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RESEARCH REPORT

# Seasonal variations in mu opiate receptor signaling in the nervous system of the blue mussel, *Mytilus edulis*: temperature controls physiological processes

**KJ Mantione, P Cadet, F Casares, W Zhu, GB Stefano**

*Neuroscience Research Institute, State University of New York at Old Westbury, NY 11568, USA*

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## Abstract

It is anticipated that invertebrate processes will be subject to seasonal variations because of their poikilothermal characteristics. In the present study we determined if the morphine coupled nitric oxide (NO) release, which is constitutive in nature, exhibits seasonal characteristics, which has previously been shown for catecholamine processes in the marine mollusc *Mytilus edulis*. In this regard, morphine induced NO release measured on a monthly basis for one year revealed a peak release value (39 ± 4 nM) during the late spring and early summer. The lowest NO release occurred during the months of January (6.0 ± 0.5 nM) through March (6.5 ± 1.1 nM). The lowest sea surface temperatures (1.3 °C) were also recorded in these same three winter months in New York. Relative mu opiate receptor gene expression was assessed by real time PCR during these seasons. The mRNA expression reached a relative peak during the month of June and was at its lowest in February and March, further demonstrating the direct coupling of morphine with this receptor. We conclude that the temperature an animal is chronically exposed to serves to control cellular processes, i.e., opiate signaling.

**Key Words**: nitric oxide; mu opiate receptor; morphine

### Introduction

Through homeostasis, living organisms maintain their survival in the face of both internally and externally generated stimuli. This balance is constantly challenged and therefore the ability to overcome these normal perturbations is essential to survival and longevity (Chrousos and Gold, 1992; Fricchione and Stefano, 1994). In this regard, subjecting invertebrate animals to temperature changes has been shown to alter the ganglionic monoamine levels as well as affecting functionality of gill cilia in *Mytilus edulis* (Stefano *et al.*, 1977a, 1977b; Stefano and Catapane, 1977b). Additionally, opiate processes respond to various types of stressors in both vertebrates and invertebrates ( Lee and Spector, 1991; Marrazzi *et al.*, 1997; Sonetti *et al.*, 1999; Zhu *et al.*, 2001; Cadet *et al.*, 2002; Guarna *et al.*, 2002).

We have previously demonstrated that endogenous morphine represents the terminal component of a successful stress response and that its actions are generally down regulating immune responses and metabolic rates (Stefano *et al.*, 2000). This down regulation occurs because of coupling of the mu opiate receptor to nitric oxide (NO) release (Cadet *et al.*, 2003). Our group has also demonstrated that opiate receptors and subsequent morphine induced NO release can be profoundly impacted by temperature changes in *M. edulis* (Cadet *et al.*, 2002; Mantione *et al.*, 2003). Furthermore, we have presented molecular evidence on the effect of rapid temperature changes on mu opiate receptor expression and morphine levels in this invertebrate's nervous system (Cadet *et al.*, 2002). In cold stressed organisms, ganglionic mu opiate receptor decreases and morphine levels increase (Cadet *et al.*, 2002). In the present study, we investigated the seasonal variation in ganglionic mu opiate receptor expression in *M. edulis*. In addition, we performed a morphine induced NO release assay to determine the functionality of the morphine signaling system in this model organism.

*Corresponding author:*
Kirk J Mantione
Neuroscience Research Institute
SUNY - College at Old Westbury
P.O. Box 210
Old Westbury, NY 11568-0210, USA
E-mail: KJMantione@sunynri.org

*List of abbreviations:*
Nitric oxide, NO; phosphate buffered saline, PBS; S-nitroso-N-acetyl-DL-penicillamine, SNAP; constitutive nitric oxide synthase, cNOS

