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ORIGINAL ARTICLE

Nitric oxide modulates the physiological control of ciliary activity in the marine mussel *Mytilus edulis* via morphine: Novel mu opiate receptor splice variants

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Abstract

OBJECTIVES: The study sought to determine how dopamine controls ganglionic processes involved with modulating lateral cilia beating via the peripheral branchial nerve.

Methods: The lateral cilia found on the gill filaments exhibit metachronal ciliary beating determined stroboscopically. Novel opiate receptors were determined pharmacologically and demonstrated by RT-PCR and sequence analysis of total RNA from *Mytilus edulis* visceral ganglia.

RESULTS: Dopamine applied to the visceral ganglion inhibits the activity of lateral cilia in a concentration and haloperidol sensitive manner. Morphine or DAMGO significantly enhances ciliary beating in a naloxone sensitive manner, whereas L-NAME, a nitric oxide synthase inhibitor, only antagonized morphine's action. SNAP, a nitric oxide donor, also enhanced lateral ciliary beating rates. Supporting the observation, i.e., morphine sensitive nitric oxide enhancement of ciliary beating and DAMGO insensitive, that two different mu opiate receptors are present in this tissue, a 602 bp fragment of the human $\mu 3$ opiate receptor and a 935 bp fragment, designated $\mu 4$ have been demonstrated.

CONCLUSIONS: The lateral epithelium of the gill is innervated by serotonergic, cilioexcitatory neurons and dopaminergic, cilioinhibitory neurons, originating in the visceral ganglion. This data supports previous reports that demonstrate inhibiting ganglionic dopamine release allows the serotonin signals to prevail uncontrolled, enhancing ciliary rates. Supporting the observation that two different mu opiate receptors are present in this tissue, evidence is presented that identifies a 602 bp fragment of the human $\mu 3$ opiate receptor and a 935 bp fragment, designated $\mu 4$. Overall, the data strongly suggests that the two alternatively spliced mu opiate receptors may be involved in the physiological regulation of lateral ciliary activity in the visceral ganglia via dopamine and nitric oxide.

Abbreviations:

RT-PCR	reverse transcriptase polymerase chain reaction
SNAP	S-nitroso-N-acetyl-DL-penicillamine
L-NAME	N omega-nitro-L-arginine methyl ester
DAMGO	(Tyr-D-Ala^2, Gly-N-Me-Phe^4, Gly(ol)^5)-enkephalin
NO	nitric oxide

Introduction

Mytilus edulis visceral ganglia contains dopamine, which is involved with inhibiting ciliary activity in the lateral epithelium of the gill of *M. edulis* via the branchial nerve[1–3]. The lateral gill cilia are stimulated by serotonin and inhibited both centrally and peripherally by dopamine [1–6]. In the past we demonstrated that the central nervous system dopaminergic processes are modulated by opioid and opiate compounds[1–6]. These compounds inhibit ganglionic dopamine release, removing the inhibitory influence of dopaminergic transmission as noted by significantly higher lateral ciliary beating rates [1–6].

The significance of these early observations coupled with recent developments necessitates a re-examination of this physiological process. Importantly, it has been demonstrated that *M. edulis* nervous tissues contain opioid peptides [7,8] as well as opiate alkaloids (e.g., morphine, morphine 6 glucuronide and morphine 3 glucuronide) and their precursors [9–12]. The ganglia also contain opiate receptors [13,14], including an opiate receptor that is opiate alkaloid selective and opioid peptide insensitive (μ3) [9,15], supporting the presence of endogenous opiate alkaloids, i.e., morphinergic signaling. Additionally, the ganglia of *M. edulis*, which contains the μ3 opiate receptor subtype, express a mu opiate receptor genetic material determined by RT-PCR [16,17]. This mu opiate receptor subtype in *M. edulis* ganglia is coupled to constitutive nitric oxide (NO) release [15,18–21].

These studies, including ones that demonstrate neural dopamine release is inhibited by opiate alkaloids, suggest that the inhibition of ganglionic dopamine release may be under the physiological control of NO [19,22]. In the present report this hypothesis is examined. The report shows that via constitutive nitric oxide synthase (cNOS) derived NO morphine initiates higher ciliary beating rates, allowing serotonergic signals to operate unchecked.

Materials and methods

Specimens of subtidal *Mytilus edulis* (2–4 cm long) were collected from Long Island Sound at Northport, New York. Healthy animals were identified as those that closed their valves when the mantle was touched. The animals were used within a 24-hr period from the time they were harvested.

