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Increased Food Intake Following Injection of the Benzodiazepine Receptor Agonist Midazolam Into the IVth Ventricle

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HIGGS, S. AND S. J. COOPER. *Increase in food intake caused by injection of the benzodiazepine receptor agonist midazolam into the IVth ventricle.* PHARMACOL BIOCHEM BEHAV **55**(1) 81–86, 1996.—Despite a prolonged period of research with benzodiazepines, the central site(s) of action for the hyperphagic effects of these compounds remains to be determined. The aim of the present studies was to examine the effect of direct administration of the benzodiazepine receptor agonist midazolam into the IVth ventricle on ingestive behavior in nondeprived rats. In Experiment 1, microinjection of midazolam (3 and 30 μg/μl) into the IVth ventricle was sufficient to increase consumption of a palatable mash. In Experiment 2, the hyperphagic effect was blocked by systemic administration of the selective benzodiazepine receptor antagonist flumazenil (20 mg/kg). The results indicate that a brainstem site of action may be important for the effects of benzodiazepine receptor agonists on ingestive behavior.

Benzodiazepines Food intake IVth ventricle Brainstem Midazolam Palatability

FOLLOWING systemic administration, benzodiazepine receptor (BZR) agonists such as chlordiazepoxide (CDP) and midazolam produce a hyperphagic response in a number of species (4,5). For example, BZR agonists have been shown to stimulate a substantial increase in food consumption in nondeprived rats trained to eat a palatable diet (6). The hyperphagic effect can be blocked by a specific BZR antagonist such as flumazenil (Ro 15-1788) (6,10).

It is unlikely that the hyperphagic effect is due to an inhibition of satiety (6). Instead, it has been proposed that the benzodiazepine-induced increase in food intake is due to an increase in the palatability or hedonic value of ingested materials (1,2,9). In support of this view, midazolam has been shown to increase sucrose sham feeding (11). The sham feeding preparation provides a means of studying the effect of oropharyngeal factors on the consumption of palatable food. In this preparation, postingestional factors are eliminated by virtue of the fact that ingested solutions are allowed to drain out of the stomach via a gastric fistula. Such fistulated animals display a pronounced satiety deficit, and it has been proposed that the technique provides a measure of palatability (27). In addition, several BZR agonists have been shown to potentiate sweet taste preference in rats (8,12,24). For example, the BZR partial agonist bretazenil has been shown to increase the consumption of a preferred saccharin solution in a two-bottle choice test, without causing a concomitant increase in water intake (7). Furthermore, CDP has also been shown to enhance positive ingestive responses in a taste reactivity paradigm (3,26). In this paradigm, first developed by Grill and Norgren (14,15), sapid solutions are infused directly into the oral cavity of rats. The orofacial responses and body movements elicited are then recorded. The pattern of responding indicates whether the animal is reacting to the infused solution in a positive, ingestive manner, or with aversive reactions. The finding that CDP increases positive reactions to infused solutions, but does not affect aversive reactions, indicates that it might be enhancing hedonic responses to tastants (3,26). Midazolam has also been shown to affect taste reactivity responses in neurologically normal rats, which are allowed to sample taste stimuli voluntarily. In this modification of the taste reactivity test, midazolam has been shown to increase the number of ingestive responses to a 3% sucrose solution (13). These data are in line with those reported for oral-cannulated animals.

Despite much progress in analyzing the behavioral mecha-

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nisms underlying the effects of benzodiazepines on ingestion, little is known about the neural substrate for benzodiazepine-induced hyperphagia. One possibility is that these drugs act on neural systems that are concerned with behavioral responses to taste stimuli. An important result, relevant to the possible location of central sites of action of benzodiazepines, is that CDP retains its ability to enhance positive taste reactions in the chronic decerebrate rat (1). This result indicates that the lower brainstem may contain the site(s) of action for the effects of CDP in the taste reactivity paradigm. In an extension of this work, Berridge and Pecina (1,23) have shown that direct administration of the benzodiazepine agonist diazepam into the IVth ventricle in rats is sufficient to enhance the positive hedonic reactions elicited by a 7% sucrose solution. Hence, sites in or near the IVth ventricle may be involved in mediating the effects of benzodiazepines on ingestive responses.