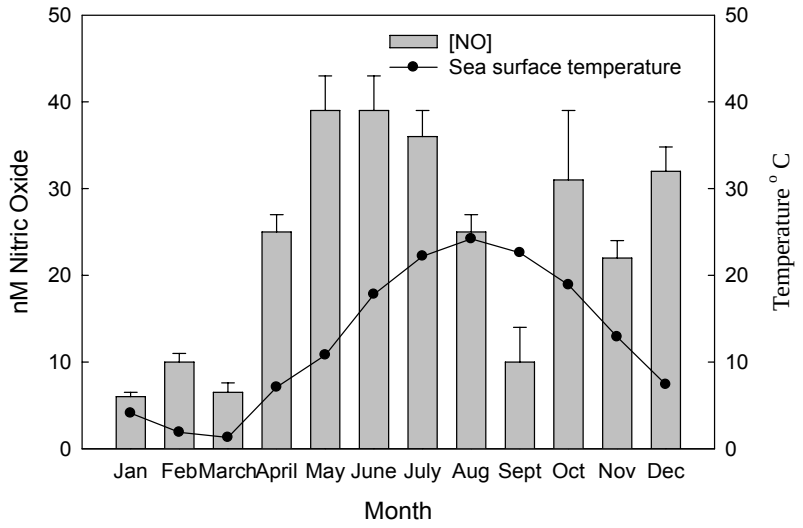


**Fig. 1** Monthly measurements of sea surface temperature and morphine (1 μM) stimulated NO release from *Mytilus edulis* pedal ganglia (20 ganglia per assay, n = 4). Paired t-tests revealed statistically significant differences between January or February or March and May or June or July ($p$ = 0.002).

## Material and Methods

*Animal collection and Nitric Oxide (NO) determination*

*Mytilus edulis* collected from Shinnecock Bay on Long Island were immediately transported to the laboratory in seawater for processing. The ambient seawater temperature was maintained using insulated coolers until dissection of animals. Samples were collected monthly on the 15 day of the month and sea surface temperature was recorded. For NO determination, approximately 20 pedal ganglia were placed in 1 ml phosphate buffered saline (PBS) at room temperature. NO release from the tissues was immediately directly measured using a 200 μm flexible NO-specific amperometric probe (World Precision Instruments, Sarasota, FL) connected to a 4-channel Biostat (ESA, Chelmsford, MA). The system was calibrated daily with S-nitroso-N-acetyl-DL-penicillamine (SNAP) in 0.1 M $Cu^{+2}$. The amperometric probe was allowed to equilibrate in PBS for at least 10 min prior to being transferred to the tube containing the tissue. Morphine-stimulated NO release was evaluated at a final concentration of $10^{-6}$ M. Each experiment was repeated four times (4 groups of 20 ganglia for each month) along with a control (PBS only). A paired student's *t*-test was performed to evaluate differences between selected months.

*Mu opiate receptor expression*

Pedal ganglia (15) were immediately processed after dissection. The ganglia were placed in 1.5 ml tubes and then washed with PBS (Invitrogen, Carlsbad, Ca). Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, Ca). Ganglia were homogenized in 600 μl lysis buffer. The samples were then processed following the manufacturer's instructions. In the final step, the RNA was eluted with 50 μl of RNase-free water. This RNA isolation process was repeated four times for each group of 15 ganglia.

First-strand cDNA synthesis was performed using random primers (Invitrogen, Carlsbad, CA). 1 μg of total RNA was denatured at 95 °C and reverse transcribed at 40 °C for 1 h using Superscript III Rnase H-RT (Invitrogen, Carlsbad, CA). Ten microliters of the RT product was added to the PCR mix containing primers for the mu opiate receptor. Primers and probes specific for the mu-opiate receptor gene (MOR) were designed by the software Primer Express (Applied Biosystems, Foster City, CA). The forward primer was 5'-ATGCCAGTGCTCATCATTAC-3' and the reverse primer sequence was 5'-GATCCTTCGAAGATTCCTGTCCT-3'. The Taqman probe was constructed with the 5'-reporter dye, 6-carboxyfluorescein (FAM), and a 3'-quencher dye, 6-carboxy-tetramethyl-rhodoamine (TAMRA). The probe sequence was 5'-CGCCTCAAGAGTGTCCGCATGCT-3'. The endogenous control gene, β-actin, was used to normalize the RT-PCR. 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 μM reverse and forward primers, 100 μM Taqman probe, 3 μl of RT product and Rnase/DNase-free water was added to the master mix to a final volume of 50 μl. The PCR reaction mixture was 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 then run for 40 cycles at 95 °C for 30 s and 60 °C for 1 min on the Applied Biosystems GeneAmp 7500 Sequence Detection System(SDS). Each PCR was performed in triplicate. The PCR results were analyzed with the GeneAmp 7500 SDS

