change in receptivity of the cell associated with the initiation of DNA synthesis, possibly expressed as the appearance of new receptors for IDS or a change in permeability of the cell membrane with altered accessibility of the cell's adenylate cyclase. Models for these alternatives have been described in other systems. For example, synchronized cultures of melanocytes show the appearance of specific cell surface receptors for melanocyte-stimulating hormone (which activates adenylate cyclase) at a precisely defined time during the G2 phase of growth (*15*). Added cyclic AMP, however, can act at any time during the cell cycle. Pardee has recently identified a "restriction point" during G1, in synchronized BHK cells; at this point nutritional factors either stimulate or inhibit cell proliferation in association with changes in intracellular cyclic AMP (*16*). Again, elevation of cyclic AMP, for example, by addition of $2 \times 10^{-3}M$ dibutyryl cyclic AMP, was effective at any time during the cycle.

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Physical Dependence on Opiate-Like Peptides

Abstract. *Methionine-enkephalin and β-endorphin, endogenous peptides with activities similar to those of opiates, were infused for 70 hours into the periaqueductal gray–fourth ventricular spaces of the rat brain. When challenged with a naloxone, a specific opiate antagonist, these animals manifested a typical morphine-like withdrawal syndrome. These results show that such peptides can cause physical dependence.*

Physical dependence on opiates is generally characterized by abstinence behavior when opiate intake is abruptly terminated or when an opiate antagonist is administered (*1*). A number of investigators using bioassays in mouse vas deferens and guinea pig ileum and stereospecific binding to purified brain extracts have discovered brain and hypophyseal peptides with opiate-like activities (*2*). The amino acid sequences of several opiate-like peptides, namely methionine- and leucine-enkephalin and α- and β-endorphin, are known and have been synthesized (*3*). Synthetic methionine-enkephalin has some central analgesic activity of short duration (*4*), whereas β-endorphin, which contains methionine-enkephalin as part of its first five NH_2-terminal residues, is, on a molar basis, at least ten times more active centrally than morphine (*5*). In considering the endogenous functions of these peptides and the development of these peptides as analgesics, it is important to determine whether opiate-like peptides can cause physical dependence (*6*). We describe here a novel method of long-term, localized, drug infusion into the brain which has enabled us to demonstrate that long-term exposure to methionine-enkephalin and β-endorphin can result in physical dependence.

Male Sprague-Dawley rats (220 to 380 g) were anesthetized with sodium pentobarbital (50 mg/kg) injected intraperitoneally; L-shaped steel cannulas, made from 21-gauge disposable needles filed to a predetermined length, were implanted into the frontal cortex or periaqueductal gray region of the rat brain (*7*). The implanted cannula, filled previously with distilled water, was secured to the skull with dental cement. To deliver drugs into the brain an osmotic minipump was utilized (*8*). The minipump, a system capable of delivering a small volume at a constant rate, was filled with

Table 1. Chronic infusion of morphine sulfate into the rat brain and the development of physical dependence.

Brain area	Morphine sulfate infusion (μg/μl)	Total estimated dose delivered in 70 hours* (μmole)	*N*	Animals showing withdrawal sign (%)		
				Teeth chattering	Escape responses	Wet shakes
Periaqueductal gray (minipump not connected to brain)	10	1.928	8	0	0	0
Frontal cortex	10	1.928	6	83	0	0
Periaqueductal gray	10	1.928	4	100	100	75
Periaqueductal gray	2.5	0.482	7	100	86	29

*Minipump flow rates of 0.92 ± 0.06 μl/hour.

Table 2. Continuous infusion of opiate-like peptides into the periaqueductal gray of the rat brain and the development of physical dependence; Met, methionine.

Chemical	Concentration of chemical in infusion (μg/μl)	Total estimated dose delivered in 70 hours* (μmole)	*N*	Animals showing withdrawal sign (%)		
				Teeth chattering	Escape responses	Wet shakes
Distilled water			4	0	0	0
Morphine sulfate	1.64	0.481	8	100	100	37
Met-enkephalin	9.00	1.537	7	100	86	14
Met-enkephalin	0.83	0.140	8	62	37	0
β-Endorphin	0.67	0.019	7	100	86	0
β-Endorphin	0.10	0.003	9	67	11	22

*Minipump flow rates of 1.40 ± 0.04 μl/hour.

the desired drug solution (*9*) and inserted subcutaneously between the scapulae in the anesthetized animal. A 21-gauge steel tube, protruding from the minipump, was then coupled to the brain cannula with plastic tubing. To avoid dislodgment of the pump by the animal, the scalp wound was closed with sutures so that the entire infusion unit was enclosed under the skin.

The drugs were infused into the brain for 70 hours, and animals were weighed and placed in 1-gallon glass jars; after an adjustment period of 10 to 15 minutes, the animals were challenged with the specific opiate antagonist, naloxone hydrochloride, 10 mg/kg delivered intraperitoneally (*10*). The resultant behavior was then observed under standardized procedures (*11*). Leaping attempts to escape from the glass jar, "wet dog shakes," and teeth chattering are examples of distinctive abstinence behavior that occur in the dependent animal after opiate antagonists are administered (*11*). If a rat made two or more escape attempts from the jar, had three or more "wet dog shakes," or made grinding noises with its teeth within 15 minutes after injection of naloxone, the animal was considered to have undergone precipitated withdrawal and was classified as manifesting the particular withdrawal sign (*11*).

