Mytilus edulis opiate processes

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Submitted: 2 November 2009
Accepted: 19 November 2009

Arch Med Sci 2009; 5, 4: 618-625

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Abstract

Mytilus edulis, a marine bivalve mollusk, exists in many coastal marine environments. It also serves as a common food with a high level of commercialization. Therefore their overall health should be of great concern and relevance to the human population. Studies in the past 30 years have demonstrated considerable conservation in opiate and opioid peptide processes between invertebrate and humans, including the chemical messengers themselves, as well as their respective receptors. These same messengers may serve as an indication of their over all health. Known evolutionary relationships with all animals and the presence of similar or identical processes, specifically in mollusks, make *Mytilus edulis* an ideal organism for the study of these processes as a model system. In this review, we mainly focus on the discovery of opiate signaling mechanisms in the mollusk *Mytilus edulis*, since they transcend pain in function.

Key words: opioid peptides, morphine, nitric oxide, opiate receptors, mu3 opiate receptor, immune, central nervous system, microglia, delta opioid receptors, methionine enkephalin.

Opioid peptides

Previous studies demonstrated the presence of Met-enkephalin, Leu-enkephalin and Met-enkephalin-Arg^6-Phe^7 in the nervous system of *Mytilus edulis* along with highly selective opioid receptors (see Leung and Stefano, 1987 [1-3]). The demonstration of Met-enkephalin-like material in the hemolymph and immunocytes of *M. edulis* was carried out by means of high pressure liquid chromatography (HPLC) and radioimmuno-assay (RIA) [4-8].

In addition it was determined, in part, how neuropeptides in the hemolymph are degraded or their action terminated, explaining the difficulty in obtaining them in the first place. In mammals CD10 (CALLA, common acute lymphoblastic leukemia antigen/neutral endopeptidase 24.11; NEP, "enkephalinase") hydrolyzes a number of naturally occurring peptides including the endogenous opioid pentapeptides Met- and Leu-enkephalin [9, 10]. This enzyme in the mammalian brain has been termed "enkephalinase". In invertebrate organisms, such as the mollusk *M. edulis*, Met-enkephalin triggers inflammatory responses by inducing morphological changes, directed migration, and aggregation of hemocytes [4-9]. *M. edulis* hemocytes express a CD10/NEP related structure and abrogation of CD10/NEP enzymatic activity reduces the amount of Met-enkephalin required for hemocyte activation by five orders of magnitude [9]. Human

CD10+ polymorphonuclear leukocytes are similarly responsive. Thus, a precise mammalian-like mechanism for degrading peptides in the invertebrate immune/defense system is present [10].

Additionally, Met-enkephalin is degraded by other peptidases present in the hemolymph fluid and hemocyte membrane suspension of *M. edulis* [11]. Degradation of Met-enkephalin is rapid in the fluid and slower in the membrane preparation [12]. Aminopeptidase activity is bestatin sensitive in hemocyte membrane and highest in the fluid of the hemolymph which appears to have a component which is insensitive to inhibition [13-16]. Angiotensin converting enzyme activity is found only in the fluid of the hemolymph. Carboxypeptidase and NEP are membrane bound and the former appears to predominate. Thus, invertebrate immune tissues contain the machinery to digest, as well as process, peptidergic signal molecules.

Importantly, opioid peptides and their precursors have been identified in invertebrate tissues and they contain either identical or close to identical sequences as found in mammals [17]. A mammalian-like proenkephalin peptide in invertebrates was surmised from studies demonstrating the presence of smaller peptides that are found within this precursor. The opioid peptides Met- and Leu-enkephalin, as well as Met-enkephalin-Arg-Phe, were isolated and sequenced from *M. edulis* neural tissues [18, 19]. Recently, a proenkephalin-like peptide was identified in the immunocytes of *M. edulis* [20, 21]. *M. edulis* proenkephalin exhibits a high sequence identity with human and guinea pig proenkephalin (39 and 50%, respectively). This proenkephalin contains Met- and Leu-enkephalin in a ratio of 3 : 1 for *M. edulis*. It also possesses Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe that are flanked by dibasic amino acid residues, demonstrating cleavage sites. Furthermore, using both sequence comparison and a specific antiserum raised against bovine proenkephalin A (209-237), the enkelytin peptide, FAEPLPSEEEGESYSKEVPEMEKRYGGFM, was identified as proenkephalin and it exhibited a sequence identity of 98% with mammalian enkelytin [22].

