diate-variety bout (mean score 10.1), but the difference was not significant ($T = 29$, $N = 14$, $P > .05$). However, the mean responses for individual females (over days) were significantly higher for eventual variety than for immediate variety ($T = 0$, $N = 6$, $P < .05$) (*18*) (Fig. 1D). Thus it appears that female song sparrows prefer eventual variety.

These results demonstrate a substantial match between the singing behavior exhibited by adult male song sparrows and the type of song structure and programming that provoke the strongest response from female song sparrows. Female song sparrows solicit more strongly to conspecific than they do to heterospecific songs. For both main elements of song structure, syllable structure and temporal pattern, female song sparrows prefer conspecific patterns to heterospecific ones. Male song sparrows sing bouts that contain multiple song types, and female song sparrows respond more strongly to a sequence of several song types than they do to a bout of a single song type. Finally, male song sparrows order their song types with eventual variety, suggesting that females prefer such an ordering to immediate variety. The match between male behavior and female responsiveness should be advantageous to both sexes; males should benefit from stimulating females to copulate with them, and females should benefit from responding to and copulating with only conspecific males. It remains to be determined whether female song sparrows also use individual difference between conspecific males in song structure and song bout structure as a basis for choosing particular males as mates.

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6. Implants of 17β-estradiol, packed into lengths of Silastic medical-grade tubing (1.96 mm outside diameter) with both ends plugged by adhesive, were placed under the skin of the back where they could be seen easily and removed later. Implants with 15 mm of hormone were given on 16 May and tests with song on 26, 28, and 30 May 1980. Three females that did not give solicitation displays on the first three trials were given a second estradiol implant of the same size on 30 May and tested again on 2, 4, 6, and 8 June (W. A. Searcy, P. Marler, S. S. Peters, *Anim. Behav.*, in press).
7. Each song bout consisted of a single song, 2 to 2.5 seconds long, repeated with 8 seconds between songs. The song sparrow and swamp sparrow songs were recorded from free-living males in Dutchess County, New York. The chaffinch song was from a commercial recording [M. North and E. Simms, *Witherby's Sound-Guide to British Birds* (Witherby, London, 1958)].
8. In copulation solicitation display, a female song sparrow arches her back and brings her tail forward and her head back. The wings are moved away from the body and vibrated. The observer awarded a score of 1 for an incomplete display, 2 for a complete display of short duration (approximately 1 second or less), and 3 for a complete, prolonged display.
9. The N is the number of bird observation days for which nontied response scores were obtained. Since all birds were tested on more than 1 day and the statistics applied to the total observation days, the results of these tests, strictly speaking, apply to the population of all possible scores of the subjects used rather than to the population of all possible individual female song sparrows. For the four subjects that gave solicitation displays in these trials, mean scores (across days) were greater for song sparrow song than for either heterospecific song.
10. The three females used in this experiment were the three given a single implant on 16 May (*6*) and not treated again. Test songs were made by entering natural syllables through the analog-to-digital converter to a PDP 11/10 minicomputer and re-editing the syllables into the desired temporal pattern [S. S. Peters, W. A. Searcy, P. Marler, *Anim. Behav.* **38**, 393 (1980); S. Zoloth *et al.*, *Z. Tierpsychol.* **54**, 151 (1980)].
11. All three subjects gave greater mean responses (across days) to the song with conspecific syllables and conspecific temporal pattern than to either of the two test songs with heterospecific elements.
12. Songs were four different song types taken from the repertoire of a single male recorded in Dutchess County. All bouts consisted of 32 songs repeated with 8 seconds between songs. A different single-song bout was used on each test day, each single-song bout was composed of one of the four song types used in the four-song bout. The four-song bout was assembled with eventual variety (that is, AAAAAAAABBB BBBBB and so on).
13. Six females were given implants (8 to 9 mm) of estradiol on 18 July 1980 and tested on 2, 3, 4, and 5 August. Two females were given implants of the same size on 7 August and tested on 20, 21, 22, and 23 August.
14. In these trials, all eight subjects responded with solicitation displays, and seven gave higher mean responses (across days) to the four-song bouts than to the single-song bouts.
15. C. Hartshorne, *Born to Sing. An Interpretation and World Survey of Bird Song* (Indiana Univ. Press, Bloomington, 1973); D. E. Kroodsma and J. Verner, *Auk* **95**, 703 (1978).
16. All songs were taken from the repertoire of a single male (not the same one used in the previous experiment) recorded in Dutchess County.
17. These were the same eight females used in the experiment of single-song versus four-song bouts. The group of six was tested on 29, 30, and 31 July and 1 August; the two were tested on 20, 21, 22, and 23 August.
18. Of the eight subjects, one failed to respond, one gave nonzero but tied mean responses, and six gave higher mean responses to eventual variety than to immediate variety tests.
19. We thank M. H. Searcy for drawing the figure. Research was supported by NIMH grant PHS MH 14651.

