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The Effects of Peptides on the Stimulus Properties of Ethanol[1]

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CHIPKIN, R. E., J. M. STEWART AND K. CHANNABASAVAIAH. *The effect of peptides on the stimulus properties of ethanol.* PHARMAC. BIOCHEM. BEHAV. 12(1) 93–98, 1980.—Male Sprague-Dawley rats were trained to discriminate ethanol (2 g/kg, PO: EtOH) from saline (10 ml/kg, PO: SAL) in a two-bar positively reinforced operant task on a VI 15 sec schedule. After the rats reached criterion performance (greater than 90% correct responses on the appropriate lever), thyrotropin releasing hormone (pyroGlu-His-Pro-NH_2: TRH), a metabolite of TRH (His-Pro diketopiperazine: HP), and a structural analog of TRH (HPCA-His-ThiaPro-NH_2: OHT) were tested for their ability to antagonize the EtOH cue. These peptides were chosen for their reported ability to reverse ethanol-induced narcosis. However, at doses that did not disrupt performance, TRH, HP, and OHT did not affect the stimulus properties of ethanol at any dose tested, nor did they change the stimulus properties of saline. Naloxone and ACTH(1–10)-NH_2 were also tested as ethanol antagonists of the training dose. Pretreatment with either of these compounds failed to alter ethanol-appropriate responding. In addition, (DAla^2-Met^5)-enkephalin-ol, (DAla^2-Met(O)^5)-enkephalin-ol, substance P, delta sleep-inducing peptide, and bombesin were tested for their ability to elicit ethanol appropriate responding. The EtOH cue generalized to none of these peptides.

Ethanol Thyrotropin releasing hormone His-Pro diketopiperazine Enkephalin Naloxone
Substance P Bombesin ACTH Delta sleep-inducing peptide Peptide Stimulus properties of drugs

THE potential role of thyrotropin releasing hormone (TRH) in the discriminative properties of ethanol (EtOH) has received no attention. This is despite the fact that a large body of evidence suggests a possible connection between these two compounds. For example, TRH has been shown to reverse ethanol's sleep-inducing properties [5,7] and to antagonize the depletion of cerebellar cyclicGMP induced by alcohol [15]. Furthermore, it has been reported [10,12] that thyroid function is depressed in alcoholism and that thyroid deficiency may be associated with increased ethanol preference in rats [21]. As a whole, these experiments imply that TRH may serve as an endogenous inhibitor of ethanol's pharmacological actions.

The structure of TRH is pyroGlu-His-Pro-NH_2. This tripeptide has both analeptic (i.e., ability to reverse sedative-hypnotic narcosis) and endocrine (i.e., ability to release TSH from the pituitary) effects. The analeptic response is presumably centrally mediated as it can occur in thyroidectomized and hypophysectomized animals [16,17]. Additionally, the effects of other analogs (e.g., HPCA-His-ThiaPro—NH_2: OHT, see Table 1) which exert arousing properties at doses below those needed to release thyroxine, also provide evidence for a differentiation of the central and endocrine effects [18,27].

In this work we have examined the ability of TRH, a proposed metabolite of TRH (His-Pro diketopiperazine; HP) [19], and OHT to reverse the ethanol discriminative stimulus. Theoretically, if TRH and related peptides can reverse the sleep-inducing properties of ethanol, they may have a similar effect on the alcohol cue. Furthermore, several other peptides were tested for their ability to serve as an agonist or an antagonist of the ethanol cue.

METHOD

Behavioral Methods

Naive, male, Sprague-Dawley rats (Charles River, Inc.) with an initial weight between 200 and 225 g were the experimental animals. The animals were trained and tested using Lehigh Valley electromagnetic behavioral equipment that controlled the programming for a Lehigh Valley operant chamber. All training and testing took place in the morning (8:30 a.m.–12:00 noon). The rats spent the remainder of the day in a temperature controlled room with a 12 hr light-dark schedule. The animals (n=10) were caged in groups of five, and each cage had access to water for only 45 min per day immediately after the behavioral sessions. Food was continuously available.

