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Enkephalin and Other Peptides Reduce Passiveness

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KASTIN, A. J., E. L. SCOLLAN, R. H. EHRENSING, A. V. SCHALLY AND D. H. COY. *Enkephalin and other peptides reduce passiveness.* PHARMAC. BIOCHEM. BEHAV. 9(4) 515–519, 1978.—Enkephalin and other brain peptides previously have been shown to be active in the dopa potentiation test which may be considered an animal model of mental depression. A recently described model of passive immobility during swimming, also sensitive to tricyclic antidepressants, was therefore used to study a large number of naturally occurring peptides and some of their analogues. It was found that several enkephalins with no opiate activity after peripheral injection reduced the immobility and thus increased the activity of swimming rats. α-MSH, but not its 4–10 core or a 4–9 analogue, also caused significantly more swimming than did the diluent control. As we have previously found in several animal and clinical studies, a smaller dose of MIF-I was more effective than larger doses. The results confirm our concept of the CNS actions of brain peptides and support the suggestion that some of them, like the enkephalins, might be useful after peripheral administration in mental depression or other CNS disorders.

Enkephalin Endorphin MSH MIF-I Peptides Blood-brain barrier Depression
Swimming Passiveness

USING THE dopa potentiation test, we showed in 1971 that brain peptides isolated from the hypothalamus could exert effects upon the central nervous system (CNS) [12,16]. Methionine enkephalin, a peptide found in several parts of the brain including the hypothalamus [21], also has been shown to be very active in the dopa potentiation test [18]. The activity of the tricyclic antidepressants in this test [7,15] has led some investigators to consider it a model for mental depression. Therefore, when another animal model of depression was described recently [19], we decided to use it to study the effects of enkephalin and other peptides. Since we had already demonstrated the CNS effects of enkephalin [13, 18, 20] and other brain peptides [10] after peripheral injections in several experimental situations, the intraperitoneal (IP) route of administration was chosen.

METHOD

Materials

All peptides were synthesized by solid-phase methods and highly purified in our laboratory with the exception of α-MSH, which was synthesized by classical methods, pentagastrin (Peptavlon, Ayerst), and the ACTH preparations (Organon). Each compound is identified in this paper by its full name except for the use of the following abbreviations: α-MSH (melanocyte-stimulating hormone); MIF-I (MSH-release inhibiting factor = Pro-Leu-Gly-NH_2); TRH (thyrotropin releasing hormone); LH-RH (luteinizing hormone releasing hormone); BPP (bovine pancreatic polypeptide); VIP (vasoactive intestinal peptide); and DSIP (delta sleep-inducing peptide). A soluble melanin purified from the ink sac of the cuttle-fish (*Sepia officinalis*) was purchased from ICN K and K Rare and Fine Chemical Co., Cleveland, OH. Amitriptyline hydrochloride was obtained in powder form from Merck, Sharpe, and Dohme, West Point, PA. Each material was dissolved in physiological saline which was slightly acidified to 0.01 M with acetic acid and kept cold until used within 30 hr.

Animals

About a thousand male albino rats were obtained from Simonsen Laboratories in Gilroy, CA. They were initially housed a minimum of 3 days in group cages with constant indirect illumination, white background noise, and unlimited access to food and water. The rats weighed 180–200 g at the time of the first of 3 injections which were made IP from coded solutions 24, 4, and 1 hr before testing.

Procedure

The method described by Porsolt *et al.* as a model of depression [19] was adapted for this study. On the first day,

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TABLE 1
IMMOBILITY TIME AFTER ADMINISTRATION OF ENKEPHALINS

Peptide	Dose (mg/kg)	Number of Rats	Difference from Control (sec)
Met-enkephalin	2.0	4	60.0*
	1.0	45	28.8*
	0.1	3	- 3.0
D-Ala^2-enkephalin-NH$_2$	1.0	28	14.4
	0.1	18	42.6†
	0.01	10	14.4
D-Ala^2-F$_5$Phe^5-enkephalin-NH$_2$	1.0	12	- 10.2
	0.1	5	- 48.0
	0.01	9	- 28.8
N$^\alpha$, N$^\epsilon$-bis(D-Ala^2-enkephalin)-Lys-NH$_2$	1.0	6	- 4.8
	0.1	3	3.0
	0.01	4	- 22.2
D-Phe^5-enkephalin	1.0	19	24.0*
Leu-enkephalin	1.0	42	17.4*

*p < 0.05
†p < 0.01

the rat was placed in a white cylindrical tank 15 cm wide and 45 cm high which was filled 15 cm deep with water at 25°C. Fifteen min later the rat was removed and put in a heated enclosure at 32°C for 15 min. It was then injected for the first time and placed in an individual cage. Twenty and 23 hr later the animal received the second and third injections of the test solution, a schedule used in the original description [19]. One hr after the last injection, the rat was returned to the tank of water for 5 min. During this period of observation, whenever the rat stopped swimming actively or trying to escape, the timer was activated. The cumulative amount of these passive or immobile periods for each rat was used for the statistical analyses.

