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Ineffectiveness of Hypothalamic Serotonin to Block Neuropeptide Y-Induced Feeding

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BROWN, C. M. AND D. V. COSCINA. *Ineffectiveness of hypothalamic serotonin to block neuropeptide Y-induced feeding.* PHARMACOL BIOCHEM BEHAV **51**(4) 641-646, 1995.—A variety of recent research has suggested that the feeding associated with enhanced neuropeptide Y (NPY) activity within the hypothalamus may operate in part by interacting antagonistically with other neural processes responsive to serotonin (5-hydroxytryptamine or 5-HT). To test this possibility further, experiments were performed to determine if the magnitude of feeding produced by injecting NPY into the paraventricular nucleus (PVN) or the perifornical hypothalamus (PFH) was diminished by coinjections of 5-HT into these two sites or peripheral injections of the 5-HT agonist, *d*-fenfluramine. Adult male Sprague–Dawley rats were implanted unilaterally with stainless steel cannulae aimed to terminate either in the PVN or the PFH. In both studies, NPY (235 pmol) produced significant feeding in both sites either 1 or 2 h after injection when compared to saline. This enhanced feeding response was significantly greater in the PFH 2 h after injection (40% in the central study; 70% in the peripheral study). Coinjection of 5-HT (6.3, 12.5, or 25.0 nmol) into either site had no effect on the induction of this NPY-induced feeding response. However, peripherally injected *d*-fenfluramine (0.32, 0.63, or 1.25 mg/kg) produced strong dose-dependent attenuation of both 1- and 2-h food intake elicited by 235 pmol NPY in either site, with the PFH being proportionately more sensitive to this effect. Viewed together, these results suggest that the feeding-suppressant effects of systemic fenfluramine on hypothalamic NPY-induced feeding may operate largely via peripheral mechanisms and/or central ones that have little to do with its 5-HT agonistic effects within the PVN or PFH.

Feeding Neuropeptide Y Serotonin Fenfluramine Paraventricular nucleus Perifornical hypothalamus

NEUROPEPTIDE Y (NPY) is widely distributed throughout neurons of the central and peripheral nervous systems, but is found in particularly high concentrations within the paraventricular nucleus (PVN) of the hypothalamus (5) where it coexists with classical neurotransmitters such as serotonin (5-hydroxytryptamine or 5-HT) (11,13). Several lines of evidence suggest that hypothalamic NPY and 5-HT may interact in an opposite fashion in their controls over food intake. In the case of NPY, it is a potent stimulus to induce feeding in otherwise satiated rats when injected directly into the PVN (20,22,30). Conversely, 5-HT and its agonists are known to inhibit feeding when injected either peripherally (4,8) or directly into the PVN (9,17,18). With regard to the consumption of specific macronutrients, PVN NPY-induced feeding appears to be selective for carbohydrates (3,28,29,37) whereas enhancing central 5-HT neural activity is associated with the selective inhibition of carbohydrate intake (38). Patterns of diurnal variations in endogenous NPY and 5-HT levels within the PVN are similar, with both exhibiting a peak at the onset of darkness (14,15,33), which is when rats normally initiate large bouts of feeding. Based on these observations, some researchers have suggested that both neurochemical substances may be involved in the regulation of food intake and energy homeostasis (14). In fact, it has been suggested that NPY may exert its hyperphagic effects by acting directly on the 5-HT system (26).

The findings summarized above can be interpreted to predict that enhancing levels of brain 5-HT may attenuate the hyperphagic effects of NPY in the PVN. So far, specific tests of this hypothesis have produced mixed results. Systemic administration of *d*-fenfluramine, a prototypic 5-HT agonist, has been shown to significantly reduce NPY-elicited feeding after its injection into the PVN (2). On the other hand, direct injections of 5-HT into the PVN have been briefly reported to potentiate rather than suppress NPY-induced feeding (13). Other studies have provided indirect support for a possible

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antagonistic effect of 5-HT on NPY-induced feeding by showing that hypothalamic NPY levels drop following treatment with 5-HT agonists (23,26,27) or increase after 5-HT antagonists (7). However, others have reported either opposing (35) or inconclusive (1,10,36) results in similar studies.