Ciliary Studies:

The animal was removed from the valves by cutting attachments of mantle and muscle and placed in artificial seawater (Aquarium Systems sea salts) at pH 7.8 and a specific gravity of 1.024, similar to the average values for Long Island Sound water. The mantle, foot, and byssus were cut off and the animal was positioned on its dorsal surface with the gills extended to the sides. To improve microscopic observation of ciliary activity in the gill, the internal (medial) lamina was cut off and the animal tilted to that side to allow the gill to float relatively flat on the side to be observed [4]. The animal was held in place by pinning through the posterior adductor muscle and between the anterior byssus retractors into a strip of rubber that had been glued to the bottom of the dish with Duco cement. Drug solutions that were added to the visceral ganglion were confined to that area by the presence of a rubber ring, also glued to the bottom and coated with silicon grease. The extreme anterior and posterior ends of the gill that came into contact with medium within the ring were removed, care being taken not to damage the dorsal attachment in which the branchial nerve runs. The preparation was washed liberally with artificial seawater to remove cellular debris and excess mucus and allowed to equilibrate for 1 hr before beginning the experiment. The pedal ganglia were not used or disturbed. The visceral commissure was cut so that the preparation was essentially intact with respect to the connections between ganglia and gill on each side.

The ciliary beating rate was determined by stroboscopic synchronization, using a General Radio Strobotac in place of the microscope lamp and observing the cilia with a 10 x objective [4]. Only lateral cilia that were beating metachronally in the range of 800 to 1,000 beats / min were examined. Each value presented was obtained from 10 organisms (N) and the mean measurement was determined from 10 different filaments from each organism. Ciliary measurements were made in quick succession and averaged to obtain one value for that preparation. Statistical comparison employed the one-tailed Student t test. All drugs and chemical were purchased from Sigma (St. Louis, Mo.).

Molecular Biology:

Excised visceral ganglia were homogenized in Tri-Reagent (Molecular Research Center, INC, Cincinnati, OH) using a polytron homogenizer. The homogenates were stored at room temperature for 5 minutes to allow complete dissociation of nucleoprotein. 0.1 ml of 1-bromo-3-chloropropane (BCP) per 1 ml of Tri-Reagent was added to the homogenates. The samples were vortexed vigorously for 15 seconds and then stored at room temperature for 7 min. After centrifugation of the samples for 15 min at 12,000 g, the aqueous phase was transferred to a fresh tube. RNA was isopropanol precipitated, washed with 75% ethanol, centrifuged and then air-dried. The pellet was then resuspended in Rnase-free water. RNA was analyzed spectrophotometrically and on a 1% agarose gel for purity and concentration.

Reverse Transcription (RT)-Coupled Polymerase Chain Reaction (PCR) of Total RNA:

First strand cDNA synthesis was performed using random hexamers (GIBCO, BRL, Gaithersburg, MD). 3 μg of total RNA isolated from visceral ganglia tissues