Since the intracerebral infusion technique for making animals dependent on opiates has not been reported, our initial studies were aimed at collecting information on responses of controls, to determine the relative sensitivity of various neuroanatomical sites for drug infusion, and to obtain data on dose-response relationships. Morphine sulfate, a standard opiate alkaloid, was used as the reference compound. In order to measure the responses of controls, either we did not connect the minipumps to the intracerebral cannula so that the morphine solution flowed onto the surface of the scalp, or we infused the periaqueductal gray with distilled water. Challenge with naloxone in these animals failed to provoke withdrawal behavior (Tables 1 and 2), an indication that the experimental procedures per se did not alter the animal's reactivity to naloxone. Infusion of morphine into the frontal cortex produced a mild degree of dependence; these animals showed teeth chattering but no escape behavior or "wet dog shakes" after naloxone (Table 1). By contrast, infusion of the same dose of morphine into the periaqueductal gray region, an area known from microinjection studies to be highly sensitive to morphine (*12*), produced acute morphine toxicity (*13*); in the survivors, it produced an intense abstinence syndrome upon treatment with naloxone (Table 1). In subsequent experiments, a lower, nonlethal dose of morphine sulfate (161 μg, total dose) was used to demonstrate the development of physical dependence by the intracerebral infusion technique (Table 1).

In the second set of experiments, osmotic minipumps with higher flow rates were used (*8*). To replicate the earlier results, an equivalent dose of morphine, but at a lower concentration, was infused into the periaqueductal gray. The results obtained with the second set of pumps confirmed the relative reliability of the intracerebral infusion technique for inducing physical dependence (Table 2). The opiate-like peptides, methionine-enkephalin and β-endorphin, were infused at two concentrations. When challenged with naloxone, the animals infused with the higher concentrations exhibited a withdrawal syndrome which was virtually indistinguishable from that observed with morphine (*14*). The average number of escape responses for animals showing this withdrawal sign were: morphine sulfate (0.481 μmole), 34.1 (range 6 to 82) escape responses; methionine-enkephalin (1.537 μmole), 24.6 (range 6 to 62) escape responses; and β-endorphin (0.019 μmole), 16.3 (8 to 38) escape responses. At lower doses of peptides, the incidences of withdrawal responses were decreased (Table 2). From these results, it is apparent that methionine-enkephalin and β-endorphin can cause morphine-like physical dependence. The potent activity of β-endorphin in producing dependence suggests that mechanisms of physical dependence may regulate the actions of this peptide in vivo (*6*).

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7. Stereotaxic coordinates for the frontal cortex were: 10.0 mm anterior from lambda, 3.0 mm lateral to the midline, and 2.0 mm vertical from the dura. The corresponding coordinates for the periaqueductal gray region were −0.5, 0.0, and 5.5 to 6 mm, respectively. The upper incisor bar was 2.4 mm below the interaural line. Cannula tracts were localized by gross dissection under a binocular microscope or by examination of stains after injection of dye into the cannula in situ. Further details have been described by E. Wei, H. Loh, and E. L. Way [*J. Pharmacol. Exp. Ther.* **185**, 108 (1973)] and E. Wei, S. Sigel, and E. L. Way [*ibid.* **193**, 56 (1975)].
8. Alzet osmotic minipump delivery system, manufactured by Alza Corp., Palo Alto, Calif. The flow rates of the pumps, 0.92 ± 0.06 μl/hour and 1.40 ± 0.04 μl/hour (mean ± standard deviation), were calibrated with F.D.&C. No. 1 blue dye dissolved in normal saline. Further information on these pumps may be obtained from Alza Corp.
9. Methionine-enkephalin (molecular weight, 574) was obtained from Peninsula Laboratories, San Carlos, Calif. Synthetic ovine β-endorphin (molecular weight, 3439) was a gift from Professor C. H. Li, University of California San Francisco Medical Center. The peptides and morphine sulfate (Mallinckrodt) were dissolved in sterile distilled water for the infusion.
10. Naloxone hydrochloride (Endo Laboratories, Garden City, N.Y.) was dissolved in saline and administered in a volume of 0.1 ml per 100 g of body weight.
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14. The withdrawal signs—salivation and profuse diarrhea—normally seen in animals made dependent by subcutaneous implantation of morphine pellets, were not observed in animals made dependent by intracerebral infusion of morphine [see E. Laschka, A. Herz, J. Blasig, *Psychopharmacologia* **46**, 133 (1976) for further discussion of centrally and peripherally induced withdrawal]. On day 5 after cannulation, when the pump was no longer functional, rats infused with morphine (0.482 μmole) or β-endorphin (0.019 μmole) underwent abrupt withdrawal; this syndrome is characterized by a high frequency of "wet dog shakes" and teeth chattering. The abrupt withdrawal syndrome was less pronounced in animals infused with methionine-enkephalin.
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