Analyses

The amount of immobility of the animals receiving the test compounds was compared with that of their concurrent controls injected with the diluent. The results were analyzed by Student's *t*-tests and analysis of variance with Duncan's or Dunnett's procedures for multiple comparisons where appropriate.

RESULTS

The mean differences (sec) in the time of passive immobility between rats injected with one of the naturally occurring enkephalins or their analogues, and rats injected with the diluent are shown in Table 1. As in the other tables, a negative number indicates that the rats injected with the peptide showed less passive immobility and more swimming than the rats receiving only diluent whereas a positive difference indicates more immobility and less swimming than the controls.

Both Met-enkephalin and Leu-enkephalin caused more active swimming than the diluent at the standard dose for each injection of 1 mg/kg initially used in these studies. D-Ala^2-Met-enkephalin-NH$_2$ was not significantly active at this dose although it was highly active at 0.1 mg/kg, a dose at which Met-enkephalin and two other analogues seemed to have no substantial effect (Table 1). The larger β-endorphin apparently was more effective at 1 mg/kg than at smaller doses (Table 2).

At the test dose of 1 mg/kg, injection of α-MSH resulted in significantly less immobility than seen after injection of the diluent (Table 3). This difference was not observed with MSH/ACTH 4–10, an analogue of ACTH 4–9, or ACTH 1–24, a preparation with full adrenal stimulating activity.

A reduction in immobility was found after administration of MIF-1 at the dose of 0.1 mg/kg but not at doses of 1, 10, or 30 mg/kg (Table 4). The smaller dose of 0.01 mg/kg MIF-1 was also ineffective. Injection of TRH did not cause a statistically significant change in swimming time after injection of 1 mg/kg or 0.1 mg/kg.

TABLE 2
IMMOBILITY TIME AFTER ADMINISTRATION OF ENDORPHINS

Peptide	Dose (mg/kg)	Number of Rats	Difference from Control (sec)
β-endorphin	1.0	14	34.8*
	0.5	3	20.4
	0.1	8	17.4
	0.01	3	22.2
Leu^5-β-endorphin	1.0	3	24.0
α-endorphin	1.0	7	31.8
γ-endorphin	1.0	6	6.6

*p < 0.05

TABLE 3

IMMOBILITY TIME AFTER ADMINISTRATION OF MSH AND ACTH COMPOUNDS

Peptide	Dose (mg/kg)	Number of Rats	Difference from Control (sec)
α-MSH	1.0	30	34.8*
MSH/ACTH$_{1-10}$	1.0	20	− 9.0
Met^{12}(O)-D-Lys^{8}-Phe^{9}-ACTH$_{1-9}$	1.0	16	−14.4
ACTH$_{1-24}$	1.0	12	1.2

*$p < 0.01$

TABLE 4

IMMOBILITY TIME AFTER ADMINISTRATION OF MIF-1 AND TRH

Substance	Dose (mg/kg)	Number of Rats	Difference from Control (sec)
MIF-1	30.0	10	− 4.8
	10.0	10	+ 23.4
	1.0	10	+20.4
	0.1	38	−24.0*
	0.01	6	−12.3
TRH	1.0	25	+ 6.0
	0.1	6	− 8.4

*$p < 0.05$

TABLE 5

IMMOBILITY TIME AFTER ADMINISTRATION OF GI RELATED HORMONES

Peptide	Dose (mg/kg)	Number of Rats	Difference from Control (sec)
bombesin	1.0	8	−32.4
BPP	1.0	7	11.4
VIP	1.0	7	−12.0
pentagastrin	1.0	4	−30.6

TABLE 6

IMMOBILITY TIME AFTER ADMINISTRATION OF OTHER BRAIN SUBSTANCES

Substance	Dose (mg/kg)	Number of Rats	Difference from Control (sec)
somatostatin	1.0	8	−36.0
melanin	1.0	13	−24.6
neurotensin	1.0	14	5.4
DSIP	1.0	14	3.6
D-Ala^{2}-DSIP	1.0	9	+28.2
LH-RH	1.0	9	+21.0
substance P	1.0	8	−29.4
amitriptyline	15.0	227	−42.6*
morphine	1.0	5	− 3.6

*$p < 0.001$

Table 5 shows that bombesin tended to cause more swimming than the controls. Three gastrointestinal (GI) hormones were without significant effect.