One way to further test the potential antagonistic relationship that may exist between central NPY and 5-HT systems in controlling food intake is to determine if such an interaction occurs at different feeding-sensitive brain sites. The perifornical region of the hypothalamus (PFH) has recently been found to be a particularly sensitive site in response to centrally administered NPY (28,31). Based in part on observations that the PVN contains extremely high concentrations of presynaptic NPY (5), and that both tissue content and extracellular levels of NPY are increased by a variety of manipulations accompanied by increased eating (6,12), Stanley (28) has proposed that the PVN and the PFH may both be involved in the feeding-stimulatory actions of NPY, but may have separate and distinct functions. With regard to 5-HT, recent microdialysis studies have shown that this monoamine is released in the PFH in response to deprivation-induced or natural meals (19,25), thereby implying some role of this substance in mediating feeding at this brain site.

Because of the inconclusive nature of previous work on the potential interactions of hypothalamic NPY and 5-HT in controlling feeding, and to examine the possible existence of such an interaction within the PFH, the present studies sought to determine: (a) if coinfusions of 5-HT suppress NPY-induced feeding from the PVN and/or the PFH, and (b) if peripheral injections of the 5-HT agonist, *d*-fenfluramine, already shown to be effective in suppressing PVN NPY-induced feeding (2) and releasing endogenous 5-HT in the PFH (24), might be effective in suppressing PFH NPY-induced feeding.

METHOD

Subjects

Thirty-one adult male Sprague–Dawley rats purchased from the Charles River Company (St. Constant, Quebec) were used. Rats were housed individually in hanging wire-mesh cages with free access to food (standard Purina Rat Chow pellets) and water at all times in a temperature (22°C)- and humidity-controlled room with lights on from 0600 to 1800 h. Upon arrival, subjects weighed between 225 and 275 g and were handled for approximately 5 min per day.

Surgery

Once animals had attained a body weight of at least 275 g, they were anaesthetized with sodium pentobarbital (50 mg/kg, IP). If, after this dose, a rat was still reacting to tail pinch, a supplemental dose of 5 mg was given. Once anaesthetized, each rat was positioned in a stereotaxic apparatus and implanted with a 15-mm stainless steel 22-ga guide cannula (Plastic Products, Roanoke, VA) aimed to terminate 4 mm dorsal to the target nucleus. There were two groups of subjects: those with unilateral cannulae aimed at the PVN ($n = 14$) and those with unilateral cannulae aimed at the PFH ($n = 17$). With the incisor bar set 3.3 mm below the interaural line, the coordinates (21) were: for the PVN, anterior-posterior (AP) −1.5 mm from bregma, midline (ML) −0.4 mm from the midsagittal sinus, and dorsal-ventral (DV) −4.2 mm from bregma's DV skull coordinate; for the PFH, −1.6 mm from bregma, ML −1.1 mm from the midsagittal sinus, and DV −4.1 mm from bregma's DV skull coordinate. Four stainless steel screws were also implanted in the skull and the entire construction was covered with dental acrylic to anchor guide cannulae in place. Following implantation, a stylet was kept inside each guide cannula to prevent occlusion.

Testing

Following at least 5 days of postoperative recovery, testing began with NPY (Sigma, St. Louis, MO; or synthesized in the lab of Dr. R. D. Myers, East Carolina Medical School, NC) and 5-HT maleate salt (Sigma) or *d*-fenfluramine (kindly donated by Servier, Neully sur Seine, France). All drug solutions to be injected centrally were dissolved in sterile physiological saline and injected in a volume of 0.4 μl using a stainless steel 28-ga injector (Plastic Products) cut to terminate 4 mm below the guide cannula. The injector was attached by a length of polyethylene tubing to a 5-μl Hamilton microsyringe. All solutions were infused manually over a period of 1 min. The injector was left inside the guide cannula for an additional 30 s to allow for diffusion of the solution away from the tip. For systemic drug studies, *d*-fenfluramine was dissolved in physiological saline.

All testing took place in subjects' home cages between 1000 and 1400 h using their standard diet as the test food. Animals were satiated prior to drug administrations by placing fresh chow pellets on home cage floors for at least 1 h before the first injection. At least 2 days separated each testing session. Subjects' body weights were measured daily throughout these testing periods.

Experiment 1

The purpose of this experiment was to examine the effects of centrally injected 5-HT on NPY-induced feeding. A fixed dose of 235 pmol NPY was used based on observations (2,32) that this dose elicits a robust feeding response in both sites, which was confirmed by pilot studies in our own lab. Nine rats with PVN cannulae and 13 rats with PFH-aimed cannulae were tested in this phase. Baseline NPY feeding responses were first obtained, then testing with NPY and 5-HT took place. Subjects received six double injections of drug solutions in a semirandomized, counterbalanced order. The drug combinations consisted of NPY + saline, NPY and one of three doses of 5-HT (6.3, 12.5, or 25.0 nmol), or double saline injections. The two injections were given approximately 10 min apart, with the NPY injection given first to account for the approximate 10-min latency of NPY's action to induce feeding observed in our pilot studies. Food intake measurements were made 1 and 2 h after the second injection.