It is also possible that benzodiazepine-induced increases in food consumption may depend, to some degree at least, on brainstem systems involved in the control of ingestive responses. Therefore, the present experiments were designed to investigate the effects of microinjection of a benzodiazepine agonist into the fourth ventricle on food consumption in nondeprived rats. In Experiment 1, midazolam was injected directly into the IVth ventricle, and its effect on palatable food intake was measured. In Experiment 2, the selective benzodiazepine antagonist flumazenil was used to establish specific benzodiazepine receptor-mediation of an enhanced feeding effect. The aim of these experiments was to determine whether hindbrain structures play an important role in the hyperphagic effect of benzodiazepines.

METHOD

Animals

Adult male Lister rats (General strain bred in the School of Psychology, University of Birmingham) weighing 300–500 g were used. Rats were housed individually in plastic cages in a room with a constant temperature of 22 ± 2°C, and were maintained on a 12 L:12 D cycle (lights on at 0800 h). Standard laboratory food pellets (Pilsbury 41B) and water were available at all times.

Drugs

The water-soluble BZR agonist midazolam maleate (Roche, Basel) was prepared for injection by dissolving in isotonic saline. The doses used in these experiments were 3 and 30 μg/μl of midazolam, which had been determined to be effective in previous pilot experiments. The vehicle used in control injections was isotonic saline. Midazolam was intracerebrally microinjected to the target site immediately prior to testing.

The selective benzodiazepine antagonist flumazenil (Ro 15-1788) (Roche, Basel) was prepared for injection by ultrasonic dispersion in distilled water to which Tween 80 had been added. The dose used in these experiments was 20 mg/kg or vehicle (distilled water to which Tween 80 had been added). This dose was used because it had been shown in previous experiments to be effective in blocking the hyperphagia induced by systemic midazolam (6,10).

Surgery

For implantation of stainless steel guide cannulae, animals were anesthetized and placed in a stereotaxic frame. The anesthetic used was pentobarbital at a dose of 60 mg/kg IP. A single stainless steel guide cannulae (21 gauge, 16 mm length) was implanted 1 mm dorsal to the IVth ventricle (coordinates L 0, A-P, −10.5 V −6). Bregma was used as a reference point and the coordinates were taken from the atlas of Paxinos and Watson (22). The cannulae were fixed to the skull using three screws and dental acrylic. Stylets were placed in the guide cannulae to prevent occlusion, and the animals were allowed 7 days to recover before behavioral testing occurred. Postoperative care involved weighing the animals daily and applying an antibiotic wound powder to the headmount if necessary.

Injection Procedure

Central microinjection of drugs was performed using an injection cannula connected by a polyethylene tube to a micrometer-driven 10 μl Hamilton syringe. The injection needles protruded 1 mm beyond the tip of the guide cannula and accuracy of injections was ensured by observing the progress of an air bubble in the tubing. The volume infused was 3 μl, injected over a period of 1 min. Each animal was then placed immediately in the test cage and food intake over 30 min was measured. In the case of pretreatment with flumazenil, the antagonist was administered via the IP route 15 min prior to central injection of midazolam. The volume injected was 1 ml/kg. Two days prior to testing each animal received a sham injection of isotonic saline to familiarize it with the microinjection procedure.

Test Meal

The sweetened mash meal was made up daily according to the following formula: 100 ml sweetened condensed milk, 400 ml ground maintenance diet (Special Diet Services Ltd., Essex, UK), and 200 ml distilled water. The constituents were mixed to produce a soft mash. This recipe has been previously shown to be readily consumed by rats (6).

