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GABA and Hypothalamic Feeding Systems. II. A Comparison of GABA, Glycine and Acetylcholine Agonists and Their Antagonists[1]

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KELLY, J. AND S. P. GROSSMAN. *GABA and hypothalamic feeding systems. II. A comparison of glycine and acetylcholine and their antagonists.* PHARMAC. BIOCHEM. BEHAV. 11(6) 647–652, 1979.—Microinjections of various compounds into the paraventricular hypothalamic nucleus (PVH) were made and the effects on feeding observed. During the light phase of the lighting cycle, injections of 0.3 μl of muscimol (100 ng) and flurazepam diHCl (20 μg) increased feeding. Similar injections of glycine (500 ng) did not influence feeding during the light phase. During the dark phase, 0. 3 μl injections of bicuculline methiodide (30 ng) and picrotoxin (160 ng) suppressed feeding. Similar injections of carbachol increased drinking during the dark phase. Injections of strychnine during this phase were without effect. Tilt box activity levels were not altered by injection of picrotoxin (160 ng) into the PVH.

Paraventricular hypothalamic nucleus GABA Muscimol Flurazepam diHCl Bicuculline methiodide Picrotoxin Glycine Strychnine Carbachol Feeding behavior Drinking behavior

THIS experiment was designed to determine the pharmacological specificity of the feeding effect produced by microinjections of muscimol into the PVH [14,17]. For this purpose, rats with cannulas in the PVH were tested during the light phase of the lighting cycle for feeding increases with injections of muscimol, the most potent GABA agonist known (see [21]), glycine, another putative inhibitory amino acid neurotransmitter, and flurazepam diHCl, a water-soluble benzodiazepine which has GABAmimetic properties (see [4] for GABAergic actions of benzodiazepines). Animals were also tested for feeding decreases during the dark phase of the cycle with bicuculline methiodide (BMI), and picrotoxin, both putative GABA antagonists (see [7] for review), as well as carbachol, a cholinomimetic, and strychnine, a putative glycine receptor blocker. The cholinergic agonist, carbachol, was used to determine whether the anticholinesterase activity of bicuculline and picrotoxin which elevate brain acetylcholine levels [27] might be related to the feeding suppression produced by these GABA blockers.

There is considerable evidence that the effects of the benzodiazepines are mediated by the direct or indirect activation of GABA receptors [2, 4, 12]. The mode of action of the benzodiazepines on GABAergic receptors is not known [4], but recent reports which utilized interacerebral injections of two benzodiazepines, chlordiazepoxide HCl [30] and flurazepam diHCl [29], have produced behavioral effects identical to similar injections of GABA agonists, muscimol and baclofen. In the present experiment, a similar behavioral comparison was made using muscimol and a behaviorally effective dose of flurazepam diHCl [29].

METHOD

Animals

Forty-seven adult female rats (230–300 g) of the Sprague-Dawley strain (Holtzman, Madison, Wisconsin) were used. The animals were maintained on ad lib food (Teklad, 6% fat diet) and tap water while housed individually in stainless steel cages. The temperature of the vivarium was maintained at 21–24°C. A 12-hour light-dark cycle (0700–1900 light) was employed.

Surgery

Twenty-three ga. stainless steel cannulas (with 30 ga inner obdurators) were implanted stereotaxically. Cannulas were fastened to the skull with dental cement such that the tips were 3.0 mm above the PVH (AP=6.0; H=1.3; L=0.5, according to deGroot [8]). Chemicals were administered with a 30 ga injector which extended 3.0 mm beyond the tip of the guide cannula. Surgery was conducted under Ketamine plus Acepromazine anesthesia.

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Post-Surgical Procedure

Following surgery, food and water intake and general motor abiltiy were monitored. Particular note was taken of ambulatory ability (the presence of akinesia) and the visual placing response (rats normally extend their forelimbs as they are brought near a surface when held in a head-down position). All drug tests were deferred until ingestive behavior and motor functions were normal.

Chemical Injections and Ingestive Behavior

Injections of various compounds into the PVH and subsequent measurements of food intake were conducted during the light or dark phases of the lighting cycle. Following each injection, feeding and drinking behavior was observed in the home cage. Ingestion of standard lab chow and tap water was monitored by weighing fresh pre-weighed food blocks (Teklad, 6% fat diet) to the nearest 0.1 g (including spillage) and reading calibrated drinking tubes (Wahmann, Baltimore, Maryland) to the nearest ml. These intake measurements were recorded every 15 min for the first hour after the injection. A final reading was recorded 2 hr post-injection.