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Opioid Inhibition of Dopamine Release from Nervous Tissue of *Mytilus edulis* and *Octopus bimaculatus*

Abstract. *Morphine and D-Ala²-Met-enkephalin as well as other opioids suppress potassium-stimulated release of ³H-labeled dopamine from nervous tissue of two marine invertebrates,* Mytilus edulis *and* Octopus bimaculatus. *Naloxone reverses the inhibitory effects in both species. Potassium-stimulated release of ³H-labeled serotonin is not altered by opioids. It is postulated that opiate receptors and their endogenous effectors play a prominent role in regulation of transmitter release in invertebrates.*

Although the importance of aminergic systems in invertebrates has been realized for some time, the role of biologically active small peptides in invertebrate nervous systems has only recently become apparent (*1*). Opioid peptides and various narcotic agents increase dopamine concentrations in certain ganglia of the marine mollusk *Mytilus edulis* (*2*), the freshwater mollusk *Anodonta cygnea* (*3*), and the land snail *Helix pomatia* (*4*). This effect, which requires relatively low concentrations of the agents, is reversed by naloxone, strongly suggesting the involvement of an opiate receptor mechanism. A naloxone-reversible influence of methionine enkephalin and morphine on activity of identified single neurons in *H. pomatia* has been reported (*5*), and opiate binding sites have been characterized in *M. edulis* (*6*), providing further indication that opiate systems play a role in the molluscan nervous system.

Opioids specifically and selectively inhibit the release of dopamine (*7*) and norepinephrine (*8*) as well as other transmitters (*7, 9*) in regions of the mammalian central and peripheral nervous systems; indeed, this may represent a major mode of action of opioid compounds. This action may also be directly mediated by presynaptically localized opiate receptors (*10*). On the basis of our previous studies of the influence of opioids on dopamine concentrations in ganglia of *M. edulis* (*2*), we have examined directly the influence of these substances on dopamine release both in ganglia of *M.*

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edulis and in the brain of the cephalopod *Octopus bimaculatus*. The octopus was chosen for investigation because of its relatively large and highly complex central nervous system (*11*) and because of the high concentration of dopamine and other amine transmitters in octopus brain (*12*). These studies demonstrate that opioids exert an inhibitory influence on transmitter release in invertebrates.

Ganglia were dissected from *M. edulis* freshly harvested from subtidal waters in Long Island Sound, Northport, New York, as previously described (*2*). *Octopus bimaculatus* (250 to 500 g), were obtained from Pacific Bio-Marine Laboratories and kept in artificial sea water (ASW, Instant Ocean) at 13°C prior to experimentation. Either the supraesophageal lobes (excluding optic lobes) were dissected out as a unit and then randomly sliced or the lobes of the brain were dissected out individually according to Young (*12*). Dissections of both *M. edulis* and *O. bimaculatus* were carried out in ASW in the cold. Tissues were then incubated with [^{3}H]dopamine or other labeled precursor at 22°C, washed, and transferred to a perfusion chamber for exposure to KCl or drugs (*13*).

