the use of endotoxin-contaminated protein preparations should be avoided in any kind of biomedical research unless it can be shown that the particular system is insensitive to endotoxins. The endotoxin content of processed biological materials should be stated by the supplier, or should be tested by the investigator before they are used in biological research.

LASZLO Z. BITO

Research Division, Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York 10032

References and Notes

1. B. A. Philip, P. Herbert, J. W. Hollingsworth, *Proc. Soc. Exp. Biol. Med.* **123**, 576 (1966).
2. S. J. Ajl, S. Kadis, T. C. Montre, Eds., *Microbial Toxins* (Academic Press, New York, 1971), vol. 5.
3. L. Z. Bito, *Invest. Ophthalmol.* **13**, 959 (1974).
4. Protein preparations were made up in pyrogen-free saline to a concentration of 100 mg/ml, and filtered through a Millipore GS 0.22 membrane; 1-ml portions were kept at 2°C until injection. The following protein preparations were used: sterile bovine albumin (BSA), 30 percent solution (Gallard-Schlesinger); ovalbumin, 2× crystallized (Worthington); rabbit and human albumin (RSA and HSA, respectively) crystallized (Pentex, Miles Laboratories); and normal serum albumin (human; HSA/USP), 25 percent solution (Abbott).
5. Five tenfold serial dilutions (0.1 ng to 10 μg/100 μl of pyrogen-free saline) of shigella endotoxin (lipopolysaccharide B, *S. flexneri*; Difco) were filtered as described above and stored at −10°C.
6. L. Z. Bito, J. C. Roberts, S. Saraf, *J. Physiol. (London)* **231**, 71 (1973).
7. Iritis was judged on the basis of the color of the iris and engorgement of its blood vessels and was rated 0 through 3 [see also (*3*)].
8. The endotoxin assay was kindly performed for us by J. Fine of Harvard University Medical School according to the limulus technique; see R. B. Reinhold and J. Fine, *Proc. Soc. Exp. Biol. Med.* **137**, 334 (1971).
9. The fact that the crystallized HSA, which is prepared from sterile human blood, also contains endotoxins suggests that processing is an important source of this contamination. Thus processed proteins in general, including enzymes of both animal and plant origin, should be suspected of endotoxin contamination.
10. There is accumulating evidence that such diverse effects of endotoxins as induction of fever [C. J. Woolf, G. H. Willis, H. Laburn, C. Rosendorff, *Neuropharmacology* **14**, 397 (1975)], pulmonary hypertension [F. L. Anderson, D. J. Tsagavis, W. Jubiz, H. Kudia, *Am. J. Physiol.* **228**, 1479 (1975)], renal hypotension [A. G. Herman and J. R. Vane, *Arch. Int. Pharmacol.* **208**, 365 (1974)], or the sensitization of fat cells to norepinephrine in vitro [J. A. Spitzer, *Proc. Soc. Exp. Biol. Med.* **145**, 186 (1974)] are mediated by stimulation of prostaglandin synthesis or the production of 3′,5′-adenosine monophosphate (cyclic AMP). Since both the prostaglandin and the cyclic AMP systems are ubiquitous and play a basic role in the control of cellular processes, the apparent ability of endotoxins to stimulate these systems further emphasizes their broad potential to affect biological processes.
11. I thank J. Fine for the endotoxin assays; and R. A. Baroody, E. V. Salvador, and D. W. Garnick for assistance. Supported by NIH grant EY 00402-09.