Discrimination training was accomplished in the following manner. First, all the rats were trained to press the right bar in a two-lever operant chamber for a sweetened milk reward on a continuous reinforcement schedule (CRF). Following acquisition of this behavior, responses were no longer reinforced on the right bar and the rats were then taught to press the left bar for CRF. When the response rates became stable, daily 15 min sessions alternating the active bar ensued until

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pressing on both levers was consistent and comparable. Following this, a variable interval 15 sec (VI 15 sec) schedule of reinforcement was introduced on both levers and the sessions alternating the active bar were continued until response rates on both bars were similar.

After both the switching of the levers and the schedule were well learned (approximately two weeks), drug discrimination training began. On Days 1 and 3, ethanol (2 g/kg, PO) in a 20% w/v solution was given 15 min before the session; on Days 2 and 4, saline (10 ml/kg, PO) was given at an equivalent time interval before the session. Half the rats were trained to associate ethanol with the right bar and saline with the left bar; for the other half the active bar relative to the solution injected was reversed. This was done to equate any side preferences in the determination of the rats' behavior.

On the days following Day 4, drug presentation was on a double alternating schedule, i.e., Sal-Sal-EtOH-EtOH. The behavioral session on these days was divided into two parts: a one-minute test session when responding had no consequences and a 14-min training session during which time appropriate responding was reinforced. Training was accomplished to a criterion level of greater than 90% correct responses for eight consecutive sessions after ethanol or saline for six out of ten rats after approximately 25 sessions. These six animals were the subjects for the following experiments. Initial suppression of response rates resulting from EtOH administration lessened with time; tolerance to the rate-depressant effects was observed, although in general after EtOH the rates were still below those seen after saline.

Test days were separated by at least two days during which the rats were retrained on ethanol and saline. If at any time after a test drug, discriminative responding was not comparable to prior data (i.e., greater than 90% correct responses), no further testing was done. This, however, did not occur at any time during the study, suggesting that the effects of the peptides were acute and dissipated before the next day's session.

Data Evaluation

Test generalization sessions with ethanol, saline, and unknown compounds were four minutes long and were done during extinction. Because the response rates were often altered by co-administration of ethanol and other drugs, the data are expressed as a percentage of correct responses on the ethanol correct lever out of the total number of responses. Since it has previously been demonstrated that the discriminability of a drug is not a function of the response rate [6] evaluating the data in this format was considered most appropriate.

Statistical evaluation of the results was done using Student's *t*-test. The data were normalized by transformation to log values prior to statistical testing.

Behavioral disruption was said to occur if the rat failed to make at least five responses during the test session.

Measurement of Ethanol-Induced Narcosis

Male mice genetically inbred to be sensitive to ethanol [11] were the experimental animals. Group 1 (n=6) was first injected with saline (0.1 ml/10 g body wt.) and immediately afterward given ethanol (4 g/kg, IP, in a 20% w/v solution). Groups 2 (n=6) and 3 (n=6) received TRH (10 and 3 mg/kg, IP, respectively) immediately before also being administered ethanol. Group 4 (n=6) was given HP in the same manner as TRH at a dose of 6mg/kg before ethanol. Group 5 (n=6) received OHT 10 min before the injection of ethanol. The time it took until the mice were able to show within thirty seconds two successful righting reflexes was recorded. The difference between the time of injection and the time to regain the righting reflex was taken as the sleeping time. No differences were observed in the onset of the ethanol effect in saline versus peptide-treated mice.

Drugs and Methods of Administration

Saline. Sterile, isotonic saline for injection (Travenol, Inc.) was acquired from the University of Colorado Hospital Pharmacy, and was given orally by stomach tube in a volume of 10 ml/kg body weight 15 min before the session.

Ethanol. Ethanol solutions (U.S.I. Chemical Co.) were prepared by diluting 25 ml of pure ethanol to a final volume of 100 ml with saline (20% w/v). The drug was given orally in the same volume as saline 15 min before the session.

Compounds Tested for their Ability to Reverse the EtOH Cue

TRH, HP, ACTH (1–10)-NH_2. These three peptides (see Table 1 for structures) were synthesized by solid phase techniques [26] in this lab and purity ascertained by thin layer chromatography, high voltage electrophoresis, and amino acid analysis. They were examined for their ability to reverse the ethanol cue. The drugs were dissolved in distilled water and injected via the intraperitoneal route (1.0 ml/kg). TRH and HP were given immediately before the oral administration of ethanol; ACTH (1–10)-NH_2 was given 2 min before EtOH. Test generalization occurred 15 min following ethanol as described above.