Experiment 2

The purpose of this experiment was to determine if peripherally injected *d*-fenfluramine would inhibit NPY-induced feeding responses. Based on previous results (2) and our own pilot data, the effects of three doses of *d*-fenfluramine (0.32, 0.63, and 1.25 mg/kg, IP) as well as saline were tested in combination with hypothalamic infusions of NPY. A total of eight PVN- and 13 PFH-cannulated rats were tested in this phase. Of these, three PVN and nine PFH animals used in Experiment 1 were employed. As described in Experiment 1, subjects were first satiated for at least 1 h prior to testing and then were injected systemically with the appropriate dose of *d*-fenfluramine or saline. After 30 min, NPY was injected centrally in the same dose and volume as described in Experiment 1. Food intake was then measured 1 and 2 h after the second injection.

Histology

At the conclusion of testing, rats were sacrificed with an overdose of sodium pentobarbital, then perfused through the heart using isotonic saline followed by a 10% formalin solution. Prior to perfusion, a 0.1-μl injection of fast green dye was injected into each hypothalamic site to facilitate localization of cannula tips. After fixation in formalin solution, brains were sliced coronally at 40-μm intervals in a cryostat. Representative sections were retained for mounting on slides, dehydration, and staining with cresyl violet before being examined by light microscopy to determine the exact location of cannula tips.

Data Analysis

All intake data were analyzed by Student's *t*-tests or appropriate analyses of variance (ANOVAs) using SYSTAT™ software. Two-tailed α levels were set at 0.05.

RESULTS

Cannulae Placements

The location of cannula tips is shown in Fig. 1. Two rats from the PFH group in Experiment 1 had inaccurate placements so their data were not included in the study. All other subjects had accurate placements or, in the occasional instance where the quality of histology did not allow for confident anatomical localizations, their feeding responses were comparable to those obtained in the other animals studied as well as by other investigators (31,32). One PVN animal became ill before completion of Experiment 2, so its data were not included in that study.

Experiment 1

Figure 2 summarizes the group food intake responses across all drug conditions tested in PVN vs. PFH animals. Compared to double-saline (control) injections, NPY + saline produced the expected feeding enhancements at both 1 and 2 h [for PVN: $t(16) = 3.664$ at 1 h, 4.029 at 2 h; $p < 0.002$ and 0.001, respectively; for PFH: $t(20) = 5.412$ at 1 h, 7.103 at 2 h; both $p < 0.001$]. To assess the effects of 5-HT infusions on NPY-induced feeding, the amount eaten by each rat during the double-saline infusions was subtracted from their individual intakes during all NPY feeding tests. These difference scores were then subjected to separate two-way ANOVAs (5-HT dose × hypothalamic site) for each time pe-

FIG. 1. Cannulae placements of rats implanted in the PVN (black circles) or the PFH (black circles with stars) shown on partial reconstructions of rostral-caudal plates 23–27 from (33) positioned top-to-bottom without frame separations. Abbreviations of anatomical structures adjacent to cannula placements are: AHN = anterior hypothalamic nucleus: c = central part, p = posterior part; BST = bed nucleus of the stria terminalis: if = posterior division, interfasicular division, pr = posterior division, principle nucleus; fx = fornix; LHA = lateral hypothalamic area; MPO = medial preoptic area; opt = optic tract; PVa = anterior periventricular nucleus hypothalamus; PVH = paraventricular nucleus hypothalamus: ap = anterior parvicellular division, dp = dorsal parvicellular part, mpd = medial parvicellular part dorsal zone, pmm = posterior magnocellular part medial zone, pv = periventricular part; SBPV = subparaventricular zone hypothalamus; V3 = third ventricle; ZI = zona incerta.