M. edulis also contains a prodyn molecule in its hemocytes [20, 23]. *M. edulis* prodyn contains, αneo-endorphin, dynorphin-A and dynorphin-B at the C-terminus, exhibiting 100, 70.5, and 85% sequence identity with the rat prodyn-derived counterparts, respectively [20, 23]. The number of Leu-enkephalins in this precursor is identical to that found in vertebrates. *M. edulis* prodyn is distinguished from that described earlier in leeches in that the N-terminus is longer. Additionally, the presence of an orphanin FQ-like peptide, exhibiting 50% sequence homology with that found in mammals, was found [23].

Briefly, besides opioid processes catecholaminergic processes are also found in *M. edulis* tissues, resembling there invertebrate counterparts [24]. Additionally, estrogen signaling exists in these tissues as well, again resembling those processes found in mammals [25-28].

Receptors

The effects of the naturally occurring opioid neuropeptide deltorphin I, isolated from amphibian skin, on immunoregulatory activities were studied in invertebrates. (D-Ala^2) Deltorphin I binding and pharmacological studies have provided evidence for a special subtype of delta opioid receptor, δ_2, on human and invertebrate immune cells [29-31]. The high potency of this compound parallels that of Met-enkephalin previously demonstrated in vertebrate plasma and invertebrate hemolymph. In cold saturation experiments a single high affinity binding site was revealed for *M. edulis* immunocyte membrane homogenates. The ability of a variety of other opioids to displace specifically bound ^{3}H-DAMA also was investigated. The opioid peptides were effective in the following decreasing order: deltorphin I = DAMA > [Met]enkephalin> DADLE > DPDPE. By contrast, the mu and kappa ligands DAGO and dynorphin 1-17 were quite weak [31]. The results obtained with deltorphin I support the view that the special role played by endogenous Met-enkephalin in immunobiological activities of vertebrates and invertebrates is mediated by a special subtype of delta opioid receptor, δ_2. This site is also sensitive to the inhibitory influence of naltrindole further documenting its identity as δ_2 [32, 33] and its role in immunocyte activation. It is of interest to note that this new receptor subtype was first demonstrated in an invertebrate and then found on human granulocytes [31]. Thus, opioid peptides appear to have an immunocyte stimulatory action (induce chemotaxis) in both groups of animals via this novel opioid receptor subtype [32, 33].

Opiate alkaloids

Morphine-like and codeine-like substances were demonstrated in the pedal ganglia, hemolymph and mantle tissues of the mollusk *M. edulis* [34]. The pharmacological activities of the endogenous morphine-like material resemble those of authentic morphine. Both substances counteracted, in a dose dependent manner, the stimulatory effect of tumor necrosis factor (TNF)-α or interleukin (IL)-1α on human monocytes and *M. edulis* immunocytes. The immunosuppressive effect of this opiate material expresses itself in a lowering of chemotactic activity, cellular velocity and adherence, as well as making active immunocytes inactive, i.e., rounded [34, 35]. Codeine mimics the activity of authentic morphine,

but only at much higher concentrations. These pharmacological effects of morphine on immunocytes are consistent with those actions attributed to opiates reported in the literature [32]. Indeed, it has been surmised that morphinergic transmission may regulate the down regulation of immune activation [32].

Furthermore, a specific, high-affinity and novel receptor site (μ_3) for morphine has been identified on human monocytes as well as *M. edulis* immunocytes [34]. Scatchard analysis revealed a single relatively high affinity binding site. A variety of opioids, tested by two methods, were found to be ineffective in displacing specifically bound 3-dihydromorphine (DHM) [34]. The discovery of this receptor site mediating opiate effects also was first found in an invertebrate and then in man, further demonstrating the value of the comparative approach [34].