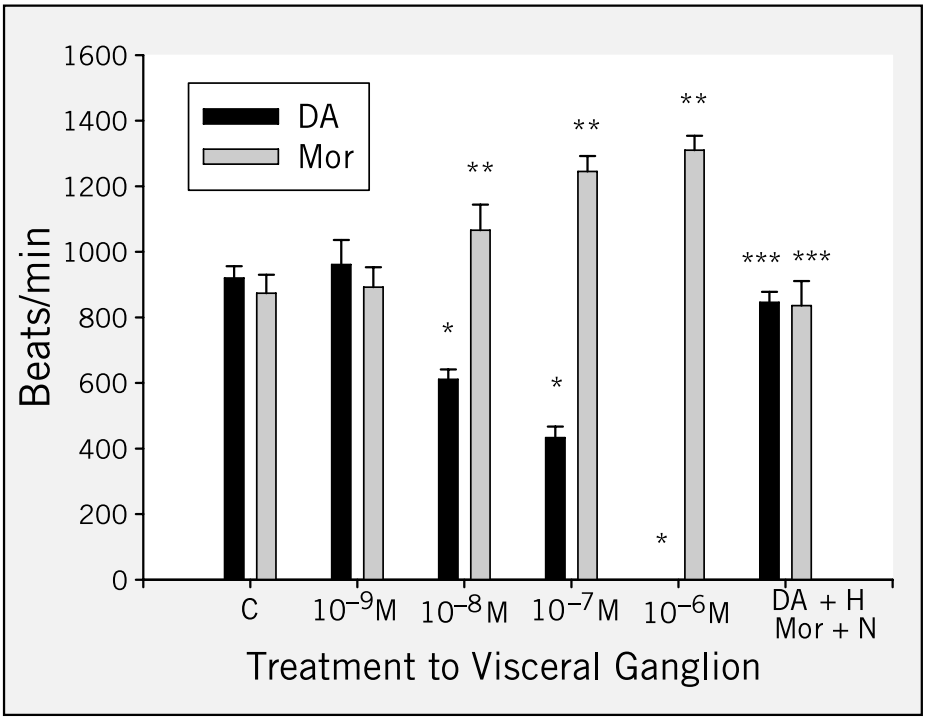

Figure 1. Exposure of visceral ganglion to either dopamine (DA) or morphine (Mor) and its effect on peripheral gill lateral ciliary activity. Activity is expressed as the mean ± SE for the rate of beating determined by stroboscopic synchronization. For the DA prep N = 9 and for the Mor prep N = 8. When either haloperidol (H; 10^{-7} M) or naloxone (N; 10^{-6} M were applied it was done fine prior to DA (10^{-7} M or Mor (10^{-6} M) application. The gill was bathed in artificial seawater at pH 7.8 and 22°C. Statistical Significance was < P 0.01 when compared to corresponding dose of other drug (*) and control (**) or (***) when compared to drug effect at corresponding dose without inhibitor present.

were denatured at 95 °C and reverse transcribed at 42 °C for 1 hr using Superscript II Rnase H-RT (GIBCO BRL, MD). Seven microliters of the RT product was added to the PCR mix containing specific primers for the μ3 opioid receptor gene and Taq DNA polymerase (GIBCO, BRL, MD). The PCR reaction was denatured at 95 °C for 5 minutes followed by 40 cycles at 95 °C for 30s, 53 °C for 30s, and 72 °C for 1 min, and then an extension step cycle at 72 °C for 10 min. PCR products were analyzed on a 2% agarose gel (SIGMA) ethidium bromide stained with the UVP gel documentation system. The μ3-specific primers used in the PCR reactions are: Forward- 5'GGTACTGGGAAAACCTGCTGAAGATCTGTG3'; Reverse- CATCCATGACCACAGTGGGCAAGGCAC, and at map position. Part of this segment of the gene encodes the third extracellular loop of the receptor which is important for μ agonist selectivity. Bands of interest were excised, purified with the StrataPrep purification kit (Stratagene), and then ligated into the PCR-Script Amp SK (+) vector (Stratagene) and transformed into XL10-Gold kan competent cells (Stratagene).

Band density readings were determined by Gel Pro Density Analysis (Media Cybemetrics, Inc., MD). Pharmacological agents were purchased from Sigma (St. Louis, MO).

Sequencing of the cloned PCR Products:

Purified plasmid DNA containing the PCR fragments were sequenced with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems-Perkin-Elmer) and the ABI Prisms 310 Genetic Analyzer and ABI 377 DNA sequencer.

Results

Mussels prepared as described above with the central nervous system still innervating the gill evidenced considerable ciliary activity. A majority of the lateral cilia were active and were beating metachronally at a rate of 967 + 68 beats/min left undisturbed, this activity continued relatively unchanged for several hours. Experiments were begun about 1 hr after setting up the preparation.

The administration of dopamine to the visceral ganglion, as previously reported [2,4], caused a dose-dependent decrease in the rate of beating, a phenomenon that was blocked by the dopamine receptor antagonist haloperidol (Figure 1). Exposure of the visceral ganglia to either morphine, in a concentration dependent manner, or DAMGO (10^{-6} M), an opioid peptide, resulted in a statistical significant higher lateral ciliary beating rate that was antagonized by the opiate receptor blocker naloxone (10^{-6} M) (Figure 1, Table 1).

L-NAME, a NOS inhibitor, exposure to the visceral ganglia followed by morphine blocked the increase in lateral ciliary rates, suggesting that morphine is exerting its action via NO. DAMGO induced lateral ciliary beat increases was not antagonized by L-NAME, suggesting that another regulatory mechanism was operating that was NO insensitive (Table 1, 2). Naloxone and L- NAME administered alone had no visible effect on peripheral lateral ciliary activity. However, adding a NO donor, SNAP, to the visceral ganglia resulted in higher beating rates, demonstrating that NO was involved in regulating beating rates via ganglionic processes (Table 3).

