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Fourth Ventricular Injection of the Bombesin Receptor Antagonist [D-Phe^6]Bombesin(6-13)methyl Ester, But Not BW2258U89, Increases Food Intake in Rats

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STRATFORD, T. R., J. GIBBS, D. H. COY AND G. P. SMITH. *Fourth ventricular injection of the bombesin receptor antagonist [D-Phe^6]bombesin(6-13)methyl ester, but not BW2258U89, increases food intake in rats.* PHARMACOL BIOCHEM BEHAV **50**(3) 463–471, 1995.—To investigate the role of endogenous bombesin-like peptides in the caudal brainstem for the short-term control of food intake, we evaluated the effects of fourth-ventricular injections of two different bombesin (BN) receptor antagonists, [D-Phe^6]BN(6-13) methyl ester and BW2258U89, on intake of sweetened, condensed milk in male rats. Although fourth-ventricular administration of BW2258U89 (0.125–20 ng) had no effect on food intake, fourth-ventricular injections of 1.0–20.0 ng of [D-Phe^{6}BN(6-13) methyl ester resulted in an inverted U-shaped, dose-response curve with a maximal effect at 2.5 ng. Microstructural analysis of the licking behavior indicated that the increase in intake was primarily the result of an increased number of licks and an increase in lick efficiency. Behavioral time sampling demonstrated that these changes in intake occurred without the appearance of any competing behavior or significant change in the overall pattern of behavior. Because [D-Phe^{6}BN(6-13) methyl ester appears to be a preferential antagonist at the GRP-preferring receptor, the increased intake that occurred after its administration suggests that an endogenous GRP-mechanism in the caudal brainstem is necessary for the normal, short-term control of sweet milk intake under these conditions.

Gastrin-releasing peptide Ingestion Microstructural analysis Central peptide receptors
Peptide receptor antagonist Behavioral analysis

PERIPHERAL administration of the amphibian peptide bombesin (BN) has been shown to reduce meal size in a dose-dependent, behaviorally specific manner in a number of species (2,10,13,16,40), including humans (26,30). It appears that the initial site of action of peripherally administered BN is in the gut, because disconnection of the neural pathways connecting the viscera and brain blocks the effect of bombesin on food intake (35).

There is, however, a convergent body of evidence suggesting that central BN-like peptides also play a role in the physiological control of food intake. The evidence comes from four kinds of observations: First, the mammalian BN-like peptides, neuromedin B (NMB) and gastrin-releasing peptide (GRP), their mRNAs and binding sites are heterogeneously distributed in the rat brain (19,23,29,31,32,37,41). Second, food deprivation and subsequent refeeding are correlated with rapid alterations of hippocampal and hypothalamic concentrations of immunoassayable GRP-like peptides (27). Third, injection of BN-like peptides into the lateral or paraventricular regions of the hypothalamus (22,34), the substantia nigra (3), the rostral pole or medial subnucleus of the nucleus of the solitary tract (9,18), or the lateral or fourth cerebral ventricles (11,15,21,25) decreases food intake. Fourth, intracerebroventricular injections of various BN-receptor antagonists increase

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food intake in rats. For example, it has been demonstrated that intracerebroventricular injection of the BN-receptor antagonist [Leu^{14}, ψ13-14]-BN increased intake of solid food in a prefeed paradigm using 17-h food-deprived rats (28). The antagonist was more potent when it was injected into the fourth ventricle than when injected into the third ventricle. The increased food intake appeared to be a relatively specific effect of the antagonist because the antagonist did not change water intake. In addition, fourth-ventricular injections of two other BN-receptor antagonists, (D-Phe^{12},Leu^{14}]BN and [D-Phe^{6}BN(6-13) methyl ester, increased intake of milk in nondeprived rats without increasing grooming or exploration (12).

These results obtained with antagonist treatment are strong evidence for a physiologic role of central, endogenous BN-like peptides in the control of meal size. They suggest that structures surrounding the fourth ventricle are the site of this action. Furthermore, because all of the antagonists that have been used are reported to be antagonists of GRP, and one, [Leu^{14}, ψ13-14]-BN, has been shown to have an extremely low affinity for the NMB receptor (17,36,38,39), these results suggest that the increased intake produced by the antagonists results from the blockade of an endogenous GRP-mechanism in this region.

To investigate this GRP-mechanism further, we examined the effect of fourth-ventricular injections of several doses of one of these BN-like peptide receptor antagonists, [D-Phe^{6}BN(6-13) methyl ester, on the intake of sweetened, condensed milk in nondeprived rats. This peptide is a member of a family of bombesin analogues with modified carboxyl terminals that have an extremely high affinity for the mammalian GRP-preferring receptor, as compared with the NMB-preferring receptor (17,24,36,38). Administration of [D-Phe^{6}BN(6-13) methyl ester has been shown to reduce vagally induced gastrin release from rat stomach and to inhibit [125]Tyr^{4}-BN binding and BN-mediated tension and inositol phosphate responses in rat myometrium by blocking GRP-preferrng receptors on these tissues (1,39). We also tested fourth-ventricular injections of a novel GRP analogue, BW2258U89 ([(de-NH_2)Phe^{19},D-Ala^{24},D-Pro^{26}ψ(CH_2NH)Phe^{27}]-GRP(19-27)). This compound has been reported to decrease BN-induced gastrin release from rat stomach in vitro and in vivo and is a potent inhibitor of [125]Tyr^{4}-BN binding at GRP-preferring receptors on rat pancreatic acinar cells (33).