Because our previous experiments [14, 16, 17] demonstrated that feeding increased following injections of GABA agonists into the PVH, the GABAergic agonists (muscimol and flurazepam) and glycine, another inhibitory neurotransmitter, were tested during the light phase. Bicuculline methiodide, picrotoxin (GABA antagonists) and strychnine were tested during the dark phase to determine if blockade of inhibitory neurotransmitters during normal feeding would be affected. BMI attenuated the feeding induced by muscimol during the light phase [14].

Prior to the injection, animals were taken from their cages, the obdurators were removed and bilateral injections were made. Before returning the animals to the home cage, the obdurators were replaced. This procedure was usually completed within 2 min. Each chemical injection was preceded by several days of control tests using vehicle injections (0.9% bactereostatic saline, Abbot and Company, Berkley, CA) until a stable feeding baseline was established. Another vehicle injection was made 24 hr after the drug test to ascertain a return to baseline.

Injections were made with a 1.0 μl Hamilton syringe. The following drugs were injected in a 0.3 μl volume when animals were tested in the light: flurazepam diHCl (20 μg [29]; pH approximately 2.0, gift from Hoffman-LaRoche, Inc., Nutley, NJ), glycine (500 ng [25]; pH approximately 6.4; Sigma, St. Louis, MO), muscimol (100 ng, pH approximately 5.6, Biosearch, San Rafael, CA). When the animals were tested in the dark the following compounds were injected in 0.3 μl volumes: bicuculline methiodide (BMI, 30 ng; pH approximately 5.5, prepared from bicuculline (Sigma, St. Louis, MO) according to [23]), carbamylcholine (carbachol, 250 ng [25]; pH approximately 5.5, Sigma, St. Louis, MO), picrotoxin (160 ng [25]; pH approximately 5.5, Sigma, St. Louis, MO), and strychnine sulfate (950 ng [11]; pH approximately 5.0, Fischer Scientific, Chicago, IL). All animals received a counterbalanced sequence of a combination of at least four of these drugs.

On the first drug day, animals were divided into seven groups. On subsequent drug days, each animal in a specific group was assigned a different drug to which it was not previously exposed such that drug sequences were distributed approximately equally.

Thirty-three of 47 rats displayed consistent significant changes in feeding when tested with various combinations of drugs. Twenty-six received injections of the GABA antagonists, bicuculline methiodide (BMI) and picrotoxin (PIC). Nine of these 26 were also tested with carbachol (CAR) and strychnine (STR). Twelve of these 26 were tested with muscimol (MUS) and flurazepam (FLU). A total of 17 of the 33 rats used in the present experiment were injected with muscimol. Of these 17, seven were tested for the effects on feeding of both flurazepam and glycine (GLY).

Activity

The effect of intrahypothalamic injections of the convulsant GABA antagonist, picrotoxin, on locomotor activity was monitored in eight tilt cages (a microswitch recorded each crossing from one side of the cage to the other) located in a sound attenuating room (see [15] for a a detailed description). The injection procedure was identical to that described above for the feeding experiments, except that food and water were not available in the tilt cages. Immediately after the injection, animals were placed in the activity chambers. Activity readings were taken at the same times as the readings in the feeding tests (i.e. every 15 min after injection for 60 min and a final reading 2 hr postinjection).

Histology

All animals' brains were examined histologically to determine the cannula placements. Rats were sacrificed with an overdose of Nembutal and perfused transcardially with isotonic saline followed by a 10% formol-saline solution. After fixation in formalin, brains were sectioned on a freezing microtome. Fifty micron sections were made and every other section in the area of the cannula tract was saved. Sections were mounted on glass slides from gelatin alcohol and stained with cresyl violet.

Statistical Analysis

T-tests for correlated samples [31] were used to compare behavior after chemical injections to baseline (vehicle injection) behavior. When multiple drug or saline injections were administered, mean scores were calculated and used for the statistical analysis. The Pearson product-moment correlation [31] was calculated to evaluate the relationship between activity and food intake.

