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# BRIEF COMMUNICATION

## Generalization of [DAla$^2$]-Enkephalinamide But Not of Substance P to the Morphine Cue

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CHIPKIN, R. E., J. M. STEWART, D. H. MORRIS AND T. J. CROWLEY. *Generalization of [DAla$^2$]-enkephalinamide but not of Substance P to the morphine cue.* PHARMAC. BIOCHEM. BEHAV. 9(1) 129–132, 1978.—Rats were trained to discriminate morphine (7.5 mg/kg, IP) from saline in a two bar positively reinforced lever pressing paradigm on a FR4 schedule. Morphine (IP) showed a naloxone reversible dose-related generalization to the training dose. [DAla$^2$]-Methionine enkephalinamide (DAE) at 1 mg/kg and Substance P (SP) at 0.1 and 0.25 mg/kg showed vehicle appropriate responding after IP injection. DAE (5 mg/kg) disrupted responding completely; SP (0.5 and 0.1 mg/kg) disrupted responding in 50% of the rats. The disruption caused by IP injection of DAE was not naloxone reversible. Intraventricular injection of morphine (5 μg/rat) and DAE (5 μg/rat) produced generalization to the opiate cue. The effect of DAE was reversed by naloxone (1 mg/kg, SC). SP (500 and 750 ng/rat, IVT) produced saline-like responding; 1 μg/rat disrupted responding completely. These data demonstrate that morphine and enkephalin, but not Substance P, share similar discriminative properties.

Morphine Enkephalin [DAal$^2$-Met$^5$]-Enkephalinamide Substance P Naloxone Discriminative stimuli

THE ABILITY of morphine and other synthetic opioids to serve as a discriminative stimulus in an operant procedure has been previously demonstrated [1,3]. However, the discovery of endogenous peptides [9] that display opiate-like physiological properties raises the question whether or not these amino acid-containing compounds will share cognitive effects similar to morphine. In order to initially evaluate this problem, we trained a group of rats to discriminate morphine from saline and thereafter tested the generalizability of either [DAla$^2$-Met$^5$]-enkephalinamide (DAE) or Substance P (SP). DAE was chosen over enkephalin itself due to its longer in vivo duration of action [11]. Both peptides have been shown to have naloxone reversible analgesic effects and to bind to opiate receptors [5, 11, 14]; although DAE and SP have clearly demonstrable differences on stimulated guinea pig ileum [2].

### METHOD

#### *Animals*

Male Sprague-Dawley rats (Charles River), N=4, approximately nine weeks old at the beginning of the experiment were used. They were without behavioral experience and were drug naive. The rats had continuous access to food but were permitted drinking water for only 15 min per day immediately following behavioral training. They were singly housed and kept on a 12 hr light-dark schedule.

In the course of the experiment one rat died from a cerebral infection resulting from implantation of the cannula. A second rat broke an injection cannula off in the guide cannula during one test session and his data are only included in experiments prior to that. Thus, for the experiments utilizing intraperitoneal injection of drugs, in all cases but one (1 mg/kg of DAE) the number of rats is four. In this other case only three rats were used. After intraventricular (IVT) administration, N=3 for morphine and saline test sessions, N=3 for all the Substance P data, and N=2 for the experiments with DAE and its reversal by naloxone.

#### *Behavioral Methods*

Rats were trained to press one lever after morphine injections and the opposite lever after saline injections in a two bar positively reinforced operant procedure, according to the methods of Hirschhorn and Rosecrans [7]. A Lehigh Valley operant chamber with two bars and Lehigh Valley programming equipment were used. Sweetened milk (0.1 mL/reinforcement) was the reinforcer. The rats were first trained to press both bars in the operant chamber on a schedule rewarding every fourth response (FR4). Following this, discrimination training began in daily 15 min sessions. On the first and second days of the training each rat was injected intraperitoneally with saline (0.1 mL/100 g body wt) 30 min before being placed in the operant chamber. For one half the rats, responding on the right bar was reinforced on saline days; for the other half, the left bar was the reinforced lever. On the third and fourth day, morphine (7.5 mg/kg, IP) was given in a similar volume 1/2 hr before the session and the active bar for each group reversed. On the days following Day 4, the rats were similarly injected with saline (S) or morphine (M) on a two-day alternating schedule (SSMM),