Using primers derived from the human neuronal mu1 opiate receptor and reverse transcription-polymerase chain reaction (RT-PCR), we found mu transcripts in *M. edulis* pedal ganglia, substantiating the earlier observation [36]. Sequence analysis of the RT-PCR products revealed 95% identity with the neuronal human mu1 receptor. Furthermore, interleukin-1 and morphine exposure to excised pedal ganglia resulted in up- and down-regulation of the mu receptor transcripts, respectively [36]. This study provides molecular evidence that mu-type opiate receptors are expressed in molluskan ganglia, suggesting that they first appear in invertebrate organisms and are retained during evolution. Interestingly, this receptor has also been identified in both human brain tissue and human white blood cells as well [37, 38]. It has also been isolated, sequenced and cloned in human tissues and determined to be a truncated mu-1 like receptor [37, 39, 40].

Opioid-cytokine link

Opioid induction of an interleukin 1-like substance in *M. edulis* pedal ganglia and immunocytes has been demonstrated [41-46]. Recombinant human IL-1 can induce the formation of a TNF-like substance, as well as initiate specific immunocyte conformational changes that are interpreted as activation in immunocytes [41]. Both the immune and nervous system of *M. edulis* contain an IL-1-like molecule [11, 46]. In nervous tissue it is apparently localized in microglial cells [11]. Opioid challenge can induce the formation of an endogenous IL-1-type molecule that stimulates immunocytes as does authentic IL-1 [11, 46-48]. This immunocyte stimulation can be blocked by specific IL-1 antibody. DAMA induced stimulation of the production of an IL-1-like substance is shared by both the immune and nervous system. In *M. edulis*, recombinant human IL-6, although not activating cells directly, potentiated IL-1 activation of immunocytes [42, 43, 45]. Furthermore a irIL-6 appears to be present in *M. edulis* and the insect *Leucophaea* hemolymph (0.82 ng/ml) [43]. It was also found that irIL-6 is produced in pedal ganglia in response to the pharmacological challenge of the Met-enkephalin analogue DAMA. These data also imply that immune signal molecules may have functions that transcend immunomodulation.

Morphine biosynthesis in *Mytilus*

Morphine and morphine-6-glucuronide, a morphine metabolite, have been identified and quantified in *Mytilus edulis* pedal ganglia by high performance liquid chromatography coupled to electrochemical detection [49]. These opiate alkaloids were further identified by both gas-chromatography mass spectrometry and nanoflow electrospray ionization double quadrupole orthogonal acceleration Time of Flight mass spectrometry. In animals that were starved, the morphine levels increased significantly compared to controls. Tetrahydropapaveroline and reticuline, isoquinoline alkaloids, morphine precursors, were purified and identified in pedal ganglia as well [50]. These studies demonstrate that opiate alkaloids are present as naturally occurring signal molecules whose levels respond to stress, i.e., starvation.

In *M. edulis*, endogenous morphine level in the ganglia statistically increased after their incubation with reticuline, tyrosine and tetrahydropapaveroline [51, 52]. Reticuline does not stimulate ganglionic NO release, as do the other morphine precursors [53]. Furthermore, in binding displacement experiments both reticuline and salutaridine (another morphine precursor) exhibit no binding affinity for the pedal ganglia mu opiate receptor subtype [53, 54]. Injection of intact, whole healthy animals with reticuline or L-DOPA also results in significantly higher endogenous ganglionic morphine levels [52]. Incubation with quinidine and/or AMPT diminished ganglionic morphine and dopamine synthesis at various steps in the synthesis process [52]. It was also demonstrated that CYP2D6 mediates the tyramine to dopamine step in this process, as did tyrosine hydroxylase in the step from tyrosine to L-DOPA [52]. Furthermore, via RT-PCR, a cDNA fragment of the CYP2D6 enzyme in the ganglia, which exhibits 94% sequence identity with its human counterpart, was identified [55, 56]. Other studies demonstrate critical link with opiate alkaloid synthesis and actions associated with morphine [55, 57].