Two other hypothalamic hormones—LH-RH and somatostatin—did not exert any statistically significant effects although there was a tendency toward less immobility after injection of somatostatin (Table 6). Similarly, neurotensin, substance P, and DSIP did not seem to change the swimming times. Melanin and morphine sulfate also were without significant effect at 1 mg/kg. These materials, like some of the others, were administered to only a few animals and at only 1 dose, so that many of the results must be considered preliminary. Our confidence in the data involving a larger sampling is obviously greater. This is most evident with amitriptyline which was used in every experiment at a dose of 15 mg/kg (Table 6).

Since anticholinergic responses can be seen at therapeutic doses of the tricyclic antidepressants and particularly amitriptyline, we tried several doses of physostigmine concurrently in an attempt to block the effects of amitriptyline. Although some reversal seemed to occur at high doses (500 μg/kg), this dose also caused immobility by itself. A smaller dose (15 μg/kg), which had no effect on the swimming time, did not seem to consistently reverse the effects of amitriptyline in these preliminary experiments. Atropine, at doses of 10 and 50 μg/kg, did not appear to duplicate the actions of amitriptyline in prolonging swimming time.

DISCUSSION

The results support the concept that peptides found in the brain can exert effects there. Unlike the studies with which we introduced this concept [12,16], hypophysectomized animals were not used. Nevertheless, it is unlikely that the results can be fully explained by a secondary action of the peptides upon the pituitary gland since there was no correlation between known hormonal effects and activity in the swimming test. This is illustrated by Met-enkephalin which was active in the swimming study (Table 1) yet is not known to cause any substantial release of pituitary hormones, and by TRH and LH-RH which were inactive in this test (Tables 4 and 6) but are known to release several pituitary hormones.

The significant differences between rats injected with peptides and rats injected with diluent as a control were observed more than an hour after the last IP injection. Because of the rapid degradation of many of the peptides and their short half lives, the demonstration of any CNS activity

after peripheral administration will continue to surprise some investigators [10]. Moreover, Met-enkephalin and some of the other brain opiates were active after IP injection at doses devoid of analgesic activity and the effect demonstrated increased rather than decreased activity (Table 1). This probably represents another example of the dissociation of the opiate and behavioral effects of enkephalin, as first described elsewhere [9,13].

The observation of CNS effects after peripheral administration of enkephalin and other peptides does not necessarily imply the passage of large amounts of unchanged material across the blood-barrier. All that may be required is the movement of a small amount of material into the brain; this need not penetrate directly from the blood into brain tissue but may enter the CSF first. Recent determination of the brain uptake index of enkephalin by an unmodified method (with L. Wade), as opposed to the modified method we used previously [11], supports the statement that only a small amount of enkephalin penetrates brain tissue directly after peripheral injection. Initial penetration into the CSF would not be detected by this method, although entry into the CSF has been included in our broader use of the term blood-brain barrier. Moreover, it is possible that the active agent might be a degradation product of the injected peptide or a different substance altogether and primary peripheral actions have not been completely ruled out. A possible advantage of peripheral injections, in addition to their convenience and eventual clinical practicability, is their potential for demonstrating effects that might differ from those observed after central injections, such as an increased permeability of the blood-brain barrier to other substances.

All peptides were initially tested in small groups of rats at the single dose of 1 mg/kg body weight. Other doses and different times of observation after the injections might have given different results in additional animals. At this dose, significant differences from controls were observed in rats injected with the following enkephalins: Met-enkephalin, D-Ala^2-Met-enkephalin-NH$_2$, Leu-enkephalin, and D-Phe^1-enkephalin (Table 1). D-Phe^1-enkephalin has negligible opiate activity in the vas deferens and opiate receptor assays [2], but appeared active in another behavioral test [13]. However, N$^\alpha$, N$^\epsilon$ bis (D-Ala^2-enkephalin)-Lys-NH$_2$ and D-Ala^2-F$_5$ Phe^1-enkephalin-NH$_2$, two enkephalin derivatives extremely potent in the tail-flick test of analgesia [3], were inactive in the swimming test (Table 1), as was morphine at the 1 mg/kg dose (Table 6). We consider these results to be further evidence for the behavioral effects of enkephalins under conditions in which the opiate actions may be irrelevant.

