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Enkephalin-Induced Depression of Single Neurons in Brain Areas with Opiate Receptors—Antagonism by Naloxone

Abstract. *Enkephalin, applied microiontophoretically, depressed spontaneous and glutamate-induced firing of single neurons in frontal cortex, caudate nucleus, and periaqueductal gray matter, where enkephalin and high concentrations of opiate receptors are found. Many of the depressions were blocked by the specific narcotic antagonist naloxone. The data are compatible with a neurotransmitter or neuromodulator role for this new brain pentapeptide.*

Stereospecific opiate binding sites have been demonstrated in mammalian brain (*1*). An endogenous morphine-like substance can be extracted from mammalian brain or cerebrospinal fluid and has a distribution similar to that of the receptors (*2*). Hughes *et al.* (*3*) identified their substance as a mixture of two pentapeptides, H-Tyr-Gly-Gly-Phe-Met-OH and H-Tyr-Gly-Gly-Phe-Leu-OH, which they named methionine-enkephalin and leucine-enkephalin, respectively (*4*). These substances have been synthesized (*3*) and, like the endogenously extracted morphine-like material, have a high affinity for the opiate receptor. Their pharmacology is similar to that of the opiates on in vitro opiate receptor model systems such as the mouse vas deferens and guinea pig ileum (*1–3*). Methionine-enkephalin is more potent than leucine-enkephalin in these systems. In this report the term enkephalin refers to the methionine terminal material.

The suggestion has been made that the opiate receptors in brain do not exist by chance but are there to receive an endogenous ligand, presumably enkephalin, which is released by a specific neural system (*2*, *5–8*). We have examined the effects of microiontophoretically applied enkephalin and morphine on single neurons in various brain areas reported to be rich both in enkephalin and in opiate receptors and have obtained support for this suggestion. Enkephalin depressed the firing rate of many neurons in these brain areas by a mechanism that could be blocked by the narcotic antagonist naloxone, but did not depress neurons in an area comparatively devoid of opiate receptors.

Male Sprague-Dawley rats (230 to 260 g) were used in these experiments. The rats were anesthetized with urethane (1.2 g/kg, intraperitoneally) and rectal temperature was monitored with a thermistor probe and maintained at 36.5° ± 0.5°C by means of a heating pad under the abdomen. Six-barrel glass microelectrodes were placed into the caudate nucleus or periaqueductal gray (PAG) region (Fig. 1) according to the atlas of König and Klippel (*9*). Neurons were studied in frontal and posterior cerebral cortex also (see Fig. 1). Immediately before use, the electrode barrels were filled (*10*) with the following solutions: NaCl (5*M*, one barrel for recording and one barrel for current balancing), monosodium-L-glutamate (0.5*M*, *p*H 9), enkephalin hydrochloride (*11*) (3.75 m*M* in 0.03*M* NaCl, *p*H 4), morphine sulfate (0.05*M* in 0.03*M* NaCl, *p*H 4), and naloxone hydrochloride (0.2*M*, *p*H 4). Glutamate was passed as an anion, while enkephalin, morphine, and naloxone were passed as cations. The unit recording and microiontophoretic techniques have been described previously (*12*). The technique of automatic balancing of current at the tip of the microelectrodes was used to prevent or minimize current effects (*13*).

The results reported here for enkephalin were obtained from 63 neurons (spontaneously firing, glutamate-accelerated, or glutamate-activated) in the caudate nucleus, the PAG, or the cerebral

