versible catatonic-like (*16*) state reminiscent of some aspects of schizophrenia. Depending on the dose level, α- and γ-endorphins and Met^5-enkephalin also exhibited subsets of the other behavioral and physiological effects of β-endorphin, in which morphine-like (that is, loss of response to noxious stimuli) actions appeared to be only a portion of a larger neuropsychopharmacological picture. As with the separate nicotinic and muscarinic actions of acetylcholine, all endorphin-mediated actions may not necessarily be explicable in terms of the alkaloid agonist morphine. Extremely puzzling in this regard is the finding that all three endorphin peptides and Met^5-enkephalin can elicit from the lateral ventricle, in drug-naive rats, the wet-dog shaking behaviors ordinarily attributable to opiate withdrawal, and that these responses are counteracted by naloxone (*21*). All of our observations suggest that normal variations—either qualitative or quantitative—in the homeostatic mechanisms regulating the postulated (*4*) conversion of β-LPH as a prohormone to its several endorphin cleavage products could constitute a system fundamentally involved in maintaining behavioral homeostasis.

Furthermore, we propose that subtle derangements in any of the biochemical or physiological mechanisms normally regulating β-lipotropin-endorphins homeostasis could lead to signs and symptoms of mental illness. Such a potential psychophysiological role of endorphins could logically be testable through the therapeutic administration of available opiate antagonists. In fact, at a recent presentation of these results and concepts (*22*), Terenius (*23*) reported Gunne and Lindström's observation that administration of naloxone to two chronic schizophrenics halted their auditory hallucinations within minutes. The ultimate identification of endorphin-sensitive behavioral events and specific treatment of their dysfunctional states may require the development of more specific "anti-endorphins" than those now available, and other naturally occurring brain peptides (*24*) have already been reported to be endorphin antagonists.

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18. Seven days or more before testing, stainless steel cannulae were stereotaxically implanted over the right lateral ventricle. Awake rats, briefly restrained by wrapping for attachment of injection tubing, were injected with 10 μl of test material administered over 60 seconds. Placements were confirmed by subsequent injection of 10 μl of methylene blue dye at the time animals were killed.
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3 August 1976

The C-fragment of β-Lipotropin: An Endogenous Neuroleptic or Antipsychotogen?

Abstract. *Microinjection of the C-fragment (also called β-endorphin), which is amino acid sequence 61-91 of the endogenous pituitary hormone, β-lipotropin (β-LPH), in the periaqueductal gray of the rat resulted in profound sedation and catalepsy, while microinjection of smaller fragments—that is, methionine-enkephalin [sequence β-LPH-(61-65)] and its related pentapeptide, leucine enkephalin, and α-endorphin [sequence β-LPH-(61-76)] resulted in attenuated forms of this behavior. This indicates that the C-fragment is an important neuromodulator of the central nervous system. The similarity of this behavior to that seen after systemic administration to experimental animals of exogenous neuroleptics suggests that a disturbance in the bioavailability of this neuropeptide to receptor sites in brain—perhaps due to lack of enzymatic cleavage from the circulating parent hormone, β-lipotropin—may be an etiological factor in those psychopathological states for which the exogenous neuroleptics exert an ameliorative influence.*

Reports have suggested that various fragments from the pituitary hormone, β-lipotropin (β-LPH), have opioid-like analgesic properties in the central nervous system (CNS). High doses of methionine-enkephalin (which is sequence 61-65 of β-LPH) and its related pentapeptide, leucine-enkephalin, were reported to have transient analgesic effects resulting from intracerebroventricular (*1*) or intracerebral injections into the periaqueductal gray (PAG) (*2*) of rats or mice. Recently, interest has shifted to larger fragments of the hormone, that is, α-endorphin [sequence β-LPH-(61-76)] and the C-fragment (β-LPH-(61-91) (also called β-endorphin); the latter has been reported to exert potent long-lasting analgesia when injected intracerebroventricularly in the cat (*3*) and rat and mouse (*4*). We now report that the C-fragment exerts a profound sedative and cataleptic influence when microinjected in the PAG, a site shown to mediate multiple morphine action, including potent analgesia (*5*), similar to the action of

systemically administered neuroleptic drugs, with the analgesia being only a secondary aspect of this profound sedation. The finding that a fragment of an endogenous pituitary peptide, β-LPH, has marked physiological effects when introduced into brain implies that this fragment is an important neuromodulator of the CNS, and suggests that this peptide may play a significant etiological role in those psychopathological states for which the exogenous neuroleptics exert a therapeutic effect.