In the previous two studies employing fourth-ventricular injections of BN antagonists (12,28), gross behavioral observations of the rats were made during the tests to determine the range of behaviors activated by administration of the antagonists. In both studies the effects appeared to be specific to feeding behavior. To verify this under our conditions, we analyzed behavior by a time-sampling method. The previous studies did not, however, attempt to determine whether the increased food intake was the result of increasing the positive feedback (palatability of diet) or decreasing the negative feedback (postingestive satiety signals) of the ingested food. A new technique, the microstructural analysis of licking behavior (4), has been employed to demonstrate that consistent changes in certain components of licking behavior are associated with variations in palatability or postabsorptive satiety signals produced by varying the concentration of carbohydrate solutions (6,8). Thus, in addition to time-sampling general behavior during our tests, we employed the technique of analyzing the microstructure of licking behavior in an attempt to determine whether blockade of hindbrain BN-like peptide receptors increases food intake by modifying positive or negative feedback signals arising from ingestion of the diet.

METHOD

Subjects

Eleven male Sprague-Dawley rats, weighing between 290–310 g, were obtained from Taconic Farms (Germantown, NY). The animals were housed individually in clear acrylic cages with wire-mesh floors and were maintained on a 12 h : 12 h light-dark cycle (lights on 0700 h) in a temperature-controlled room (approximately 20°C) with food (Purina Lab Chow, St Louis, MO) and tapwater available ad lib, except as noted subsequently.

Surgery

The rats were anesthetized with an IP injection of a solution of chloral hydrate and sodium pentobarbital (85 and 18 mg/kg, respectively). Using standard stereotaxic techniques, 26-ga stainless-steel guide cannulas (Plastics One, Roanoke, VA) were implanted on the midline at a point 2.5 mm anterior to the external occipital crest and 5.5 mm below the surface of the dura. These coordinates result in a cannula placement that terminates immediately dorsal to the roof of the fourth ventricle (12). The cannulas were secured to the skull with dental cement and stainless-steel skull screws, and 33-ga obturators, extending 0.5 mm beyond the end of the guide cannulas, were inserted to maintain patency. Immediately after surgery, all rats received an intramuscular injection of sterile penicillin ($\approx$30,000 U).

Testing Apparatus

Behavioral testing was performed in cages equipped with lickometers (DiLog Instruments, Tallahassee, FL). Each tongue contact with the stainless-steel drinking tube completed a circuit of less than 60 nA. This signal was detected, amplified, and sent to a computer in which the time of each contact was determined to the nearest millisecond and stored in a variable array for later processing.

Procedure

All rats began behavioral testing following a 7-day recovery period, during which time they were handled daily. Food and water were removed 1 h before the intraventricular injection to prevent any ingestion immediately before the 60-min test that began at 0900 h. Intraventricular injections were made through a 33-ga cannula terminating 1 mm below the end of the guide. The injection cannula was attached via fluid-filled polyethylene tubing to a hand-held Hamilton microsyringe (Reno, NV). The 5-μl infusion was made over a 30-s interval, and the injection cannula was left in place for an additional 30 s to reduce the amount of drug drawn up the cannula track. After the infusion, the obturator was replaced and the animal was immediately placed in the test cage for behavioral testing. A calibrated drinking tube filled with sweetened, condensed milk (Eagle Brand, Columbus, OH; diluted 1 : 1 with deionized water) was attached to the front of each cage. Intakes were measured to the nearest milliliter every 5 min for the duration of the test.

The experimenter also recorded the behavior displayed by each subject at 1-min intervals using a tone-cued technique noted for its high interrater reliability (14). Behavior was categorized as either feeding (licking spout or lips), exploring (sniffing, locomotion, rearing), grooming, or resting. The test period was divided into 5-min intervals, and the number of behaviors observed in each of the four categories was ex-

pressed as the percentage of total behaviors observed in each interval.

To acclimate the rats to the injection procedure and test diet, each rat was tested on three occasions with a 5-μl intraventricular injection of sterile, 0.15 M saline. After the acclimation trials, each rat was tested a total of 13 times (four doses of [D-Phe^{6}BN(6-13) methyl ester (1.0–20.0 ng), six doses of BW2258U89 (0.125–20.0 ng), and three intraventricular injections of the isotonic saline vehicle). The drugs were administered in random order, with two of the three saline trials bracketing the series of antagonist treatments. Three rats did not drink at all during the acclimation trials and were not included in the remainder of the experiment. Seventy-two hours elapsed between injections, and the rats were not tested during that period.