Procedure

Following recovery from surgery the animals were adapted to eating the palatable sweetened mash. Familiarization continued until a steady baseline intake of mash was observed. Each rat was given 30 min access to 50 g portions of the diet placed in a clear plastic dish inside an individual stainless steel test cage. The consumption of the sweetened mash was measured to the nearest 0.1 g, with corrections made for any spillage. During testing animals did not have access to water or maintenance diet. All testing was carried out during the light cycle to insure relatively low baseline levels of consumption.

Histology

At the end of each experiment rats were deeply anesthetized with pentobarbital and a small quantity of methylene blue dye was injected through the cannula. Each animal was then perfused transcardially with isotonic saline followed by a 10% formalin solution. After decapitation, the excised brains were fixed in a 10% formalin solution for 1 week. The fixed brains were then frozen and sectioned sagitally on a freezing microtome and the correct placement of the cannulae was verified histologically. The histological work was conducted blind with respect to the behavioral results.

Statistical Design and Analysis

A repeated-measures design was used throughout in which each animal served as its own control and 24 h elapsed between

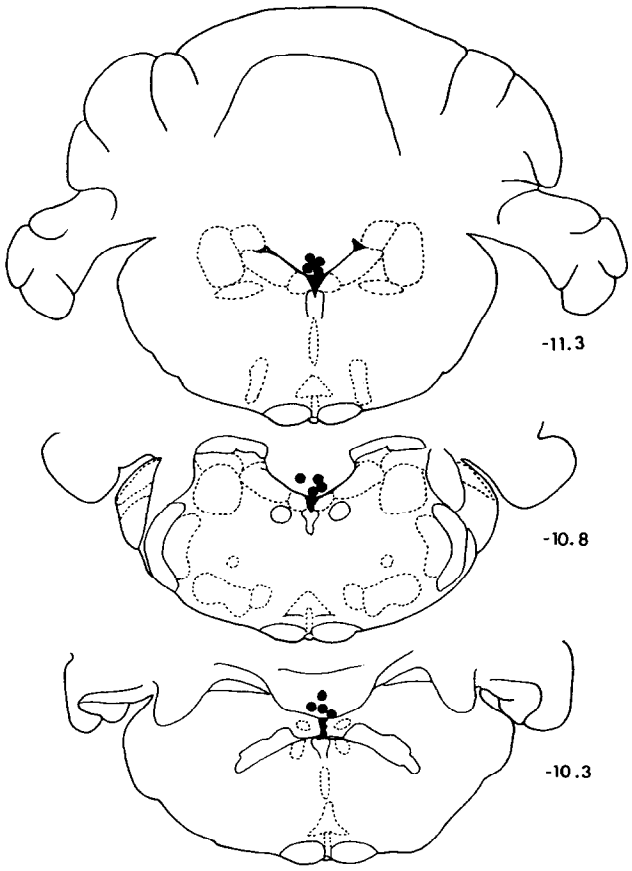

FIG. 1. The distribution of injection sites in the IVth ventricle for rats used in Experiment 1. Sections are redrawn from Paxinos and Watson (22). Each shows the location of the injection cannula tip for one animal. Section number refers to mm from bregma.

successive injections. A 24 h washout period was considered sufficient because midazolam is a short-acting drug with a half-life of 27 min + 1 when injected peripherally in rats. The data were analyzed using a one-way analysis of variance (ANOVA) for repeated-measures. Post hoc comparisons between means were carried out using Dunnett's t-test or a Newman–Keuls multiple comparisons test. Statistical tests were performed using Statview SE+graphics (Abacus Concepts Inc., Berkeley, CA) and a result was considered statistically significant if $p < 0.05$.