Male albino rats (250 to 350 g) were anesthetized with chloral hydrate (380 mg/kg), and mounted on a Baltimore stereotaxic instrument (with ear plugs); using standard methods, we implanted into the brains bilateral cannulae (mounted in parallel on a single pedestal, with 1.0 to 1.5 mm separation), the tips of which were aimed at a site 2 mm dorsal to the PAG (the stereotaxic coordinates were: 1 mm anterior to lambda, 0.5 to 0.75 mm lateral to the midline, and 6 mm ventral to the skull surface). These guide cannulae were made of 30-G stainless steel tubing (outside diameter, 0.30 mm) and the injection needle was made of 35-G stainless steel tubing (outside diameter, 0.13 mm) and calibrated to extend precisely 2 mm beyond the tip of the guide cannula. All injections were made at least 5 days after surgery. The rate of injection was 0.1 μl per 15 seconds, and the injection volume was 0.5 to 1.0 μl per site.

Separate small groups of rats (Fig. 1) were tested (*6*) after microinjections of either methionine-enkephalin (80 or 150 μg) or leucine-enkephalin (54 or 100 μg), α-endorphin (50 μg), or the C-fragment (2 or 4 μg; Peninsula Co.). Two sets of behavioral observations were carried out immediately after injection, and again within the hour. These were (i) analgesia testing, consisting of pinches, pinpricks, a modified hot plate testing procedure, and ice water test (*5*), and (ii) a three-point rating of the status of reflexes, degree of sedation, immobility, and catalepsy (as shown in Table 1). Animals were given microinjections 2 days later with morphine (either 10 or 20 μg) to ascertain that the injection site was indeed a morphine-sensitive site (*7*). A counterbalanced design—giving morphine first and then peptide—was not used, since previous work (*5*) had shown that tolerance developed rapidly to intracerebral injections of morphine, and therefore, a second injection would show, if anything, a weaker opiate effect than the first, with the bias being in favor of optimizing the effects of the first injection over the second.

Table 1. Three-point rating scale of behavioral effects after microinjection of peptides in the periaqueductal gray of the rat.

Item	Leucine-enkephalin	Methionine-enkephalin	α-Endorphin	β-Endorphin
Reflexes*	+	+	++	+++
Sedation†	++	++	++	+++
Immobility‡	++	++	++	+++
Catalepsy§	+	+	+	+++

*The reflexes examined were as follows: Righting was tested by placing the animal on its back; grasping was tested by placing the wooden end of a Q-tip on the pad of a forepaw; biting was tested by thrusting the wooden end of a Q-tip in the animal's mouth; contact-placing was tested by lightly running the dorsal aspect of the forepaws or hindpaws (separately) along the edge of a table and seeing if the animal placed the paws on the surface; and reaching was tested by lowering the animal head-first toward a surface to ascertain whether the paws reached out for the surface. Three points indicate maximal impairment in that behavior. †Sedation was rated according to lack of emotionality of the animal when painful or sudden stimulation was applied. ‡Immobility was rated according to spontaneous locomotor movements of the animal. §Catalepsy was rated according to how soon the animal returned to a normal position after being placed in awkward postures.

With the exception of the C-fragment, none of the above peptides had analgesic activity when microinjected in the PAG (Fig. 1). This is in contrast to reports from several laboratories where transient analgesia was detected by means of the tail-flick test after intracerebroventricular or intracerebral (PAG) injections of methionine- or leucine-enkephalin, with the analgesia fading rapidly within 1 to 10 minutes (*1, 2*). However, we feel that the tail-flick test by itself may not be an appropriate test for analgesia, since the observed small delay in flicking the tail away from thermal stimulation may reflect an impairment of the efferent motor system without necessarily involving the sensory system in general, and the C and A-delta pain fibers in particular. Moreover, the other (and more notable) CNS effects that were observed after microinjection of each of these two peptides had a more prolonged period of action, lasting approximately 30 minutes. This makes it unlikely that analgesia alone of all the CNS effects observed would be the single effect to be so transient.

When methioine- and leucine-enkephalins were incubated with rat brain homogenates (at 37°C) and the breakdown products were analyzed after separation on an autoanalyzer as described (*8*), both peptides were found to undergo breakdown within 1 to 5 minutes with release of the tyrosine residue. The C-fragment was found to be more stable (unpublished results), indicating a more significant role for this peptide, and a more prolonged period of action (as we observed in our in vivo bioassay described below).