23 November 1976

An Analog of Enkephalin Having Prolonged Opiate-Like Effects in vivo

Abstract. *Intraventricular administration of the enkephalin analog, [D-Ala²]-Met-enkephalin, induces profound and long-lasting analgesia, as well as other opiate-like behavioral effects in the rat. This analgesia was highly dose dependent, of much greater magnitude, and about 30 times longer lasting than that produced by the naturally occurring peptide, methionine-enkephalin. The behavioral effects of the [D-Ala²] analog could be completely reversed by the opiate antagonist, naloxone, suggesting that these effects were mediated by opiate receptors. Systemic administration of naloxone alone resulted in a significant increase in pain sensitivity. These findings support the view that endogenous opiate systems may play an important role in modulating pain sensitivity.*

Endogenous peptides with strong opiate agonist properties have been isolated from the mammalian central nervous system and identified by Hughes as the pentapeptides methionine-enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) and leucine-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) (*1*). The enkephalins were shown to have potent opiate-like activity in a number of assay systems (*2*). Similarly, behavioral tests have confirmed the opiate-like properties of enkephalin. A very short-lasting but significant analgesia was demonstrated with the D'Amour-Smith tail-flick test (*3*) after intraventricular or localized midbrain injections of methionine-enkephalin (100 to 200 μg) in the rat and mouse (*4*). The relative selectivity of the tail-flick test for opiate agonists (*5*) and the reports that enkephalin-induced analgesia can be antagonized by naloxone (*4*) suggest that the analgesic effects of this peptide are due to its actions on opiate receptors. These results, in conjunction with reports that electrical stimulation of localized regions in the brainstem can induce profound analgesia which appears to be mediated by opiate receptors (*6*), have prompted suggestions that endogenous enkephalins may act to modulate pain systems.

The brief analgesia produced by the enkephalins, however, stands in marked contrast to the much more prolonged effects of similar administrations of morphine (*7*). Structurally, methionine-enkephalin resembles morphine and morphine-related narcotics most closely when a type I β-bend conformation is assigned to the H-Tyr-Gly-Gly sequence of the pentapeptide (*8*). Additional evidence (*9*) suggests the possibility that the preferred type I β-bend conformation may be stabilized by the substitution of a D-amino acid for the second glycine in this sequence.

We now report a profound and long-lasting analgesia induced by intracerebroventricular administration of [D-Ala²]-methionine-enkephalin, a finding consistent with a recent report on a similar analog published since the submission of this report (*10*). Further, we found that naloxone, a blocker of opiate receptors, produced the opposite effect of increased sensitivity in otherwise untreated animals. These findings provide evidence for a functional role of endogenous opiate systems in the modulation of pain sensitivity.

Permanent intraventricular cannulas were surgically implanted in six male albino (Holtzman) rats 120 to 180 days old (*11*). Examination of cannula placements after injection of a marker dye revealed the presence of dye in the ventricles of all animals. After 1 week for postoperative recovery, each rat was tested for analgesia by the tail-flick test both before and after intraventricular administration of 200 μg of methionine-enkephalin, 200 μg [D-Ala²]-methionine-enkephalin, or the injection vehicle. Peptide synthesis has been described (*12*). Peptides were dissolved in Ringer's solution just before injection and were then administered in 10-μl volumes over a period of 1 minute by means of a microsyringe. Control injections consisted of the vehicle alone adjusted to a *p*H of 4.0 to approximate the *p*H of the drug solution. Each rat received all treatments with tests separated by at least 48 hours. Control tests with the vehicle alone were given first and last, with the two drug treatments intervening in counterbalanced order. Each test consisted of a baseline period during which the latency to withdraw the tail from a radiant heat source (*3*) was recorded every 2 minutes. A maximum latency of 10 seconds was used since the intense heat created by this procedure could cause lasting tissue damage. After the establishment of stable tail-flick latencies (3.5 to 5.5 seconds), baseline latencies were recorded for a period of 10 minutes. The drug or control was then administered, and tail-flick latencies were recorded every 2 minutes for a minimum of 10 minutes or until latencies had returned to within 30 percent of baseline values.