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but the time in the box was subdivided into a Test and a Training session. During the test session (1.5 min) neither bar was active and the total number of responses on each bar was recorded. During the training session (13.5 min) the appropriate bar (relative to the solution injected) was active and the number of responses on each bar was observed. No test drugs were given until the rats reached a criterion of greater than 90% correct responses on the appropriate bar during the test session for eight consecutive sessions. This took approximately 50 sessions. Thus, the stimulus properties of the drug/no-drug states became reliably discriminable, separate and quantifiable.

Stimulus generalization tests took place after the rats reached criterion behavior. Test days were separated by 3–5 days, and no compound was tested if any of the animals displayed abnormal behavior on the intervening training days. For stimulus generalization tests, the rat received the test compound (saline, morphine, DAE or SP) via either the intraperitoneal or intraventricular route immediately before entering the operant chamber. All drugs were prepared freshly each day. The number of responses on each lever within the 1.5 min no reinforcement period was recorded. If the rat failed to press either lever, another 1.5 min test session began. Following this the rat was removed from the box and returned to his cage. At 30 min post-injection all rats were replaced in the chamber and the testing procedure as outlined above was repeated. Data reported here were from 30 min post-injection trials, as there was no difference before the 1 and 30 min time points. If an animal failed to make more than five responses within 3 min his behavior was considered to be totally disrupted. All data reported represent at least this minimum number of responses; in the majority of experiments responding was usually several times greater than this. The data are expressed as a percentage of responding on the morphine correct bar (i.e., number of presses on morphine correct lever/total number of presses × 100%) and were evaluated utilizing Student's $t$ test.

*Cannulae Implantation*

Rats were implanted stereotaxically in the lateral ventricle with commercially available cannulae (Plastic Products, Inc.). A 22 ga guide cannula was implanted at the following coordinates: AP 5.8, Lateral 1.5, Ventral 3.0 [10]. The injection cannula was a 28 g hypodermic needle cut to extend 1 mm beyond the guide cannula tip. The injection volume was 5 $\mu$l in all experiments. Histological examination following sacrifice confirmed a ventricular locus of administration.

*Drugs*

Morphine Sulfate (Lilly) was dissolved in distilled water for peripheral injection and in sterile isotonic saline for central administration. Naloxone hydrochloride (Endo) was dissolved in distilled water. Drug doses refer to the salt.

[DAla$^2$–Met$^5$]–Enkephalinamide and Substance P were synthesized by standard techniques [15]. Purity of the peptide was determined by thin layer chromatography and paper electrophoresis. Upon hydrolysis, amino acid analysis of all peptides gave the expected ratios. Both compounds were tested on guinea pig ileum and found to be biologically active. DAE was dissolved in distilled water for peripheral injection and in sterile saline for the IVT injections. SP was dissolved in pure propylene glycol for intraperitoneal injections. For intraventricular injections SP was first dissolved in pure propylene glycol and then diluted (1:4) to the proper concentration with sterile saline. See text for molar concentrations used.

## RESULTS

After intraperitoneal injection of morphine, a dose-related, naloxone reversible generalization was observed to the training dose. [DAla$^2$–Met$^5$]-enkephalinamide was given at two dosage levels. At 5 mg/kg (7.10 $\mu$M/kg) (IP) none of the rats responded at 1 or 30 min post-injection. By 60 min two rats responded and pressed >80% on the saline correct lever. This disrupting ability of DAE was not reversed by naloxone (1 mg/kg, SC), nor did the rats display any analgesia (as measured by tail-flick latency) following administration. On the other hand, 1 mg/kg (1.42 $\mu$M/kg) (IP) of DAE caused only 11.9 ± 6.2% ($\bar{x}$ ± SEM) responding on the morphine correct bar.

