Opioid Peptides May Excite Hippocampal Pyramidal Neurons by Inhibiting Adjacent Inhibitory Interneurons

Abstract. *The atypical excitation by opiates and opioid peptides of hippocampal pyramidal cells can be antagonized by iontophoresis of naloxone, the γ-aminobutyric acid antagonist bicuculline, or magnesium ion. The recurrent inhibition of these cells evoked by transcallosal stimulation of the contralateral hippocampus is blocked by enkephalin but only shortened by acetylcholine. The results suggest that the opioids excite pyramidal neurons indirectly by inhibition of neighboring inhibitory interneurons (probably containing γ-aminobutyric acid). This mechanism may be pertinent to the electrographic signs of addictive drugs.*

The isolation and characterization of a group of endogenous brain peptides (the endorphins) and their specific opioid receptors (*1*) has lead to an intense search for their physiological role. The endorphin peptides, including enkephalin pentapeptides, are distributed histochemically in diverse nerve networks throughout the brain, including a patchy distribution of enkephalin fibers and opiate receptors in the hippocampal formation (*1, 2*). When applied by iontophoresis, opiate alkaloids and opioid peptides predominantly depress neurons in most brain regions; the depression is reversed by the opiate antagonist naloxone (*3*). A notable exception is the hippocampal pyramidal cell (HPC), which is usually (*4, 5*) [but not always (*6*)] excited by the opioids; these excitations are also naloxone-sensitive (*4, 5*). The HPC excitations suggest a possible relationship to the epileptiform activity seen in electroencephalographic (EEG) recordings of hippocampus after administration of opioid peptides intraventricularly (*7*) or iontophoretically (*8*). Neither the single unit excitations nor the seizure activity require an intact septal cholinergic projection to the hippocampus (*8*), and both are retained in hippocampus transplanted in oculo (*9*); this suggests a mechanism intrinsic to the hippocampus. We now report that the excitations may be produced indirectly by diffusion of the opioids to, and inhibition of, neighboring inhibitory interneurons, resulting in disinhibition of HPC's (*10*).

Rats (N = 64), 200 to 300 g, were anesthetized with 0.5 to 1.0 percent halothane, tracheotomized, and allowed to breathe spontaneously. They were studied with single unit recording and microiontophoretic techniques already described in detail (*11-15*). A micropressure ejection system (*13*) was also often used, in conjunction with iontophoretic retention currents.

In contrast to the inhibitory responses exhibited by a majority of cells in the overlying cerebral cortex, the majority of HPC's were excited (*16*) by Met^5-enkephalin, D-Ala^2-enkephalin, or β-endorphin (N = 150; 128 excited, 14 not affected, 8 depressed). This is roughly the same percentage of excitations as reported by Nicoll *et al.* (*4*); these excitations are naloxone-sensitive (*4, 16*).

In other studies higher percentages of cells were specifically inhibited by the opioids (*6*); this discrepancy may be explained by differences in the type of anesthetic used, type and content of the iontophoretic electrodes, or hippocampal region tested. However, the inhibitory responses may also arise from iontophoretic tests of hippocampal neurons other than HPC's. In the current study, 32 non-HPC's located near HPC's showed tonic, nonburst-like discharge patterns with firing rates between 10 and 40 Hz. The non-HPC's (*17*) clearly were inhibited by the same doses of opiate peptides that excited neighboring HPC's. Experiments using two recording barrels glued at separations of 30 to 180 μm for simultaneous recording of the two neuron types (16

