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REVIEW ARTICLES

Opioid peptides in invertebrates: Localization, distribution and possible functional roles

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In this review the opioids that have been found in the invertebrates are discussed. Major emphasis is placed on the arthropods, molluscs and annelids. The article deals largely with the identification and functional roles of these peptides. These endogenous opioids are involved in a wide variety of physiological processes, often serving as neurotransmitter substances. Among the putative roles of opioids in invertebrates are mediation of the release of neurohormones, initiation of proper behavioural responses, control of thermoregulatory activities and mediation of immune responses. This review concludes with a section that suggests areas of future investigation, along with possible practical applications of these compounds.

IN recent years a number of vertebrate-type neuropeptides have been discovered in the invertebrates. The discovery of these vertebrate-type peptides in invertebrate tissues has influenced research in comparative endocrinology in a major way. A class of such neuropeptides is known as the opioid family, based on the fact that among vertebrates these peptides show a potent capacity to mimic some key actions of morphine[1]. In mammals the enkephalins originate from at least two precursors, preproenkephalins A and B. Preproenkephalin A gives rise to one copy of leucine enkephalin (Leu-Enk), four copies of methionine enkephalin (Met-Enk) (Leu-Enk and Met-Enk are pentapeptides) and two C-terminally extended enkephalins, Met-Enk-Arg^6-Phe^7 (met-7) and Met-Enk-Arg^6-Gly^7-Leu^8 (Met-8)[2]. Preproenkephalin B contains no Met-Enk sequence but has three Leu-Enk-containing peptides, α/β-neoendorphin, dynorphin and rimorphin. The first enkephalins discovered, Met-Enk and Leu-Enk, were originally purified from the porcine brain[3]. There are a variety of opiate receptors (mu, delta, iota, kappa, epsilon) each of which appears to be the preferential ligand for one of the endogenous opioid peptides in mammals[4]. Endogenous opioids have been detected in all vertebrate species investigated[5,6]. These substances serve widely throughout the body as neuromodulatory substances.

The search for the presence of endogenous opioids in invertebrates initially met with some uncertainty. Early reports did not support the presence in several invertebrates of opiate-receptor-binding sites[7,8]. However, the development of appropriate techniques, especially immunocytochemistry, radioimmunoassay (RIA) and high-performance liquid chromatography (HPLC), made it possible to discover opioid peptides in several invertebrates. Among the invertebrates, studies have been undertaken mainly with arthropods, molluscs and annelids, and in this review we emphasize the studies that have been done on the localization, distribution and possible roles of the endogenous opioids in these groups.

Crustacea

Localization and distribution

The first opioid study on a crustacean was reported in 1981 by Mancillas *et al.*[9]. Through immunocytochemistry they found Leu-Enk-like immunoreactivity in all the retinular cells of the spiny lobster *Panulirus interruptus* and the red swamp crayfish *Procambarus clarkii*. In addition, such immunoreactivity was also apparent in nerve fibres in chiasm 3 that run from the medulla interna to the medulla terminalis. Since then immunocytochemical mapping studies, which not only detect the presence of opioid peptides but also give their distribution, have been reported in a variety of crustaceans. Table 1 summarizes the peptides detected so far in crustaceans and the techniques used to demonstrate their presence[9–11,14–22]. Marino *et al.*[12] and Piccoli *et al.*[13], by using a receptor-binding assay, reported the presence of opioid-like peptides in the suboesophageal ganglion and brain of *Squilla mantis* and *Carcinus maenas*. There are a few reports about the presence of enzymes that degrade the opioid peptides in Crustacea. Colleti-Previero *et al.*[23] found three peptidases in the haemolymph of the crayfish *Astacus fluviatilis* that rapidly degrade Leu-Enk. Watabe *et al.*[24] reported evidence for the presence of β-endorphin-degrading enzymes in the cells of the hepatopancreas of the crayfish *Procambarus clarkii*.

Functions

Behaviour. The first study of a possible role of an opioid in any crustacean was that by Maldonado and Miralto[25] with *Squilla mantis*. They found that the defensive response, a rapid flexure of the abdomen, which can be experimentally induced by electric shock, can be modified by morphine. This drug increases the threshold current required to elicit the response. The loss of sensitivity is dose-related. Naloxone blocks the action of this opioid. Naloxone is a competitive opioid antagonist, binding to opioid receptors. Martinez *et al.*[26] found that when the crab *Gecarcinus lateralis* was placed into the activity-monitoring chambers, the locomotor activity increased. FK 33 824, a stable Met-Enk analogue, markedly enhances this initial activity but naloxone blocks this excitatory action of the opioid. In studies with another crab, *Chasmagnathus granulatus*, Lozada *et al.*[27] found that this crab assumes a defensive posture, with both chelae extended and the body elevated when an electric shock of 50 Hz, 1 s duration and at least 8 V is given. Morphine produces a dose-dependent reduction in the sensitivity of this crab to the electric shock. But when naloxone was coinjected with morphine, no reduction in sensitivity occurred, which suggests that the morphine was acting through opioid receptors and that such receptors are indeed involved in this reduction of sensitivity to the electric shock. In other behavioural studies with this crab[28,29], it was found that a danger stimulus in the form of a passing shadow elicits an escape response that habituates after repeated stimulation, and that this habituation appears to be mediated by endorphins. Support for this suggestion that habituation is the result of endorphin release is the observation that naloxone increases the responsiveness to the danger signal and after a habituation session which reduces the response level to the danger signal there is also an analgesic effect on the defensive response of this crab to an electrical shock. Very recently, Godoy and Maldonado[30] reported that the synthetic opioid analogue (D-Ala^2) Met-enkephalin (DAME) significantly reduces the escape response to a danger stimulus in the crab *Chasmagnathus granulatus* when administered within a dose range of 0.01–1.0 μg/g. A 0.1 μg/g dose of naloxone has no effect *per se* on the response, but when it is administered together with DAME, it completely blocks the decremental effect of this drug.