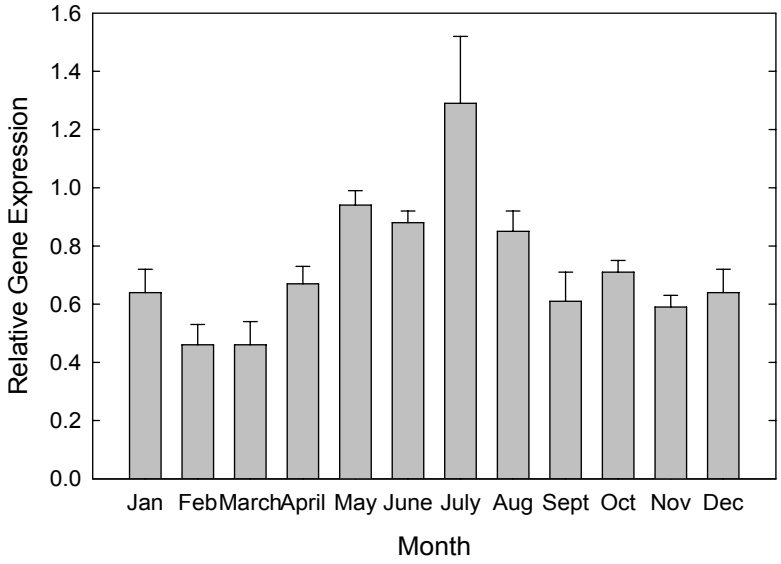


**Fig. 2** Relative mu opiate receptor gene expression in *Mytilus edulis* pedal ganglia determined by real time PCR for each monthly sample (n = 4). Paired *t*-tests revealed statistically significant differences between February or March and May or June or July ($p$ = 0.004).

software (Applied Biosystems). Relative gene expression was calculated using the method of Yoshikawa *et al.* (2001). Standard curves were generated by serial dilution of the June cDNA sample. R values were calculated and used to directly compare the monthly measurements. A paired student's *t*-test was performed to evaluate differences between selected months.

**Results**

Morphine induced NO release measured on a monthly basis for one year revealed a average peak value of 39 ± 4 nM, during the late spring and early summer (Fig. 1). The lowest NO release occurred during the months of January (6.0 ± 0.5nM) through March (6.5 ± 1.1nM) (Fig. 1). The lowest sea surface temperatures (1.3 °C) were also recorded in these same three months (Fig. 1). Student's *t*-tests revealed a statistically significant difference ($p$ = 0.002) between the warm season high NO values and cold season low NO values.

Relative gene expression was assessed by real time PCR. The mRNA expression reached a relative peak during the month of June and was at its lowest in February and March (Fig. 2). Student's *t*-tests revealed a statistically significant difference ($p$ = 0.004) between the warm season high R values (1.2 ± 0.10) and cold season low R values (0.46 ± 0.052).

A regression analysis using mu opiate receptor expression as the independent variable and NO release as the dependent variable showed a correlation between the measurements. The calculated R value was 0.729.

**Discussion**

*M. edulis* neural tissues contain the typical biogenic amines, which includes dopamine (Stefano *et al.*, 1976). Biogenic amines display variations in their ganglionic levels, which corresponds to the seasons and temperature, being high in warmer months and low in the winter months (Stefano *et al.*, 1977a, 1977b; Stefano and Catapane, 1977a, 1977b). Interestingly, this same relationship occurs with opioid peptide expression along with their receptors (Stefano *et al.*, 1980; Stefano and Leung, 1986). As demonstrated in this report, this same phenomenon involves NO release, which is coupled to opiate receptor activation, namely via μ3 ( Liu and Stefano, 1996; Liu *et al.*, 1996; Magazine *et al.*, 1996; Stefano and Scharrer, 1996; Stefano *et al.*, 1996).