As shown in Fig. 1, 50 m*M* KCl caused the release of [^{3}H]dopamine from pedal ganglia of *M. edulis*. In the absence of drugs the release caused by KCl was approximately 10 percent of the tissue content of radioactivity. Addition of morphine or D-Ala^2-Met-enkephalin (DALA) to the superfusing medium at the time of the K^+ application resulted in concentration-dependent inhibition of the evoked release of dopamine. Essentially complete inhibition of K^+-evoked release was achieved with 5 μM concentrations of either agent. Superfusion with naloxone produced a concentration-dependent reversal or prevention of the effects of morphine and DALA (Table 1). Naloxone alone at 10 μM to 100 μM had no effect on spontaneous of K^+-evoked release of dopamine. In other studies dextrorphan did not affect the process in any observable manner at concentrations up to 40 μM. However, etorphine and β-endorphin each caused complete inhibition at 1 μM, and these effects were also reversed by naloxone in a concentration-dependent manner (data not shown).

In addition to the pedal ganglion, the other ganglia of *M. edulis* were also influenced by opioids. Morphine at 5 μM inhibited K^+-evoked dopamine release from cerebral and visceral ganglia by 80 and 90 percent, respectively. DALA also inhibited release from cerebral and vis-

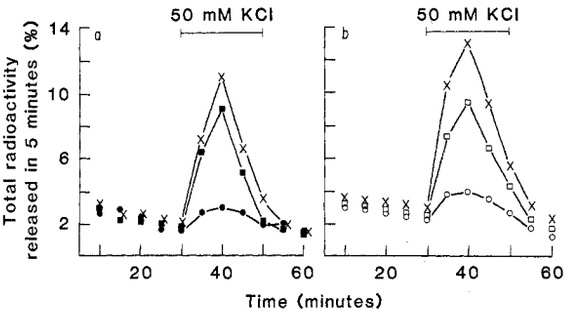

Fig. 1. Inhibition of K^+-stimulated release of [^{3}H]dopamine by (a) morphine and (b) DALA (D-Ala^2-Met-enkephalin) in the pedal ganglion of *M. edulis*. Ganglia were incubated at 22°C for 30 minutes in 1 ml of ASW containing 0.1 percent ascorbic acid and [^{3}H]dopamine (0.7×10^6 dis/min; specific activity, 35.6 Ci/mmole) (New England Nuclear) with constant shaking. After being incubated, the ganglia were washed twice in 2 ml of ASW and then each ganglion (average wet weight, 1.8 mg) was transferred to a Plexiglas perfusion chamber containing 2 ml of ASW. An average of 3200 count/min was present in the ganglion at the start of the superfusion. A rabbit four-channel peristaltic pump (Rainin) maintained the flow rate at 1 ml/5 min to an inflow opening at the bottom of the chamber. The perfusing solution was altered at the desired intervals by manually transporting the inflow tubes to the appropriate beaker. The superfusate was collected from an outflow opening near the rim of the chamber with one superfusate fraction collected every 5 minutes. Ganglia were first superfused with ASW for 30 minutes. Then, for the next 15 minutes ganglia were perfused with ASW containing 50 m*M* KCl alone (control) or together with drug (morphine or DALA). Finally, the ganglia were again perfused with ASW for the remainder of the experiment. Radioactivity of the superfusate solutions was determined by liquid scintillation spectrometry. The values shown represent the percentage of the total radioactivity in the ganglion that was released during the 5-minute period ending at the time indicated. Each value is the mean of five separate experiments. Symbols: x, control; ●, 5 μM morphine; ■, 1 μM morphine; ○, 5 μM DALA; and □, 1 μM DALA. The standard error of the mean was less than ±1.0 percent for all values.

