Different Brain Areas Mediate the Analgesic and Epileptic Properties of Enkephalin

Abstract. *Single injections of 120 micrograms of methionine-enkephalin were made into various midbrain and forebrain structures in the rat. Analgesia was observed after injections into or near the ventral, caudal midbrain periaqueductal gray matter. Seizures and other pathological electroencephalogram (EEG) changes were seen with injections into or near the forebrain dorsomedial nucleus of the thalamus. No animals with midbrain injection sites showed EEG changes, and none with forebrain injection sites were analgesic. These data, taken together with other lines of evidence, suggest that enkephalin-induced analgesia and enkephalin-induced seizures are mediated by opiate receptors that are located in different brain areas and that are pharmacologically different.*

Since the initial report by Hughes and co-workers (*1*) that two structurally similar pentapeptides (leucine- and methionine-enkephalin) may be endogenous ligands for opiate receptors in the brain, several studies have appeared suggesting roles for these substances in mediating certain normal and pathological functions (*2*-*5*). Inevitably, greatest attention has focused on the possible analgesic action of these peptides. Both [Leu]- and [Met]enkephalin have been shown to induce clear but short-lived analgesia in the rodent tail-flick test after intraventricular administration (*6*, *7*). Similar analgesia has been reported in rats with microinjections of the enkephalins directly into the periaqueductal gray matter (PAG) (*8*), an area known to support stimulation-produced analgesia (*9*, *9a*) and thought to be part of a larger medial brain-stem system normally serving an endogenous pain-inhibitory function (*10*). Several groups, however, have failed to obtain analgesia after intraventricular or intracerebral microinjections of the enkephalins (*2*, *11*); and we (*5*) have reported analgesia in only 8 of 19 rats with intraventricular injections of 200 μg of [Met]enkephalin.

In our study (*5*), although analgesia was only observed in some rats, the same enkephalin injections caused powerful and long-lasting seizures in the EEG of almost every animal tested. These seizures were accompanied by myoclonic twitches and wet-dog shakes. We have subsequently found (*12*) that such seizures could be as readily induced by [Leu]- as by [Met]enkephalin. In fact, the EEG effects of [Leu]enkephalin lasted significantly longer than those of [Met]enkephalin. Both compounds reliably caused seizures at doses of 100 and 25 μg; and even at 10 μg, seizures were occasionally seen. Analgesia was virtually never obtained at these doses. Seizures of extremely similar morphology were recorded after [Leu]enkephalin, [Met]enkephalin, and morphine at 100 μg, and such seizures were reliably blocked by prior administration of naloxone at 10 mg/kg. In view of the similar morphology of the opiate and opioid seizures, the fact that the enkephalins caused seizures at such low doses, and the finding that naloxone effectively blocked these effects, it was suggested (*5*, *12*) that such seizures result from an interaction of these compounds with opiate receptors in the brain (*13*).

Opiate receptors are widely distributed in the brain (*14*), and it is generally assumed that different brain areas mediate the various pharmacological actions of opiate drugs. In our study, we sought to determine whether enkephalin's analgesic and epileptic effects were mediated in the same or different brain regions. We find that the same dose of enkephalin that causes analgesia without seizures when injected near the PAG causes seizures without analgesia when administered near the dorsomedial nucleus of the thalamus.

Forty-three male Sprague-Dawley rats weighing 400 to 450 g were anesthetized and prepared with single stainless steel guide cannulas (22 gauge) aimed 2 mm above the PAG or rostral thalamus. Cannulas were sealed and kept patent by insertion of a 27-gauge stylet at the time of surgery. Four stainless steel jeweler's screws were threaded into the skull bilaterally over the frontal cortex and unilaterally over the sensorimotor and occipital cortices to serve as epidural electrodes for EEG recording. Teflon-coated wires led from the screws to a connector plug, and the assembly was cemented to the skull with dental acrylic. Experimentation began 1 week or more after surgery.

Microinjections were made by replacing the stylet with a 27-gauge injection needle designed to protrude 2 mm beyond the guide cannula. The needle was attached to a microsyringe by polyethylene tubing. The microsyringe was driven by an infusion pump that delivered the 1-μl volume of drug over a 20-second period. Each animal was tested only once with a single 1-μl injection of 120 μg of [Met]enkephalin (*15*).