RESULTS

The Effect of IVth Ventricular Midazolam on Ingestion of Sweet Mash

Histological analysis revealed that all cannulae were correctly targeted and, therefore, all 12 animals were included in the data analysis (Fig. 1). Midazolam produced a dose-dependent increase in food consumption, $F(2, 22) = 5.61, p < 0.01$. The baseline intake of 13.75 g was increased to 18 g after 30 min (Fig. 2). Individual comparisons with a Dunnett's t-test revealed that a significant increase occurred at the 30 μg/μl dose ($p < 0.01$). Midazolam had an effect early in the test session and a significant increase in intake was evident after 10 min, $F(2, 22) = 7.25, p < 0.01$. The difference between treatment groups did not reach significance at 20 min, $F(2,$

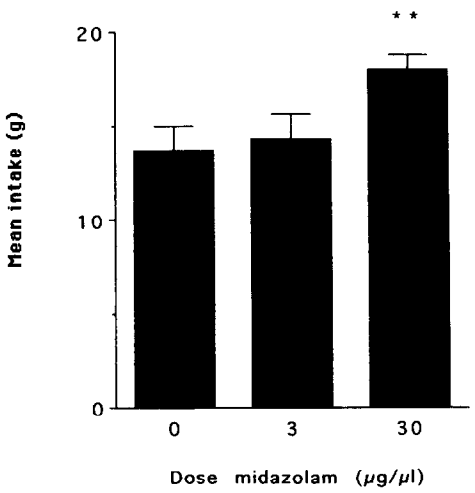

FIG. 2. Direct injection of midazolam into the IVth ventricle dose dependently increased the ingestion of a sweet wet mash in nondeprived rats. Levels of significance for individual dose comparison against the vehicle control. $^{**}p < 0.01$ (Dunnett's t-test). Results are shown as mean intake (g) after 30 min.

TABLE 1

THE EFFECTS OF IVTH VENTRICULAR MIDAZOLAM ON THE INTAKE OF SWEET WET MASH

	Mash Consumed (g)		
Treatment	10 min	20 min	30 min
Vehicle	5.93 + 0.75	10.567 + 1.08	13.75 + 1.32
3 μg/μl midazolam	7.02 + 0.70	11.711 + 1.15	14.29 + 1.344
30 μg/μl midazolam	9.608* + 0.59	11.68 + 1.26	17.98* + 0.8

Results are shown mean intake (g) + SEM. $n = 12$ rats per condition. Levels of significance: $^*p < 0.01$ (Dunnett's t-test).

22) = 0.228, $p = 0.7$, but was evident at the end of the 30 min session. A summary of the results is shown in Table 1.

The Effect of Flumazenil on Hyperphagia Induced by IVth Ventricular Midazolam

Histological analysis revealed that all cannulae were targeted correctly (Fig. 3). A one-way repeated-measures ANOVA revealed a significant effect of drug treatment, $F(3, 18) = 7.66$, $p < 0.01$. A Newman–Keuls multiple comparison test showed that the dose of 30 μg/μl of midazolam significantly increased consumption of the mash compared with the control condition ($p < 0.01$). The baseline intake of 11.7 g was increased by 50% to 16.8 g (Fig. 4). This increase was almost completely blocked by pretreatment with flumazenil (20 mg/kg). Flumazenil administered alone had no significant effect on the ingestion of sweet wet mash. The effect of midazolam (30 μg/μl) was observed within the first 10 min of the test. A significant increase in food intake was already evident after 10 min, $F(3, 18) = 9.934$, $p < 0.001$. This early increase was also effectively blocked by pretreatment with flumazenil. The hyperphagic effect of midazolam was maintained after 20 min, $F(3, 18) = 8.064$ $p < 0.01$. The results are shown in Table 2.

DISCUSSION

These experiments demonstrate that direct injection of a benzodiazepine agonist into the IVth ventricle is sufficient to induce a significant hyperphagic response in nondeprived rats. The increase in food intake observed by this route of administration is comparable to that obtained following systemic administration of benzodiazepines (6). Antagonism of the hyperphagic effect by flumazenil is an indication that the effect is mediated by specific benzodiazepine receptors. Hence, these results argue for central mediation of benzodiazepine-induced hyperphagia, and point to lower brainstem structures as possible locations of the site of action of the drug.