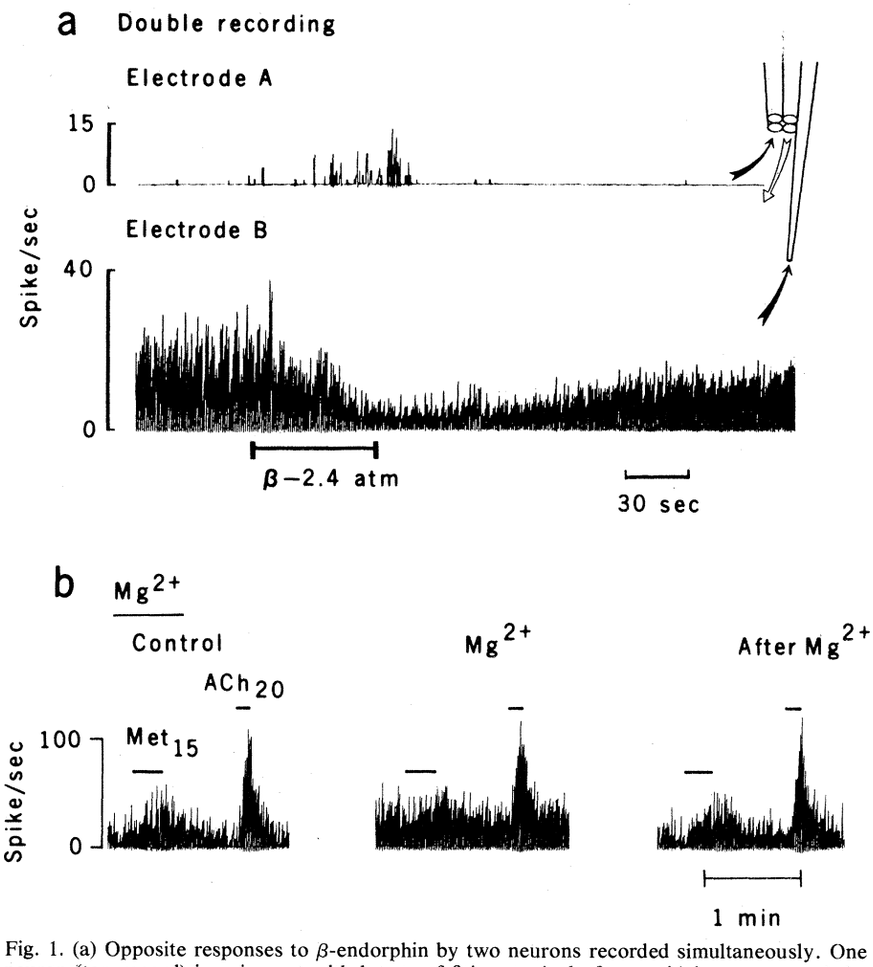

Fig. 1. (a) Opposite responses to β-endorphin by two neurons recorded simultaneously. One neuron (top record) is quiescent with bursts of firing, typical of pyramidal neurons, and the second is a nonbursting, spontaneously firing cell, a presumptive interneuron (lower record). Each cell was recorded in isolation by gluing a second recording barrel in advance of the standard recording barrel of a four-barrel assembly (schematic on right). Both recording electrodes were filled with 3*M* NaCl. Endorphin (β) was applied by the micropressure method (duration indicated by bar under records) from another barrel of the same assembly. Endorphin speeds firing of HPC and concurrently slows that of non-HPC neurons. (b) Excitatory responses of an HPC to iontophoretic pulses of Met^5-enkephalin (*Met*) and acetylcholine (*ACh*) before, during, and after iontophoretically applied Mg^{2+}. Subscripts after each drug indicate iontophoretic currents; bars above record show duration of application. Note that Mg^{2+} (35 to 70 nA) has little effect on the ACh response while excitation in response to enkephalin is greatly reduced and even reversed to inhibition during the early injection phase. Breaks in records are approximately 5 minutes each; repeated pulses of Met and ACh were continued at precise intervals throughout the test period.

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double recordings) made these reciprocal effects more obvious (Fig. 1a).

Histochemical (*18*) and neurophysiological (*19, 20*) studies have shown that the HPC is most likely under inhibitory control from inhibitory interneurons with γ-aminobutyric acid (GABA) as neurotransmitter. To test the hypothesis that opiate peptides excite pyramidal cells by altering the release of some transmitter such as GABA, iontophoresis of Mg^{2+} was used to block ongoing synaptic transmission (*21*). Low currents of Mg^{2+} antagonized the excitations elicited by the peptides (Fig. 1b) in 25 of 35 neurons tested, without affecting excitations induced by acetylcholine (ACh). Often such Mg^{2+} applications (5 to 30 nA, 1 to 10 minutes) directly slowed or, less frequently, accelerated firing after 20 to 60 seconds (Fig. 1b). In two neurons Mg^{2+} converted excitations to inhibitions.