Both methionine-enkephalin and the

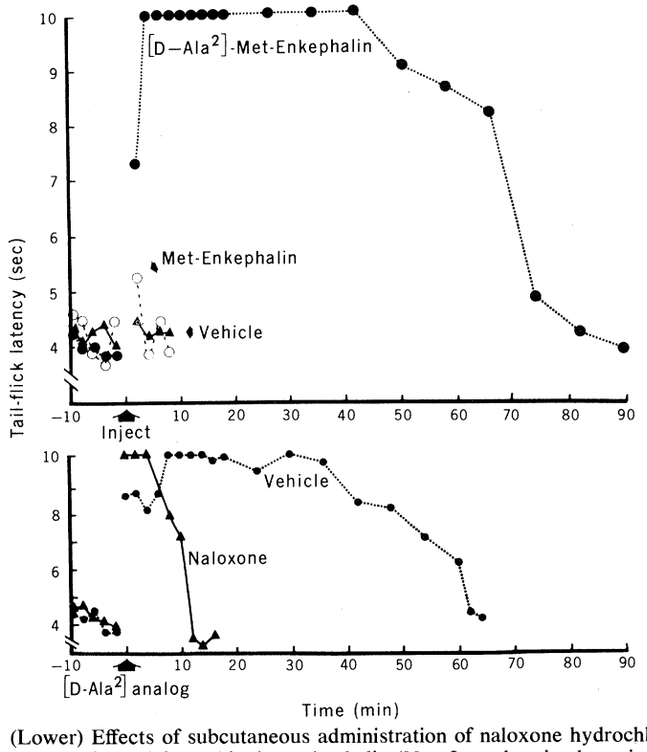
[D-Ala²] analog produced elevations greater than control values in the latency to tail-flick (Fig. 1). Although elevations after control injections were nonsignificant, both methionine-enkephalin and the [D-Ala²] analog produced significant increases over their baseline values and over the control values. All comparisons were significant at the .03 level (two-tailed randomization tests). Figure 1 also illustrates that the [D-Ala²] analog produced a much more profound effect than did methionine-enkephalin, as evidenced by a significantly greater magnitude (the maximum increase in tail-flick latency, mean ± standard error of the mean, after administration of [D-Ala²] was 5.7 ± 0.18, and after methionine-enkephalin it was 2.0 ± 1.0, $P = .03$); the duration of the analgesia—that is, the mean time (minutes) of more than 30 percent elevation in tail-flick latency was, for [D-Ala²], 51 ± 6.8, and for methionine-enkephalin it was 4 ± 2.89, $P = .03$.

Analgesia produced by both methionine-enkephalin and the [D-Ala²] analog was generally accompanied by other behavioral changes, especially at the higher doses. Shortly after injection, the rats often exhibited a coarse tremor (*13*) followed by a marked immobility and a stuporous appearance. These motor effects were prolonged and dramatic after treatment with the [D-Ala²] analog. In this condition many animals would lie motionless on their backs for an hour or more and show no response to exceedingly noxious pinches to the face or tongue. An additional observation was that a spontaneous seminal emission often occurred. Naloxone completely reversed the analgesia as well as the other behavioral effects of the [D-Ala²] analog, strongly suggesting that these effects were mediated by opiate receptors. Two rats each received intraventricular injections of 200 μg of [D-Ala²]-methionine-enkephalin in two separate tests, and tail-flick latencies were measured as described above. When the latency to tail-withdrawal exceeded baseline values by 30 percent for three consecutive tests, either naloxone hydrochloride (Endo Laboratories), at 10 mg/kg, or the saline vehicle was administered subcutaneously. One animal received the saline injection on the first test and naloxone on the second; the other received treatments in the opposite order. The tail-flick latencies returned to baseline values within a few minutes after naloxone injections (Fig. 1, lower panel), while latencies remained elevated for more than 50 minutes after saline injections.

A systematic dose comparison of the [D-Ala²] analog revealed a high degree of dose dependency for the analgesic effects of the peptide. A separate group of six rats was used for these tests. Surgery and testing were performed as outlined above with the exception that animals received counterbalanced administrations of three levels of a single drug as well as two control tests with the vehicle alone. As illustrated in Fig. 2, higher doses of [D-Ala²]-methionine-enkephalin resulted in both a greater magnitude and duration of analgesia ($P = .01$ and .04, respectively; Friedman's analysis of variance).