Pigmentary effectors. Crustaceans have two types of pigmentary effectors: (a) the chromatophores, which are responsible for colour changes and (b) the retinal pigments, which control the amount of light impinging on the rhabdom. Translocation of the pigments in the chromatophores and at least in the distal and reflecting pigments is clearly regulated by neurohormones.

(a) *Chromatophores.* The physiology of crustacean chromatophores has been reviewed recently[31]. In several species dual control of chromatophores by pigment-dispersing and pigment-concentrating neurohormones has been demonstrated. In this laboratory we have been particularly interested in identifying the neuroregulators that control the release of crustacean neurohormones. In the fiddler crab *Uca pugilator*, 5-hydroxytryptamine (5-HT) stimulates the release of red-pigment-dispersing hormone while norepinephrine (NE) stimulates the release of black-pigment-dispersing hormone. Met-Enk, but not Leu-Enk, was found to stimulate the concentration of the black and red pigments in intact *Uca pugilator*[32]. In *in vitro* experiments Met-Enk stimulated the release of black- and red-pigment-concentrating hormones from isolated eyestalks[32,33]. Naloxone blocked the action of Met-Enk in intact crabs and on isolated eyestalks. Met-Enk, like the classical aminergic neurotransmitters, has no direct effect on these chromatophores, as evidenced by the lack of effect on the chromatophores in isolated legs. Presumably, therefore, 5-HT, NE and Met-Enk exert their effect only indirectly, by stimulating the release of the appropriate specific chromatophorotropic neurohormones. However, surprisingly, β-endorphin was found to produce black-pigment dispersion in both intact *Uca pugilator* and in isolated legs[32]. Additional evidence for the involvement of enkephalinergic substances

Table 1. Identification and localization of opioid-like peptides in crustaceans

Peptide	Species	Localization	Techniques	References
Leu-Enk-like	*Cancer pagurus*	PO	Immunocytochemistry	15
	Carcinus maenas	SG, LG, ME, MI, MT, PO, segmental nerve connections to the thoracic ganglia, thoracic ganglia	Immunocytochemistry HPLC	10, 14, 17, 18
	Gecarcinus lateralis	Brain	RIA, HPLC	19
	Maja squinado	PO	Immunocytochemistry	15
	Panulirus interruptus	Retinular cells, nerve fibres in chiasm 3	Immunocytochemistry	9
	Portunus puber	PO	Immunocytochemistry	15
	Procambarus clarkii	Retinular cells, nerve fibres in chiasm 3	Immunocytochemistry	9
	Uca pugilator	Retinular cells, LG, SG, OP, MT, ovary, testis	Immunocytochemistry	11, 21
Met-Enk-like	*Carcinus maenas*	SG, hepatopancreas thoracic ganglia	HPLC, immunoflurescence, RIA, sequence analysis	10, 16–18
	Gecarcinus lateralis	Eyestalk, brain	HPLC, RIA	19
	Uca pugilator	Retinular cells, SG, LG, ,OP, MT, x-organ of MT, ovary, testis	Immunocytochemistry	11, 21
Met-Enk-Arg^6-Phe^7	*Carcinus maenas*	SG	HPLC	10
Met-Enk-Arg-Phe-amide (YGGFMRFamide)	*Homarus americanus*	Nervous system	Immunocytochemistry	20
β-endorphin-like	*Procambarus clarkii*	Hepatopancreas	RIA, Immunocytochemistry	22

HPLC: high-performance liquid chromatography; LG: lamina ganglionaris; MT. medulla interna, ME: medulla externa; MT. medulla terminalis; OP: optic peduncle, PO pericardial organ, SG· sinus gland, RIA. radioimmunoassay.

in crustacean colour changes was provided by Martinez *et al.*[34], who found that although injection of the stable Met-Enk analogue FK 33 824 alone into *Gecarcinus lateralis* has no effect on the chromatophores, coinjection of this analogue with eyestalk extract strongly potentiates the black-pigment-dispersing and red-pigment-concentrating actions of the eyestalk extract. This potentiation is blocked by naloxone.

(b) *Distal retinal pigment.* NE produces light adaptation of the distal retinal pigment of *Uca pugilator* whereas dopamine (DA) induces dark adaptation[35,36], presumably by stimulating the release of the light-adapting and dark-adapting neurohormones, respectively. Met-Enk, like DA, produces dark adaptations of this pigment, but Leu-Enk has no effect on this pigment[37].