Substance P was tested IP at four dose levels: 0.10, 0.25, 0.50 and 1.0 mg/kg (0.057, 0.13, 0.29 and 0.57 $\mu$M/kg). At the two lower doses percent responding on the morphine correct bar never significantly exceeded the effect of the vehicle alone. The two higher doses caused total disruption of bar pressing in two out of four rats. The other two responded with depressed rates and pressed entirely on the saline correct lever.

Figure 1 shows the response to intraventricular administration of these drugs. Morphine, 5 $\mu$g/rat (6.6 nM/rat) caused significant ($p<0.01$, compared to saline, $t$ test, Morphine vs. saline) generalization to the peripheral training dose. This result suggested an approximately 1000-fold potency difference between central and peripheral administration. DAE, 5 $\mu$g/rat (7.1 nM/rat), produced similar generalization (90%) to the morphine cue; the effect of DAE was reversed by naloxone to a level of responding comparable to saline. SP at 0.5 or 0.75 $\mu$g/rat (0.29 or 0.43 nM) showed virtually no ability to generalize to the morphine cue; 1 $\mu$g/rat (0.57 nM/rat) of SP completely disrupted responding.

## DISCUSSION

Two important ideas emerge from these data. First, rats responded to intracerebral DAE as they did to the internal cue of morphine. This establishes still another property of enkephalins in common with morphine and is the first demonstration of an endogenous peptide generalizing to an exogenous drug stimulus. There are several possible explanations for the observation that DAE is active after intraventricular but not peripheral administration. First, DAE may be metabolized before it can reach its sites of action in the central nervous system (CNS). Second, DAE may not cross the blood brain barrier after intraperitoneal injection. Last, it may successfully penetrate the brain intact, but because of unknown distributional factors, may not be accessible at the sites necessary for generalization to occur. The most likely explanation is probably the first. Other enkephalin analogs with greater enzymatic resistance have been shown to be active analgesics peripherally [12], whereas DAE has not. Thus, the activity of enkephalin analogs IP is most likely based on the extent to which they are enzymatically degraded, rather than on their distribution. Intraventricular administration makes DAE immediately accessible to the CNS and not subject to extensive breakdown in the periphery. Thus, generalization of DAE to morphine after IVT but not after IP administration is probably a func-