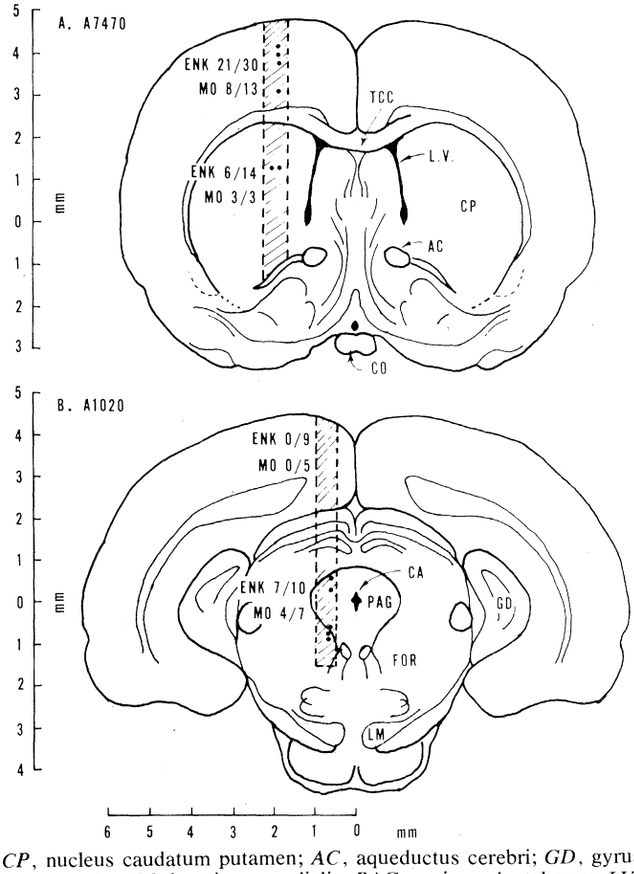

Fig. 1. Frontal sections of rat brain showing location of studies with multibarrel microelectrodes. Hatched areas indicate regions where cells were recorded and tested with microiontophoretic enkephalin. These sections were drawn from the atlas of König and Klippel (*9*). (A) Section at anterior 7470 μm including the caudate nucleus; (B) section at anterior 1020 μm including the PAG matter. The number of cells depressed by morphine and enkephalin over the total number of cells tested in each case is indicated just outside each hatched area. Filled circles in hatched areas show approximate locations of cells on which depression of activity by enkephalin was antagonized by naloxone. Abbreviations: *TCC*, truncus corporis callosi; *CA*, commissura anterior; *CO*, chiasma opticum; *CP*, nucleus caudatum putamen; *AC*, aqueductus cerebri; *GD*, gyrus dentatus; *FOR*, formatio reticularis; *LM*, lemniscus medialis; *PAG*, periaqueductal gray; *LV*, lateral ventricle; *ENK*, enkephalin; and *MO*, morphine.

cortex. Twenty-eight of the 63 cells were tested with morphine as well as with enkephalin. Morphine and enkephalin had the same effect on 23 of these 28 cells.

The specific regions where neurons were studied are indicated in Fig. 1. The number of cells for which the firing rate was changed, over the total number of cells tested, for enkephalin and for morphine are given next to each of the regions studied. Cells in the posterior cerebral cortex above the PAG did not respond to either drug, but responsive cells were found in all other areas. More than 90 percent of the cells that did respond (54 percent of all the cells tested for each drug) experienced a depression of neuronal firing rate. Figure 2 shows the dose-dependent depression by enkephalin of a glutamate-fired cell in the frontal cerebral cortex above the caudate nucleus. This depression by enkephalin was antagonized by naloxone. Figure 3 shows the effect of enkephalin on a spontaneously firing neuron (accelerated by glutamate leak, with the backing current reduced to zero) in the PAG. Enkephalin at 80 na completely shut this cell off and a burst of glutamate was necessary to start it firing again. Naloxone at 60 na blocked this depressant action of enkephalin. Morphine applied in the presence of enkephalin and naloxone slightly increased the firing rate of this cell. This cell was atypical in the duration of its response to enkephalin. Recovery from depressions induced by enkephalin was generally very rapid, whereas cells depressed by morphine were often relatively slow to recover. An example of this is shown in Fig. 4, which refers to a glutamate-fired cell in the PAG. This cell was depressed by enkephalin. The cell recovered immediately when enkephalin was turned off, and the depression was blocked during the application of naloxone. Morphine, at a higher current, also depressed this cell. This depression was of a much longer duration and had to be reversed with naloxone. Only 5 of the 63 cells examined were excited by either morphine or enkephalin. These excitatory actions were probably nonspecific (*14*, *15*).

Naloxone antagonized the depressant action of enkephalin on 11 of 14 cells tested in this manner. Such antagonism is essential in order to conclude that the effects of enkephalin or morphine were due to action on specific opiate receptors. The approximate vertical locations of these 11 neurons in the appropriate brain regions are indicated in Fig. 1. Naloxone antagonized the depressant action of morphine on four of six cells so tested. Satoh *et al.* (*14*) reported naloxone-sensitive depressions of neuronal firing by morphine in sensorimotor cortex of rat.

Our data support the idea that enkephalin may play a role as an inhibitory neurotransmitter in particular brain regions. Specific opiate receptors have been demonstrated in frontal cerebral cortex, caudate nucleus, and PAG (*1*, *2*, *16*), regions from which enkephalin has been extracted (*2*) and in which we report naloxone-sensitive depressions of single neuronal activity by enkephalin and by morphine.