Given the presence of a NO sensitive morphinergic process and a NO insensitive DAMGO mechansism affecting ciliary activity regulation in the visceral ganglia the tissue was examined for opiate receptor

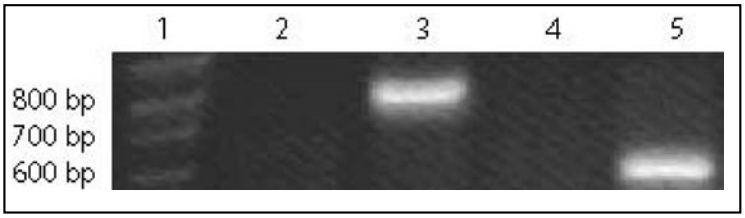

Figure 2. Identification of two novel mu opioid receptor variants in *Mytilus edulis* visceral ganglia. cDNA made from visceral ganglia total RNA was amplified with primers specific for the mu3 variant. Lanes: 1, 100 bp DNA marker; 2, negative reagent control; 3, novel mu 935 bp fragment; 4, empty; 5, expected mu3 602 bp fragment.

Table I. The Effect on Lateral Cilia of Applying DAMGO and **Morphine to** the Visceral Ganglion After Having Applied L-NAME

Agent applied to visceral ganglion	N	Rate (beats/min)[a]
None	7	936 ± 62
Morphine (10^{-7} M)	7	1,187 ± 38*
DAMGO (10^{-6} M)	8	1, 213 ± 53*
DAMGO (10^{-6} M) + Naloxone (10^{-6} M)	7	793 ± 75
Morphine (10^{-7} M) + L-NAME (10^{-4} M)	7	883 ± 62
DAMGO (10^{-6} M) + L-NAME (10^{-4} M)	7	1,185 ± 59*

[a] Before testing, 91 ± 9% of the filaments were active. Morphine was added to the visceral ganglion of each, following **L-NAME (10^{-4} M)** exposure 5 min earlier. Ciliary activity was measured after 20 min following morphine exposure and is expressed as t he mean ± SE.
* $P < 0.01$ for comparison of this value to the control (top line).

Table II. The Effect on Lateral Cilia of Applying Dopamine and other agents to the visceral ganglion

Concentration applied (M)	N	Rate (beats/min)[a]
None	10	920 ± 36
DA 10^{-7} M + Haloperidol (10^{-7} M)	10	1178 ± 57
DA 10^{-7} M + Morphine (10^{-7} M)	10	397 ± 42
DA 10^{-7} M + DAMGO (10^{-6} M)	10	433 ± 34
DA 10^{-7} M + L-NAME (10^{-7} M)	10	403 ± 41
DA 10^{-7} M + SNAP (10^{-6} M)	10	476 ± 51

[a] Activity is expressed as the mean ± SE for the percentage of filaments with active lateral cilia and for the rate of beating determined by stroboscopic synchronization after exposure of the ganglion to dopamine for 20 min. The gill was bathed in artificial seawater at pH 7.8 and 22°C.

Table III. The Effect on Lateral Cilia of Naloxone, L-NAME and SNAP Applied to the Ganglion of Preparations Evidencing High Endogenous Rates of Beating

Treatment	N	Ciliary rate (beats/min)[a]	
		Initial	After treatment
Artificial seawater	8	985 ± 52	947 ± 62
Naloxone(10^{-6} M)	7	966 ± 61	1007 ± 68
L-NAME (10^{-4} M)	7	971 ± 54	1009 ± 69
SNAP (10^{-6} M)	8	993 ± 65	1,287 ± 46*

[a] The initial rate was measured first, followed by drug exposed ganglia after 20 min.
* $P < 0.01$ for comparison with pre-exposed.

expression. To determine if μ3 opiate receptor is expressed in excised visceral ganglia the tissue was examined with specific primers designed to amplify a 602 bp fragment of the coding region of the μ3 opiate receptor [23]. RT-PCR and sequence analysis revealed two bands at a molecular weight of 602 (μ3, expected size) and 935 base pairs (designated, μ4) as observed on the agarose gel stained with ethidium bromide (Fig. 2). Sequence analysis of the PCR products demonstrate that the μ4 fragment exhibits 98% sequence identity with the human μ3 opiate receptor (Figs. 3,4, and 5). However, they were different downstream of the conserved region where μ4 contains 333 additional sequences.