After the injection needle was inserted, and a recording cable to the connector plug was attached, animals were placed in Plexiglas restraining tubes from which their tails protruded. The tail-flick test (*16*) was used to assess baseline pain threshold and analgesia. To prevent tissue damage to the tail, the radiant heat source was automatically shut off after 7.3 seconds if the animal failed to respond. Before the drug was injected, three tail-flick trials were run at 1-minute intervals, and the mean baseline latency to tail withdrawal was determined. Latencies varied among animals between

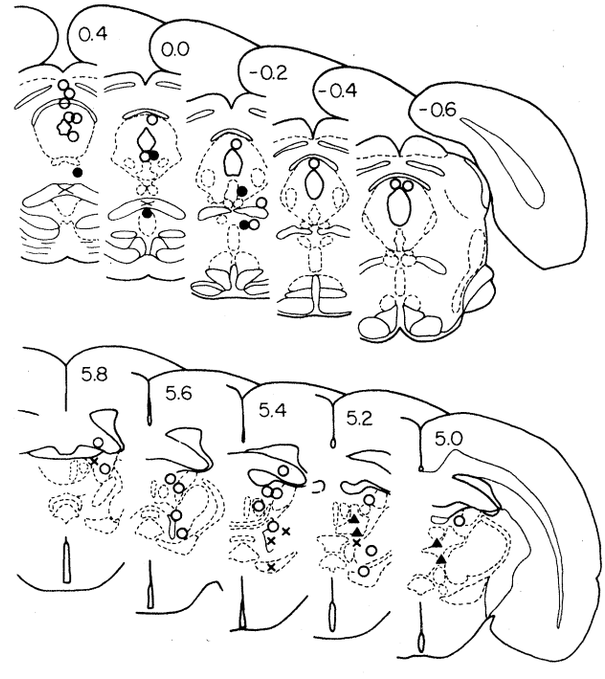

Fig. 1. Midbrain and forebrain sites of injection of 120-μg [Met]-enkephalin. Injections caused analgesia without EEG changes (●), seizures without analgesia (▲), other pathological EEG changes without analgesia (×), or had no effect (○). The diagrams are modified from the stereotaxic atlas of Pellegrino and Cushman (*27*).

0036-8075/78/0421-0335$00.50/0

3.0 and 4.0 seconds. One minute after drug infusion was completed, tail-flick trials were resumed and continued at 1-minute intervals for the remainder of the session (10 minutes). Analgesia was defined by the absence of tail withdrawal during the 7.3-second trial. No animal failing to attain this criterion showed a change in tail-flick latency exceeding 20 percent plus or minus its baseline.

With the aid of a Grass model 6 electroencephalograph, differential EEG recordings were made between frontal-occipital and sensorimotor-occipital leads, the second frontal electrode serving as ground. Recording was begun immediately after determination of baseline tail-flick latency and was continued without interruption until the end of the experimental session.

After the tests were completed, animals were given a lethal dose of anesthetic, and the heart was perfused first with normal saline and then by 10 percent formalin solution. Frozen brain sections were stained with thionine to ascertain the injection site.

Injection of 120 μg of [Met]enkephalin caused analgesia in five animals. The effect lasted for 2 to 6 minutes. The site of injection in all five analgesic animals was the ventromedial portion of the PAG or subadjacent regions of the tegmentum (Fig. 1). No alterations in EEG pattern were seen after enkephalin administration in these or any other animals with midbrain injection sites.

In four animals, the enkephalin injection induced EEG seizures accompanied by intermittent myoclonic twitches and wet-dog shakes. In these four animals, injection sites were evident in the dorsomedial nucleus of thalamus (Fig. 1). The seizures were indistinguishable from those induced by intraventricular administration of either [Leu]- or [Met]-enkephalin at doses between 10 and 200 μg or by intraventricular injection of 100 μg of morphine (*5, 12, 17*). Enkephalin infusion at 5 additional sites (Fig. 1) also caused pathological EEG changes (but not seizures) lasting 3 to 10 minutes. In these animals, high-amplitude hypersynchronous waves (1 to 2 per second) were seen, accompanied in one case by occasional spiking. Myoclonic twitches and wet-dog shakes were not observed. Four of these placements lay ventral to the dorsomedial nucleus; one was rostral and close to the ventricular border. No animals with forebrain injection sites manifested enkephalin-induced analgesia.