Histology

At the end of the experiments, each rat was placed under deep pentobarbital anesthesia and received a 5-μl injection of india ink into the fourth ventricle. The rat was then perfused transcardially with 100 ml of isotonic saline followed by 400 ml of 10% phosphate-buffered formalin. The cannula and dental cement mount were removed and the brain was dissected free from the skull and placed in the fixative for 48 h. The brain was frozen in a cryostat, and 60-μm parasagittal sections were taken across the midline (≈ ± 0.6 mm), mounted on chrome-alum-coated slides, and stained with cresyl violet. The sections were examined using a dissecting microscope (40×), and the placement of the cannula tip and extent of ink diffusion were determined and recorded for each rat.

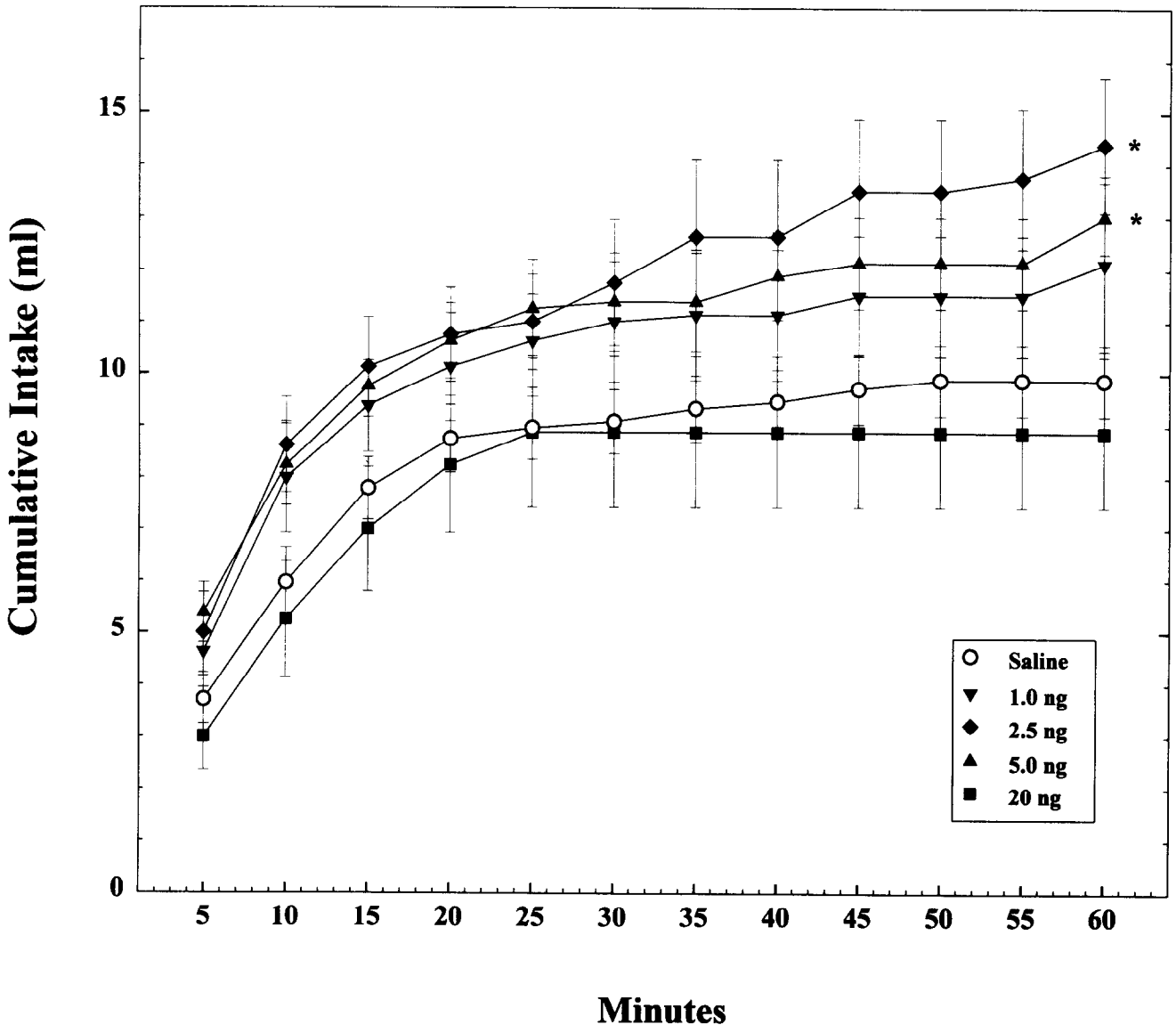

FIG. 1. Cumulative intake (mean ± SE) of sweetened, condensed milk by eight nondeprived rats after fourth-ventricular injection of various doses of [D-Phe^{6}BN(6-13)methyl ester. Intake after treatment with the three lowest doses was consistently higher than that after saline injections. This increase was evident throughout the 60-min test. *The 2.5 and 5.0 ng doses increased 60-min intake significantly compared with saline treatment ($p < 0.05$).

Data Analysis

Sixty-minute intakes were compared across all doses for each antagonist using a one-way analysis of variance with repeated measures. Because we did not find a significant difference in intake between the saline trials, the results of those three trials were combined into a single control group against which the drug groups were compared.