Discussion

The data demonstrate that the application of DA to the visceral ganglion of M. edulis initiates processes leading to cilioinhibitory of the lateral gill cilia that can be blocked by the DA antagonist haloperidol, demonstrating a receptor mediated ganglionic mechanism. Morphine and DAMGO application to the visceral ganglia up regulate lateral gill ciliary activity in a concentration and naloxone sensitive manner. Exposure of the visceral ganglion to L-NAME, a NOS inhibitor, followed by morphine blocked the cilioexcitatory effect of morphine. However, L-NAME pretreatment did not block the cilioexcitatory effect of DAMGO,

5′ggtactgggaaaacctgctgaagatctgtgttttcatcttcgccttcattatgccagtgctcatcattaccgtgtgcta
tggactgatgatcttgcgcctcaagagtgtccgcatgctctctggctccaaagaaaaggacaggaatcttcgaaggatcac
caggatggtgctggtggtggtggctgtgttcatcgtctgctggactcccattcacatttacgtcatcattaaagccttggt
tacaatcccagaaactacgttccagactgtttcttggcacttctgcattgctctaggttacacaaacagctgcctcaaccc
agtcctttatgcatttctggatgaaaacttcaaacgatgcttcagagagttctgtatcccaacctcttccaacattgagca
acaaaactccactcgaattcgtcagaacactagagaccacccctccacggccaatacagtggatagaactaatcat**cagaa
ttattatataattcatagatgttctgcaataccctcttatttctcaaaagccagtcttgctctggtttctggattaaaga
gagagggtgagtgccttgcccactgtggtcatggatg-3′**

Figure 3. Sequence analysis of a 602 bp fragment of the mu3 gene in *Mytilus edulis* visceral ganglia. PCR fragment of the RT-PCR of visceral ganglia was excised and sequence analysis was performed. The underlined sequences represent MOR1 region at map position 895–1373, and Mu3 at map position 403–881. The bold letters represent a segment of the mu3 transcript at map position 882–1005.

5′ggtactgggaaaacctgctgaagatctgtgttttcatcttcgccttcattatgccagtgctcatcattaccgtgtgcta
tggactgatgatcttgcgcctcaagagtgtccgcatgctctctggctccaaagaaaaggacaggaatcttcgaaggatcac
caggatggtgctggtggtggtggctgtgttcatcgtctgctggactcccattcacatttacgtcatcattaaagccttggt
tacaatcccagaaactacgttccagactgtttcttggcacttctgcattgctctaggttacacaaacagctgcctcaaccc
agtcctttatgcatttctggatgaaaacttcaaacgatgcttcagagagttctgtatcccaacctcttccaacattgagca
acaaaactccactcgaattcgtcagaacactagagaccacccctccacggccaatacagtggatagaactaatcat**caggt
acgcagtctctagaattaggtatatctactggggatgacataaaaattataaggctttgtgctaaactaggagtttaatcc
attatagaggatgagaatggagggaagggaaagcaaattgtggtttaagggttaaagaagaggtttgtatataaactgggg
tcctttaaatttgcctgtacatattcattaaggtttaaggatccccaatgggnaaaaccatggaacttttcaaaatacctt
ttttatggcctttacttttatgcaaaatttatgactttagcacattatagaaataattctgatctagaatccttttcattt
tccc**cagaattattatataattcatagatgttctgcaataccctcttatttctcaaaagccagtcttgctctggtttctg
gattaaagagagagggtgagtgccttgcccactgtggtcatggatg**-3′**

Figure 4. Sequence analysis of a novel alternatively spliced 935 bp fragment of the MOR transcript in *Mytilus edulis* visceral ganglia referred to mu4. PCR fragment of the RT-PCR product of visceral ganglia was excised and sequence analysis was performed. The underlined sequences represent MOR1 region at map position 895-1373, and Mu3 at map position 403-881. The bold letters represent the new sequences of the mu4 transcript. The other sequences represent a segment of the mu3 transcript at map position 882-1005.

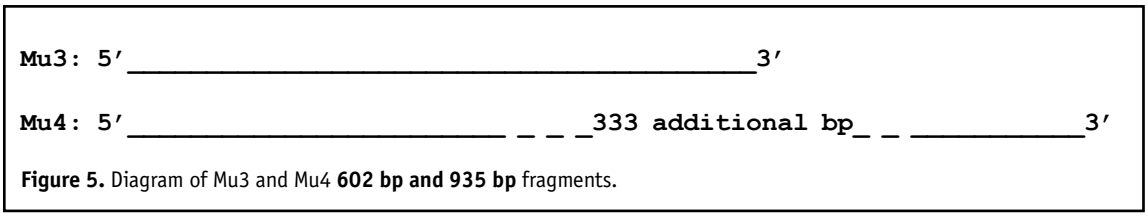

Figure 5. Diagram of Mu3 and Mu4 **602 bp and 935 bp** fragments.