Each of the four peptides had moderate to profound effects on other measures of CNS activity (Table 1) (*9*). Reflexes were diminished or abolished, animals remained immobile (without motor paralysis, since coordinated movements were possible when the animal was startled), and appeared sedated, with

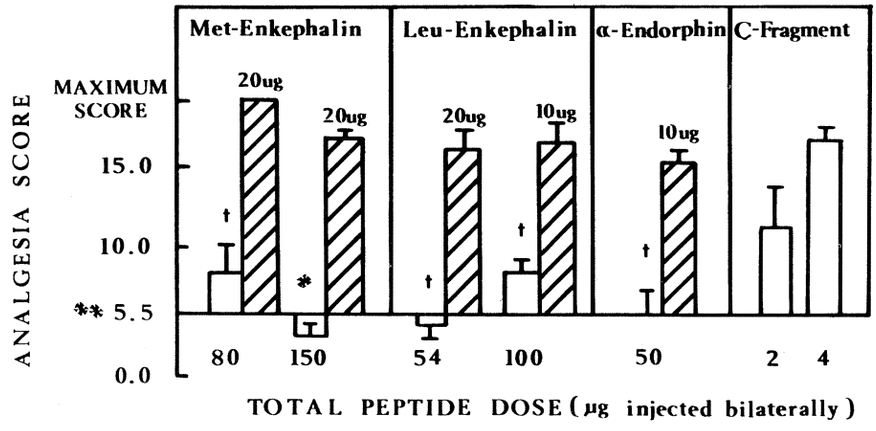

Fig. 1. Mean analgesia scores following intracerebral injection of peptides (open bars) or morphine (hatched bars) into the periaqueductal gray (PAG) of rats. Each peptide was injected in 1 to 2 μl of vehicle, 0.5 to 1 μl being injected into each bilateral PAG site. The morphine dose was either 10 or 20 μg. The effects of 2 and 4 μg of the C fragment (0.6 and 1.2 nmole) resulted in an analgesic response comparable to 10 and 20 μg of morphine sulfate (26 and 52 nmole of morphine) and is thus 50 times more potent than morphine on a molar basis. Abbreviations: $^{*}P < 02$; $^{+}P < 01$; two-tailed t-test for correlated means ($N = 3$ or 4), **Baseline of 5.5 represents saline control level (± S.E. = 0.89).

blunted affect as if in a "dissociated" state (for example, animals would often react to painful stimuli with a barely audible squeak after a long delay, and without attempting to remove the affected limb, even though fully coordinated movements were possible), and were often catatonic, exhibiting a "waxy flexibility" in which state they could be molded in any position, maintaining awkward postures for long periods (more than 1 hour) without any attempt to return to more normal positions (Fig 2). Naloxone given intraperitoneally (1 mg/kg) fully reversed all behavioral effects of the C-fragment.

This behavioral syndrome was elicited only in an attenuated form by high doses of methionine-enkephalin, leucine-enkephalin, and α-endorphin, while it was most fully elicited by a moderate dose of the C-fragment (4 μg). For example, after microinjection of the shorter fragments in the PAG, loss of righting occurred only if the animal were blindfolded, whereas after microinjection of 4 μg of the C-fragment loss of righting occurred if the animal was not blind-folded. Mild to profound sedation was induced by each of the four peptides, with the degree of sedation increasing with the size of the fragment, with the C-fragment at 4 μg exhibiting the most profound sedation. Comparison of the analgesic effects indicates that the C-fragment was 50 times more potent than morphine on a molar basis. In general, the analgesia observed after microinjection of the C-fragment appeared to be a secondary product of this profound sedation. It should be stressed, however, that the behavioral effects of the C-fragment were distinctly different from those that follow morphine microinjection into the identical CNS sites (*9*).

These observations suggest that of the four peptides, the C-fragment offers the best "fit" for the receptor which mediates these physiological functions. The fact that smaller fragments in high doses induce the same behavior but in an attenuated form suggests that they offer a partial fit which incompletely activates the receptor. Data from binding studies where the C-fragment was shown to bind more potently with brain homogenates than methionine-enkephalin (*3*) would provide some support for this view (*10*).

Several laboratories have shown that various fragments of β-LPH bind to so-called "opiate receptors" (*11*), suggesting that these may be the receptors for endogenous neuroactive peptides rather than for exogenous compounds such as opiates. Significantly, neuroleptics have been reported to bind to "opiate receptors" (*12*). The CNS regional distribution of these receptors would not be inconsistent with the view that these are receptors for an endogenous neuroleptic peptide, such as the C-fragment. Effective antipsychotic neuroleptic drugs characteristically produce Parkinson-like extrapyramidal side effects, a syndrome in which the striatum has been implicated. Interestingly, of all CNS regions investigated, the striatum has been shown to have the highest opiate binding (*13*), whereas no known physiological effects of direct opiate injection in the striatum could be found (*14*).