Blood glucose. The crustacean hyperglycaemic hormone (CHH) is found in the sinus gland. In several crustaceans CHH release has been found to be triggered by 5-HT (ref. 38). Recently, Lüschen *et al.*[17] and Rothe *et al* [18] provided evidence that synthetic Leu-Enk, endogenous sequenced Leu-Enk isolated from thoracic ganglia of *Carcinus maenas* and purified Leu-Enk-like material from sinus glands of this crab, when injected into intact *Uca pugilator*, the test organism for these bioassays, produce a decrease in the haemolymph glucose concentration. Also, in CHH bioassay done again by injecting into intact *Uca pugilator*, it was found that eyestalks of *Carcinus* incubated in the presence of synthetic Leu-Enk release less than the basal level of CHH was compared to eyestalks incubated in saline alone. Furthermore, injection of naloxone into intact *Uca pugilator* prior to injection of Leu-Enk blocks the hypoglycaemic action of this opioid in this crab. In contrast, Leu-Enk does not affect the haemolymph glucose concentration in eyestalkless fiddler crabs, which is consistent with the hypothesis that the enkephalin acts by reducing CHH output from the sinus glands in the eyestalks. In the crayfish *Astacus leptodactylus*, Met-Enk decreases the haemolymph glucose level as well as the nocturnal peak in the circadian rhythm of haemolymph glucose[39]. Sarojini *et al.*[40] provided evidence that both L-Enk and DA are involved in the regulation of blood glucose in the crayfish *Procambarus clarkii*. *In vitro*, both L-Enk and DA reduced the rate of release of CHH from isolated eyestalk neuroendocrine tissue. These results suggest that both L-Enk and DA act to inhibit the release of CHH by affecting the eyestalk neuroendocrine complex and that the

Table 2. Identification and localization of opioid-like peptides in insects

Peptide	Species	Localization	Techniques	References
Leu-Enk-like	*Calliphora vomitoria*	Brain, thoracic ganglia, retrocerebral complex	Immunocytochemistry	50
	Locusta migratoria	Brain	RIA	42
	Eristalis aeneus	Brain	Immunocytochemistry	48
	Manduca sexta	Brain	Immunocytochemistry	49
Met-Enk-like	*Calliphora vomitoria*	Brain, thoracic ganglia, retrocerebral complex	Immunocytochemistry	50
	Drosophila melanogaster	Brain, ovaries, testes	Immunocytochemistry	47, 57
	Leucophaea maderae	Corpora cardiaca	Immunocytochemistry	46
	Locusta migratoria	Brain, corpora cardiaca, spermatogonia, spermatids, spermatocytes, ovarian follicles	Immunocytochemistry, RIA	42, 52, 54, 55
	Periplaneta americana	Brain, nerves leading to corpora cardiaca and corpora allata, midgut endocrine cells	Immunocytochemistry	45, 53
	Nauphoeta cinera	Midgut endocrine cells	Immunocytochemistry	54
	Sarcophage bullata	Spermatogonia, spermatids, spermatocytes, ovarian follicles	Immunocytochemistry	55, 56
	Schistocera gregaria	Brain, thoracic ganglia, subesophageal ganglia, abdominal ganglia	Immunocytochemistry	51
Met-7	*Calliphora vomitoria*	Brain, thoracic ganglia, retrocerebral complex	Immunocytochemistry	50
Met-8	*Calliphora vomitoria*	Brain, thoracic ganglia, retrocerebral complex	Immunocytochemistry	50
Endorphin	*Bombyx mori*	Neurosecretory cells in the pars intercerebralis	Immunocytochemistry	59
	Calliphora vomitoria	Neurosecretory cells in the pars intercerebralis	Immunocytochemistry	60
	Eristalis aeneus	Neurosecretory cells in the pars intercerebralis	Immunocytochemistry	60
	Leptinotarsa decemlineata	Neurosecretory cells in the pars intercerebralis	Immunocytochemistry	58
α-Endorphin	*Bombyx*	Median cells of suboesophageal ganglion	Immunocytochemistry	61–63
	Thaumetopoea gityocampa	Median cells of suboesophageal ganglion	Immunocytochemistry	61, 62
MSH	*Leucophaea maderae*	Corpora cardiaca, corpora allata	Immunocytochemistry	65
	Locusta migratoria	Cell bodies and nerve fibres in the central nervous system	Immunocytochemistry	64
	Sarcophaga bullata	Cell bodies and nerve fibres in the central nervous system	Immunocytochemistry	64
ACTH	*Leucophaea maderae*	Corpora cardiaca, corpora allata, Malpighian tubules	Immunocytochemistry, ACTH antiserum	65, 66
	Locusta migratoria	Corpora cardiaca	Immunocytochemistry	64

RIA: radioimmunoassay.

enkephalinergic inhibitory neuron follows the dopaminergic inhibitory neuron in the chain of neurons which leads to the neuroendocrine cells that secrete CHH.

Reproduction. The potential involvement of an endogenous opioid system in the regulation of ovarian development in the *Uca pugilator* was investigated *in vivo*[41]. Injection of synthetic Met-Enk into female crabs slowed ovarian maturation significantly. The inhibition was dose-dependent. The mean ovarian index and the mean oocyte diameter of the crabs that received the opioid were significantly smaller than the corresponding values for the saline-injected concurrent control specimens. Injection of naloxone produced dose-related ovarian maturation[41].