Opiate alkaloid function in *Mytilus*

As noted earlier, given the presence of morphine select receptors as well as the opiate alkaloid itself functions associated it also became evident. In an

effort to demonstrate possible functional roles of morphine in *M. edulis*, its presence was examined in the course of a stressful situation brought about by experimental intervention [34, 47]. In animals having been subjected to electrical stimulation of the pedal ganglia morphine down regulated immune and motor responses caused by the stimulus in a naloxone sensitive manner. Examination of the levels of endogenous morphine-like material revealed a significant increase in the hemolymph and in the pedal ganglia taken from the 30-h post electrical stimulation group. The deactivating influence of opiate substances in both systems has to be interpreted in context with the body of information on immunoregulation, particularly immunostimulating activities of opioid peptides and other messenger molecules. The need for a functional interaction of regulatory factors with opposite effects can be considered to be enhanced under abnormal conditions. This speaks for a general and yet specific role of morphine in calming or terminating the state of alertness created by the initial release of endogenous opioids and/or cytokines [32]. This same role of naturally occurring morphine as an immune down-regulating molecule has been proposed for morphine in CNS [32]. It also may represent a common protective mechanism via the stress followed by relaxation, if appropriate [58].

In another study the concept of the existence in insects and mollusks of a distinctive class of neuroglial cells, comparable to vertebrate microglia, was examined during surgical stress [59]. The evidence presented was as valid as that used in reference to the separate status of vertebrate microglia, i.e., the demonstration of a close structural and functional relationship of these cells with cells of the immune system. The excision of ganglia from three invertebrate species (the mollusks *Planorbarius corneus* and *Mytilus edulis*, and the insect *Leucophaea maderae*), and their maintenance in incubation media, led to an exodus of small cells and their accumulation in the culture dish [59]. During this process, they underwent conformational changes from stellate to rounded, and then to more or less ameboid, comparable to those indicative of the process of activation in the animals' immunocytes. Functional characteristics, which these translocated microglia-like cells share with immunocytes, are motility, phagocytotic activity, and adherence to the culture dish. An additional phenomenon of particular interest for the classification of microglial elements is their response to morphine [59]. At a concentration of 10^{-6} M, this drug inhibits not only the number of cells emerging from the excised ganglia, but also the degree of their transformation to the "active" ameboid form. This dose-dependent and naloxone-sensitive effect of morphine on microglial cells parallels that on activated immunocytes of the same species [34]. Corresponding results demonstrating an inhibitory effect of morphine on mobilized microglial cells of the frog *Rana pipieus* indicate that this relationship between the two cell types under consideration also exists in vertebrates [59]. Binding and displacement experiments with membrane homogenates of microglial cells as well as immunocytes of *M. edulis* have shown that the effects of morphine on both cell types are mediated by the same special opiate receptor μ_3 [59].

Opiate coupled nitric oxide signaling

Nitric oxide (NO) serves many functions, including free radical scavenger, anti-bacterial and - viral and widespread gaseous messenger actions [60-62]. In numerous reports we also have demonstrated that the novel opiate receptor mu3, which is opiate alkaloid selective and opioid peptide insensitive, is coupled to constitutive nitric oxide release (cNOS) in *M. edulis* tissues [34, 63-66]. In *M. edulis*, microglia egress from ganglia, which morphine inhibits, was found to operate via NO release [67]. Taken together, these data demonstrate that morphine can stimulate NO release in cells obtained from an invertebrate that represents an animal 500 million years divergent in evolution from man, underscoring the significance of this process and further substantiating the critical importance of morphine as a naturally occurring signal molecule [68].

The morphine metabolite morphine-6-glucuronide (M6G) also stimulates pedal ganglia cNOS-derived NO release at identical concentrations and similar peak levels as morphine [69, 70]. However, the classic opiate antagonist, naloxone, only blocked the ability of morphine to stimulate cNOS-derived NO release and not that of M6G. CTOP, a mu-specific antagonist, blocked the ability of M6G to induce cNOS-derived NO release, as well as that of morphine, suggesting that a novel mu opiate receptor was present and selective toward M6G.

Subjecting the marine bivalve *M. edulis* to an immediate temperature change has been shown to rapidly alter the animals' ganglionic monoamine levels, opiate processes as well as its ciliary activity [71]. After 12 h cold exposure the estimated relative mu opiate receptor (MOR) gene expression in *M. edulis* pedal ganglia, measured by real-time PCR, did not differ significantly from the control group whereas 24 h of cold exposure significantly down regulated their mu opiate receptor mRNA expression and limiting nitric oxide release. Interestingly, morphine and DAMGO significantly enhances ciliary beating in a naloxone sensitive

manner, whereas L-NAME, a nitric oxide synthase inhibitor, only antagonized morphine's action [72]. This study 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.