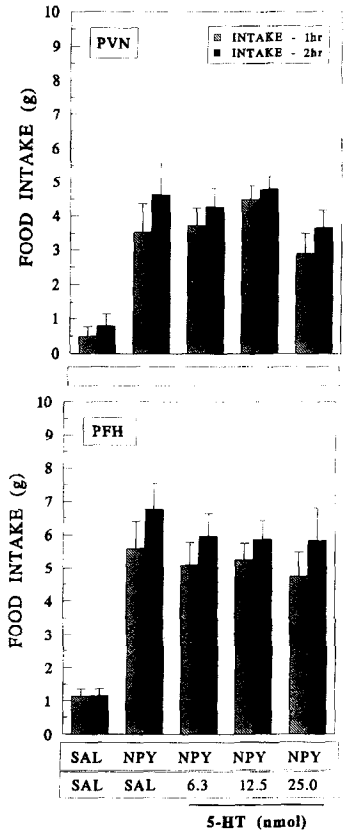

FIG. 2. The effects of central injections of 5-HT or saline in the PVN (upper frame; $n = 9$) or the PFH (lower frame; $n = 11$) on the feeding response elicited by 235 pmol of NPY injected 10 min previously. Values represent mean ± SEM g food intakes for 1 and 2 h after the second injection.

riod using repeated-measures corrections on the intake data. For 1-h feeding there was no effect of 5-HT dose but a trend, $F(1, 72) = 3.080$, $p = 0.084$, for PFH rats to have higher NPY-induced feeding in general. No interaction was found between 5-HT dose and site. For 2-h feeding there again was no significant effect of 5-HT dose nor dose × site interaction. However, the effect of injection site was now significant, $F(1, 72) = 6.994$, $p = 0.01$, indicating that PFH rats generally ate more than PVN rats. In essential confirmation of these separate two-way ANOVAs, one three-way ANOVA conducted on these difference scores (5-HT dose × hypothalamic site × hour of feeding) revealed significant effects for only site, $F(1, 72) = 5.324$, $p = 0.024$, and hour, $F(1, 72) = 20.748$, $p < 0.001$, with a trend for the site × hour interaction, $F(1, 72) = 3.625$, $p = 0.061$. This again indicated that PFH rats ate more than PVN rats.

Experiment 2

Figure 3 summarizes the group feeding responses for all drug conditions tested in PVN vs. PFH rats. To first determine if the basic NPY feeding response was quantitatively the same as that seen in Experiment 1, two two-way ANOVAS (experiment number × hypothalamic site) were performed on the 1- vs. 2-h intakes recorded after NPY + central saline in the first study vs. NPY + systemic saline in the second study. For 1-h intakes there were no reliable effects of experiment number nor interaction between experiment number and site, but a trend for a reliable site effect of PFH rats eating more than PVN rats, $F(1, 34) = 3.074$, $p = 0.089$. For 2-h intakes there again were no reliable effects of experiment number or an interaction between experiment number and site, but now the site effect was significant, $F(1, 34) = 5.477$, $p = 0.025$.

Turning to the results of Experiment 2 alone, separate two-way ANOVAs (fenfluramine dose × hypothalamic site) were performed for each time period on the feeding scores obtained by subtracting the amount eaten per animal after NPY + systemic saline from the amounts eaten after each of the three doses of hypothalamic NPY + systemic *d*-fenfluramine. For 1-h feeding, the results revealed a reliable effect of fenfluramine dose in suppressing feeding, $F(2, 57) = 5.962$, $p = 0.004$, and a trend in hypothalamic site for PFH rats to show less feeding, $F(1, 57) = 3.297$, $p = 0.075$, but no interaction between these factors. For 2-h feeding the two previous main effects were supported. That is, there was still a very reliable effect of fenfluramine dose to suppress feeding as dosage increased, $F(2, 57) = 6.449$, $p = 0.003$, with the hypothalamic site effect of less PFH feeding now reaching significance, $F(1, 57) = 6.421$, $p = 0.014$, but still no interaction between these two factors. In confirmation of these results, a three-way

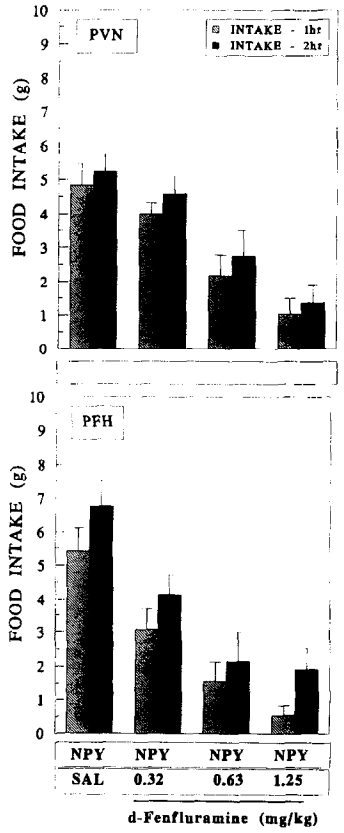

FIG. 3. The effects of peripheral injections of *d*-fenfluramine or saline on the feeding response elicited by injecting 235 pmol of NPY 30 min later into the PVN (upper frame; $n = 8$) or the PFH (lower frame; $n = 13$). Values represent mean ± SEM g food intake for 1 and 2 h after combined injections.