The microstructural variables were derived using the Quick Lick computer program (5) (DiLog Instruments, Tallahassee, FL) based on the occurrence of interlick intervals (ILIs) of predetermined duration (4,8). In addition to volume ingested, the variables we examined were: a) total number of licks emitted during the test; b) number of licks emitted per milliliter of intake (licking efficiency); c) latency to begin licking; d) duration of the feeding episode; e) mean duration of the intervals between licks while the rat was engaged in a burst of licking; f) mean size of uninterrupted bursts of licking (groups of licks separated by ILIs < 250 ms); g) mean number of bursts; h) mean duration of intervals separating bursts (ILIs between 250 and500 ms); i) mean size of clusters of licks (groups of licks separated by ILIs > 500 ms); j) mean number of clusters; and k) mean duration of intercluster intervals. These measures were analyzed using a two-tailed, paired Student *t*-test to compare variable means between the 2.5-ng [D-Phe^{6}BN(6-13) methyl ester trial and the subsequent vehicle trial. This dose of antagonist was used because it produced the largest increase in intake. Because latency, burst size, and cluster size have been reported to have non-Gaussian distributions (4), these measures were also compared using the Wil-

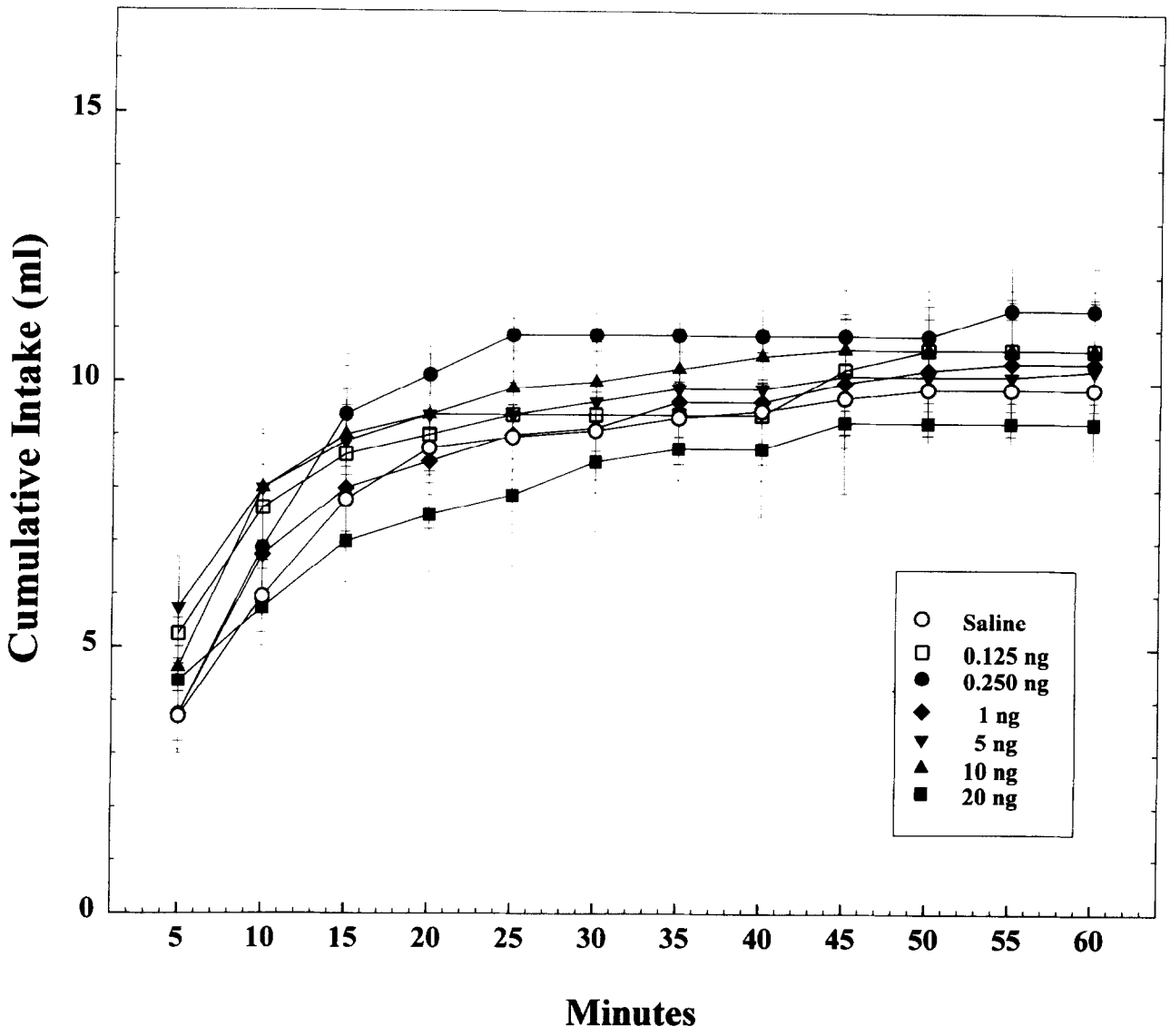

FIG. 2. Cumulative intake of sweetened, condensed milk after fourth-ventricular administration of six doses of BW2258U89. At no point during the test did the intake after drug treatment differ significantly from that observed after saline treatment.

coxon matched-pairs signed-ranks test. Finally, we also examined minute-to-minute changes in the rate of licking during the first 15 min of the test.