suggesting the presence of another opioid mediated process in producing ganglionic derived peripheral cilioexcitation. Exposure of the visceral ganglion to SNAP, a NO donor, resulted in peripheral cilioexcitation, supporting the view that morphine was acting via a NO mediated mechanism. Additionally, two mu opiate receptor subtypes were found to be expressed in the visceral ganglion. Interestingly, one was shorter than the other, i.e., $\mu 3$. Taken together, the most plausible mechanism for these results is that the pathway through the ganglion involves dopaminergic synapses, which are regulated by opioid peptides and by, yet another mechanism, opiate alkaloids via NO that inhibit dopamine transmission. Furthermore, given earlier reports that demonstrate opioid peptides and opiate alkaloids inhibit ganglionic presynaptic DA release and not presynaptic serotonin release, I surmise that inhibiting DA release allows serotonin release to go unchecked, since they are in balance controlling ciliary beating, resulting in higher peripheral ciliary beating rates [2,4,24].

There is ample biochemical and histochemical evidence for dopamine-opioid interactions in the central nervous system of *M. edulis*. Morphine, DAMGO, etorphine, and β-endorphin block the release of dopamine but not serotonin from the pedal ganglion in response to 50mM KCl [24]. The release was blocked by naloxone. In the present study, comparable concentrations of opioid and opiate signal molecules elicited measurable changes in ciliary activity, suggesting that similar mechanisms were involved.

Regarding the variation of mu opiate receptor subtypes presence in invertebrate ganglia there is the following information. Displacement of ^{3}H-dihydromorphine (DHM) by opioid and non-opioid ligands in *M. edulis* ganglia membrane suspensions revealed the following: a) delta ligands (DAMGO, Deltorphin, Met-enkepahlin. DADLE and DPDPE) were ineffec-

tive; b.) mu opioid peptides (endomorphin-1 &-2 and DAMGO) were ineffective; c.) kappa agonists were also ineffective; whereas d.) opiate alkaloids, morphine, dihydromorphine displaced at 28 $\pm$ 3.3 and 30 nM $\pm$ 41., respectively [9]. These experiments demonstrate that the opioid peptide insensitive and opiate alkaloid selective mu opiate receptor, designated μ3, is present in *M. edulis* pedal ganglia membrane homogenates [25]. This report now extends this observation to the visceral ganglion where this receptor appears to modulate the physiological responsivity of the lateral gill cilia.

Regarding the two mu opiate receptor subtypes expressed in *M. edulis* visceral ganglia, the following information in mammalian tissues becomes important. Human μ receptor gene expression has found a μ1 specific mRNA (see [26]). These data were obtained by using a set of primers which amplified a small region of the third extracellular loop of the 7 transmembrane domain of the human brain opiate receptor, on the same exon as the genomic clone of human μ receptor (see [27]) and therefore any variants could not be demonstrated by PCR with this primer pair alone. Thus, a nested PCR method, in which the outer primer pair was based on the 5' and 3' non coding region and amplified a 1447 bp long fragment, which included two intron/exon boundaries identified in the human genomic clone was used for this study. With this strategy a putative splice variant based on the difference in base pair length of the amplified products was found in this study. In the second round amplification, using the same primers as Chuang et al. [26], a 441 bp fragment with 100% homology to the published μ1 receptor was detected [28]**.** This demonstrates that with this primer the bands identified were identical, indicating that they cannot be used to determine the presence of splice variants.

Previous studies have demonstrated that opiate alkaloids, but not opioid peptides, release NO via the μ3 receptor [18,29,30]. Taken together, these results strongly suggest that the μ3-like receptor we identified is indeed the opiate receptor subtype designated μ3 [9]. Our binding studies indicate that this μ receptor is opiate alkaloid selective and opioid peptide insensitive. It is important to note that μ3 receptor is coupled to NO release in the tissues that express this receptor, including invertebrates, see [25,31]. The tentative identification of the other, bigger μ4 opiate receptor subtype may actually be the one that is NO insensitive and interacting via opioid peptides. Overall, similar to *M. edulis* pedal ganglia, it appears that μ3 opiate receptor is playing a role in regulating ciliary activity physiology in the visceral ganglia. Additionally, the same is true for μ4, suggesting that each becomes operational based on variations of stimulation.

Acknowledgments

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