OHT. This peptide (see Table 1 for structure) was a gift from Merck, Sharpe, and Dohme, Inc., and was tested as an antagonist of ethanol. The drug was given (IP) 10 min before ethanol and was dissolved in distilled water. Testing took place 15 min after ethanol as described above.

Compounds Tested for their Ability to Mimic the EtOH Cue

(DAla^2-Met^5)-Enkephalin-ol, (DAla^2-Met(0)^5)-Enkephalin-ol, Substance P, Delta Sleep-Inducing Peptide, Bombesin, and Pentobarbital. The peptides (see Table 1 for structures) were synthesized in this lab and pentobarbital acquired commercially (Napental®, Massengill Co.). The peptides were dissolved in distilled water. Pentobarbital was diluted from its vehicle (propylene glycol: alcohol: water, 2:1:7) with saline. All injections were given IP in a volume of 1.0 ml/kg. (DAla^2-Met^5)-Enk-ol, (DAla^2-Met(0)^5)-Enk-ol, bombesin, and delta sleep-inducing peptide were given 15 min before testing; substance P was given 30 min prior to testing. Pentobarbital was given at either 45 or 120 min before testing.

RESULTS

In order to establish an initial dose range of TRH, HP, and OHT suitable to use in other operant studies, varying concentrations of these peptides were examined for their ability to alter the cuing properties of the ethanol training dose (2 g/kg, PO). These data are summarized in Table 2. Ethanol and saline were clearly discriminable by these rats, with the training dose resulting in 94.5 ± 2.6% (mean ± SEM) responding on the ethanol correct lever, while after saline fewer than five percent of the rats' responses were on

TABLE 1

STRUCTURES OF PEPTIDES USED IN THE FOLLOWING EXPERIMENTS

TRH	<Glu-His-Pro-NH_2
His-Pro diketopiperazine	His-Pro
OHT	L-N-(2-oxopiperidine-6-YL-carbonyl)-His-ThiaPro-NH_2
(DAla^2-Met^5)-Enkephalin-ol	Tyr-dAla-Gly-Phe-Met-ol
(DAla^2-Met(O)^5)-Enkephalin-ol	Tyr-dAla-Gly-Phe-Met-ol ↓ O
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Bombesin	<Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
Delta Sleep-Inducing Peptide	Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-COOH
ACTH (1–10) NH_2	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-NH_2

TABLE 2

ANTAGONIST EFFECTS OF TRH, HP, OHT, ACTH (1–10)-NH_2, AND NALOXONE ON THE ETHANOL DISCRIMINATIVE STIMULUS IN RATS

Drugs and Doses	n*	Per Cent Responses on the EtOH Lever (Mean ± S.E.M.)
SAL		
10 ml/kg	6/6	2.4 ± 1.1
EtOH		
2.0 g/kg	6/6	94.5 ± 2.6
EtOH + TRH		
2.0 g/kg + 10.0 mg/kg	4/6	77.3 ± 19.3
3.0	5/6	82.5 ± 17.5
1.0	6/6	86.2 ± 10.0
0.6	6/6	86.8 ± 6.5
EtOH +HP		
2.0 g/kg + 3.0 mg/kg	2/6	100.0 ± 0.0
1.0	5/6	74.6 ± 17.8
0.6	6/6	91.2 ± 4.2
EtOH +OHT		
2.0 g/kg + 1.0 mg/kg	1/6	90.0 ± 0.0
0.3	5/6	98.5 ± 1.5
0.1	6/6	94.5 ± 3.8
EtOH + ACTH (1–10)-NH_2		
2.0 g/kg + 1.0 mg/kg	6/6	90.3 ± 4.9
EtOH + Naloxone		
2.0 g/kg + 5.0 mg/kg	6/6	86.0 ± 9.5

n*=number of animals responding out of number of animals tested.

the ethanol lever. The blood alcohol levels of these rats were 212.8 ± 12.8 mg% (mean ± SEM) at the time of generalization testing. The response rates during the 4 min test session for ethanol and saline were, respectively, 6.7 ± 1.9 and 9.3 ± 1.6 responses/min (mean ± SEM).

TRH was tested at four concentrations: 10, 3, 1 and 0.6 mg/kg (IP). The highest dose tested disrupted responding completely (less than five responses during the four minute test session) in four out of six rats tested, whereas, only one rat was disrupted at the 3 mg/kg dose. At doses of 1 mg/kg and less, all the rats responded.