RESULTS

Food Intake

When compared with the averaged saline trials, the three lowest doses of [D-Phe^{6}BN(6-13)methyl ester increased intake at every point during the 60-min test (Fig. 1). In contrast, intake was slightly attenuated throughout the test by the 20-ng dose of this antagonist. Overall, there was a significant effect of [D-Phe^{6}BN(6-13)methyl ester on intake of the milk during the 60-min test [$F(4, 28) = 8.1, p < 0.001$]. Posthoc comparisons (Dunnett's method) revealed that the rats had eaten significantly more after the 2.5-ng [$F(1, 7) = 39.8, p < 0.001$] and 5.0-ng [$F(1, 7) = 8.7, p = 0.021$] doses when compared with the control trials. The maximally effective (2.5-ng) dose of [D-Phe^{6}BN(6-13)methyl ester first increased food intake significantly at 15 min; this increase remained significant throughout the test.

In contrast to the effect of [D-Phe^{6}BN(6-13)methyl ester, no dose of BW2258U89 significantly changed food intake [$F(6, 42) = 0.65, p = 0.700$] (Fig. 2).

Behavioral Observations

No dose of either antagonist resulted in a marked change in the relative percentages of behaviors elicited or in the sequence of their maximal expression. The general behavioral profile of the rats after injections of [D-Phe^{6}BN(6-13)methyl ester was similar to that observed after saline injections (Fig. 3). The rats began the test period by feeding for a large percentage of the time. Over the initial 25 min, the incidence of feeding gradually decreased and the incidence of exploring increased. At 25 min, the meal was essentially finished and the

