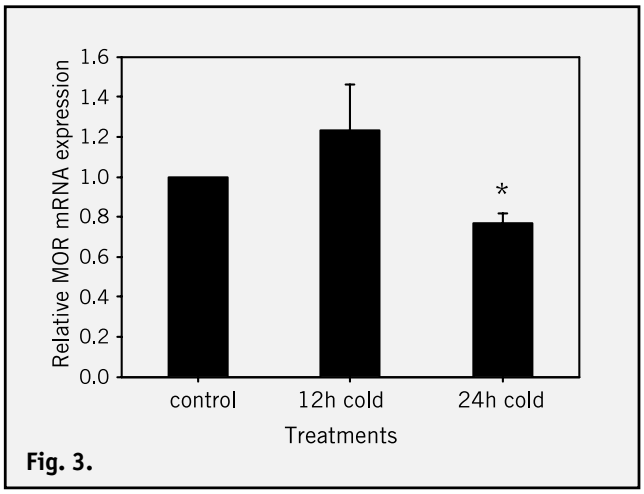

Fig. 3.

Fig. 2. Real time PCR amplification plot generated by the ABI GeneAmp 5700 Sequence Detection System for μ-opiate receptor gene expression. **Inset**: Calibration curve using SHY neuroblastoma cells total RNA. All samples were within the standard range.

Figure 3. Temporal variations in the effect of cold stress on μ-opiate receptor expression in *M. edulis*. Relative μ-opiate receptor gene expression of pedal ganglia was determined by real-time RT-PCR and was normalized to control animals.
* Statistically significant difference from control (P< 0.001).

by the organism. With this in mind, it would appear that opiate signaling is involved in this organism's response to thermal stress.

Additionally, in this study, we demonstrate that mu opiate receptor expression as a result of cold stress exposure, also results in a significantly diminished NO release from the ganglionic tissue. Previously, in *M. edulis* and human tissues, we demonstrated the coupling of NO to mu opiate receptors [13,16,17,18,19]. Hence, it appears that the relationship of opiate alkaloid signaling via mu opiate receptor coupling to NO is further supported by these findings. Furthermore, this action appears to have additional significance in this stress response to cold exposure.

In another study involving mollusks and physical stress, we demonstrated that a morphine-like compound in different tissues of a freshwater snail (*Planorbarius corneus*) rose significantly after 24 and 48 hours [20]. Additionally, *M. edulis* starvation also results in higher ganglionic morphine levels [21]**.** This has led us to believe that, given morphine's general immune and neural down regulating actions, it functions to limit the extent of excitation caused by an initial stressor, i.e., thermal, restoring homeostasis [13,17,22]. In the present report, we surmise that the down regulation of mu opiate receptor expression and the diminished capability to produce NO, occurs because coldness, in general, down regulates physiological processes (i.e. lowers metabolic rates). Hence, the general down regulation of these processes, and the removal of the opiate influence, allows these tissues to become more excitable than if general cold and opiate down regulation were both present. In short, we surmise that the "removal" of opiate down regulation in reality does allow for greater tissue excitability.

In conclusion, opioid processes appear to have evolved much earlier than previously thought. In this regard, opioid peptide and opiate alkaloid signaling are evolutionarily conserved. This conservation is noted by the high sequence identity of the actual signaling molecules as well as the μ opiate receptor. We conclude that this signaling "family" is quite important over the

course of evolution based on the fact that it has been highly conserved. Furthermore, the significance of this signaling family can be ascertained by the fact that it can simultaneously coordinate, in a time dependent manner, both immune and neural signaling appropriate to a non-cognitive and/or cognitive threat [17,22,23].

Acknowledgements

This work was supported by the following grants: NIMH COR 17138, NIDA 09010 and the Research Foundation and Central Administration of the State University of New York, NIH Fogarty INT 00045 (GBS).