Our results confirm earlier observations that methionine-enkephalin produces moderate levels of analgesia for short periods of time. Furthermore, our data together with a recent independent report (*10*) indicate that a closely related analog, [D-Ala²]-methionine-enkephalin, can induce a much more profound and long-lasting analgesia at a comparable dose level. These findings are consistent with a recent demonstration of the greater potency of this analog on the dopa potentiation test (*14*) and our report of its greater potency in two bioassay systems (*12*). Furthermore, we have found that the [D-Ala²] analogs of α and γ endorphins also have much more potent analgesic and sedative effects than the naturally occurring peptides (*15*).

In this and previous reports, enkepha-



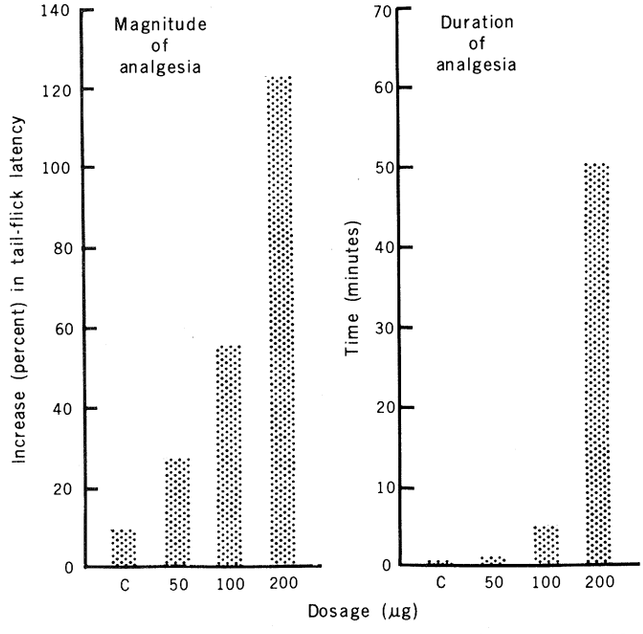


Fig. 1 (left). (Upper) Median time course of analgesia, as measured by the tail-flick test, after intraventricular injections of methionine-enkephalin (200 μg), [D-Ala²]-methionine-enkephalin (200 μg), or the Ringer's vehicle ($N = 6$; each animal received all treatments). (Lower) Effects of subcutaneous administration of naloxone hydrochloride, or the saline vehicle, on the time course of analgesia induced by 200 μg of [D-Ala²]-methionine-enkephalin ($N = 2$; each animal received both treatments). Fig. 2 (right). Dose-response comparison of the magnitude and duration of analgesia produced by [D-Ala²]-methionine-enkephalin or a control injection of the vehicle alone (C). Duration is the time from the first to the last criterion elevation (30 percent above baseline) in the latency to tail-withdrawal. Magnitude is taken as the average latency within this period ($N = 6$; each animal received all treatments).

lins or their analogs have been shown to exert profound effects upon behavioral systems. Nevertheless, there has been relatively little direct evidence concerning the role of endogenous opiate systems in normal behavioral regulation. If endogenous opiate systems are normally active in reducing pain, then blockade of opiate receptors should enhance pain sensitivity. To test this possibility, we examined the effects of naloxone, a specific blocker of opiate receptors, on sensitivity to pain as measured by the tail-flick test. Twelve male albino (Holtzman) rats were given a single tail-flick test according to the general procedures outlined above. After baseline testing, six animals were injected with naloxone (2 mg/kg, subcutaneously) and the other six received subcutaneous injections of the saline vehicle alone. After drug treatments, tail-flick latencies were redetermined (3-minute intertrial interval) over the course of the subsequent 20 minutes. We found that while baseline latencies did not differ between the two groups and while saline produced no significant change in latencies, naloxone induced a significant decline in the latency to tail-withdrawal relative both to baseline values before injection (mean baseline latency, 4.42 seconds; latency after injection, 3.39 seconds; t, 5.81; 5 d.f.; $P < .01$) and saline control latencies (mean latencies after saline injections, 4.50 seconds; after naloxone, 3.39 seconds; t, 3.37, 10 d.f., $P < .01$). Thus, blockade of opiate receptors in otherwise untreated animals increased their sensitivity to the thermal stimuli used in the present test, indicating that opiate systems may act normally to suppress sensitivity to certain classes of stimuli. This suggestion is supported by a report in which a different measure of thermal sensitivity (the hot plate test) was used (*16*), although other workers have challenged this view because of their failure to obtain enhanced pain reactions to electric shock after naloxone administration (*17*). In view of our findings it seems highly possible that endogenous opiate systems may exert differential tonic influences on different sensory modalities.