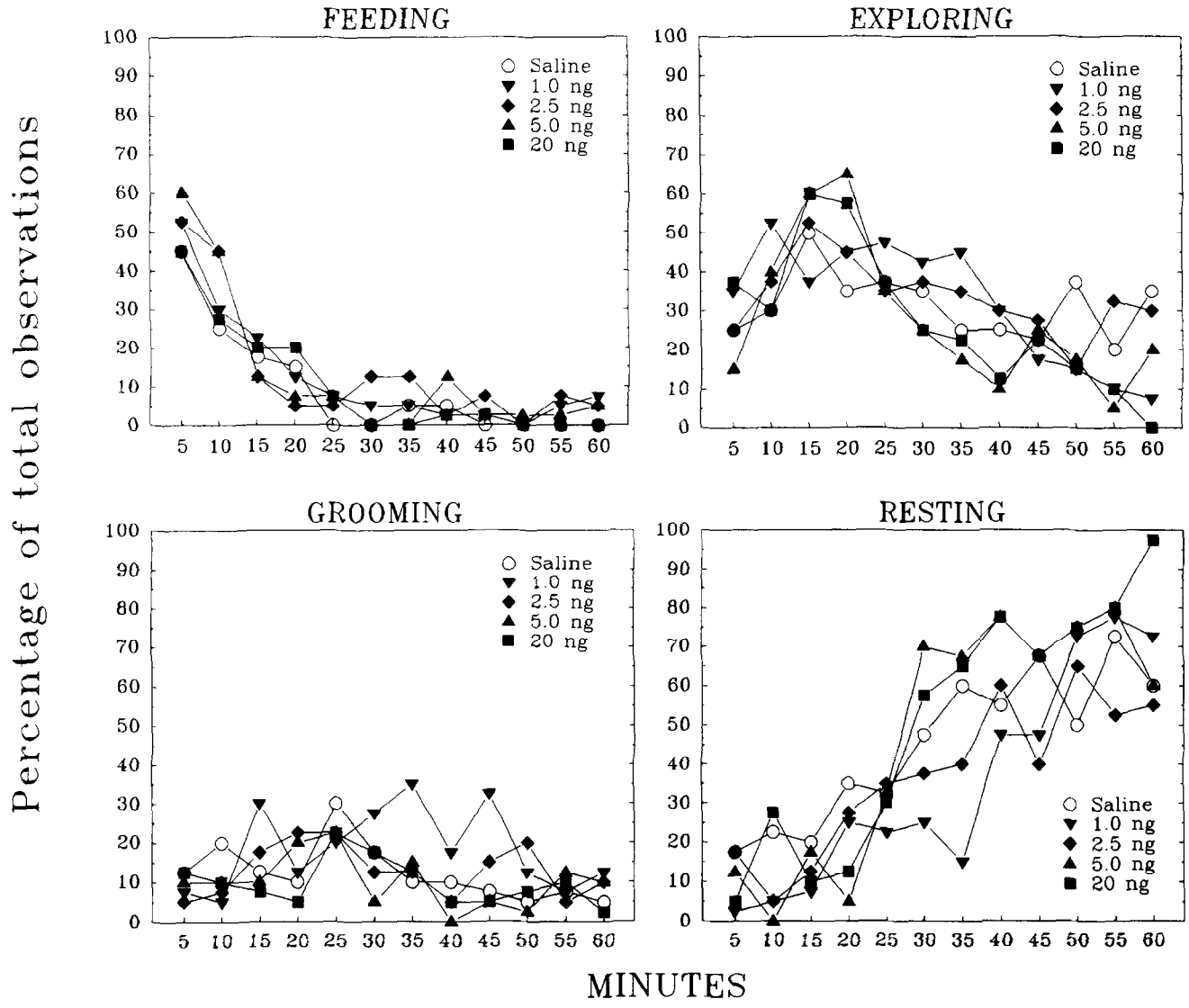

FIG. 3. Feeding, exploring, grooming, and resting behaviors expressed as a percentage of the total behavioral observations recorded after injections of various doses of [D-Phe^{6}BN(6-13)methyl ester into the fourth ventricle.

TABLE 1

LICKING BEHAVIOR AFTER INJECTION OF SALINE OR 2.5 ng [D-Phe^6]BN(6-13)METHYL ESTER INTO THE FOURTH VENTRICLE

Measures	15-Min Saline	15-Min Methyl Ester	60-Min Saline	60-Min Methyl Ester
Volume (ml)	7.8 ± 0.9	10.1 ± 1.0*	10.3 ± 1.6	14.4 ± 1.4*
Total licks	1596 ± 172	1847 ± 200†	2162 ± 367	2574 ± 334†
Licks/ml	209.2 ± 9.5	182.7 ± 8.5†	212.0 ± 8.8	178.0 ± 9.5†
Latency (s)	100.6 ± 72.4	95.2 ± 48.0		
Duration (min)	10.9 ± 1.4	10.7 ± 1.2	25.3 ± 5.0	35.7 ± 5.3
Interlick interval (s)	0.153 ± 0.004	0.149 ± .003†	0.153 ± 0.004	0.150 ± 0.003†
Burst size (licks)	26.5 ± 6.0	33.1 ± 7.9	19.9 ± 3.4	26.7 ± 5.9
No. of bursts	67.9 ± 11.4	68.4 ± 16.0	106.8 ± 24.5	119.6 ± 33.8
Interburst interval (s)	0.323 ± 0.005	0.318 ± 0.006	0.320 ± 0.006	0.317 ± 0.006
Cluster size (licks)	47.4 ± 12.0	61.3 ± 16.5	40.4 ± 10.5	55.6 ± 17.2
No. of clusters	40.5 ± 6.8	39.8 ± 9.0	61.9 ± 16.5	70.6 ± 20.7
Intercluster interval (s)	12.6 ± 3.5	14.5 ± 3.8	26.5 ± 8.0	39.6 ± 10.4

Mean scores (±SE, n = 8) for total intake and 11 measures of licking behavior after fourth-ventricular injections of the maximally effective dose of [D-Phe^{6}BN(6-13)methyl ester (2.5 ng) and the subsequent vehicle treatment. Microstructural variables were derived for both the initial 15 min and for the entire 60-min test. Treatment means significantly different from the respective control value: *$p < 0.01$, †$p < 0.05$.

rats spent the remainder of the test period exploring, grooming, and, increasingly, resting. Although not illustrated, the activity profile of rats receiving injections of BW2258U89 was basically the same as those receiving [D-Phe^{6}BN(6-13)methyl ester.

Microstructural Analysis of Licking Behavior

[D-Phe^{6}BN(6-13)Methyl ester (2.5 ng) increased the intake of sweetened, condensed milk by increasing the total number of licks emitted [$t(7) = 2.46$, $p = 0.044$] and by increasing the efficiency of licking [reducing the number of licks per milliliter; $t(7) = 3.33$, $p = 0.012$] (Table 1). The interlick interval was also significantly reduced, which indicated that the rate at which the rats licked during their uninterrupted bursts of licking was increased by [D-Phe^{6}BN(6-13)methyl ester. None of the other microstructural measures differed significantly between the treatments (Table 1).

In addition to comparing the microstrutcural variables derived from the 60-min data, we also examined licking behavior using data from the initial 15 min of the test. This interval represents a period of intense, relatively sustained licking during which 70–75% of the total volume of diet was consumed. Again, the significant increase in volume ingested seen after administration of 2.5-ng [D-Phe^{6}BN(6-13)methyl ester [$t(7) = 4.77$, $p = 0.002$] (Table 1) could be accounted for solely by increases in total licks [$t(7) = 2.96$, $p = 0.020$] and lick efficiency [$t(7) = 3.02$, $p = 0.020$]. The interlick interval was also reduced by the antagonist during this interval [$t(7) = 2.64$, $p = 0.034$].

We also examined the minute-by-minute rate of licking for the initial 15 min of the test. Overall, the rate of licking and the decay function for the curve appeared to be similar for the two conditions (Fig. 4). A paired Student's t-test was used to compare treatment means at the five intervals showing the largest difference between means (min 1, 3, 8, 9, and 13). [D-Phe^{6}BN(6-13)Methyl ester significantly increased the rate of licking during the first [$t = 2.78$, $p = 0.027$] and ninth [$t(7) = 2.53$, $p = 0.039$] minutes of the test.