Insecta

Localization and distribution

The first demonstration of the presence of an opioid in an invertebrate came from an RIA study with the brain of a locust, *Locusta migratoria*[42]. No β-endorphin was found but Leu-Enk and Met-Enk were both present, with Met-Enk present in larger quantity than Leu-Enk. Since then immunocytochemical studies based on antisera raised against opioid peptides have been carried out in about 35 species of insects belonging to eight orders[43,44]. Table 2 summarizes the results so far obtained regarding the localization and distribution of opioid peptides in insects as well as the methods employed to detect these peptides[45–66]. A few investigators have demonstrated the presence of opioid-binding sites in insect neural tissues. Pert and Taylor[68] showed that membranes prepared from heads of *Drosophila melanogaster* bind Leu-Enk, but naloxone does not bind well to these membranes. D-ala-2-met-5-enkephalinamide (DAMA) also binds extensively to cerebral ganglion membrane suspensions from *Leucophaea maderae*[67]. In the same insect, high-affinity stereospecific opioid binding was demonstrated in midgut preparations[69]. Santoro *et al.*[70] reported that tissues of *Drosophila* appear to contain kappa-like opioid receptors. These results suggest that insects possess both opioid substances and their receptors, thus revealing the existence of a complex opioid mechanism similar to mammals. Recently, Lamango and Isaac[71] characterized an endopeptidase from the heads of *Musca domestica* that can degrade a range of neuropeptides, including enkephalins and substance P.

Functions

Neuromodulation. Morphological evidence suggests the existence of specific enkephalinergic pathways within the central nervous system (CNS) of insects[43]. Immunoreactive nerve fibres originating in the brain were seen in nerves issuing from the corpora cardiaca (CC) and running along pathways leading to the anterior sympathetic nervous system and corpora allata (CA). These opioid peptides may be functioning as neurotransmitter or neuromodulatory substances[43]. In *Calliphora vomitoria*, the heart has a direct enkephalinergic innervation, particularly by Met-7 immunoreactive terminals in the vicinity of the ostia; the enkephalin-like material presumably acts as a neuromodulator of the intrinsic heart rhythm[43]. In addition, in this insect the phasic secretory activity of the CA is regulated by enkephalin-like peptide[43].

Neurohormone. The presence of enkephalins in the neurohaemal areas of the dorsal neural sheath of the thoracic ganglia of *Calliphora vomitoria* suggests a hormonal role for these peptides[43]. Furthermore, a close association between 5-HT and a variety of peptidergic substances, including Leu-Enk and Met-7, was observed. Met-7 at a concentration of as low as 10^{-12} M displayed a powerful stimulatory influence on salivary gland secretion in the rat. In contrast, Leu-Enk and Met-Enk were completely without effect. Naloxone failed to inhibit this response of the rat on salivary glands to Met-7. Thorpe and Duve[43] suggested that Met-7 may act through kappa receptors, where naloxone has only a weak binding capacity.

Behaviour. A behavioural role for an enkephalin in regulating locomotor activity in the cockroach *Leucophaea maderae* was demonstrated by Ford *et al.*[72]. When the enkephalin analogue D-ala-2-met-5-enkephalinamide (DAMA) was applied topically to the cerebral ganglia, it resulted in a decrease in locomotor activity. Naloxone can block this effect as well as the depressant effect of morphine on locomotor activity. In contrast, another analogue, D-ala-2-leu-5-enkephalinamide (DALA) and also dynorphin increased the locomotor activity. This acceleratory effect can also be antagonized by concomitant naloxone treatment. In another cockroach, *Periplanata americana*, enhanced locomotor activity was induced by morphine, a mu opioid agonist[73]. In the same species, it was shown that the kappa opiate agonist U-50,488H was able to stimulate the ingestive responses of free-feeding animals. These results on opioid-induced behaviours indicate the presence of multiple opiate receptor types in insects.

Thermoregulation. Survival of an animal is dependent on its ability to avoid thermal extremes. Many ectotherms show at least a primitive ability to control their body temperature. The behavioural thermoregulatory responses of at least some insects are sensitive to opiates. Kavaliers and Hirst[74] studied the thermal responses of larvae of the mealworm *Tenebrio molitor*. Fourth instar larvae were injected with morphine, Met-Enk, β-endorphin,

Table 3. Identification and localization of opioid-like peptides in molluscs

Peptide	Species	Localization	Techniques	References
Leu-Enk-like	*Aplysia californica*	Central nervous system	Immunocytochemistry	83
	Cepaea nemoralis	Brain neurons, central nervous system	Immunocytochemistry enzyme assay	84, 89
	Lepidopleurus asellus	Central nervous system	Immunocytochemistry	84
	Lymnaea magnalis	Central nervous system	Immunocytochemistry	83
	Lymnaea stagnalis	Central nervous system, peripheral nervous system	Immunocytochemistry	82, 87
	Mytilus edulis	Pedal ganglia, cerebral ganglia	Immunocytochemistry, HPLC, amino acid sequencing	90, 91
Met-Enk-like	*Achatina fulica*	Cerebral ganglia, visceral ganglia, nerves and ganglion cells of posterior intestine, parietal ganglia	Immunocytochemistry	80, 81
	Acmaea testudinalis	Peripheral nervous system	Immunocytochemistry	84
	Aplysia californica	Central nervous system	Immunocytochemistry	83
	Cepaea nemoralis	Brain neurons, peripheral nervous system	Immunocytochemistry	84
	Helix aspersa	Gonads, peripheral nervous system	Immunocytochemistry	84, 85
	Helix pomatia	Central nervous system	Immunocytochemistry	83
	Lepidopleurus asellus	Central nervous system	Immunocytochemistry	84
	Littorina littorea	Peripheral nervous system	Immunocytochemistry	84
	Lymnaea magnalis	Central nervous system	Immunocytochemistry	83
	Lymnaea stagnalis	Cerebral ganglia, pedal ganglia, central nervous system	Immunocytochemistry, HPLC	79, 87
	Mytilus edulis	Pedal ganglia, cerebral ganglia	Immunocytochemistry, HPLC, amino acid sequencing	90, 91
	Octopus vulgaris	Vena cava neurohaemal organ, neurosecretory cells of the brain	Immunocytochemistry	93, 94
Met-Enk-Arg^6-Phe^7	*Mytilus edulis*	Cerebral ganglia, pedal ganglia	Immunocytochemistry, HPLC	92
	Octopus vulgaris	Vena cava neurohaemal organ, neurosecretory cells of the brain	Immunocytochemistry	94, 95
ACTH	*Lymnaea stagnalis*	Central nervous system	Immunocytochemistry	87
1–24 ACTH	*Helix aspersa*	Retina, ganglionic cells, some spermatogenic cells	Immunocytochemistry	85, 88
	Planorbis corneus	Brain	Immunocytochemistry	86
17–39 ACTH	*Helix aspersa*	Young oocytes, nerve fibres surrounding each gonadal acinus and hermaphrodite duct	Immunocytochemistry	85
α-MSH	*Helix aspersa*	Retina, ganglionic cells, young oocytes	Immunocytochemistry	85, 88
	Lymnaea stagnalis	Central nervous system	Immunocytochemistry	87
	Planorbis corneus	Brain	Immunocytochemistry	86