A direct involvement of GABA in the opioid responses was tested with bicuculline, a GABA antagonist (*20*). As would be expected if endogenous GABA were tonically released, iontophoretically applied bicuculline (10 to 80 nA, 10 to 100 seconds) initially excited 34 of 41 pyramidal cells. In 8 of 13 HPC's, bicuculline antagonized and occasionally reversed (three cells) the opioid excitations (Fig. 2a). Although bicuculline could have nonspecific speeding actions, equivalent elevations in rate elicited by ACh did not alter enkephalin excitations; therefore, the direct speeding effect of bicuculline per se (whether or not due to GABA antagonism) cannot explain the blockade of opioid-induced excitation.

To assess directly the role of inhibitory interneurons we stimulated the alveus of the contralateral hippocampus to evoke excitatory-inhibitory responses in ipsilateral pyramidal cells (*22*). These biphasic responses (Fig. 2b) are believed due to an excitatory antidromic and orthodromic action [presumably amino acid–mediated (*23*)] on pyramidal cells followed by a recurrent inhibition through inhibitory, presumably GABAergic interneurons (*19, 20*). In poststimulus time histograms, the inhibitory period was completely overriden by Met-enkephalin in five of ten HPC's. This antagonism is not due solely to the excitatory action of the peptide: ACh excites the cells to the same extent or more, yet could only shorten and never completely overcome the inhibitory phase. During Met-enkephalin applications the primary excitatory responses usually show more spikes (Fig. 2b), which should lead to a more pronounced inhibitory phase but does not.

These experiments suggest that opiates excite HPC's indirectly by means of a direct inhibition of neighboring tonically active inhibitory interneurons (disinhibition) (Fig. 2c). We postulate that the interneurons release GABA tonically since the nonbursting neurons inhibited by the opiates fire at moderately high rates, and since bicuculline directly excites HPC's. Diffusion of opioids from drug pipettes to these interneurons would then inhibit them and facilitate firing of HPC's by disinhibition.

The circuitry of hippocampus features a strong tonic inhibitory control by interneurons over pyramidal cells. The hippocampus of the rat also contains a patchy distribution of opiate binding sites (*1, 24*) and enkephalin immunoreactivity (*25*), as well as enkephalin-reactive fibers (*2*) whose target cells have not yet been identified. If the GABAergic interneurons were the enkephalin-receptive target cells for these fibers, then a slight depressant effect upon the interneurons could result in a net excitation of HPC's (by feed-forward mechanisms and synchronization). This mechanism could explain the epileptogenic effects seen with very low concentrations of β-endorphin (*7, 8*). Brainstem neurons also show naloxone-sensitive excitatory responses in a region where nearby neurons are inhibited (*4, 26*). Thus, while specific opiate receptors mediate neuronal inhibition in most brain regions (*3*), the ultimate action of exogenous opiates on a given region may depend upon the local cytoarchitectonics.

Recently, several other euphorigenic drugs have also been shown to produce distinctive changes in limbic EEG's (*27*). Thus, if limbic excitation, such as that