RESULTS

Histology

Figure 1 shows the location of the cannula tips in the 40 animals that were used in the present experiment. Seven animals (not represented in Fig. 1) died before or during the test period. Many, but not all, of these fatalities demonstrated the autonomic syndrome (e.g. copious salivation, and lacrimation as well as expanded or acid-filled gastrointestinal tracts) described by Kelly *et al.* [14].

Of the 40 animals represented in Fig. 1, seven did not respond to any of the compounds injected. Although all rats were not injected with all seven drugs, the remaining 33 responded consistently to whatever drugs they were exposed to. The seven rats that failed to respond to any injection (see Fig. 1) had cannula placements which bordered the muscimol-sensitive region of the PVH as described by Kelly *et al.* [14] or were in close proximity to the feeding suppres-

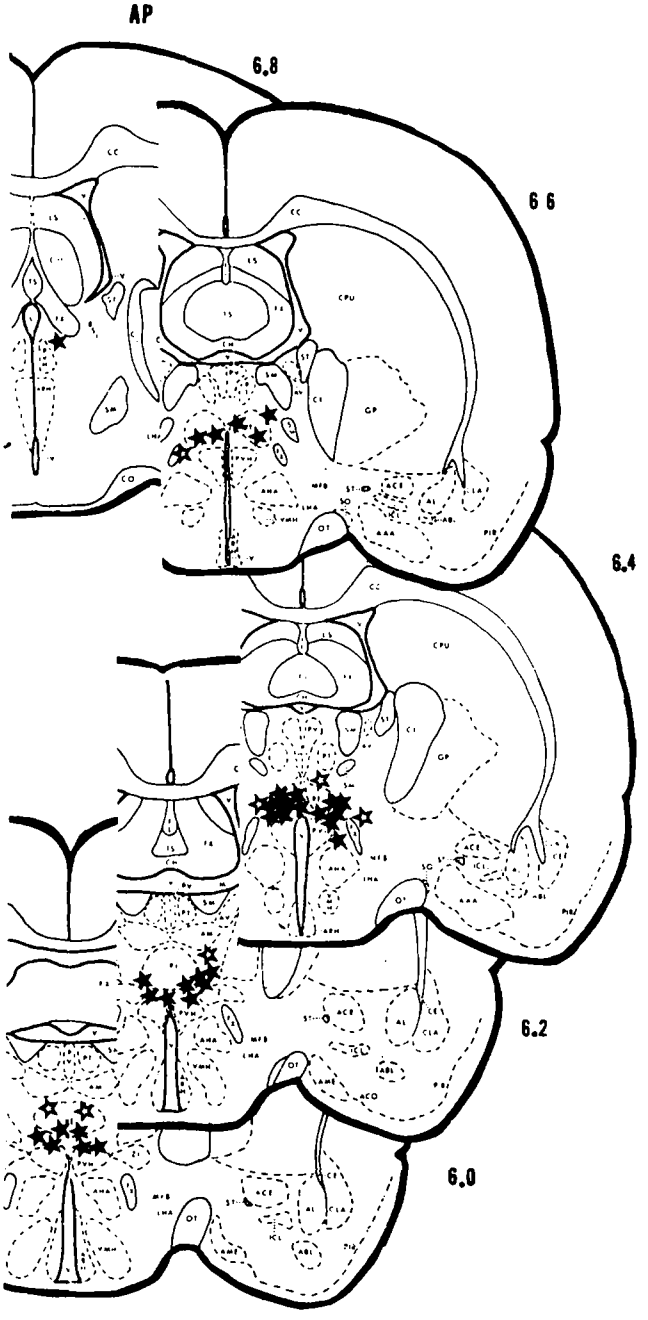

FIG. 1. Schematic representation of injection sites in the PVH where muscimol increased food intake (solid star) or produced no change in food intake (open star) (after DeGroot [8]).

sion zone near the perifornical area (PFA) [14]. Behaviorally responsive rats had cannula placements situated just dorsal to the PVH from AP=6.8 to 6.0, an area previously [14] shown to be specifically sensitive to muscimol.

Feeding Response to Various Pharmacological Agents

When tested during the day, muscimol increased ingestion of solid food by more than a gram above saline baselines ($p<0.005$, 15, 30, 45, 60 and 120 min after the injection; see Fig.2). Flurazepam, a member of the benzodiazepines whose effects are thought to be mediated by GABA, caused a small, but significant, increase in feeding. However, this effect was short-lived ($p<0.005$ at 15 min; $p<0.025$ at 30 min, not significant thereafter).