The demonstration that the naturally occurring opiate-like peptide methionine-enkephalin or its potent analog [D-Ala^2]-methionine-enkephalin can produce analgesia, whereas the opiate receptor blocker, naloxone, induces hypalgesia, supports the suggestion that endogenous opiate systems may function to regulate tonic sensitivity of central pain systems. The previous demonstration that physical stress can induce a release of endogenous enkephalin and a corresponding marked analgesia in the rat further indicates that the tonic activity of opiate systems can be modulated by environmental influences.

J. MICHAEL WALKER
GARY G. BERNTSON
CURT A. SANDMAN
Department of Psychology, Ohio State University, Columbus 43210
DAVID H. COY, ANDREW V. SCHALLY
ABBA J. KASTIN
Tulane University School of Medicine and Veterans Administration Hospital, New Orleans, Louisiana 70112

References and Notes

1. J. C. Hughes, T. W. Smith, H. W. Kosterlitz, L. Fothergill, B. A. Morgan, H. R. Morris, *Nature (London)* **258**, 577 (1975). The abbreviations for the amino acid residues are: Tyr, tyrosine; Gly, glycine; Phe, phenylalanine; Leu, leucine; Met, methionine.
2. J. C. Hughes, T. Smith, B. Morgan, L. Fothergill, *Life Sci.* **16**, 1753 (1975); R. Simantov and S. H. Snyder, *ibid.* **18**, 781 (1976).
3. F. E. D'Amour and D. L. Smith, *J. Pharmacol. Exp. Ther.* **72**, 74 (1941).
4. J. D. Belluzzi, N. Grant, V. Garsky, D. Sarantakis, C. D. Wise, L. Stein, *Nature (London)* **260**, 625 (1976); J. Chang, T. W. Fong, A. Pert, C. Pert, *Life Sci.* **18**, 1473 (1976); H. H. Buscher, R. C. Hill, R. Deitmar, F. Cardinaux, A. Closs, D. Hauser, J. Pless, *Nature (London)* **261**, 423 (1976).
5. L. Graumbach, in *Pain*, R. S. Knighton and P. R. Dumke, Eds. (Little, Brown, Boston, 1966), p. 163.
6. J. C. Liebeskind, in *Proceedings, First World Pain Congress* (Raven, New York, 1976).
7. K. Tsou and C. S. Jang, *Sci. Sin.* **13**, 1099 (1964).
8. A. F. Bradbury, D. G. Smyth, C. R. Snell, *Nature (London)* **260**, 165 (1976); C. Garbay-Jaureguiberry, B. P. Roques, R. Oberlin, M. Anteunis, A. K. Lala, *Biochem. Biophys. Res. Commun.* **71**, 558 (1976); B. P. Roques, C. Garbay-Jaureguiberry, R. Oberlin, M. Anteunis, A. K. Lala, *Nature (London)* **262**, 778 (1976); C. R. Jones, W. A. Gibbons, V. Garsky, *ibid.*, p. 779.
9. F. A. Momany, *J. Am. Chem. Soc.* **98**, 2990 (1976); *ibid.*, p. 2996; W. W. Monahan, M. S. Amoss, H. A. Anderson, W. Vale, *Biochemistry* **12**, 4616 (1973); D. H. Coy, E. J. Coy, A. V. Schally, J. A. Vilchez-Martinez, Y. Hirotsu, A. Arimura, *Biochem. Biophys. Res. Commun.* **57**, 335 (1974).
10. C. B. Pert, A. Pert, J. K. Chang, B. T. W. Fong, *Science* **194**, 330 (1976).
11. S. Ritter and L. Stein, *J. Comp. Physiol. Psychol.* **85**, 443 (1973).
12. D. H. Coy, A. J. Kastin, A. V. Schally, O. Morin, N. G. Caron, N. Faure, F. Labrie, J. M. Walker, R. Fertel, G. G. Berntson, C. A. Sandman, *Biochem. Biophys. Res. Commun.* **73**, 632 (1976).
13. This behavior resembles an opiate withdrawal syndrome commonly referred to as the "wet dog shakes." The functional significance of this behavior is uncertain, and we are hesitant to suggest a common mechanism. A description of the syndrome may be found in G. Gianutsos, R. Drawbaugh, M. Hynes, H. Lal, in *Methods in Narcotic Research*, S. Ehrenpreis and A. Neidle, Eds. (Dekker, New York, 1975), p. 297.
14. N. P. Plotnikoff, A. J. Kastin, D. H. Coy, C. W. Christensen, A. V. Schally, M. A. Spirtes, *Life Sci.* **19**, 1283 (1976).
15. J. M. Walker, G. G. Berntson, C. A. Sandman, D. H. Coy, A. V. Schally, A. J. Kastin, unpublished observations.
16. J. J. Jacob, E. C. Tremblay, M. Colombel, *Psychopharmacologia* **37**, 217, 1974.
17. A. Goldstein, G. T. Pryor, L. S. Otis, F. Larsen, *Life Sci.* **18**, 599 (1976); A. El-Sobky, J. V. Dostrovsky, P. D. Wall, *Nature (London)* **263**, 783 (1976).
18. H. Akil, J. Madden, R. L. Patrick, J. D. Barchas, in *Opiates and Endogenous Opioid Peptides*, H. W. Kosterlitz, Ed. (North-Holland, New York, 1976), p. 63.