Several doses of some of the compounds found to be more active were examined. In some cases (e.g. D-Ala^2-enkephalin-NH$_2$ and MIF-1), a smaller amount seemed to be more effective than the initial dose of 1.0 mg/kg (Tables 1 and 4). This does not appear to be explained simply by an excessive potency of some materials exceeding an effective ceiling since the potent opiates D-Ala^2-F$_5$ Phe^1-enkephalin-NH$_2$, N$^\alpha$N$^\epsilon$-bis (D-Ala^2-enkephalin)-Lys-NH$_2$, and β-endorphin were not as active at smaller doses (Tables 1 and 2). In this regard, β-endorphin might not be expected to penetrate the blood-brain barrier as readily as D-Ala^2-enkephalin-NH$_2$, but the pentafluorinated enkephalin analogue should enter the brain more easily.

We have previously observed this biphasic or "inverted U-shaped" pattern of response to brain peptides in several animal and clinical studies, as reviewed elsewhere [5,10]. Two of these studies involved trials of MIF-1 in what might be considered animal models of mental depression, namely the dopa-potentiation test [16] and reserpine reversal test [17], and 2 additional studies involved patients with mental depression [5,6]. As in the dopa potentiation test, MIF-1 was found to be active in the swimming test with a dose (0.1 mg/kg) at which TRH was inactive (Table 4), although TRH has also been tried in mental depression [12]. This is consistent with our impression that MIF-1 shows promise as an antidepressant [5, 6, 12].

The test system used in the present investigation measured the swimming time of a rat in a situation from which there was no escape. After initial swimming activity, it became relatively immobile, barely treading water enough to keep its nose and mouth in the air. This situation of "passiveness", "resignation", "helplessness", or "despair" was shown to be reversed by tricyclic antidepressants, but not several tranquilizers, and therefore was described as a new animal model for mental depression [19]. The contribution of the rat being housed individually for the 24 hr before testing was not ascertained; it is possible that some of the peptides protect against the effects of this "social deprivation".

Amitriptyline was used as the standard in every trial of these studies at the dose of 15 mg/kg (Table 6). The similarity in activity between this tricyclic antidepressant and several of the peptides tested provides additional support for the possibility of clinical trials of enkephalin and some other peptides in patients with mental depression or other CNS disorders. Perhaps analogues less active in opiate tests but more active in tests such as these should be considered for the trials. Regardless, our results suggest that D-Ala^2-Met-enkephalin-NH$_2$ might act as an antidepressant and we are proceeding to test this hypothesis with Drs. D. Gonzalez-Barcena and A. Macias. The most preliminary results from this study are in the predicted direction, but because of the nature of depression and the high placebo response in this condition, nothing can be determined with any certainty until a large, double-blind clinical trial is performed.

The significant activity of natural α-MSH, but not its 4–10 amino acid core or the newer 4–9 analogue, in the swimming test (Table 3) coincides with other instances of divergent activity of these related peptides, e.g. [4]. Moreover, in the determination of structure-activity relationships of MSH-related compounds [8], our results emphasize the danger of generalizing from only a single animal test. Although other explanations are possible, this may be part of the reason why α-MSH or MSH/ACTH 4–10 was inactive in some rats [1] or normal human subjects [14] tested in a conditioned avoidance response. Dissociations such as this support the experimental approach adopted by our group about ten years ago of using a number of animal systems in an attempt to interpret the meaning of the CNS effects of naturally occurring peptides. At the least, this approach has resulted in a profile or "fingerprint" pattern of responses which in the case of MSH, for example, resulted in the concept of its actions on attention [10,12].

As a logical extension of our teleological reasoning that if a peptide is present in the brain it should be able to exert an effect there, we tested in rats for the first time the CNS effects of several gastrointestinal peptides which are being found in the brain (Table 5). Although no statistically significant effects were observed in the swimming test, there seemed to be a tendency for bombesin to reduce the time of

immobility. Regardless of these preliminary observations, we expect that CNS actions of the GI hormones and perhaps the related skin peptides will be found which do not involve the GI tract. It is reasonable to expect that these may eventually be tried in CNS disorders.