The results we obtained in the PAG are of particular interest. Henry (*17*) has described a depression of ongoing activity and also of glutamate excitation of neurons in the PAG of cat by microiontophoretic morphine. In the studies reported here we observed depressions

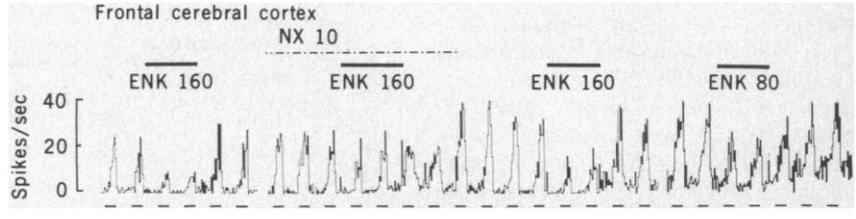

Fig. 2. Ratemeter record of glutamate-fired cell at a depth of 620 μm in the frontal cerebral cortex. Glutamate was applied to the cell with a current of −60 na during the times indicated by the bars under the trace, which represent 10 seconds each. The ordinate gives the firing rate. Enkephalin (*ENK*) was applied to the cell at 80 or 160 na during the times indicated by the solid bars above the trace. Naloxone (*NX*) was applied at 10 na during the time indicated by the dotted line above the trace. Enkephalin depressed the firing rate of the cell, and this effect was antagonized by naloxone.

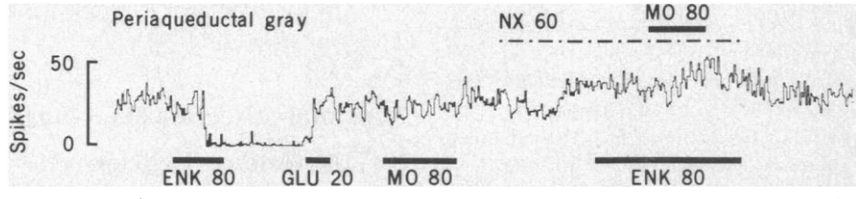

Fig. 3. Ratemeter record of a spontaneously firing cell located dorsally in the PAG. The firing rate of this cell was accelerated by turning off the glutamate backing current and allowing a diffusional leak of glutamate. The ordinate gives the firing rate. Enkephalin (*ENK*, 80 na) and morphine (*MO*, 80 na) were applied during the times indicated by the solid bars above or below the trace. Glutamate (*GLU*, 20 na) was applied at the time shown to start the cell firing again after a depression induced by enkephalin. Naloxone (*NX*, 60 na) was applied during the period indicated by the dotted bar above the trace. Enkephalin depressed this cell, and this effect was antagonized by naloxone. For time calibration the glutamate bar represents 14 seconds.

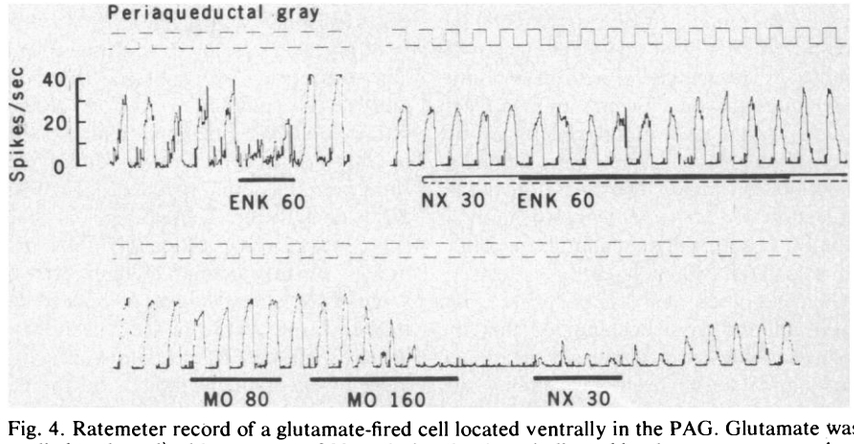

Fig. 4. Ratemeter record of a glutamate-fired cell located ventrally in the PAG. Glutamate was applied to the cell with a current of 20 na during the times indicated by the square-wave pulses above the trace, which have a duration of 15 seconds each. The ordinate gives the firing rate. Enkephalin (*ENK*) was applied to the cell at 60 na during the times indicated by the solid bars under the upper trace. Naloxone (*NX*) was applied during the period indicated by the dotted line under the upper trace. Morphine (*MO*) was applied to the cell at 80 and 160 na, and naloxone was applied at 30 na, during the times indicated by the solid bars under the lower trace. Enkephalin depressed the firing rate of this cell, and this effect was antagonized by naloxone. Morphine also depressed this cell, and this effect was reversed by naloxone.