HPLC, high-performance liquid chromatography.

or naloxone. Their behaviour and temperature preference were recorded following their return to pretreatment locations in a thermal gradient with a low temperature of 20°C and a high temperature of 40°C. Prior to drug treatment, the larvae showed a preference for a temperature of about 32°C. After injection, animals given morphine or Met-Enk moved towards higher temperatures whereas those receiving naloxone did not. Larvae receiving β-endorphin displayed no consistent changes in thermal preference. The changes in preferred

temperatures that took place were evident for approximately 1 h, after which animals returned to the pretreatment preference temperature[74].

Immune response. Among recent developments in the elucidation of opioid activities in nonnervous cells and tissues, endogenous opioids in the immune system are receiving the greatest attention in insects. The endogenous opioids in *Leucophaea maderae* appear to influence the autoregulatory activities of immunocompetent cells. These activities include the adherence and migratory behaviour of these cells[75]. There is evidence for the presence in immunocytes, as well as in cell-free haemolymph, of Met-Enk, which appears to be involved in immunoregulatory activities, perhaps by interaction with a special subtype of delta receptor. Moreover, the naloxone has an inhibitory effect on these processes, which indicates that they are receptor-mediated[75]. Thus, there appears to be a link between the neuroendocrine and the 'immune' system in insects[76].

Mollusca

Identification and localization

Tremblay *et al.*[77] seem to have been the first investigators to demonstrate the action of an opiate peptide in a mollusc. Injection of morphine into the gastropod *Aplysia californica* decreased the available neurotransmitter, acetylcholine. Erdélyi *et al.*[78] demonstrated that in the snail *Helix pomatia* Leu-Enk induces hyperpolarization of some neurons in the brain, and naloxone administration antagonizes the action of this opioid. These results suggest the presence of an endogenous opioid system in gastropod molluscs. By use of immunocytochemical techniques, a very detailed study of the immunoreactive material present in the central nervous system of the pond snail *Lymnaea stagnalis* has been performed by Schot *et al.*[79]. However, Met-Enk-like material was observed only in the cerebral and pedal ganglia. In each cerebral ganglion a cluster of 10–15 immunoreactive cells lies in the mediolateral part of the ganglion; another cluster (6–10 cells) is just dorsal to the lateral lobe. In addition, two positively reacting cells were observed in the medioventral area. Numerous positive axonal terminals were seen along the entire periphery of the cerebral commissure. In each pedal ganglion a group of 31–40 immunoreactive cells, located laterally, show a positive reaction for Met-Enk. A second group (8–10 cells) lies in the ventromedial area of the ganglion. Such immunocytochemical studies have been extended to other molluscs. Table 3 summarizes the evidence for the presence of opioids in various molluscs so far investigated[79 81,83-95]. Using HPLC, Leung *et al.*[82] identified a substance in the abdominal ganglion of *Aplysia californica* that was able to displace DAMA. In the nervous system of *Mytilus*, evidence was obtained for two classes of opioid-binding sites in nervous tissue[96,97]. It was suggested that the two binding sites may be associated with presynaptic and postsynaptic receptor sites for the opioids, or possibly with different pharmacological subtypes of receptors[98].