Histology

All injector tips terminated in the fourth ventricle. The ink was predominantly confined to the fourth ventricle with only a small amount diffusing rostrally into the ventral aspect of the recess of the inferior colliculus and dorsally along the canula track into the ventral cerebellum. The ink did not reach the third ventricle in any of the rats.

DISCUSSION

Injection of the GRP antagonist, [D-Phe^{6}BN(6-13)methyl ester, into the fourth ventricle increased the intake of sweetened milk. The dose-response relationship was inverted-U shaped with the maximal effect (≈45% increase) obtained with 2.5 ng (Fig. 5). The stimulation of eating by [D-Phe^{6}BN(6-13)methyl ester was not accompanied by any marked changes of other behaviors, and thus appears to be a specific effect on feeding.

Although the antagonist (2.5 and 5.0 ng) increased intake volume, it did not increase the incidence of feeding behavior measured by time sampling (Figure 3). In contrast, microstructural analysis of licking revealed that 2.5 ng of the antagonist significantly increased the total number of licks, the rate of licking within bursts, and the efficiency of licking (Table 1). It is interesting that the antagonist increased the number of licks per minute significantly in only the first and ninth minutes (Fig. 4). These microstructural results are the first to be reported after central injection of a BN-like peptide antagonist, and they suggest how the antagonist changes the organization of the ingestive response to sweet milk under these conditions.

The amount of diet ingested during a given time period depends on the interaction of the palatability of the diet and the amount of negative feedback exerted on feeding behavior as a consequence of its ingestion. For example, increasing the palatability of a sucrose solution by increasing the concentration results in a higher initial rate of licking and increases burst size and cluster size. In contrast, eliminating the postin-

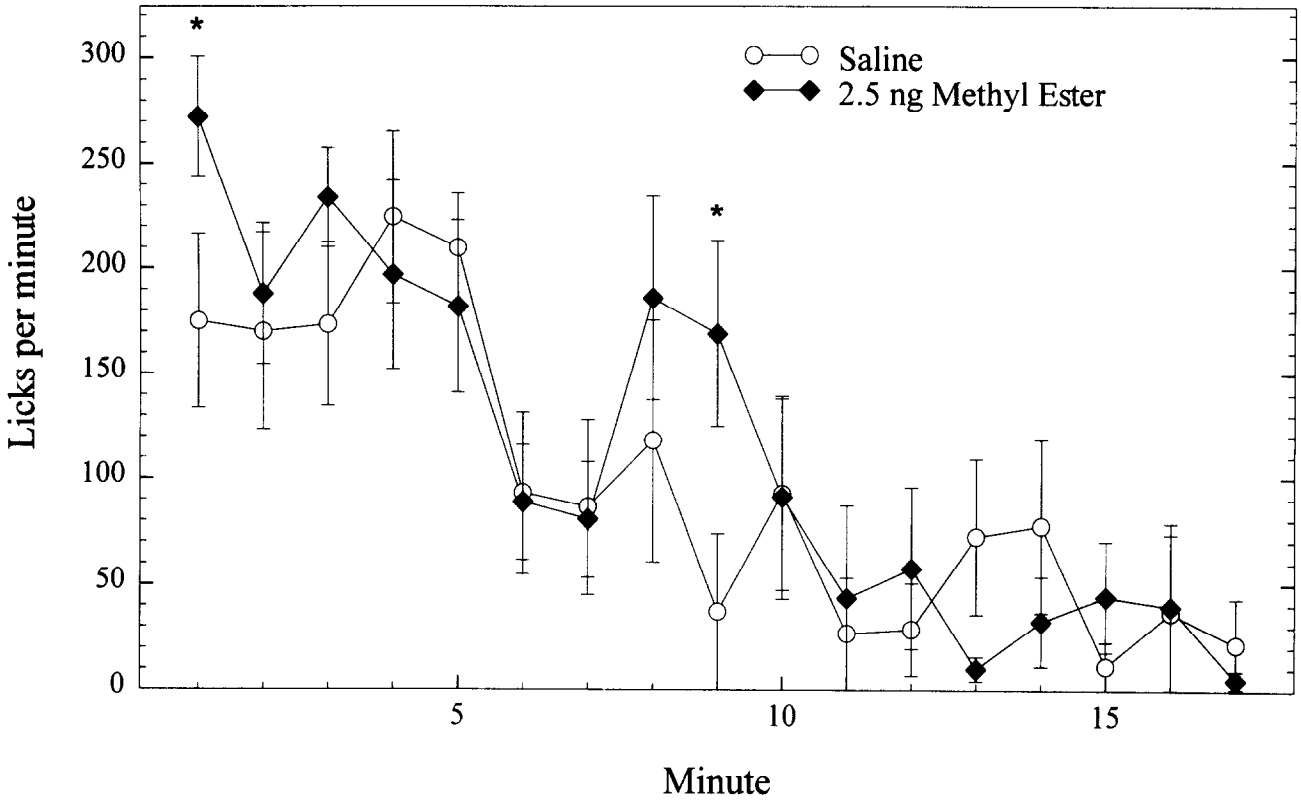

FIG. 4. Mean rate of licking (±SE) after fourth-ventricular injections of 2.5 ng [D-Phe^{6}BN(6-13)methyl ester or the subsequent saline treatment. *Significant differences between means ($p < 0.05$).