Evidence from previous studies suggests that both enkephalin-induced analgesia (*6–8*) and enkephalin-induced seizures (*12*) are mediated by an interaction of these peptides with opiate receptors in the brain. Our findings demonstrate further that such analgesic and epileptic effects are mediated by opiate receptors in different brain areas—the ventromedial midbrain and the dorsomedial thalamus, respectively, among possible others. This site specificity is in good accord with the findings of earlier reports in which other techniques were used. For example, the ventromedial midbrain (especially the ventral, caudal PAG) is known to be a particularly effective substrate for stimulation-produced as well as morphine analgesia in the rat (*9, 9a, 10, 18*); and Segal *et al.* (*4*) have reported that the ventromedial region is the most effective PAG site for eliciting analgesia with microinjections of β-endorphin. In contrast, electrical stimulation in the rat of the thalamic dorsomedial nucleus gives rise to seizures but not reliably to analgesia (*9a, 19*). Both PAG and dorsomedial regions of thalamus contain opiate binding sites, enkephalin, and β-endorphin (*14, 20*).

Our previous results (*12*), taken together with the present findings, suggest that the opiate receptors mediating analgesia and seizures not only are located in different brain areas but may also differ from one another pharmacologically. The concept of functionally different opiate receptors in the central nervous system has been promulgated chiefly by Martin and co-workers (*21*). More recently, evidence has been presented indicating a heterogeneity of opiate-receptor types in the brain (*22*). Lord *et al.* (*23*) showed that two smooth-muscle preparations (the guinea pig ileum and the mouse vas deferens) had different relative sensitivities to enkephalin and morphine and hence were heterogeneous with respect to opiate-receptor populations. Morphine had a much greater potency in the guinea pig ileum than in the mouse vas deferens, whereas the opposite was true for enkephalin (*23*). The enkephalins were also more potent than morphine in the mouse vas deferens assay (*23*). On the basis of these and other findings, they proposed the existence of a μ receptor in the guinea pig ileum and a mixed population of μ and δ receptors in the mouse vas deferens. We have reported (*12*) that intraventricular injections of enkephalin cause seizures at much lower doses than required for analgesia, whereas similar injections of morphine cause seizures only at doses greatly exceeding the analgesic dose. Our results in combination with these earlier findings (*12, 22, 23*) suggest an interesting hypothesis: that enkephalin-induced seizures are mediated by δ receptors in the dorsomedial thalamus and that enkephalin-induced analgesia is mediated by μ receptors in the PAG (*24*).

Although definitive studies are lacking and the operation of other factors cannot be ruled out (*25*), some additional evidence is available to support this hypothesis. Higher doses of naloxone appear to be required for blocking the epileptic as compared to the analgesic effect of intraventricularly administered enkephalin (*26*); and it has previously been shown that enkephalin binding to the putative δ receptor is more difficult to antagonize with naloxone than is its binding to the μ receptor (*22, 23*). Moreover, we observed (*12*) that [Leu]enkephalin has greater epileptic potency than [Met]-enkephalin, and it has similarly been shown that [Leu]enkephalin has greater agonist potency than [Met]enkephalin in the mouse vas deferens where δ receptors are thought to be present (*23*). Again, although alternative explanations could be advanced, these data can be interpreted as supportive of the view that the opiate receptors mediating analgesia and seizures are pharmacologically different. This working hypothesis has an important implication of potential clinical significance. If, as we have previously suggested (*5, 12*), certain forms of human epilepsy are mediated by opiate receptors in the forebrain, then discovery of a potent δ antagonist, so far lacking, might be expected to provide a novel and specific therapeutic approach to this disease.