REFERENCES

1 Chrousos GP, Gold PW. The concepts of stress and stress system disorders: Overview of physical and behavioral homeostasis. JAMA 1992; **267**:1244–52.
2 Fricchione GL, Stefano GB. The stress response and autoimmunoregulation. Adv Neuroimmunol 1994; **4**:13–28.
3 Stefano GB, Catapane EJ, Stefano JM. Temperature dependent ciliary rhythmicity in *Mytilus edulis* and the effects of monoaminergic agents on its manifestation. Biol Bull 1977; **153**:618–29.
4 Stefano GB, Catapane EJ. The efffects of temperature acclimation on monoamine metabolism. J Pharmacol Exp Ther 1977; **203**:449–546.
5 Stefano GB, Hiripi L, Catapane EJ. The effect of short- and long-term temperature stress on serotonin, dopamine and neuroepinephrine metabolism in molluscan ganglia. J Thermal Biol 1978; **3**:79–83.
6 Cadet P, Zhu W, Mantione K, Baggerman G, Stefano GB. Cold stress alters *Mytilus edulis* pedal ganglia expression of μ opiate receptor transcripts determined by real-time RT-PCR and morphine levels. Brain Research: Molecular Brain Research 2002; **99**:26–33.
7 Guarna M, Bianchi E, Bartolini A, Ghelardini C, Galeotti N, Bracci L et al. Endogenous morphine modulates acute thermonociception in mice. J Neurochem 2002; **80**:271–7.
8 Stefano GB, Zhu W, Cadet P, Mantione K, Bilfinger TV, Bianchi E et al. A hormonal role for endogenous opiate alkaloids: Vascular tissues. Neuroendocrinol Lett 2002; **23**:21–6.
9 Cardinale GJ, Donnerer J, Finck AD, Kantrowitz JD, Oka K, Spector S. Morphine and codeine are endogenous components of human cerebrospinal fluid. Life Sci 1987; **40**:301–6.
10 Lee CS, Spector S. Changes of endogenous morphine and codeine contents in fasting rat. J Pharmacol Exp Ther 1991; **257**:647–50.
11 Marrazzi MA, Luby ED, Kinzie J, Munjal ID, Spector S. Endogenous codeine and morphine in anorexia and bulimia nervosa. Life Sci 1997; **60**:1741–7.
12 Molina PE, Hashiguchi Y, Meijerink WJHJ, Naukam RJ, Boxer R, Abumrad NN. Modulation of endogenous opiate production: effect of fasting. Biochem Biophys Res Commun 1995; **207**:312–7.
13 Stefano GB, Goumon Y, Casares F, Cadet P, Fricchione GL, Rialas C et al. Endogenous morphine. Trends in Neurosciences 2000; **9**:436–42.
14 Zhu W, Baggerman G, Goumon Y, Casares F, Brownawell B, Stefano GB. Presence of morphine and morphine-6-glucuronide in the marine mollusk Mytilus edulis ganglia determined by GC/MS and Q-TOF-MS. Starvation increases opiate alkaloid levels. Brain Res Mol Brain Res 2001; **88**:155–60.
15 Yoshikawa M, Nakayama H, Ueno S, Nishimine N, Furuya H. Novel quantitative reverse-transcribed polymerase chain reaction of mu opioid receptor mRNA level. Brain Research Protocols 2001; **7**:147–53.
16 Liu Y, Shenouda D, Bilfinger TV, Stefano ML, Magazine HI, Stefano GB. Morphine stimulates nitric oxide release from invertebrate microglia. Brain Res 1996; **722**:125–31.
17 Stefano GB, Scharrer B, Smith EM, Hughes TK, Magazine HI, Bilfinger TV et al. Opioid and opiate immunoregulatory processes. Crit Rev in Immunol 1996; **16**:109–44.
18 Stefano GB, Hartman A, Bilfinger TV, Magazine HI, Liu Y, Casares F et al. Presence of the mu3 opiate receptor in endothelial cells. Coupling to nitric oxide production and vasodilation. Journal of Biological Chemistry 1995; **270**:30290–3.
19 Stefano GB, Liu Y. Opiate antagonism of opioid actions on immunocyte activation and nitric oxide release. Anim Biol 1996; **1**:11–6.
20 Sonetti D, Mola L, Casares F, Bianchi E, Guarna M, Stefano GB. Endogenous morphine levels increase in molluscan neural and immune tissues after physical trauma. Brain Res 1999; **835**:137–47.
21 Zhu W, Baggerman G, Goumon Y, Casares F, Brownwell B, Stefano GB. Presence of morphine and morphine-6-glucuronide in the marine mollusk Mytilus edulis ganglia determined by GC/MS and Q-TOF-MS. Starvation increases opiate alkaloid levels. Mol Brain Res 2001; **88**:155–60.
22 Stefano GB, Scharrer B. Endogenous morphine and related opiates, a new class of chemical messengers. Adv Neuroimmunol 1994; **4**:57–68.
23 Stefano GB, Salzet B, Fricchione GL. Enkelytin and opioid peptide association in invertebrates and vertebrates: immune activation and pain. Immunol Today 1998; **19**:265–8.