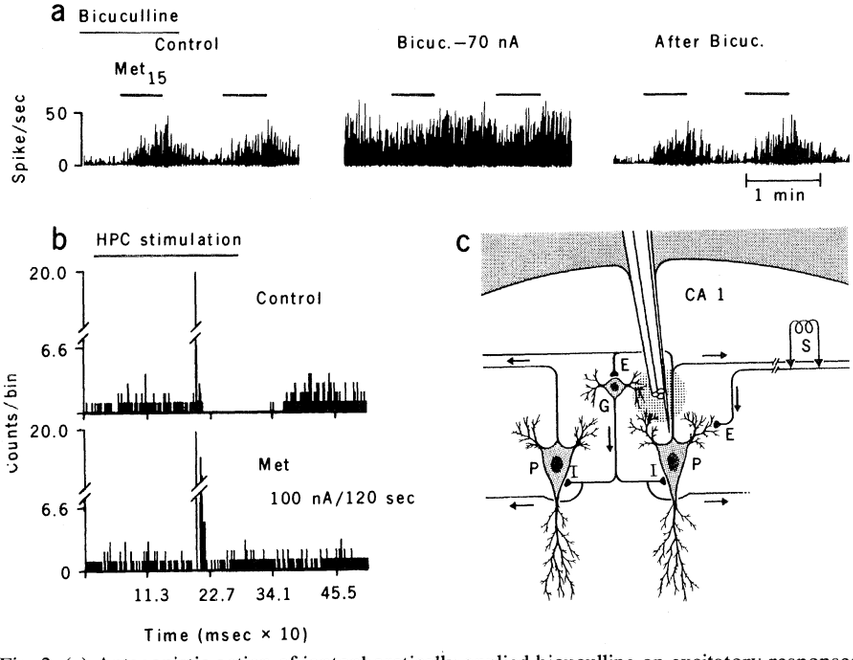

Fig. 2. (a) Antagonistic action of iontophoretically applied bicuculline on excitatory responses of an HPC to Met^5-enkephalin (*Met*) (15-nA iontophoretic current). Bars above record indicate duration of enkephalin application. Enkephalin-induced excitations are reversed to inhibitions during early phases of the bicuculline ejection pulse. Repeated pulses of enkephalin were automatically delivered at precise intervals throughout the entire test period, including the two 6-minute breaks in the record. (b) Effect of iontophoretic Met^5-enkephalin on responses of an HPC to transcallosal stimulation, analyzed by computer-generated poststimulus time histograms. A stimulus applied 20 msec after synchronization pulse (time zero) evokes occasional single spikes (short latency) followed by cessation of firing for 130 msec. Enkephalin applied for 120 seconds increases the number of stimulus-activated spikes (peak shortly after stimulus artifact) yet completely prevents the evoked inhibition, a result in keeping with the mechanism formulated in (c). These effects are reversible (not shown). Bin size, 1 msec; stimulus rate, 0.5 Hz; each histogram represents five sweeps. (c) Schematic of mechanism proposed to account for opioid-induced excitations of hippocampal pyramidal neurons (*P*). Diffusion of opioids (or naloxone) from iontophoretic pipette (stippled area) not only reaches HPC dendrites, but also those of neighboring inhibitory GABAergic interneurons (*G*), where ongoing spontaneous activity is inhibited. This interrupts the tonic release of an inhibitory transmitter (*I*), probably GABA, onto the pyramidal cell, causing it to fire faster. Recurrent activation of the interneuron via the excitatory HPC collateral (*E*) is also blocked by the opioid. The transcallosal stimulus paradigm is indicated by the contralateral stimulating electrode (*S*), which activates both orthodromic excitatory (*E*) and antidromic connections to the pyramidal cell, also resulting in recurrent inhibition through the interneuron (*G*). This "disinhibition" explanation for excitation evoked by iontophoretic opioids could also underlie epileptiform effects of systemic and intraventricularly administered opioids (*7, 8*).

seen with β-endorphin, were relevant to the euphoric component of drug self-administration, then the indirect excitatory effects on hippocampus merit further examination.