Berridge (1) has shown that benzodiazepine effects in the taste reactivity test remain intact in midbrain-transected animals, and more recently, Berridge and Pecina (1,23) have shown that direct administration of diazepam into the IVth

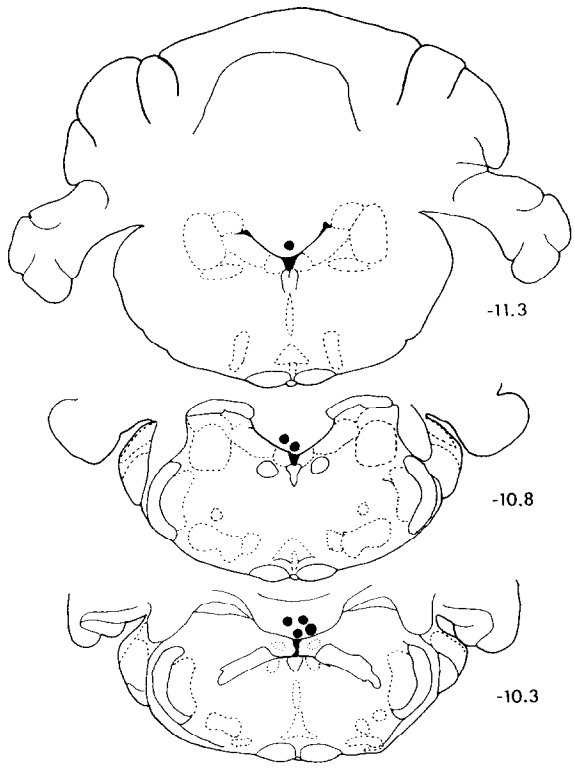

FIG. 3. The distribution of injection sites in the IVth ventricle for rats used in Experiment 2. Sections are redrawn from Paxinos and Watson (22). Each shows the location of the injection cannula tip for one animal. Section number refers to mm from bregma.

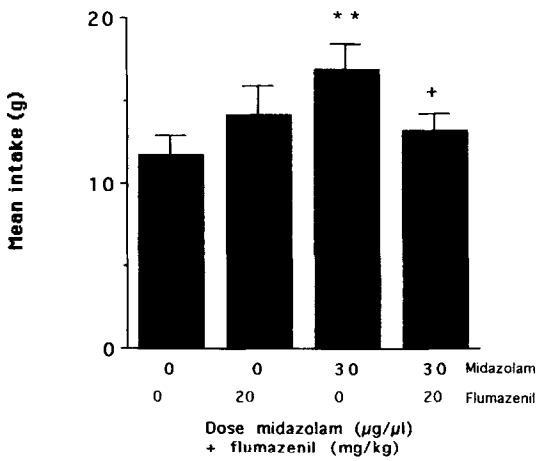

FIG. 4. Pretreatment with flumazenil blocked the hyperphagia induced by injection of midazolam into the IVth ventricle. Levels of significance for pair-wise comparisons $^{**}p < 0.001$, significantly different from control condition. $^{+}p < 0.01$, significantly different from vehicle/midazolam condition. Results are shown as mean intake (g) after 30 min.