During the dark phase, bicuculline methiodide and picrotoxin each produced a long-lasting highly significant ($p<0.005$ at all time periods) suppression of feeding in these animals (see Fig. 2).

In seven rats tested with muscimol, flurazepam and glycine, glycine failed to facilitate feeding behavior while muscimol produced large (i.e. more than 1.0 g) and flurazepam produced small (approximately 0.4 gm) increases in feeding (Fig. 3). The feeding induced by muscimol was highly significant at all test intervals ($p<0.005$ at 15, 30, 45, 60 and 120 min). The flurazepam-feeding effect lasted only 30 min ($p<0.025$ at 15 min, $p<0.025$ at 30 min and not significant thereafter) and was significantly smaller than the feeding induced by muscimol at 15 (<0.025) and 30 min ($p<0.025$) postinjection. Glycine produced small but significant ($p<0.05$) suppression of feeding at the 60 min time period only.

In nine rats injected with bicuculline methiodide, picrotoxin, carbachol and strychnine during the dark phase, only bicuculline methiodide and picrotoxin produced consistent suppression of feeding across all time intervals (Fig.4). Carbachol inhibited feeding significantly during the first 15 min postinjection. However, this was probably due to the increased drinking which competed with feeding behavior, mean($\pm$ SE)=0.85 ($\pm$ 0.14) ml for saline and mean ($\pm$ SE)=4.67 ($\pm$ 1.05) for carbachol during the first 15 min postinjection, $p<0.005$. A significant negative correlation between feeding and water intake ($r=-0.68$, $p<0.05$) supports the suggestion that the inhibition of feeding which occurred following injections of carbachol was primarily due to the drinking induced by such injections. Strychnine, a putative glycine antagonist, not only failed to suppress feeding behavior during the dark phase of the diurnal cycle, but produced a small but significant increase in feeding 15 min ($p<0.05$) and 30 min ($p<0.05$) after the injection.

Effect of Picrotoxin on Tilt Box Activity

Because both bicuculline methiodide and picrotoxin are convulsants when administered systemically (see [26]) and because these compounds increase activity when injected into various regions of the extrapyramidal system [15, 19, 20, 22, 28], twelve of the rats tested in the feeding paradigm with picrotoxin were tested in the tilt box activity apparatus. Although some animals showed moderate increases in tilt box activity after injections of 500 ng of picrotoxin in 0.3 μl of vehicle, overall there was a small, non-significant mean decrease in activity, mean ($\pm$ SE)=24.15 ($\pm$ 5.55) crossings for saline and mean ($\pm$ SE)=21.31 ($\pm$ 4.02) crossings for picrotoxin during the first 15 min. There was no correlation between the decreases in feeding and activity levels ($r=0.07$).