At the highest dose of TRH tested there may have been some slight antagonism of ethanol. However, this did not reach statistical significance and at doses which did not abolish responding, TRH was unable to influence the ethanol discriminative stimulus.

Prasad *et al.* [19] have suggested that TRH may be metabolized by the enzymatic cleavage of the pyroglutamyl-histidyl bond resulting in a histidylprolineamide dipeptide, which can cyclize to the diketopiperazine. This compound (His-Pro diketopiperazine) has been shown by them to be more potent than TRH in its ability to reverse ethanol-induced narcosis, and as such seemed an appropriate compound to test in this task.

Table 2 shows that HP, like TRH, at doses that did not abolish responding, was not able to alter the efficacy of the ethanol discriminative stimulus. However, the potency of HP is greater than TRH, as seen in the fact that it is disruptive at lower doses.

Veber *et al.* [27] and Porter *et al.* [18] have shown that OHT (a derivative of TRH) has more potent analeptic properties than endorcine effects, i.e., doses that are able to reverse ethanol's depressant property have little or no effect on thyroid hormone release. This hypothetical dissociation of the central and hormonal effects of this class of peptides allowed an opportunity to test whether the site of action of the behavioral effect was central or peripheral.

The data in Table 2 show the effect of varying doses of OHT on the ethanol discriminative stimulus. OHT, similar to the other analogs, was ineffective at influencing the alcohol cue. However, this peptide was the most potent in terms of its ability to disrupt responding.

If the percentage of rats responding out of the number of animals tested (see Table 2) is plotted against the dose of TRH, HP, or OHT, a typical dose-response relationship emerges, and an estimated behaviorally disruptive ED50 can be determined. The approximate ED50's for these three compounds are 30, 2.0 and 0.5 mg/kg, respectively. This order of potency agrees well with other published data [7, 18, 19] and points out the possible utility of operant procedures for evaluating the relative potencies of unknown compounds for their ability to interact with ethanol.

However, despite the fact that TRH, HP, and OHT did not alter the discriminative stimulus properties of the training dose of ethanol, a possibility existed that the dose response curves for ethanol in the presence and absence of the peptide might be different. In order to test this possibility the

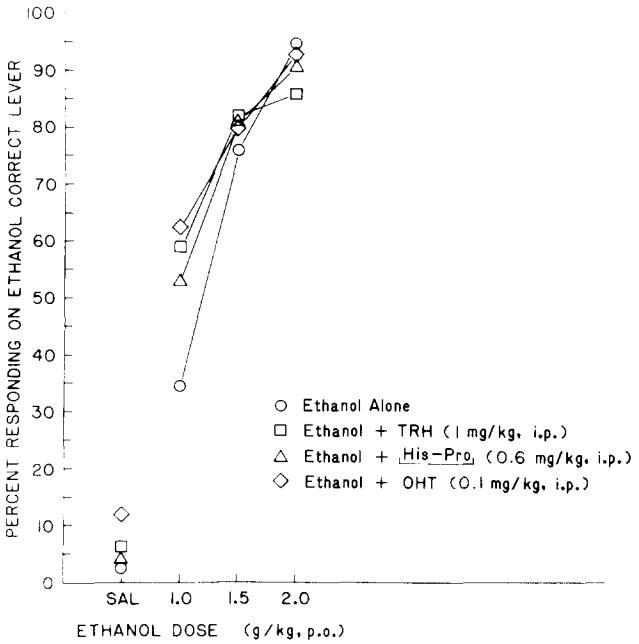

FIG. 1. The effect of intraperitoneal administration of TRH, His-Pro diketopiperazine, and OHT on the dose-response curve for the stimulus properties of ethanol. Each point represents the mean performance of six rats. Standard error bars have been omitted for clarity.

rats were pretreated with a constant dose of peptide and the concentration of ethanol administered varied. The dose of peptide chosen (as determined from the previous data) was one that did not abolish lever pressing when given in combination with ethanol.

The effect of TRH, HP and OHT on the dose response curve for the ethanol cue can be seen in Fig. 1. These peptides had no effect on saline responding. Similarly, they did not significantly alter the ethanol dose response curve.