The coupling of catecholamine, NO and morphinergic signaling has recently been reviewed (Kream *et al.*, 2009; Stefano and Kream, 2009; Stefano *et al.*, 2009; Zhu and Stefano, 2009). It is important to note that dopamine is a morphine precursor in this animal, which synthesizes endogenous morphine ( Zhu *et al.*, 2005; Kream and Stefano, 2006). The significance of this precursor status of dopamine emanates from previously noted reports in this document showing seasonal and temperature alterations of catecholamine levels, which can now be directly compared to morphinergic phenomena, including the ability of morphine to release constitutively derived NO. We surmise that at colder "winter" temperatures all processes appear to be down regulated, including the homeostasis between an opiate receptor and its ligand levels, as currently demonstrated. This probably occurs because very cold temperatures influence all metabolic processes to decrease their activity levels, including those involved in various survival processes. This homeostasis mechanism occurs in all living organisms, including those with a pathogenic ability. Thus, in both types of organisms processes providing a survival benefit are not required. This

probably extends into the energy metabolic processes found in mitochondrial-like structures where morphine exerts actions (Kream and Stefano, 2009).

In conclusion, the therapeutic value of performing medical operations, maintaining food, etc., at very low temperatures probably arises from the ability of low temperatures to disassociate adaptive cellular processes, allowing for a rather universal down regulation, which depending on the organism has tremendous survival advantage. In the case of *Mytilus*, if bacteria, viruses can't survive or have a decreased infectious characteristic, why have a full functioning immune process with the activation of cytokines and opiate components at low temperature ( Stefano and Scharrer, 1994; Stefano and Salzet, 1999; Stefano *et al.*, 2000, 2008; Stefano and Kream, 2008).

**References**


Cadet P, Mantione KJ, Stefano GB. Molecular identification and functional expression of mu3, a novel alternatively spliced variant of the human mu opiate receptor gene. J. Immunol. 170: 5118-5123, 2003.

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 Res. Mol. Brain Res. 99: 26-33, 2002.

Chrousos GP, Gold PW. The concepts of stress and stress system disorders: Overview of physical and behavioral homeostasis. JAMA 267: 1244-1252, 1992.

Fricchione GL, Stefano GB. The stress response and autoimmunoregulation. Adv. Neuroimmunol. 4: 13-28, 1994.

Guarna M, Bianchi E, Bartolini A, Ghelardini C, Galeotti N, Bracci L, *et al.* Endogenous morphine modulates acute thermonociception in mice. J. Neurochem. 80: 271-277, 2002.

Kream RM, Mantione KJ, Sheehan M, and Stefano GB. Morphine's chemical messenger status in animals. Activitas Nervosa Superior Rediviva 51: 2009.

Kream RM, Stefano GB. De novo biosynthesis of morphine in animal cells: An evidence-based model. Med. Sci. Monit. 12: RA207-RA219, 2006.

Kream RM, Stefano GB. Endogenous morphine and nitric oxide coupled regulation of mitochondrial processes. Med. Sci. Monit. 15: RA263-RA268, 2009.

Lee CS, Spector S. Changes of endogenous morphine and codeine contents in the fasting rat. J. Pharmacol. Exp. Ther. 257: 647-650, 1991.

Liu Y, Shenouda D, Bilfinger TV, Stefano ML, Magazine HI, and Stefano GB. Morphine stimulates nitric oxide release from invertebrate microglia. Brain Res. 722: 125-131, 1996.

Liu Y, Stefano GB. HIV Gp120 inhibits human and invertebrate immunocyte phagocytosis. Chinese J. Immunol. 12: 139-142, 1996.

Magazine HI, Liu Y, Bilfinger TV, Fricchione GL, Stefano GB. Morphine-induced conformational changes in human monocytes,granulocytes, and endothelial cells and in invertebrate immunocytes and microglia are mediated by nitric oxide. J. Immunol. 156: 4845-4850, 1996.

Mantione K, Hong R, Im R, Nam JH, Simon M, Cadet P, *et al*. Effects of cold stress on morphine-induced nitric oxide production and mu-opiate receptor gene expression in Mytilus edulis pedal ganglia. Neuroendocrinol. Lett. 24: 68-72, 2003.

Marrazzi MA, Luby ED, Kinzie J, Munjal ID, Spector S. Endogenous codeine and morphine in anorexia and bulimia nervosa. Life Sci. 60: 1741-1747, 1997.

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. 835: 137-147, 1999.

Stefano GB, Catapane EJ. Seasonal monoamine changes in the central nervous system of *Mytilus edulis*. Experientia 33: 1341-1342, 1977a.