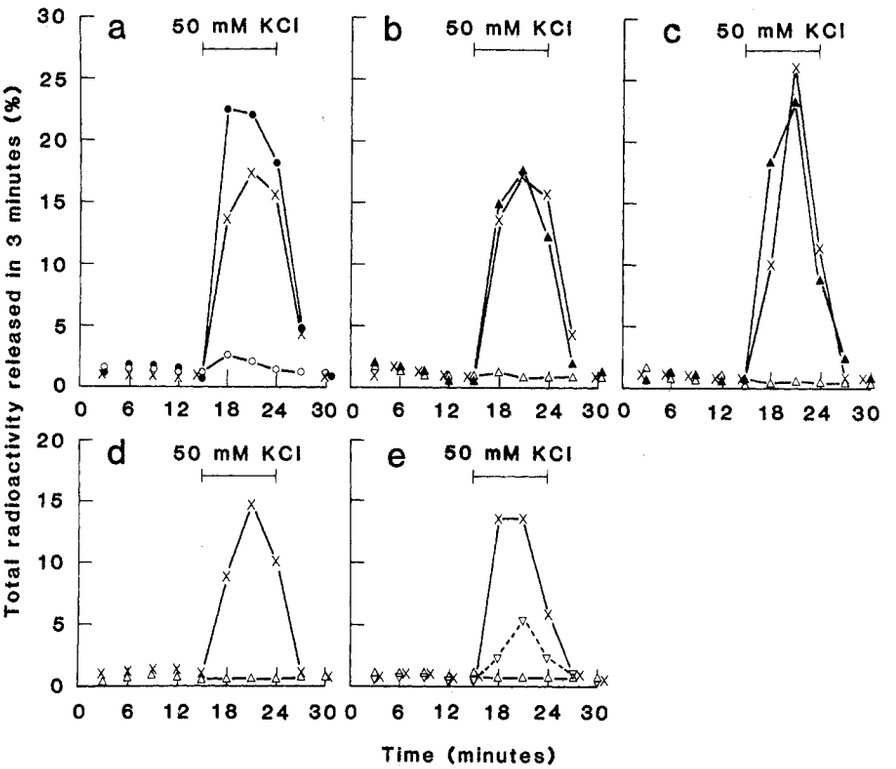

Fig. 2. Potassium-stimulated release of [^{3}H]dopamine in brain of *O. bimaculatus*. Inhibition by (a) morphine and (b to e) DALA in slices of supraesophageal lobe tissue (a and b) and of subdissected (c) vertical, (d) basal, and (e) frontal lobes of brain. Experimental details were as described in the legend to Fig. 1 and in the text with the following modifications. The tissue slices (5 to 15 mg, wet weight) were incubated with [^{3}H]dopamine (2×10^6 dis/min; specific activity, 40.7 Ci/mmole) in 1 ml of ASW. An average of 10,300 count/min was present in the combined (a and b) supraesophageal lobe tissue and 19,500, 7,700, and 12,900 count/min in the (c) vertical, (d) basal, and (e) frontal lobes, respectively, at the start of the superfusion. The flow rate through the perfusion chamber was 1 ml/3 min and the superfusate fractions were collected every 3 minutes. Brain tissue was first superfused with ASW for 15 minutes, then with 50 m*M* KCl alone or with drugs (*5*) for 9 minutes and then again with ASW for the remainder of the time. Values represent means of three to five separate experiments. Symbols: x, control; ○, 25 μM morphine; ●, 25 μM morphine plus 50 μM naloxone; △, 10 μM DALA; ▲, 10 μM DALA plus 50 μM naloxone; and ▽, 1 μM DALA. The standard error of the mean was less than ±1.5 percent for all values.

ceral ganglia and naloxone antagonized this inhibition (Table 1).

In brain of *O. bimaculatus*, KCl produced a marked stimulation of [^{3}H]dopamine release (Fig. 2). Opioid compounds inhibited this release, with complete suppression by 10 μM morphine (Fig. 2a) or DALA (Fig. 2b). Superfusion with naloxone completely reversed the effects of morphine and DALA, again demonstrating the reversibility and specificity of the opioid action. The opioids were found to be inhibitory not only in brain tissue comprising all of the supraesophageal lobes, but also in separately incubated vertical (Fig. 2c), basal (Fig. 2d), and frontal lobe (Fig. 2e) tissue.