The inability of TRH, HP, and OHT to affect discriminative responding led us to examine the effects of these peptides in another behavioral test. This was done to validate the biological efficacy of our samples. Table 3 shows the ability of these compounds to successfully antagonize ethanol-induced narcosis in mice. TRH displayed dose-responsive characteristics, and (as in the operant studies described above) OHT was the most potent agent tested. These data attest to the in vivo activity of these peptides.

In order to determine whether or not the ethanol cue could be altered by other peptides, various compounds were tested. Pentobarbital was chosen as a positive control because it has previously been shown to generalize to alcohol [14]. The enkephalins and naloxone were chosen to test for the possible interaction of the opiate peptide system in the subjective effects of ethanol (although naloxone has been shown not to effect the cue [29]). Substance P, delta sleep-inducing peptide, and bombesin have all been reported to have central nervous system depressant actions and therefore might participate in the alcohol cue complex [2, 24, 25]. Finally, Bissette, *et al.* [4] have reported that ACTH(4–7)-NH_2 could reverse ethanol-induced narcosis; hence, a peptide containing that sequence (i.e., ATCH (1–10)-NH_2) was examined for its potential antagonism of the alcohol cue.

TABLE 3

THE EFFECT OF TRH, HP AND OHT ON ETHANOL INDUCED NARCOSIS. SEE TEXT FOR DETAILS OF ADMINISTRATION. N=6 FOR ALL GROUPS

Drug	Sleeping Time in Min (Mean ± SEM)
EtOH (4 g/kg) + Saline	101.50 ± 2.05
EtOH + TRH (10 mg/kg)	53.83 ± 11.09*
EtOH + TRH (3 mg/kg)	89.17 ± 12.19*
EtOH + HP (6 mg/kg)	63.67 ± 19.77*
EtOH + OHT (1 mg/kg)	28.17 ± 10.52*

*$p<0.05$, t test, compared to saline treated mice.

TABLE 4

AGONIST EFFECTS OF PENTOBARBITAL, (DAla²-Met⁵)-Enk-ol, (DAla²-Met(O)⁵)-Enk-ol, SUBSTANCE P, DELTA SLEEP-INDUCING PEPTIDE, AND BOMBESIN ON THE ETHANOL DISCRIMINATIVE STIMULUS IN RATS

Drugs and Doses	n*	Time before Testing (min)	Per Cent Responses on the EtOH Lever (Mean ± SEM)
Pentobarbital			
15 mg/kg	6/6	45	70.0 ± 14.4
20	6/6	120	80.0 ± 16.3
(DAla²-Met⁵)-Enkephalin-ol			
1 mg/kg	6/6	15	12.5 ± 6.8
(DAla²-Met(O)⁵)-Enkephalin-ol			
0.2 mg/kg	1/3	15	0.0 ± 0.0
Substance P			
1 mg/kg	6/6	30	6.5 ± 2.3
Delta Sleep Inducing Peptide			
1 mg/kg	3/3	15	2.5 ± 1.3
Bombesin			
1.0 mg/kg	3/3	15	4.2 ± 4.2
0.1	0/3	15	—
0.1 (repeat)	1/3	15	12.5
0.01	1/3	15	0.0
0.005	2/3	15	35.7 ± 35.7
0.001	3/3	15	0.0 ± 0.0

n*=number of animals responding out of number of animals tested.

Table 4 shows that the positive control—pentobarbital—at two different doses and times was able to generalize to the alcohol cue. However, neither (DAla²-Met⁵)-Enk-ol, (DAla²-Met(0)⁵)-Enk-ol, substance P, bombesin, nor delta sleep-inducing peptide were able to generate ethanol appropriate behavior (Table 4). Additionally, ACTH (1–10)-NH_2 and naloxone were both ineffective in blocking the training dose of ethanol (Table 2).

DISCUSSION

Alcoholism is a major social problem, and the elucidation of a specific ethanol antagonist would have beneficial effects both clinically and experimentally. By using an operant pro-