Stefano GB, Catapane EJ. The effects of temperature acclimation on monoamine metabolism. J. Pharmacol. Exp. Ther. 203: 449-546, 1977b.

Stefano GB, Catapane EJ, Aiello E. Dopaminergic agents: Influence on serotonin in the molluscan nervous system. Science 194: 539-541, 1976.

Stefano GB, Catapane EJ, Stefano JM. Temperature dependent ciliary rhythmicity in *Mytilus edulis* and the effects of monoaminergic agents on its manifestation. Biol. Bull. 153: 618-629, 1977a.

Stefano GB, Goumon Y, Casares F, Cadet P, Fricchione GL, Rialas C, *et al.* Endogenous morphine. Trends Neurosci. 9: 436-442, 2000.

Stefano GB, Hiripi L, Catapane EJ. The effects of short and long term temperature stress on serotonin, dopamine and norepinephrine concentrations in molluscan ganglia. J. Thermal Biol. 3: 79-83, 1977b.

Stefano GB, Kream R. Endogenous opiates, opioids, and immune function: evolutionary brokerage of defensive behaviors. Semin. Cancer Biol. 18: 190-198, 2008.

Stefano GB, Kream RM. Dopamine, morphine, and nitric oxide: an evolutionary signaling triad. CNS Neurosci. Ther. 2009 [Epub ahead of print].

Stefano GB, Kream RM, Mantione KJ, Sheehan M, Cadet P, Zhu W, *et al.* Endogenous morphine/nitric oxide-coupled regulation of cellular physiology and gene expression: implications for cancer biology. Semin. Cancer Biol. 18: 199-210, 2008.

Stefano GB, Kream RM, Zukin RS, Catapane EJ. Seasonal variation of stereospecific enkephalin binding and dopamine responsiveness in *Mytilus edulis* pedal ganglia. In: Rozsa KS (ed), Neurotransmitters in Invertebrates, Pergamon Press, London, pp 453-459, 1980.

Stefano GB, Leung MK. Opioid aging and seasonal variations in invertebrate ganglia: Evidence for an opioid compensatory mechanism. In:

Stefano GB (ed), Comparative opioid and related neuropeptide mechanisms, CRC Press, Inc., Boca Raton, pp 233-242, 1986.

Stefano GB, Salzet M. Invertebrate opioid precursors: Evolutionary conservation and the significance of enzymatic processing. Int. Rev. Cytol. 187: 261-286, 1999.

Stefano GB, Salzet M, Ottaviani E. Neuroimmune chemical messengers and their conservation during evolution. In: Rinkevich B, Matranga V. (eds), Stem cells in marine organisms, Springer, Netherlands, pp 139-164, 2009.

Stefano GB, Scharrer B. Endogenous morphine and related opiates, a new class of chemical messengers. Adv. Neuroimmunol. 4: 57-68, 1994.

Stefano GB, Scharrer B. The presence of the μ3 opiate receptor in invertebrate neural tissues. Comp. Biochem. Physiol. 113C: 369-373, 1996.

Stefano GB, Scharrer B, Smith EM, Hughes TK, Magazine HI, Bilfinger TV, *et al.* Opioid and opiate immunoregulatory processes. Crit. Rev. Immunol. 16: 109-144, 1996.

Yoshikawa M, Nakayama H, Ueno S, Nishimine N, Furuya H. Novel quantitative reverse-transcribed polymerase chain reaction of mu opioid receptor mRNA level. Brain Res. Brain Res. Protoc. 7: 147-153, 2001.

Zhu W, Baggerman G, Goumon Y, Casares F, Brownawell B, and 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. 88: 155-160, 2001.

Zhu W, Mantione KJ, Shen L, Cadet P, Esch T, Goumon Y, *et al*. Tyrosine and tyramine increase endogenous ganglionic morphine and dopamine levels *in vitro* and *in vivo*: CYP2D6 and tyrosine hydroxylase modulation demonstrates a dopamine coupling. Med. Sci. Monit. 11: BR397-BR404, 2005.

Zhu W, Stefano GB. Comparative aspects of endogenous morphine synthesis and signaling in animals. Ann. NY Acad. Sci. 1163: 330-339, 2009.