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25. The enkephalins are known to be enzymatically degraded very rapidly in the brain. Their greater epileptic potency (compared to their analgesic potency) could be explained, therefore, by assuming that they have more ready access to thalamic sites mediating seizures than to midbrain sites mediating analgesia. However, we have noted (*5*) that the latencies to enkephalin-induced analgesia and seizures are, in fact, very similar.
26. We were unable to block enkephalin seizures with systemic injections of naloxone at 2 mg/kg in four rats tested (*5*), although naloxone at 10 mg/kg has subsequently proved effective (*12*). By contrast, Belluzzi *et al.* (*6*) reported that naloxone at 2 mg/kg did block enkephalin analgesia.
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Neurotrophic Protein Regulates Muscle Acetylcholinesterase in Culture

Abstract. *Skeletal muscles lose acetylcholinesterase in culture as a result of denervation. A protein fraction isolated from peripheral nerves maintained the level of acetylcholinesterase in cultures of aneural embryonic muscle or denervated adult chicken muscle. These results indicate that trophic regulation of muscle acetylcholinesterase might be mediated by a protein produced by nerves.*

In normally innervated skeletal muscles, acetylcholinesterase (AChE) is highly localized at the motor end plate (*1*). After denervation the end plate AChE decreases rapidly (*2, 3*) and, conversely, reinnervation of the denervated muscle restores its AChE (*4*). Furthermore, AChE appears in developing and regenerating muscles in relation to innervation, in vivo as well as in vitro (*5*). Thus, the spinal motor neuron is believed to exert a trophic effect on the end plate AChE.

We do not know how the motor neuron regulates muscle AChE activity, but several mechanisms have been proposed, including muscle activity (*6*), acetylcholine (ACh) release (*7*), and neurotrophic substances (*8*). Recent evidence indicates that a neurotrophic substance transported by axoplasmic flow might be responsible for regulation of muscle AChE. Blockage of axoplasmic flow by colchicine or vinblastine causes a decrease in muscle AChE without disturbing ACh release and consequent muscle activity (*9*).

Although organ culture of adult muscles results in a loss of muscle AChE as a result of denervation (*10, 11*), addition of nerve homogenates to the nutrient medium of muscle cultures prevents the decrease in AChE activity (*10*). An aqueous extract of adult peripheral nerves increases the AChE activity of cultured aneural embryonic muscle (*12*). These results thus indicate that the trophic effect on muscle AChE is mediated by a substance produced by nerves. Recently, we isolated and partially purified a protein fraction from peripheral nerves which enhances morphological differentiation, stimulates protein synthesis, and increases the creatine kinase (CK) activity of cultured muscle cells (*13*). We now report that this protein fraction regulates AChE activity in cultured muscle.

Myogenic cells (3×10^5) were obtained from trypsin-dissociated thigh muscles of 11-day chick embryos (*14*) and were cultured in a collagen-coated plastic dish (*13*). Also, slow muscles [anterior latissimus dorsi (ALD)] and fast muscles [posterior latissimus dorsi (PLD)] were isolated aseptically from 41-week-old chickens (White Leghorn) and cultured in collagen-coated dishes. The cultures were maintained in a medium of 65 percent Dulbecco's modified Eagle's medium, 10 percent horse serum, 2.5 percent chick embryo extract (brain and spinal cord excluded), 20.5 percent Hanks balanced salt solution, and 2 percent glucose (20 percent stock solution), and fed with fresh medium every 3 days. An active peripheral nerve (PN) protein fraction was obtained from chicken sciatic nerve extracts by gel filtration on Sephadex G-200 (*15*). The AChE activity of muscle homogenates was measured by a modification (*3*) of the method of Ellman *et al.* (*16*) in the presence of $10^{-4}M$ iso-OMPA (tetraisopropylpyrophosphoramide), an inhibitor of nonspecific cholinesterase. Noncollagen protein was determined as described by Rifenberick *et al.* (*17*), using the method of Lowry *et al.* (*18*).

Figure 1 shows the change in AChE activity during differentiation of embryonic muscle cells in culture. The AChE activity increased markedly during the period of fusion (between 28 and 72 hours) (*19*). Thereafter it decreased rapidly as muscle maturation progressed. By day 8, muscle fibers exhibited cross-striations and spontaneous contractions. The AChE activity decreased moderately between 8 and 12 days in culture. Addition of an active PN protein fraction (61 μg/ml) to cross-striated mature muscle cultures prevented a further decrease in muscle AChE activity, whereas the inactive PN protein fractions (*15*) were ineffective (Fig. 1).

To determine whether this phenomenon resulted from simple enzyme induc-

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