In contrast to the above results with [^{3}H]dopamine, opioids did not alter K^+-evoked release of radioactivity from *M. edulis* ganglia previously incubated with [^{3}H]serotonin. Thus, when tested at concentrations capable of completely inhibiting [^{3}H]dopamine release, morphine, DALA, etorphine, and β-endorphin were each completely ineffective in inhibiting [^{3}H]serotonin release. In studies of *O. bimaculatus*, 10 μM DALA was also entirely without effect on K^+-evoked release of [^{3}H]serotonin.

These experiments indicate that opioids can exert a profound inhibitory influence on K^+-evoked [^{3}H]dopamine release from neuronal tissue in the two marine invertebrates examined. The pharmacological characteristics of the response, including reversal by naloxone and inactivity of dextrorphan, strongly suggest that opiate receptors mediate the response. Also, the sensitivity to opioid compounds is similar to that reported for inhibition of transmitter release from mammalian nervous tissue incubated in vitro under similar conditions (*7, 9*). It appears that essentially all neurons capable of K^+-evoked dopamine release in the invertebrate tissues studied are sensitive to the opioid compounds. While the specific neuronal population accumulating and releasing [^{3}H]dopamine remains to be established, it seems likely that a major component of this response involves dopaminergic neurons. Thus, release of [^{3}H]serotonin was not affected by opioids. Also, in other studies not presented here [^{3}H]norepinephrine release was not affected by opioids in the cerebral ganglia and was only partially inhibited by even high concentrations of opioids in pedal and visceral ganglia of *M. edulis*. It seems likely that the inhibition of dopamine release by opioids may at least in part account for the previously reported increases in dopamine levels in various invertebrates after opioid administration (*2–4, 6*).

Table 1. Blockade by naloxone of opioid inhibition of K^+-evoked dopamine release in ganglia of *M. edulis*. The experimental conditions were as described in the legend to Fig. 1 and in the text. Each value is the mean of four separate experiments. The standard error of the mean was ±3 percent or less for all values.

Drug additions	Release (% of control)
Pedal ganglion	
None (control)	(100)
Morphine (5 μM)	4
Plus naloxone (8 μM)	4
Plus naloxone (14 μM)	22
Plus naloxone (22 μM)	50
Plus naloxone (30 μM)	68
Plus naloxone (40 μM)	99
DALA (5 μM)	5
Plus naloxone (8 μM)	13
Plus naloxone (14 μM)	35
Plus naloxone (18 μM)	53
Plus naloxone (22 μM)	67
Plus naloxone (27 μM)	98
Cerebral ganglion	
None (control)	(100)
DALA (5 μM)	10
Plus naloxone (10 μM)	64
Visceral ganglion	
None (control)	(100)
DALA (5 μM)	9
Plus naloxone (10 μM)	62

Thus, dopamine release in *M. edulis* and *O. bimaculatus* appears to be influenced by opiate alkaloids (morphine) as well as by both small (enkephalin) and large (endorphin) peptides. While this provides no strong indication as to the possible chemical nature of endogenous opioid substances in the invertebrate tissues studied, a recent report (*14*) indicates the presence of methionine enkephalin–like immunoreactivity in a neurophil layer in the vena cava of *Octopus vulgaris*. The present study suggests that the octopus and mussel contain in their nervous systems a fairly widely distributed opiate system that may resemble in many respects that present in mammals. The present study also indicates the involvement of opiates in presynaptic release mechanisms in invertebrates. Although transmitter release has been extensively utilized as a means for elucidating relations between transmitter systems in vertebrates, modulation of the release of one transmitter by another transmitter in invertebrates has not, to our knowledge, previously been reported. The occurrence of opiate systems in invertebrate phyla other than Mollusca is suggested by the presence of opiate binding sites in *Drosophila* heads and of enkephalin- and endorphin-like immunoreactivity in earthworm neurons (*15*). The finding that opioids modulate dopaminergic systems in invertebrates may be important for our further understanding of the functional roles of each of these systems in invertebrates as well as in the evolution of transmitter relationships.

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