Functions

Behaviour. In the land snail *Helix pomatia* morphine induces a state of immobilization and muscle rigidity, resulting in a loss of the righting reflex. These effects are reversible by naloxone treatment[99,100]. This behavioural effect of morphine is reduced as treatments progress for four days and reoccurs if a higher dose of morphine is given on the fifth day, thus demonstrating that tolerance has occurred. It was suggested that in these molluscan behavioural responses enkephalins can mediate sensory neurotransmission[101]. Electrophysiologically, opioids are known to elicit a variety of actions in molluscan neurons, including both excitatory and inhibitory responses, some of which are naloxone-reversible and some that are not[102–104]. The first report of the effect of an opioid in an invertebrate appears to be that of Stefano and Hiripi[105], in which they describe an increase in the intraganglionic level of DA in response to Met-Enk and Leu-Enk in *Mytilus edulis*. Naloxone blocked the effects of Leu-Enk and Met-Enk. These responses displayed a time-dependent desensitization to Met-Enk. With the freshwater bivalve *Anodonta cygnea*, Stefano and Hiripi[106] also found an increase in the DA level in the cerebral ganglia after injection of Met-Enk or morphine. Furthermore, both agents increased the levels of cyclic GMP and depressed the levels of cyclic AMP. The pharmacological effects on DA and the cyclic nucleotide levels are blocked by prior treatment of the cerebral ganglia with naloxone. The data suggest that the decrease in the cyclic AMP concentration caused by the opiates may be a result of inhibition of adenylate cyclase[107]. Aiello and Hager[108] showed that the application of morphine to the visceral ganglion of *Mytilus edulis* is cilioexcitatory to the lateral cells of the gills, and this is most likely a result of decreasing the release of DA from cilioinhibitory fibres in the ganglion. Acetylcholine (ACh) has been proposed as the excitatory neurotransmitter for stimulation of tonic contraction of the anterior byssus retractor muscle of *Mytilus*. Morphine at concentrations of 10^{-9}–10^{-4} M increased markedly the contraction induced by ACh (ref. 109).

In the snail *Helix pomatia*, a number of identified neurons regulate visceral functions, and they react selectively to Leu-Enk and morphine; some become depolarized whereas others are hyperpolarized[110]. These peptides modulate the effect of ACh and 5-HT. It is known that a combination of low molecular weight neu-

rotransmitters and peptides modulates the elementary form of learning in *Helix*, such as regulation of various rhythmic processes, including movements of the pneumostoma[111]. In this snail, Leu-Enk modulates the 5-HT effect on the habituating central neurons through a cyclic 3′-5′-AMP system both *in situ* and *in vitro*. The habituating cells are connected to the regulation of various rhythmic processes, including pneumostoma (respiratory orifice) movements. In the pteropod mollusc *Clione limacina*, sensitivity to some tactile inputs can be decreased by naloxone, and it is suggested that in these behavioural responses enkephalins mediate sensory transmission[112].

Respiration. Moroz[113] reported that 5-HT activates the respiratory rhythm in the snail *Lymnaea stagnalis*, while enkephalin acts to limit this feedback system. In the marine gastropod *Aplysia californica*, which uses gill respiration, Met-Enk inhibits gill movements[114]. Furthermore, in this species it is known that 5-HT is able to activate gill movements[115]. Dyakonova[116,117] presented clear evidence for central systemic effects of Met-Enk and 5-HT in *Helix pomatia*. She showed that Met-Enk and 5-HT are able to induce slow oscillation of the membrane potential in a group of respiratory neurons. Synchronization of the activity between the neurons in the cluster appeared to be due to an increase of electrical coupling between the cells[117]. This suggested that Met-Enk might be involved in modulation of respiratory programmes, including the central respiratory generator. For another snail, *Lymnaea stagnalis*, Moroz and Winlow[118] reported that Met-Enk inhibits the 5-HT-induced respiratory rhythm during the first few minutes after this opioid is administered. Later, Met-Enk (5–30 min after its administration) induces slow oscillation of the membrane potential in central neurons related to the respiratory programme. More recently, Dyakonova *et al.*[119] studied the effect of Met-Enk on electrical coupling between molluscan neurons, using isolated brains of *Helix* and *Lymnaea*. In the presence of both 5-HT and Met-Enk, nonrectifying electrical coupling is strongly facilitated between identified respiratory neurons in *Helix*, while coupling between 5-HT-containing ciliomotoneurons in *Lymnaea* is facilitated by Met-Enk alone. These data suggest that Met-Enk can modulate different groups of electrically coupled cells and may be involved in coordination of motor patterns[119].

Feeding. Administration of morphine resulted in significant dose-dependent increases in the ingestive responses of food-deprived slugs *Limax maximus*, and in the initiation of feeding in satiated animals[120]. These effects could be blocked by naloxone, with naloxone by itself causing a significant decrease in feeding by food-deprived slugs. These results suggest that opioids are involved in the control of feeding behaviour of the slugs.

Thermoregulation. Terrestrial snails are ectotherms, their body temperatures being dependent on the ambient temperature. The terrestrial snail *Cepaea nemoralis*, when placed on a 40°C hot plate, lifts the anterior portion of its foot. The latency of this response is affected by morphine and naloxone[121]. Administration of morphine resulted in a significant dose-dependent increase in the temperature selected by the snail in a thermal gradient. These thermoregulatory effects could be blocked and reversed by naloxone, with the opiate antagonist by itself causing a significant decrease in the preferred temperature[122]. After 6–10 days of daily administration of morphine, *Cepaea nemoralis* displayed tolerance; the effect of morphine decreased and the thermal preferences of the snails administered morphine became similar to those of saline-treated individuals. These results indicate that the opiate system plays a thermoregulatory role in molluscs. With the same species of snail, Kavaliers and Hirst[74] observed temperature responses similar to those produced by morphine when the snails were treated with Met-Enk or β-endorphin. Naloxone antagonized the effects of these peptides. Recently, Kavaliers and Ossenkopp[123] reported that repeated naloxone treatments and exposure to weak (60 Hz) magnetic fields brought about a hypoalgesic response to thermal nociceptive stimuli in *Cepaea nemoralis*.