15 September 1976; revised 12 November 1976

Angiotensin Injected into the Neostriatum After Learning Disrupts Retention Performance

Abstract. *Angiotensin II, injected into the dorsal neostriatum of rats 5 minutes after they had learned a passive avoidance task, disrupted the retention of the task 24 hours later. Identical neostriatal injections given 22 hours after learning (2 hours before retention) were without effect on retention performance. Ventral neostriatum or posterior thalamus were ineffective sites for injection of angiotensin. Injection of thyrotropin releasing hormone or lysine-8-vasopressin into the dorsal neostriatum was ineffective. These findings indicate a possible role for endogenous angiotensin in the neostriatum on retention performance and suggest potential involvement in mnemonic processes.*

The nigro-neostriatal system has recently been implicated in learning and memory processes. Lesions and electrical stimulation of the neostriatum or the substantia nigra (*1*) produce deficits in the acquisition or retention of active and passive avoidance tasks or both. Manipulation of the synaptic transmitters within this system also affects learning and memory (*2*). Thus the nigro-neostriatal system, in addition to its well-known role in motor functions, may also be involved in memory mechanisms.

Recent evidence indicating that peptides play an important role in learning and memory (*3*) prompted us to investigate the effects of angiotensin II, a peptide that is endogenous to the nigro-neostriatal system. In particular, this octapeptide is present in the neostriatum along with its precursors and metabolic enzymes (*4*); angiotensin-converting enzyme is present, indeed, in highest concentrations in the neostriatum (*5*). This enzymatic system is isolated from circulating angiotensin II by the blood-brain barrier (*4*), suggesting a separate functional role for this peptide in the brain. Although other peptides affect avoidance learning when injected intracerebrally