ANOVA (fenfluramine dose × hypothalamic site × hour of feeding) revealed a highly reliable effect of fenfluramine dose in suppressing feeding, $F(2, 57) = 7.042$, $p = 0.002$, as well as the hypothalamic site effect for relatively less intake (i.e., greater suppression) seen in PFH rats, $F(1, 57) = 5.372$, $p = 0.024$. No other reliable effects were found.

DISCUSSION

Two general findings emerged from these studies. First, in agreement with previously published work (20,22,30,31), NPY increased feeding when it was injected into either the PVN or the PFH. The second finding was that, in agreement with the recently published work of Stanley and coworkers (31), we obtained consistent evidence of relatively enhanced feeding in response to PFH injections of NPY as compared to PVN injections.

One of the primary questions posed in these studies was to determine if the robust feeding induced by infusing NPY into either hypothalamic site might be attenuated by coadministration of 5-HT. The results of our dose-response studies revealed that 5-HT failed to reduce the strong NPY-mediated feeding effects in either site. However, peripheral injections of *d*-fenfluramine, a 5-HT agonist, were highly effective in dose-dependently reducing NPY-induced feeding from either site. Indeed, at the highest dose used (1.25 mg/kg), the blockade of NPY's feeding-stimulant effects was complete if compared to the minimal feeding observed in Experiment 1 after double-saline (control) infusions.

The results obtained after coinfusions of NPY and 5-HT into the PVN can be seen as agreeing to some extent with those of Kyrkouli et al. (14), who also failed to find any attenuating effect of 5-HT coinjections into the PVN on NPY-induced feeding. However, whereas Kyrkouli et al. found some evidence that 5-HT might actually potentiate NPY feeding responses, such a pattern was not demonstrated here. In addition to these findings in the PVN, the present study reports the novel observation that coinfusions of 5-HT with NPY into the PFH are also ineffective in modifying the robust feeding elicited by this peptide. This is particularly interesting inasmuch as NPY in the PFH was more effective in eliciting feeding than were equivalent injections into the PVN. Thus, the inability of 5-HT to suppress NPY-induced feeding from either hypothalamic site suggests a more generalized nonresponsiveness of this axis to the putative anorexigenic properties of local 5-HT neurocircuitry.

The results of Experiment 1 are all the more informative in light of findings obtained from Experiment 2. The latter study showed that *d*-fenfluramine, when injected peripherally, attenuated NPY-induced feeding when this peptide was injected into either the PVN or the PFH. This attenuating effect of *d*-fenfluramine was clearly dose dependent, with the highest dose producing virtual complete blockade of the NPY feeding response as mentioned above. These findings generally parallel those reported previously (2) in which a dose range of 0.63 to 2.5 mg/kg *d*-fenfluramine significantly attenuated the enhanced feeding elicited by injecting 235 pmol NPY into the PVN. The present study not only replicated this finding and extended it to include the PFH, but provided additional evidence that this dose-dependent feeding suppression appears to be reliably greater in the PFH. This site difference owes solely to the fact that NPY + systemic saline was a more potent feeding stimulus when the peptide was applied to the PFH. Because previous work (24) has shown that systemic *d*-fenfluramine releases endogenous 5-HT in the PFH, albeit in higher doses (i.e., 3 or 10 mg/kg) than used here, the fact that direct injections of 5-HT into the PFH had no effect on NPY feeding in Experiment 1 leads us to suspect that the potent anorexigenic effects of systemic fenfluramine have little to do with such local 5-HT release in either hypothalamic site. It remains to be determined if these fenfluramine-induced suppressions of feeding elicited by NPY in the PVN or PFH are wholly peripheral (e.g., gastrointestinal) in origin or come about because of some combination of both peripheral and nonhypothalamic central action(s).

In summary, the results of these two experiments suggest that although the PFH response to NPY was significantly more enhanced than that of the PVN, the 5-HT controls over NPY feeding are likely to be similar within these two regions. When 5-HT was injected into either site it did not attenuate the feeding induced by coinjections of NPY. However, because peripherally injected *d*-fenfluramine dose-dependently attenuated NPY-induced feeding from either site, it seems that the peripheral and/or central mechanisms that mediate such feeding suppression interact in common, but as yet unspecified, ways with the feeding signal(s) that emanates from both hypothalamic regions.

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