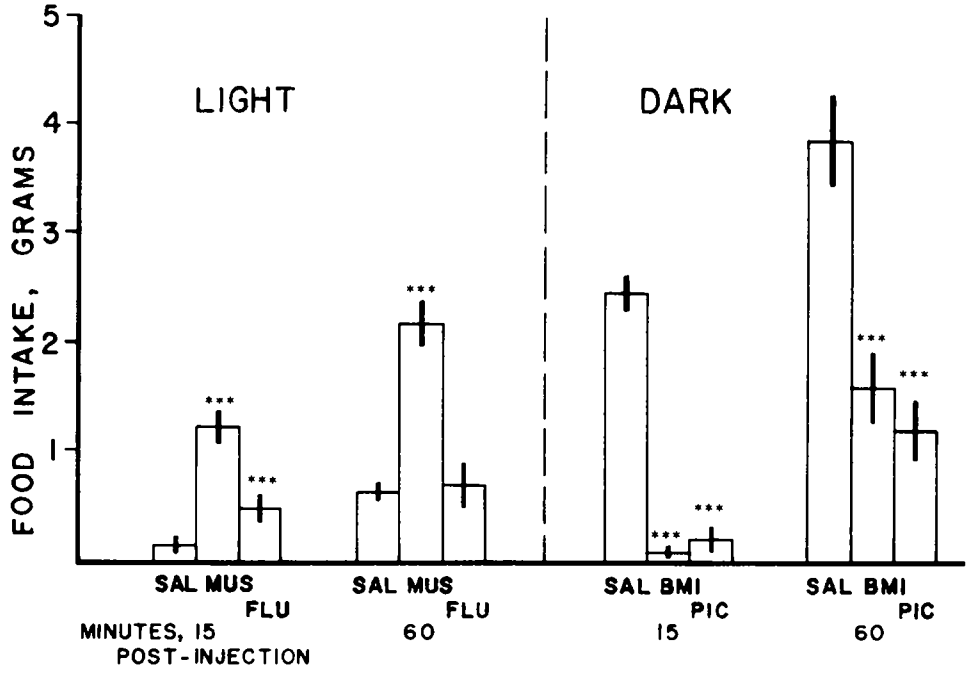

FIG. 2. Food intake after injections into the PVH of saline (SAL), muscimol (MUS) or flurazepam (FLU) in the light (left) and saline, bicuculline methiodide (BMI) or picrotoxin (PIC) in the dark (right) at 15 min and 60 min postinjection. Significantly different from isotonic saline (*** :$p<0.005$).

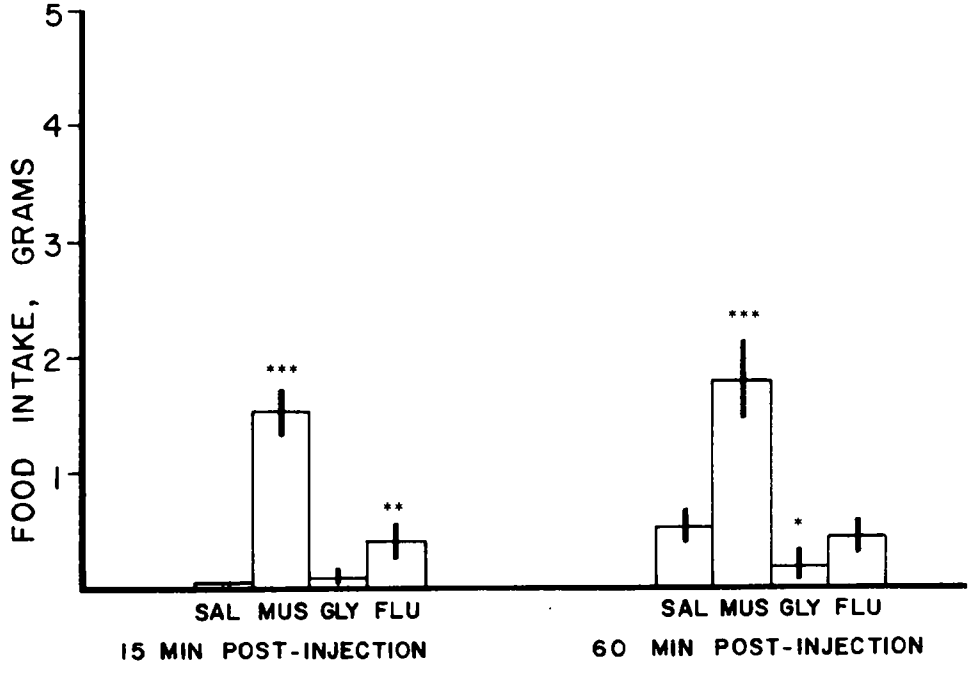

FIG. 3. Food intake after injections into the PVH of saline (SAL), muscimol (MUS), glycine (GLY) or flurazepam (FLU) in the light at 15 min and 60 min postinjection. Significantly different from isotonic saline (* :$p<0.05$; ** : $p<0.025$; *** : $p<0.005$).