Opioids in the aging process. Age-related changes in the opioid system of *Mytilus edulis* were detected by Stefano[124]. He studied the opioid binding profile in aging pedal ganglia. The high-affinity binding site density is significantly lower in older animals than in younger ones, whereas the low-affinity site density remains unchanged. The estimated Met-Enk and Met-Enk-Arg^6-Phe^7 levels were significantly higher for the older animals than for the younger ones. The decrease in the high-affinity binding site density and the corresponding increase in endogenous enkephalin levels suggest the existence of an opioid compensatory mechanism associated with the aging process[125].

Immune responses. Ottaviani *et al.*[126] have demonstrated that in the gastropod *Planorbarius corneus* ACTH (1–24) and β-endorphin can stimulate haemocyte locomotion and phagocytosis under stress.

Annelida

Localization and distribution

The earliest information concerning the presence of opioid peptides in annelids was produced by Alumets *et al.*[127] and Rémy and Dubois[128], who studied earthworms. Using immunocytochemistry, Alumets *et al.*[127] demonstrated the presence of immunoreactive Leu-Enk-like material in the cerebral ganglia and cerebral connectives

Table 4. Identification and localization of opioid-like peptides in annelids

Peptide	Species	Localization	Techniques	References
Leu-Enk-like	*Eisenia foetida*	Neurons of the brain	Immunocytochemistry	132
	Haemopis marmorata	Segmental ganglia	Immunocytochemistry	135, 137
	Lumbricus terrestris	Cerebral ganglia, cerebral connectives, suboesophageal ganglia, ganglia of the nerve cord, central nervous system, gut, seminal vesicle, body wall tissue, epidermis, nerve cord	Immunocytochemistry, RIA	127, 130, 133
	Nereis diversicolor	Central nervous system, peripheral nerves, oocytes, spermatocytes	Immunocytochemistry	140–144
	Theromyzon tessulatum	Central nervous system, proboscis, foregut	Immunocytochemistry	138
Met-Enk-like	*Eisenia foetida*	Neurons of the brain	Immunocytochemistry	132
	Hirudo medicinalis	Brain neurosecretory cells, fibres within the neuropil of the brain	Immunocytochemistry	136
	Lumbricus terrestris	Central nervous system, gut, seminal vesicles, body wall tissue, epidermis, nerve cord	Immunocytochemistry, RIA	130, 133
	Nereis diversicolor	Central nervous system, peripheral nerves	Immunocytochemistry	140–142
α-Endorphin	*Dendrobaena subrubicunda*	Suboesophageal ganglia	Immunocytochemistry	128
	Nereis diversicolor	Central nervous system, peripheral nerves	Immunocytochemistry	140–142
β-Endorphin	*Lumbricus terrestris*	Cerebral ganglia, cerebral connectives, suboesophageal ganglia, ganglia of the nerve cord	Immunocytochemistry	127, 131
	Nereis diversicolor	Central nervous system, peripheral nerves	Immunocytochemistry	140–142
α-Neoendorphin	*Theromyzon tessulatum*	Central nervous system, proboscis	Immunocytochemistry	138
Dynorphin	*Eisenia foetida*	Neurons of the brain	Immunocytochemistry	132
	Nereis diversicolor	Central nervous system peripheral nerves, oocytes, spermatocytes	Immunocytochemistry	140–142
	Theromyzon tessulatum	Central nervous system, proboscis	Immunocytochemistry	138
ACTH-like	*Dendrobaena subrubicunda*	Cerebral ganglion	Immunocytochemistry	129
	Lumbricus terrestris	Epidermis, nerve cord	Immunocytochemistry	134
	Nereis diversicolor	Central nervous system	Immunocytochemistry	144
	Theromyzon tessulatum	Brain	Immunocytochemistry	139
MSH	*Lumbricus terrestris*	Epidermis, nerve cord	Immunocytochemistry	134
α-MSH	*Theromyzon tessulatum*	Brain	Immunocytochemistry	139
β-MSH	*Nereis diversicolor*	Central nervous system	Immunocytochemistry	144

RIA radioimmunoassay

of *Lumbricus terrestris*. They also observed in some, but not all, of the specimens immunoreactive Leu-Enk-like material in the suboesophageal ganglion and the ganglia of the nerve cord. Cells immunoreactive to β-endorphin have a distribution similar to those of the enkephalin, but differ in number and size. A summary of such localization studies[127 144] as well as distribution of opioid peptides in other annelids are presented in Table 4.

Physiological evidence for the existence of opioid receptors in the leech has been reported by Flanagan and

Zipser[137]. Homogenates of the nerve cord of the leech, *Haemopis marmorata*, display saturable binding of 3H-(D-Ala^2-)-Met-Enk.

Functions

Behaviour Leeches respond behaviourally to externally applied morphine and to morphine injections. Kaiser[145] reported that morphine produces a biphasic hyperkinetic–quiescent locomotory response in free-swimming *Hirudo medicinalis*. Flanagan and Zipser[137] found a similar biphasic response in another leech, *Haemopis marmorata*, that was injected with morphine. Control animals injected with saline were initially hyperactive after injection, but rapidly reattached themselves to the wall of the holding tank and remained highly responsive to subsequent tactile stimulation. Morphine-injected leeches were also initially hyperactive, but after 5–10 min they fell to the bottom of the tank and remained unresponsive to all but the most extreme tactile stimulation for a period of 4 days. These animals fully recovered by day 5. This behavioural effect appears to have been due to inhibition of synaptic transmission by morphine. Gardner and Walker[146] reported an inhibitory effect of morphine on the Retzius cells of *Hirudo medicinalis*. Other evidence from *Hirudo medicinalis* and *Haemopis marmorata* suggests that the enkephalins are acting either as primary neuromodulators or as sensory neurotransmitters[147].