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REFERENCES

1. Beckwith, B. E., C. A. Sandman, D. Hothersall and A. J. Kastin. Influence of neonatal injections of α-MSH on learning, memory and attention in rats. *Physiol. Behav.* **18:** 63–71, 1977.
2. Coy, D. H., A. J. Kastin, A. V. Schally, O. Morin, N. G. Caron, F. Labrie, J. M. Walker, R. Fertel, G. G. Berntson and C. A. Sandman. Synthesis and opioid activities of stereoisomers and other D-amino acid analogs of methionine-enkephalin. *Biochem. Biophys. Res. Commun.* **73:** 632–638, 1976.
3. Coy, D. H., A. J. Kastin, J. M. Walker, R. F. McGivern and C. A. Sandman. Increased analgesic activities of a fluorinated and a dimeric analogue of D-Ala^2-methionine-enkephalinamide. *Biochem. Biophys. Res. Commun.* **83:** 977–983, 1978.
4. Dunn, A. J., P. M. Iuvone and H. D. Rees. Neurochemical responses of mice to ACTH and lysine vasopressin. *Pharmac. Biochem. Behav.* **Suppl. 1, 5:** 139–145, 1976.
5. Ehrensing, R. H. and A. J. Kastin. Melanocyte-stimulating hormone-release inhibiting hormone as an anti-depressant: a pilot study. *Arch. Gen. Psychiat.* **30:** 63–65, 1974.
6. Ehrensing, R. H. and A. J. Kastin. Dose-related biphasic effect of prolyl-leucyl-glycinamide (MIF-I) in depression. *Am. J. Psychiat.* **135:** 562–566, 1978.
7. Everett, G. M. The dopa response potentiation test and its use in screening for antidepressant drugs. *Excerpta Med. Int. Cong. Ser. No.* **122:** 164–167, 1966.
8. Greven, H. M. and D. de Wied. The influence of peptides derived from corticotrophin (ACTH) on performance. Structure activity studies. *Prog. Brain Res.* **39:** 429–441, 1973.
9. Kastin, A. J., D. H. Coy, R. D. Olson, J. Panksepp, A. V. Schally and C. A. Sandman. Behavioral effects of the brain opiates enkephalin-endorphin. In: *Central Nervous System Effects of Hypothalamic Hormones and Other Peptides*, edited by R. Collu. New York: Raven Press, 1978 (in press).
10. Kastin, A. J., D. H. Coy, A. V. Schally and L. H. Miller. Peripheral administration of hypothalamic peptides results in CNS changes. *Pharmac. Res. Commun.* **10:** 293–312, 1978.
11. Kastin, A. J., C. Nissen, A. V. Schally and D. H. Coy. Blood-brain barrier, half-time disappearance, and brain distribution for labeled enkephalin and a potent analog. *Brain Res. Bull.* **1:** 583–589, 1976.
12. Kastin, A. J., C. A. Sandman, A. V. Schally and R. H. Ehrensing. Clinical effects of hypothalamic-pituitary peptides upon the central nervous system. In: *Clinical Neuropharmacology*, edited by H. L. Klawans. New York: Raven Press, 1978, pp. 133–152.
13. Kastin, A. J., E. L. Scollan, M. G. King, A. V. Schally and D. H. Coy. Enkephalin and a potent analog facilitate maze performance after intraperitoneal administration in rats. *Pharmac. Biochem. Behav.* **5:** 691–695, 1976.
14. Miller, L. H., S. C. Fischer, G. A. Groves, M. E. Rudrauff and A. J. Kastin. MSH/ACTH$_{1-10}$ influences on the CAR in human subjects: a negative finding. *Pharmac. Biochem. Behav.* **7:** 417–419, 1977.
15. Plotnikoff, N. P. and A. J. Kastin. Neuropharmacological review of hypothalamic releasing factors. In: *Neuropeptide Influences on Brain and Behavior Mechanisms*. New York: Raven Press, 1977, pp. 81–107.
16. Plotnikoff, N. P., A. J. Kastin, M. S. Anderson and A. V. Schally. DOPA potentiation by a hypothalamic factor, MSH release-inhibiting hormone (MIF). *Life Sci.* **10:** 1279–1283, 1971.
17. Plotnikoff, N. P., A. J. Kastin, M. S. Anderson and A. V. Schally. Deserpidine antagonism by a tripeptide, L-prolyl-L-leucylglycinamide. *Neuroendocrinology* **11:** 67–71, 1973.
18. Plotnikoff, N. P., A. J. Kastin, D. H. Coy, C. W. Christensen, A. V. Schally and M. A. Spirtes. Neuropharmacological actions of enkephalin after systemic administration. *Life Sci.* **19:** 1283–1288, 1976.
19. Porsolt, R. D., M. Le Pichon and M. Jalfre. Depression: a new animal model sensitive to antidepressant treatments. *Nature* **266:** 730–732, 1977.
20. Smith, D. A., R. D. Olson, A. J. Kastin, G. A. Olson, D. H. Coy and G. F. Michell. Neuropeptides and the blood-brain barrier in goldfish. *Abstr. Soc. Neurosci.* **4:** 414, 1978.
21. Yang, H.-Y., J. S. Hong and E. Costa. Regional distribution of leu and met enkephalin in rat brain. *Neuropharmacology* **16:** 303–307, 1977.