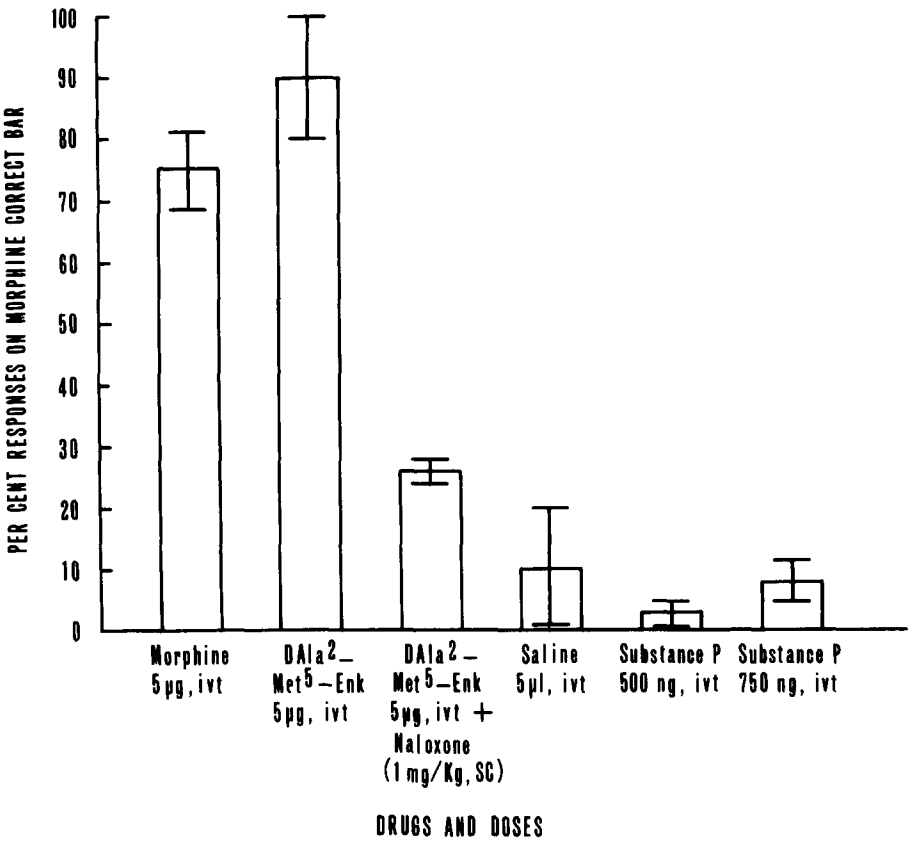


FIG. 1. Percent responses ($\bar{x} \pm$ SEM) on the morphine correct lever 30 min after intraventricular injections or morphine (N=3), Saline (N=3), Substance P (N=3), DAla$^2$–Met$^5$–Enkephalinamide (N=2), and [DAa$^2$–Met$^5$]–Enkephalinamide plus naloxone (N=2). Each point represents a single observation per rat.

tion of the stability of the compound in the CNS compared with the periphery.

In these experiments, by using an enzyme resistant derivative, we have been able to show generalization of DAE to a narcotic cue, whereas Colpaert *et al.* [4] could not. The inability of those researchers to observe generalization of enkephalin itself to fentanyl is most likely explained by the extremely rapid in vivo metabolism of the peptide [6]. They injected the compound 15 min before testing. Presumably, within this time period, unprotected enkephalin derivatives are completely degraded and hence, exert minimal pharmacological or CNS effects. We believe the discrepancy between our data and that of Colpaert *et al.* can be fully explained by the differences in the in vivo stability of the two compounds, although other differences (e.g., procedural) may contribute.

The second important point is that Substance P, although possessing some characteristics in common with other endorphins (e.g., naloxone reversible analgesia), does not appear to have subjective effects similar to morphine or DAE. As with DAE, SP's failure to generalize after peripheral injection may be related to enzymatic degradation before it reaches the CNS. This seems less likely for SP than for DAE since SP has been shown to be an analgesic after IP injection [14] and reportedly does cross the blood brain barrier [13]. It is, therefore, not as evident that SP's inability to generate responding on the morphine correct lever after IP injection is due to degradation as it is with DAE.

Moreover, the saline appropriate responding seen after IVT administration of Substance P seems to indicate that SP and morphine are perceived differently—at least by rats. SP-containing neurons have been identified around the lateral ventricles [8] and these would theoretically be accessible to interact with exogenous SP. The behavioral disruption seen at higher doses of SP may be caused by stimulation and/or depression of the SP-containing neurons adjacent to the ventricle.

Possibly SP is acting as a mixed agonist-antagonist similar to cyclazocine or nalorphine. These compounds have been shown not to generalize to the morphine cue, although both are analgesics [7]. Thus, it may be useful to determine more precisely the discriminative stimulus characteristics of Substance P and to seek more analgesically potent analogs with minimal behavioral effects.