The observation that the C-fragment of the endogenous pituitary peptide β-LPH has profound physiological effects when introduced in brain clearly implies that this fragment is an important neuromodulator of the CNS. The striking similarity of the behavioral effects with those of certain neuroleptic drugs suggests that a disturbance in the availability of this neuropeptide to receptor sites located in this region of the CNS as well as other sites (perhaps as a consequence of failure of cleavage from the parent hormone in vivo due to enzymatic inactivity) might be involved in those psychopathological states for which the exogenous neuroleptic drugs offer therapeutic relief. The implications are indeed far-reaching, and need to be thoroughly followed up.

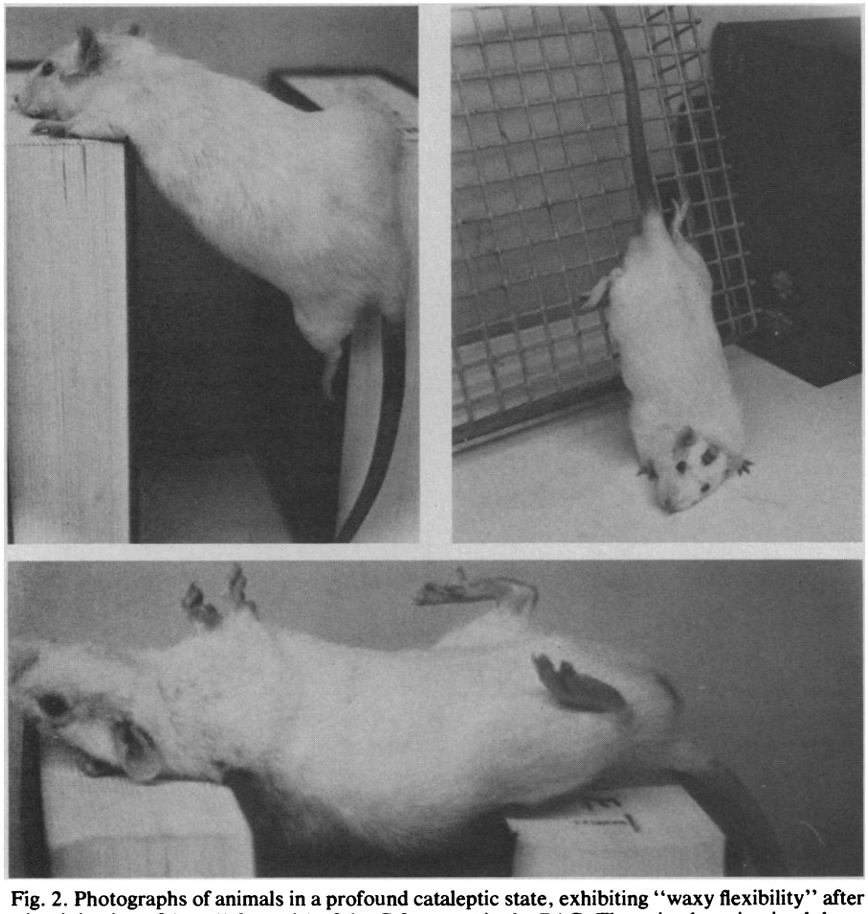

Fig. 2. Photographs of animals in a profound cataleptic state, exhibiting "waxy flexibility" after microinjection of 4 μg (1.2 nmole) of the C-fragment in the PAG. The animals maintained these postures for long periods (more than 1 hour if not startled); however, a sudden stimulus, such as a flash from the light bulb of the camera, or a sudden noise, would arouse the animal and cause it to resume a normal posture.

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Regulation of Acetylcholine Synthesis: Does Cytoplasmic Acetylcholine Control High Affinity Choline Uptake?

Abstract. *When brain synaptosomes are obtained from animals that have been injected intravenously with [$^2H_4$]choline 1 minute before being killed, their high affinity [^3H]choline uptake is correlated inversely with their acetylcholine content and directly with the rate at which they synthesize [$^2H_4$]acetylcholine. The control of such choline uptake by the cytoplasmic acetycholine concentration is proposed as a mechanism regulating acetylcholine synthesis in cholinergic nerve terminals.*

The relatively small changes in acetylcholine (ACh) concentrations that are caused by alterations in neuronal impulse flow (*1, 1a*) suggest that a regulatory feedback system controls the rate of ACh synthesis in response to changing demands. The nature of this regulatory mechanism has been the subject of much investigation and speculation. It has recently been proposed that precursor availability may regulate ACh synthesis, since the increase in plasma and brain choline concentrations following oral or parenteral administration of choline is associated with an increase in brain ACh concentration (*2*). Feedback mechanisms that have been suggested include inhibition of choline acetyltransferase (E.C. 2.3.1.6) by ACh (*3*); maintenance by choline acetyltransferase of a mass action relationship between choline, acetyl coenzyme A (acetyl CoA), ACh and coenzyme A (CoA) (*4*); and, most recently, regulation of the high affinity choline uptake system (*5, 6*) which appears to be coupled to ACh synthesis (*7*) and release (*1a, 8*). On the basis of the properties of synaptosomal fractions isolated from animals subjected to various treatments before being killed, it was suggested (*5, 6*) that the high affinity choline transport system is regulated by neuronal impulse flow.