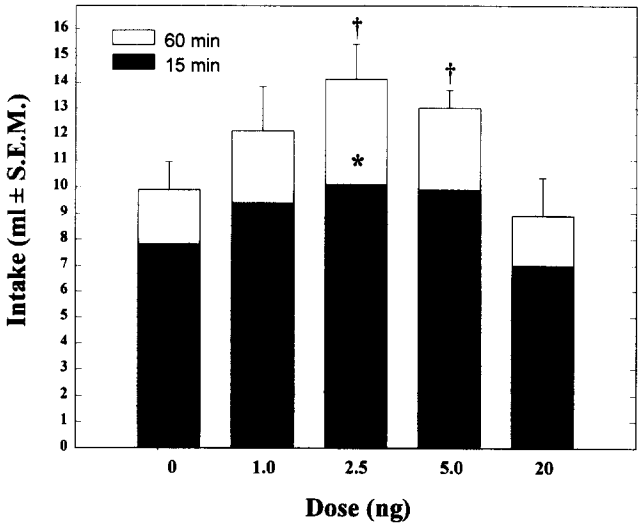

FIG. 5. Intake of sweetened, condensed milk after fourth-ventricular injections of isotonic saline or various doses of [D-Phe^{6}BN(6-13)methyl ester. *After 15 min, the 2.5-ng dose significantly increased intake compared with the saline treatment. †Conditions under which 60-min intakes differed significantly from those observed after saline injections.

gestive action of a carbohydrate solution through the use of gastric fistulas results in longer meal duration and an increase in the number, but not size, of both bursts and clusters (6–8). Administration of [D-Phe^{6}BN(6-13)methyl ester significantly increased the rate of ingestion during the first minute of the test and produced larger bursts and clusters during the initial 15 min and the entire 60-min test. Because the increases of burst size and cluster size were not statistically significant and the difference in initial lick rate was very transient, it appears that the antagonist had at most only a marginal effect on increasing palatability. The failure of the antagonist to increase the duration of the meal or the number of bursts or clusters suggests that [D-Phe^{6}BN(6-13)methyl ester did not increase intake by decreasing the central processing of postingestive satiety signals.

The 2.5-ng dose of [D-Phe^{6}BN(6-13)methyl ester significantly decreased the interlick interval during sustained bouts of licking (Table 1), demonstrating an increase in mean lick rate (6.67 licks/s) compared with saline treatment (6.54 licks/s). It is interesting to note that 17-h food deprivation has been reported to significantly decrease the latency of rats to begin feeding, increase the initial rate of licking, and increase mean interlick interval during sustained bursts of licking (5). After administration of [D-Phe^{6}BN(6-13)methyl ester, we observed no change in latency, a transient increase in lick rate during the first minute of the test, and a significant decrease in the mean within-burst interlick interval. This suggests that the GRP antagonist is not simply mimicking a physiologic state similar to that occurring after food-deprivation. The analysis also revealed that intake is increased because: a) the rats emit

more licks during the test session and b) there is an increase in licking efficiency (indicated by the significantly fewer licks emitted per milliliter of diet ingested). Each of these factors accounts for approximately one-half of the 40% increase in intake observed following administration of the 2.5-ng dose of [D-Phe^{6}BN(6-13)methyl ester. Thus, the major effect of the antagonist appears to be on the central neural mechanisms, presumably in the caudal brainstem, that determine the rate and efficiency of licking.

Because our fourth-ventricular infusions remained relatively confined to that structure and did not appear to pass forward to the third ventricle, it is reasonable to assume that the site of action of [D-Phe^{6}BN(6-13)methyl ester is located in a paraventricular region of the hindbrain. However, more localized injections will be required to determine the specific site(s) of action of [D-Phe^{6}BN(6-13)methyl ester.

Although the novel GRP analogue BW2258U89 has been shown to be a potent antagonist at GRP-like peptide receptors (33), fourth-ventricular injections of this peptide did not significantly increase food intake at any of the doses tested. This was surprising, inasmuch as peripheral administration of this antagonist completely blocked the satiating effect of peripherally administered bombesin (20). Although the highest dose we tested was 160 times larger than the lowest (0.125 vs. 20 ng), it is still possible that our doses did not fall within the effective range of this antagonist. Further work is required to explain the differential efficacy of BW2258U89 after central and peripheral administration.

In summary, fourth-ventricular injections of 2.5 and 5.0 ng of the GRP-receptor antagonist, [D-Phe^{6}BN(6-13)methyl ester, significantly increased intake of sweet milk in nondeprived rats. This confirms and extends the previous reports of Flynn (12) and Merali et al. (28). An analysis of the microstructure of the rats' licking behavior indicated that the drug had no effect on postingestive signals involved in the termination of the meal, and at most, a minor influence on the perceived palatability of the diet. Instead, the antagonist appears to increased intake by increasing both the number of licks emitted by the rats and the efficiency of licking.

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