TABLE 2

THE EFFECT OF FLUMAZENIL ON HYPERPHAGIA INDUCED BY IVTH VENTRICULAR MIDAZOLAM

	Mash Consumed (g)		
Treatment	10 min	20 min	30 min
Vehicle	7.39	10.76	11.78
Vehicle	+ 1.38	+ 1.07	+ 1.18
Flumazenil (20 mg/kg)	7.62	12.78	14.05
Vehicle	+ 1.13	+ 1.73	+ 1.83
Vehicle	12.41*	15.31*	16.7*
Midazolam (30 μg/μl)	+ 0.99	+ 1.51	+ 1.6
Flumazenil (20 mg/kg)	8.56†	11.44†	13.14†
Midazolam (30 μg/μl)	+ 1.16	+ 1.08	+ 1.038

Results are shown mean intake (g) + SEM. $n = 7$ rats per condition. Levels of significance: $^{*}p < 0.01$ Different from vehicle/vehicle condition; $^{\dagger}p < 0.05$ Different from vehicle/midazolam condition. Newman-Keuls multiple comparisons test.

ventricle of the rat enhances hedonic reactions to a 7% sucrose solution. The present data are consistent with these results in showing that the lower brainstem appears to be important for the effects of benzodiazepines on ingestive responses.

The precise location of the brainstem receptors remains uncertain, but the parabrachial nucleus (PBN) in the pons may be a possible candidate for mediation. In an autoradiographic binding study, we have shown that there is a population of benzodiazepine receptors in or near the PBN (16). Hence, benzodiazepine receptors in this area may be involved in the effects of benzodiazepines on ingestive behavior. There are several reasons for asserting that the PBN may be an important central site of action for the effects of benzodiazepines on food intake. First, the neuroanatomy of the gustatory system has been carefully mapped, and it is well established that the PBN contains the second central relay for the taste pathway (18,20,21). Because taste is an important determinant for the control of food intake, this evidence is consistent with the notion that the PBN is important for the control of feeding behavior. Second, it has been established that the PBN makes extensive connections to many areas of the brain that are involved in the control of ingestive behavior, such as the hypothalamus (17,18,25) and amygdala (18), and this prompts the notion that the PBN itself may contribute to the execution of such behaviors. It is also possible that benzodiazepine receptor agonist modulation of feeding may occur even earlier on the taste pathway in the nucleus of the solitary tract (NTS), which is the first relay in the gustatory pathway (19). This hypothesis remains to be tested, but a role for the NTS seems less likely given that the autoradiographic binding study failed to reveal any benzodiazepine receptors in this nucleus (16).

Presently, we cannot rule out the possibility that structures rostral to the IVth ventricle are also involved in benzodiazepine-induced hyperphagia. Direct injection of benzodiazepine into the forebrain would help to address this issue, but the present results do serve to draw attention to brainstem participation in the control of ingestive behavior, particularly in relation to benzodiazepine effects. These data suggest that the continued investigation of brainstem mechanisms of ingestion would prove to be of great value.

An important question arising from the present studies is whether the effects of benzodiazepines in the taste-reactivity test are mediated by the same receptor populations underlying benzodiazepine-induced hyperphagia. It is possible that the increase in hedonic reactions following benzodiazepine administration may be dissociable from the effect of these drugs on food consumption. This dissociation could occur either in terms of distinguishable sites of action, or in terms of receptor subtypes within a specific location, or both. Alternatively, it may be that the hyperphagic effect depends upon and is secondary to the enhancement of hedonic reactions to taste stimuli. This would suggest that whenever an increase in hedonic reactions is achieved (e.g., by benzodiazepine treatment) an increase in food consumption would follow. This idea is consistent with the possibility that benzodiazepines enhance food palatability. Integrating the results of the present study with the data of Berridge and Pecina (1,23) provides some support for this view, because IVth ventricular administration of a benzodiazepine agonist has been shown to be increase both hedonic reactions to taste stimuli and food consumption. This conclusion is reinforced by the observation that the increase in consumption of the palatable mash caused by midazolam was observed after just 10 min of the test session had elapsed. This suggests that the hyperphagic effect was probably not caused by a reduction in satiety occurring at the end of the feeding period.

In conclusion, the present studies indicate that the neural substrate for benzodiazepine-induced hyperphagia may be located in the brainstem, although specifically which structures are involved remains to be elucidated.

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