cedure based on the discriminative effects of the drug, an evaluation of a theoretically central effect of alcohol can be accomplished in alert, conscious animals [1, 23, 31]. Hypothetically, a drug that could block the alcohol cue might be useful in both the treatment of alcoholism and the understanding of ethanol's biochemical effects in a manner analogous to the use of opiate antagonists in narcotic research. The ability of TRH and related peptides to reverse the sleep-inducing effects of sedative-hypnotics suggested the possibility that such an antagonist might be available. Unfortunately, the data presented herein do not support the thesis that TRH is capable of blocking all of ethanol's properties. TRH, its proposed metabolite (His-Pro diketopiperazine), and a potent TRH analog (OHT) were all unable to alter significantly the dose response curve for the ethanol discriminative stimulus (Fig. 1). Presumably, the doses of peptides chosen were appropriate, as seen in the fact that higher concentrations would have disrupted performance (see Table 2). The times and routes of administration of the peptides were chosen from the literature [7, 18, 19] as being suitable for reversing the sleep-inducing properties of ethanol. Thus, at behaviorally relevant times, doses, and routes of administration, the stimulus properties of alcohol were not changed by TRH. Additionally, a group of other peptide compounds (see Table 2 and 4) were unable to act as either agonists or antagonists of ethanol. There is a possibility that an interaction of these peptides with ethanol may be evident under different testing parameters (e.g., different training doses of EtOH, routes of administrations, animals, times, etc.), although this seems unlikely in light of the results found here. However, these more exhaustive experiments remain to be done. Nevertheless, these data taken as a whole imply that the stimulus effects of ethanol are not mediated by a TRH, substance P, or enkephalinergic neurotransmitter system.

The analeptic effect of TRH has been shown to be similar in both rats and mice [7]. The data in Table 3 clearly demonstrate the efficacy of these peptides to attenuate hypnotic doses of alcohol. However, the inability of TRH, HP, and OHT to affect discriminative based responding while successfully altering ethanol-induced narcosis suggests differential underlying mechanisms subserving these two behaviors.

An important aspect of the dissociation of the sleep-inducing and stimulus effects of ethanol is the implication that different neurotransmitter systems may control each of these properties. The sleep-inducing effects of ethanol have been reversed by a variety of drugs, whereas reversal of the ethanol cue has only been accomplished by very few. For example, although bemegride could reverse the sedative-hypnotic stimulus, other stimulants such as picrotoxin and *d*-amphetamine could not [13]. Schechter [23] has noted that whereas propranolol or caffeine could not affect the ethanol cue, *d*-amphetamine was active, and furthermore, parachlorophenylalanine (a serotonin synthesis inhibitor; PCPA) blocked the cue for up to five weeks—suggesting a strong serotonergic component to the cue. However, Winter [30] could not repeat this finding. On the other hand, whereas TRH, HP, OHT, naloxone, and ACTH (4–7)-NH_2 have been shown to reverse sedative-hypnotic narcosis [3, 4, 7, 18, 19] they do not affect the stimulus properties of ethanol. Therefore, the fact that a variety of substances can alter the depressant effects of alcohol without changing its stimulus characteristics strongly suggests the existence of distinguishable underlying systems. The potential of manipulating these separate effects to achieve selective therapeutic actions may be of considerable importance.

It was interesting to note that combinations of low doses of ethanol (1 g/kg) and the TRH-related peptides may have synergized. As can be seen in Figure 1, there is a tendency toward greater responding on the ethanol correct lever when these drugs are co-administered than when ethanol is given alone (although this did not reach statistical significance). This same potentiation has also been observed by others [9,28]. These authors report that TRH enhances the anti-conflict effects of ethanol. Thus, it is likely that there is not a simple agonist-antagonist relationship between TRH and ethanol, and that the effects seen may vary with doses. This would be analagous to the results of Rech, *et al.* [20] which show a similar discontinuity when testing interactions of stimulants (e.g., amphetamine and cocaine) and sedative-hypnotics (e.g., ethanol and diazepam).

The behavioral effects of bombesin in these rats were somewhat surprising. This peptide—isolated from frog skin—has been shown to be a potent hypothermic agent [2]. In these animals, it was interesting to note that although 100% of the rats could respond at 1 mg/kg (IP), at 0.1 mg/kg (IP) there was a substantial disruption in the rats' lever pressing ability. This behavioral disruption was persistent to doses as low as 0.005 mg/kg and dissipated at a dose five fold less than that (see Table 4). These behavioral effects were notable both because of their occurrence at such low concentrations and because of the biphasic nature of the dose response curve. The significance of these results is not clear and awaits further study. Similarly, it is not evident why (DAla^2-Met(0)^5)-enkephalin-ol was able to disrupt performance at doses below those needed to produce analgesia [22]. However, it agrees with other data [8] which suggest that the analgesic and behavioral effects of the endorphins are separable.

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