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11. Body temperature was kept between 37.5° and 38.5°C. Stereotaxic coordinates for the dorsal HPC recordings were 1.5 to 3.0 lateral to midline, 3.0 to 3.7 mm posterior to bregma, and 2.0 to 3.5 mm in depth. Electrodes for microiontophoretic applications (four- and five-barreled pipettes) were constructed either as complete five-barrel assemblies or from fused omega-dot capillaries (American Glass Co.). The latter were glued alongside a tungsten or a 3*M* NaCl-filled glass recording electrode (*12*). The vertical separation between recording and iontophoresis electrodes was 30 to 180 μm (Figs. 1a and 2c). The drug barrels were filled immediately before the electrode was used with the following solutions: Met^5-enkephalin or D-Ala^2-enkephalin, 0.03*M*, *p*H 5 in 0.9 percent NaCl solution; β-endorphin, 0.03*M*, *p*H 5 in 0.9 percent NaCl; naloxone hydrochloride, 0.1*M*, *p*H 5; l-monosodium glutamate, 0.5*M*, *p*H 8; acetylcholine chloride, 0.5*M*, *p*H 5; bicuculline, 0.05*M*, with or without 0.9 percent NaCl, *p*H 5; and MgCl, 1.0*M*, *p*H 6. The compounds were applied by cationic currents except for L-glutamate which required anionic current. With the micropressure method (*13*), *p*H of the endorphin solutions was 6.5 to 7.0, and the same barrel as for iontophoresis was used. This system allows a better estimation of the concentration reached at the tip orifice than does iontophoresis, and is advantageous with poorly ionized or relatively dilute solutions. Cells were considered responsive when (i) their firing rates were changed by more than 30 percent, (ii) the effect could be reproduced at least three times, and (iii) the response was not mimicked by polarizing current passed through a barrel containing 2*M* NaCl. Current neutralization (*14*) was used continuously by means of the NaCl barrel. Responses were considered antagonized by a substance if they were reduced by more than 50 percent. Poststimulus time histograms were constructed on a PDP-11/40 computer (*15*).
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16. Pyramidal cells (sometimes identified by transcallosal stimulation) were characterized by their burstlike firing pattern (*17*). The administration of the opiate agonist at 10 to 150 nA or 1 to 3 atm for 60 to 120 seconds generally led to an excitation after 2 to 10 seconds; responses were dose-related, and outlasted the application by 20 to 50 seconds. Naloxone, administered either by iontophoresis (20 to 100 nA for 60 to 120 seconds before or simultaneously with the opioid peptides) or subcutaneously (1 mg per kilogram of body weight) antagonized the excitatory action of the peptides in 30 of 40 HPC's. About half the HPC's tested by iontophoresis were excited slightly by naloxone.
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Sensitization in *Aplysia*: Restoration of Transmission in Synapses Inactivated by Long-Term Habituation

Abstract. *Long-term habituation of a simple withdrawal reflex in* Aplysia *leads to an inactivation of synaptic transmission between identified sensory and gill motor neurons that persists for more than 3 weeks. A single sensitizing stimulus rapidly reactivates both the depressed behavioral response and the inactivated synaptic transmission. Thus sensitization, a simple competitive form of learning, provides a mechanism whereby changing environmental demands can rapidly override the long-term memory of habituation.*

Recent investigations of the neural mechanisms of memory in the marine mollusc *Aplysia californica* have shown that long-term habituation training of the defensive withdrawal reflex produces a profound depression of synaptic transmission leading to functional inactivation of the synapses between identified sensory neurons and gill motor neurons. This depression persists for more than 3 weeks (*1*). A variety of studies in both vertebrates in invertebrates indicate that sensitization, a simple competitive form of learning, can rapidly counteract the short-term memory for habituation (*2, 3*). We were therefore interested to know whether sensitization can override the long-term memory of habituation and if so, whether it leads to a functional reactivation of the synapses between the sensory and motor neurons.

Using a combined behavioral and cellular approach, we find that a single sensitizing stimulus reactivates both reflex function and synaptic transmission between sensory neurons and gill motor neurons. Thus, even long-term memory, which normally requires more than 3 weeks to recover, can be rapidly counteracted by a competitive short-term learning process.

We used 43 *Aplysia californica*, weighing 100 to 300 g (Pacific Bio Marine Co., Venice, California). All animals were housed individually in a 200-gallon aquarium for at least 5 days before an experiment.

In behavioral experiments, we first examined the effects of a single sensitizing stimulus on the reflex response of animals that had received long-term habituation training. Twenty-three animals were given ten trials of habituation training per day for 5 days (*4*). The animals exhibited a significantly habituated reflex response ($P < .01$) on day 5 compared to that on day 1 (*5*). This training procedure produces long-term habituation that persists unchanged for 1 week and is only partially recovered after 3 weeks (*6*). On day 5, the habituation scores of that day were ranked and the

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