Feeding. Kaloustian and Rzasa[131], using radioimmunoassay found the highest concentrations of Met-Enk-like, Leu-Enk-like and β-endorphin-like material in the region of the gut which contains high digestive enzyme activity of *Lumbricus terrestris*. Isolated gut treated with these opioids showed significant decreases in its contractile properties. It appears that these opioids exert their effects by modulating the action of DA, resulting thereby in an increased transit time for food in areas of the gut with high digestive enzyme activities, hence enhancing the digestive process.

Reproduction. Experiments were done with *Nereis diversicolor* to determine whether dynorphin may have a role in the control of reproduction[144]. The basis for these experiments was the presence of this opioid in the infracerebral neurohemal area of the cerebral ganglia. Parapodia in which spermatogenesis was proceeding were incubated in sea water containing dynorphin. However, no effect on spermatogenesis was observed. Thus, it seems likely that if dynorphin-like peptides have a role in reproduction, they do not act directly on the spermatocytes, in contrast to the direct action of 'nereidin' (a brain neuropeptide that inhibits the maturation of spermatocytes in nereids)[148]. Therefore, if these dynorphin-like peptides have any role in reproduction, it is only to function as neurotransmitters to control the release of reproductive hormones.

Other invertebrates

Immunocytochemical evidence for the occurrence of opioid peptides in invertebrates other than annelids, arthropods and molluscs is rather scarce. Compounds similar to ACTH (1–39) and β-endorphin have been detected in the protozoan *Tetrahymena pyriformis*[149]. Roth *et al.*[150] reported that opioid peptides alter the feeding behaviour of the amoeba, with the response being blocked by naloxone. Venturini *et al.*[151,152] detected Met-Enk-like immunoreactivity in neuronal perikarya and in neuropil in the planarian *Dugesia gonocephala*. In this planarian naloxone induces a dose-dependent rise in the cAMP level while morphine decreases cAMP. Morphine treatment decreases the motility of this planarian whereas naloxone treatment induces screw-like hyperkinesis. Treatment with dopaminergic agonists (L-dopa, apomorphine) induces typical screw-like hyperkinesis, whereas after treatment with reserpine and haloperidol all the planarians became motionless, a response similar to that after treatment with morphine. A model for interactions between dopaminergic and enkephalinergic neurotransmission and/or neuromodulation was proposed that involved inhibitory modulation of opiates on DA release[152]. Romero *et al.*[153] reported that Met-Enk decreased the mitotic rate of adult planarians during regeneration while substance P was stimulatory. Immunocytochemical studies showed the presence of Met-Enk within the central and peripheral nervous systems of this planarian.

Piccoli *et al.*[13] made a study of the presence of endogenous Met-Enk and Leu-Enk in 11 species of marine organisms ranging from sponges to tunicates. Extracts from whole-animal tissues of the sponge *Dysidea avaria*, the cnidarians *Anemonia sulcata*, *Pelagia noctiluca*, *Rhyzostoma pulmo*, and the tunicate *Ciona intestinalis* were found to contain opioid peptides. Rémy and Brossard[154] observed immunoreactivity to Met-Enk in the cephalic nervous system of two species of nemerteans, *Lineus viridis* and *Lineus ruber*, but in the ventral ganglia, Met-Enk-like material is rather scarce. Colon-Urban *et al.*[155] suggested that endogenous opioids in various bryozoans (*Bugula neritina* and *Membranipora* sp.) play a role in the organisms' motor mechanism. Morphine inhibits tentacle activity, an effect which can be blocked by prior or concomitant naloxone application. Engbretson and Chamberlain[156] reported positive immunoreactivity to Leu-Enk in fibres of the optic medulla of the horseshoe crab *Limulus polyphemus*.

Conclusions

Although the study of endogenous opioid peptides in invertebrates is still at an early stage, the data presented in this review support the view that such molecules are present throughout the invertebrate phyla and have a

long evolutionary history. The application of immunocytochemical techniques has greatly influenced opioid research in invertebrates. Despite an impressive amount of research in invertebrates, for many of the species studied the functional significance of these opioid peptides and the mechanisms underlying their action remain elusive. The biochemical, physiological and pharmacological characterization of opioid peptides needs a careful study in order to strengthen our knowledge about their actual role in the physiology of invertebrates. Another important area that merits much attention is the identification and purification of the receptors for opioid peptides in invertebrates. Elucidation of the molecular structures of the opioid receptors will open new avenues for the evaluation of the molecular aspects of opioid receptor function.

A challenging phenomenon of current opioid research in higher animals, including mammals, is the colocalization of opioid peptides with nonopioids and classical neurotransmitters in the same nerve terminal. The rapidly increasing data on this issue show a bewildering complexity, which is presently difficult to understand in the mammalian nervous system. Simple models using invertebrates may be more suitable to unravel the nature of the putative interactions between opioid peptides and classical neurotransmitters. Finally, invertebrate opioid research may have practical applications to help control harmful organisms, such as insect pests and barnacles. Knowledge of the neurochemistry of the opioids in the invertebrates could lead to the discovery of novel ways to inhibit reproduction and growth of these organisms. If opioid peptides are important regulatory agents in metabolism, then it might be possible to devise methods of breaking particular links in the synthesis of these compounds, and research directed along these lines could provide us with much-needed compounds for the control of invertebrate pests.

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