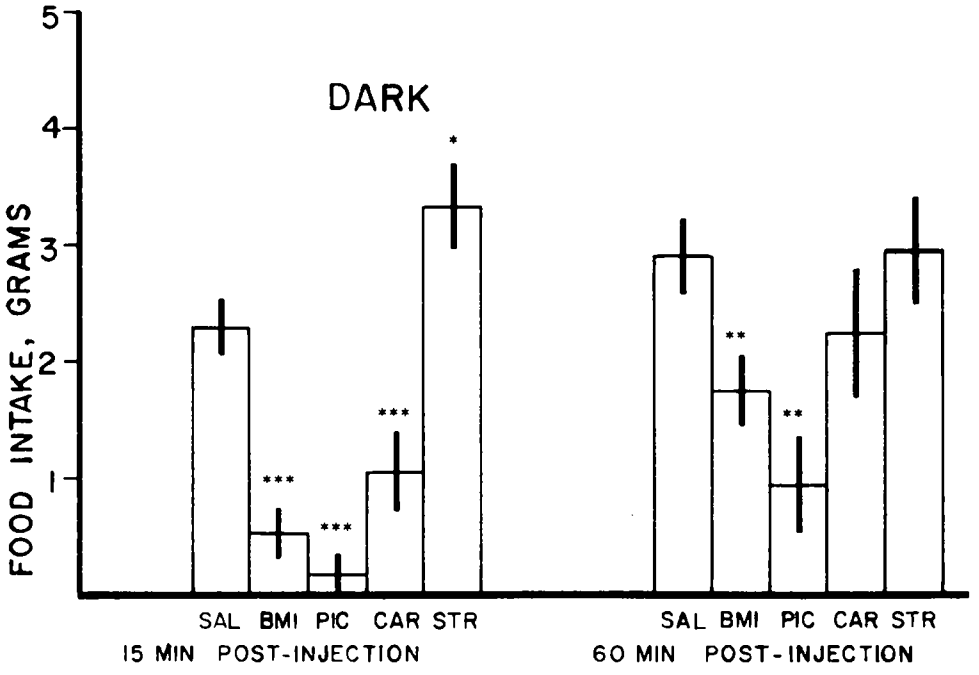

FIG. 4. Food intake after injections of isotonic saline (SAL), bicuculline methiodide (BMI), picrotoxin (PIC), carbachol (CAR) or strychnine (STR) in the dark at 15 min and 60 min postinjection. Significantly different from isotonic saline (* : $p<0.05$; ** : $p<0.025$; *** : $p<0.005$).

DISCUSSION

The results of these studies provide support for the hypothesis that the feeding induced by injections of muscimol into the medial hypothalamus is mediated through GABAergic mechanisms. Muscimol, when injected into the dorsomedial hypothalamus during the light phase of the lighting cycle, increased feeding. In the dark when most feeding occurs in nocturnal rodents such as rats, feeding was reduced or abolished by injections of the GABA antagonists, bicuculline methiodide or picrotoxin. These effects were apparently unrelated to glycine, another putative inhibitory neurotransmitter, because neither glycine nor its receptor blocker, strychnine, produced changes in feeding when injected into the PVH.

The feeding suppression induced by hypothalamic injections of picrotoxin and bicuculline methiodide were probably unrelated to the anticholinesterase properties of these agents. Injections of carbachol produced only a short-lasting inhibition of feeding that appeared to be related to competitive drinking which followed these injections. The relationship between increased drinking and inhibition of feeding was demonstrated by a significant negative correlation between these two variables. Furthermore, it can be argued that if the increase in acetylcholine levels produced by bicuculline methiodide or picrotoxin were significant, these compounds would have elicited drinking. Instead, drinking paralleled the effects of BMI and picrotoxin on feeding (i.e. decrease) throughout these experiments.

Injections of flurazepam, a benzodiazepine, into the PVH produced small but significant increases in feeding during the light phase of the lighting cycle. As in the substantia nigra [29,30], the effects of benzodiazepines mimicked the effects of muscimol, a potent GABA agonist. These observations support the hypothesis that the effects of the benzodiazepines are mediated by a direct or indirect activation of GABA receptors.

Our behavioral results agree with the biochemical data (see [4]) in suggesting that the benzodiazepines (e.g. flurazepam) interact with GABAergic mechanisms in a manner similar to muscimol. The feeding induced by flurazepam was less intense and of a shorter duration, suggesting that its putative GABA stimulating effects were of an indirect nature.

In summary, this report provides further evidence for the involvement of GABAergic mechanisms in medial hypothalamic satiety systems (see also [14, 16,17]. In addition to the pharmacological specificity of the muscimol feeding effect demonstrated in this communication, feeding induced by injections of muscimol into the medial hypothalamus is dose dependent, attenuated by pretreatment with BMI and limited to a region of the hypothalamus medial to the fornix (see [14]). These GABAergic mechanisms may interact with adrenergic feeding systems (see [10,18]), both of which have similar anatomical substrates [14,18]. Furthermore, the present experiment suggests that the PVH may be a part of the neurocircuitry involved in the mediation of the anorexic properties of the benzodiazepines (e.g. flurazepam).

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