The fact that both DAE (5 mg/kg) and SP (0.5 and 1 mg/kg) disrupted responding after IP administration is interesting, albeit confusing. Since DAE's disruption was neither naloxone reversible nor associated with analgesia, it may have been the result of a peripheral, non-specific effect of the compound itself (or some non-peptide contaminant). On the other hand, since there are no antagonists of SP available, the specificity of SP's effect cannot be definitively stated. As

noted above, SP has been reported to cross the blood brain barrier. In the present studies disruption occurred after both peripheral and central administration, suggesting a central locus for SP's (or an SP metabolite's) disrupting effect. Arguing against a direct effect of SP in the CNS and for a simple irritant effect are earlier reports which suggest that SP is an algogen, i.e., a pain-producing substance. However, these observations are from experiments utilizing an impure preparation of SP (contaminated with histamine and/or bradykinin), and more recent work with synthetic SP [14] has contradicted this. Further work is needed to clarify whether the behavioral disruption caused by SP is related to a central or a peripheral mechanism.

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## REFERENCES

1. Barry, H. Classification of drugs according to their discriminable effects in rats. *Fedn Proc.* **33:** 1814–1824, 1974.
2. Chipkin, R. E. and J. M. Stewart. Effect of Substance P and analogs on stimulated and non-stimulated guinea pig ileum. *Pharmacologist* **19:** 30, 1977.
3. Colpaert, F. C. Narcotic cue and narcotic state. *Life Sci.* **20:** 1097–1108, 1977.
4. Colpaert, F. C., C. J. E. Niemegeers, P. A. J. Janssen and J. M. van Ree. Narcotic cueing properties of intraventricularly administered sufentanil, fentanyl, morphine and Met-Enkephalin. *Eur. J. Pharmac.* **47:** 115–119, 1978.
5. Czlonkowski, A., V. Höllt and A. Herz. Binding of opiates and endogenous peptides to neuroleptic receptor sites in the corpus striatum. *Life Sci.* **22:** 953–962, 1978.
6. Frederickson, R. C. A. Enkephalin pentapeptides—A review of current evidence for a physiological role in vertebrate neurotransmission. *Life Sci.* **21:** 23–42, 1977.
7. Hirschhorn, I. D. and J. A. Rosecrans. Generalization of morphine and lysergic acid diethylamide (LSD) stimulus properties to narcotic analgesics. *Psychopharm.* **47:** 65–69, 1976.
8. Hokfelt, T., O. Johansson, J. O. Kellerth, A. Ljungdahl, G. Nilsson, A. Nygards and B. Pernow. Immunohistochemical distribution of Substance P. In: *Substance P,* edited by U. S. Von Euler and B. Pernow. New York: Raven Press, 1977, pp. 117–146.
9. Hughes, J., T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan and H. R. Morris. Identification of two related pentapeptides from brain with potent opiate agonist activity. *Nature* **258:** 577–579, 1975.
10. Pellagrino, L. J. and A. J. Cushman. *A Stereotaxic Atlas of the Rat Brain.* New York: Appleton-Century-Crofts, 1967.
11. Pert, C. B., A. Pert, J. K. Chang and B. T. W. Fong. DAla$^2$–Met–Enkephalinamide: A potent, long lasting synthetic pentapeptide analgesic. *Science* **194:** 330–332, 1976.
12. Roemer, D., H. H. Buescher, R. C. Hill, J. Pless, W. Bauer, F. Cardinaux, A. Closse, D. Hauser and R. Huguenin. A synthetic enkephalin analogue with prolonged parenteral and oral analgesic activity. *Nature* **268:** 547–549, 1977.
13. Stern, P. and J. Hadzovic. Pharmacological analysis of central actions of synthetic Substance P. *Arch. int. Pharmacodyn.* **202:** 259–262, 1973.
14. Stewart, J. M., C. J. Getto, K. Neldner, E. B. Reeve, W. A. Krivoy and E. Zimmermann. Substance P and analgesia. *Nature* **262:** 784–785, 1976.
15. Stewart, J. M. and J. D. Young. *Solid Phase Peptide Synthesis.* San Francisco: W. H. Freeman and Co., 1969.