One of the simpler mechanisms by which some of the properties of synaptosomes in vitro could be dependent upon their immediate antemortem history would be through the content of choline and ACh of the synaptosomes at the time they were prepared by homogenation of brain tissue. We present evidence that the choline and ACh content of synaptosomes reflect cholinergic events in the brain at the time the synaptosomes were prepared. We have confirmed that the high affinity choline uptake by synaptosomes is influenced in a predictable manner by the way in which the animal is treated before it is killed; we show that this uptake is consistently related to the synaptosomal ACh content and synthesis rate. We propose that the factor controlling the high affinity uptake of [^3H]choline by synaptosomes in these experiments may be their content of ACh.

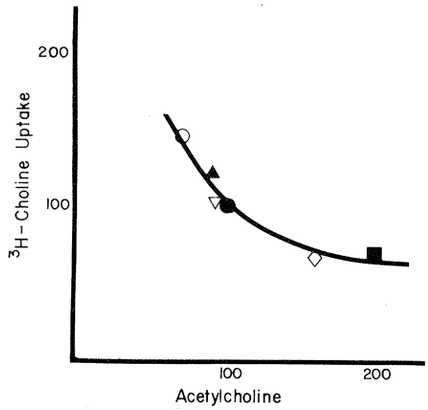

Fig. 1. Acetylcholine concentration and [^3H]choline uptake by synaptosomes prepared from animals subjected to various treatments before being killed. Experimental details were as indicated in Tables 1 and 2. The rate of the high affinity uptake of 0.54 μ*M* [^3H]choline was measured as described by Atweh *et al.* (*6*) and is presented as a percentage of the rate of uptake by synaptosomes prepared from saline-treated mice. The concentration of ACh in the synaptosomes is presented as a percentage of the concentration in synaptosomes prepared from saline-treated mice. Symbols: • saline; ○ atropine; ▲, pentylenetetrazole; ▽, electroshock; ◇, pentobarbital; and ■, oxotremorine, at the doses given in Table 2. There is a highly significant correlation (r = .970; P = .0014) between [^3H]choline uptake and the reciprocal of the ACh concentration.

Synaptosomes prepared from mouse brain at various time intervals after the animals are killed show changes in their content of choline and ACh (Table 1) which parallel those observed previously in whole brain (*9*). A progressive increase in choline content and a decrease in ACh content are observed in both systems. When [$^2H_4$]choline is injected intravenously 1 minute before the animals are killed, the [$^2H_4$]ACh formed in brain provides an estimate of relative turnover rate of ACh (*10, 10a*). The mole fractions of [$^2H_4$]choline and [$^2H_4$]ACh in synaptosomes parallel the corresponding values in whole brain (Table 1, columns 5 and 9).

To examine the effect that synaptosomal choline and ACh content may have on high affinity [^3H]choline uptake in vitro, mice were treated with drugs known to influence cholinergic activity and with electroshock. Synaptosomes were prepared from the whole brains and were used to measure: (i) the rate of [^3H]choline uptake under conditions favoring high affinity uptake, (ii) total choline and ACh concentrations in the freshly isolated synaptosomes, and (iii) mole fractions of [$^2H_4$]choline and [$^2H_4$]ACh in synaptosomes prepared from animals into which [$^2H_4$]choline was injected intravenously 1 minute before they were killed. The relationships between these variables were consistent, regardless of the measures used to perturb the normal state before death (Table 2).

High affinity choline uptake (Fig. 1) was reduced in synaptosomes prepared from animals treated with pentobarbital or oxotremorine, as reported earlier (*6*). The synaptosomal acetylcholine content was in both cases significantly increased, like that in whole brain (*11*). In contrast, prior treatment of the animals with atropine increased the high affinity choline uptake by synaptosomes; such synaptosomes contained lower concentrations of ACh, again in agreement with reported effects in whole brain (*11*). Treatment with pentylenetetrazole or electroshock produced similar but smaller changes in both the high affinity uptake of choline and the concentration of ACh.