Incubation of *M. edulis* excised gill filaments reveal spontaneously lateral cilia beating in a metachronal wave, which was significantly decreased by morphine in a concentration dependent and naloxone reversible manner [73]. Exposure of the spontaneously beating cilia to SNAP, a NO donor, also diminished the beating rate. Exposing the cilia to L-NAME blocked the morphine induced cilioinhibition, demonstrating that morphine was working to inhibit the cilia via NO. Furthermore, the gill tissue contained mu opiate receptor transcripts, which was mu_3 in nature [73]. As in mammals, opiate signaling is not confined to neural tissues. This report demonstrates the occurrence of opiate signaling for the first time in an invertebrate's respiratory tissue.

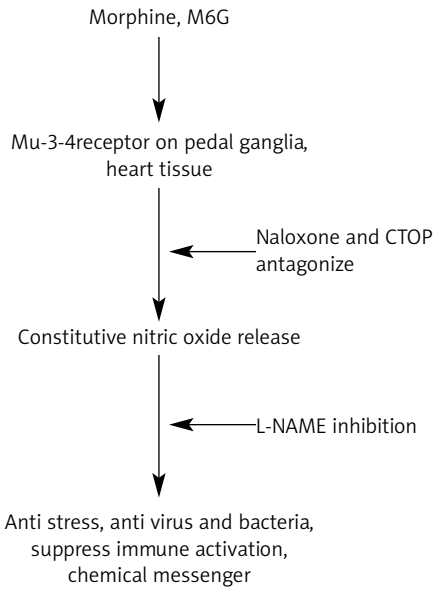

Figure 1. Opiates coupled nitric oxide signaling

Mytilus edulis as a model for substance abuse screening

Based on the experimental data that *Mytilus edulis* ganglia make morphine and that perturbation of its environment, such as cold stress, bacteria infection, starvation, can alter its synthesis make it a likely model for testing substances of abuse [74]. We have designed an assay that can actually measure the effect of substances of abuse on morphine release from *M. edulis* ganglia [75-78]. Incubation of pooled *M. edulis* pedal ganglia with ethanol, cocaine or nicotine, substances with high substance abuse potential, resulted in a statistically significant enhancement of ^{125}I-trace labeled morphine release [75-85]. Taken together, besides demonstrating the suitability of *M. edulis* as a model animal for influencing opiate processes Mytilus studies introduce the hypothesis that substance of abuse may all work, in part, by releasing endogenous morphine, which impacts the CNS reward and motor systems.

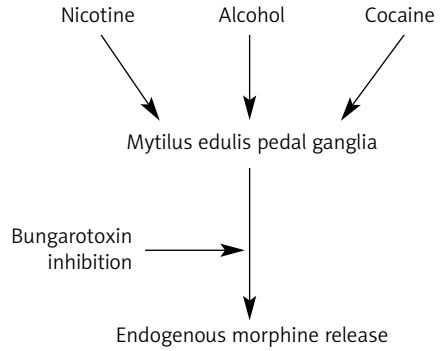

Figure 2. Substances of abuse stimulate endogenous morphine release

Conclusions

Thus, opioid peptides and opiate alkaloids, functioning in an autocrine/paracrine manner have not only been conserved during the course of evolution but their activities in immuno- and neuroregulation have been conserved as well. Stereoselect degradation and dynamic receptor mechanisms also exist in invertebrates to insure that their signals exhibit the highest fidelity. The potential for immunocytes to communicate with neural and immune elements using opioid, opiate, cholinergic, GABAnergic and catecholamininergic, as well as cytokine, signal molecules also exists. Furthermore, "opiates" may be used, in part, by parasites to escape immune surveillance [86-88]. Thus, the comparative study, emphasizing *M. edulis* in this review, of chemical messenger associated regulation and its involvement in autoimmunoregulation represents a scientific area of timely interest, which will be the subject of future endeavors. These primitive receptors and the morphinergic signal molecules associated with them are likely affecting many underlying biochemical processes, including endocrine and within the scope of substance abuse [81, 89-91].

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