but no excitations of PAG neurons by both enkephalin and morphine (morphine excited one cell in the presence of naloxone). Naloxone antagonized all depressions in this area, caused by morphine or enkephalin, against which it was tested. The PAG is an area with a large number of opiate binding sites (*1, 16*). Focal electrical stimulation of this area of brain produces an analgesia which can be at least partly blocked by naloxone (*6–8*). Mayer (*6*) and also Akil *et al.* (*7*) have suggested that such stimulation-produced analgesia may result, at least in part, from the release of an endogenous morphine-like neurochemical modulator, presumably enkephalin or a closely related substance, onto the opiate binding sites on postsynaptic elements here. Furthermore, several groups (*18, 19*) have shown that focal injection of morphine into the PAG of rat, particularly the ventrolateral aspect, produces analgesia that is reversible by naloxone. Yaksh *et al.* (*19*) postulated the existence within the PAG of a morphine-sensitive inhibitory interneuron which is tonically active. According to this hypothesis, opiate action depresses these interneurons, thus disinhibiting a second-order neuron which provides ascending and descending modulation of sensory transmission. Our data are compatible with such an enkephalinergic system within the PAG providing inhibitory modulation of the inhibitory interneuron. Thus the opiate receptors at this site would actually be receptors for the endogenous morphine-like inhibitory substance. Enkephalin-responsive cells were not restricted to the PAG itself but were found also in the reticular formation ventral to the PAG.

Although a physiological role for enkephalin remains to be proved (*20*), the data that now exist make enkephalin almost as promising a neurotransmitter candidate as the biogenic amines (such as serotonin and noradrenaline) or the amino acids (such as glutamic acid and γ-aminobutyric acid). The resolution of this question requires the demonstration of the presence of enkephalin in nerve terminals from which it can be released. The cell bodies must also be located, and confirmation must be obtained that the pharmacology of the released substance is identical to that of the exogenously administered peptide. The development of a radioimmunoassay for enkephalin will facilitate such studies.

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Neuronal-Visceral GM_1 Gangliosidosis in a Dog with β-Galactosidase Deficiency

Abstract. *A 9-month-old dog with a history of progressive motor dysfunction was shown to have a deficiency in brain β-galactosidase activity. The canine disease, like that of children with GM_1 gangliosidosis, is characterized by accumulation of GM_1 ganglioside in the brain, liver, and spleen, and membranous cytoplasmic bodies in neurons. The dog's pedigree suggests an autosomal recessive pattern of inheritance.*

Gangliosidoses of man comprise a group of rare inherited lipid storage diseases resulting from deficiencies in activity of glycosidases involved in the degradation of normal sphingolipids that contain *N*-acetylneuramic acid (*1, 2*). Large intracytoplasmic accumulations of GM_1 or GM_2 monsialogangliosides (*2a*) in neurons and, in some types of gangliosidoses, in cells of visceral organs, is characteristic of these ultimately fatal disorders. A few animal counterparts of some of the gangliosidoses have been described (*3*). Because of their usefulness in investigations of the pathogenesis of these diseases and as models for the development and evaluation of potential methods of therapy, current efforts are centered on the identification and biochemical characterization of gangliosidoses in nonhuman species. We now report what we believe to be the first confirmed occurrence of GM_1 gangliosidoses in dogs.

A crossbred male dog of predominantly beagle phenotype developed progressive signs of head tremors, hyperactivity, dysmetria, hypermetria, and visual impairment over a 4-month period, and finally an inability to stand. The dog was humanely killed with sodium barbital at 9 months of age, and the tissues were prepared for biochemical analyses and light and electron microscopy (EM) studies. At autopsy, the gross lesions were limited to the brain and consisted of thickening of the cerebrocortical gray matter with attenuation of the myelinated tracts.

Histologic examination of hematoxylin and eosin–stained tissues disclosed marked enlargement and occasional vacuolation of neurons in the brain, spinal cord, spinal root ganglia, and retina. Neuronal cytoplasmic Nissl's substance was typically reduced to a basophilic dustlike dispersion surrounding the nuclei (Fig. 1A). The remainder of the cy-