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Pharmacology, Biochemistry and Behavior 71 (2002) 681–690

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Parabrachial infusion of D-fenfluramine reduces food intake Blockade by the 5-HT_{1B} antagonist SB-216641

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Received 26 July 2001; received in revised form 11 October 2001; accepted 18 October 2001

Abstract

Systemic administration of the serotonin (5-HT) releaser/reuptake inhibitor, D-fenfluramine decreases consumption of food in mammals. This hypophagic action involves loci at several levels of the neuraxis. Indirect evidence implicates the parabrachial nucleus (PBN) of the pons as one of these regions. Consistent with this hypothesis, unilateral infusion of D-fenfluramine (200, 280, and 400 nmol/0.5 μl) directly into the lateral PBN (LPBN) of male rats reduced food intake by 33%, 56%, and 66% from baseline (7.3 ± 0.7 g) during a 30-min test with chow. Infusions lateral, medial, and dorsal to the PBN were ineffective. Stimulating 5-HT_{1B} receptors in the PBN also reduces feeding. Administration of the selective 5-HT_{1B} agonist CP-93,129 (3-(1,2,5,6-tetrahydropyrid-4-yl)pyrrolo[3,2-*b*]pyrid-5-one) (0, 0.625, 2.5, and 10 nmol/0.5 μl) into the PBN reduced food intake by 25–79%. The selective 5-HT_{1B} antagonist SB-216641 (*N*-[3-[3-(dimethylamino(ethoxy]-4-methoxyphenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1′-biphenyl]-4-carboxamide) (2.5 nmol) completely blocked the hypophagic action of the approximate ED_{50} doses of CP-93,129 (2.5 nmol) and D-fenfluramine (280 nmol). These data strongly suggest that directly or indirectly activating 5-HT_{1B} receptors in the LPBN inhibits feeding and implicates this pontine region in the serotonergic regulation of eating and satiation.

Keywords: Ingestive behavior; Satiety; Anorexia; Serotonin; Brainstem; Parabrachial nucleus; 5-HT_{1B} receptors; D-Fenfluramine; CP-93,129; SB-216641

1. Introduction

Fenfluramine has been the prototypical compound for investigating the role of serotonin (5-HT) in controlling eating since the 1970s (e.g., Blundell et al., 1976). This interest came from the clinical relevance of the compound, from the demonstration that nonselective 5-HT antagonists blocked the hypophagic action of the drug (Jespersen and Scheel-Krüger, 1973; see Rowland and Carlton, 1986, for review), and from the inference that fenfluramine−especially its D-isomer—probed serotonergic function by releasing 5-HT and blocking its reuptake (Mennini et al., 1991; Crespi et al., 1997). Despite this attention, however, neither the critical anatomical site(s) nor the pharmacological mechanism(s) for the hypophagic action of fenfluramine are resolved.

In early anatomical studies, rats consumed less food after administration of fenfluramine or its active metabolite norfenfluramine into the lateral ventricles (Kruk, 1973; Rowland and Carlton, 1984; Rowland et al., 1985). Injection of fenfluramine into the lateral hypothalamus also reduced intake, although only after a protracted delay and using a large volume for delivery (Blundell and Leshem, 1973). In contrast, direct injections of smaller volumes and doses of fenfluramine, norfenfluramine or their D-isomers into the paraventricular nucleus (PVN) and other medial hypothalamic nuclei inhibited feeding more quickly in a variety of testing paradigms (Shor-Posner et al., 1986; Weiss et al., 1986, 1990; Smith et al., 1999). These results suggested that the medial hypothalamus was a primary target for fenfluramine. Nonetheless, extracellular levels of 5-HT in the paraventricular area did not correlate with the hypophagic actions produced by systemic administration of these drugs in controls or after inhibition of 5-HT synthesis (Oluyomi et al., 1994; Raiteri et al., 1995). Further, electrolytic lesions of the lateral hypothalamus enhanced (Blundell and Leshem, 1974) and radiofrequency lesions of the PVN did not change (Fletcher et al., 1993) the anorectic actions of systemic racemic or D-fenfluramine. Thus, stimulation of the hypothalamus by fenfluramine or

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0091-3057/02/$ – see front matter
PII: S0091-3057(01)00740-7

its metabolite may be sufficient but not necessary for these compounds to inhibit feeding.

More recently, Grill et al. (1997) demonstrated that systemically administered D-fenfluramine reduced food intake in decerebrate rats with supracollicular transections. Systemic fenfluramine did not inhibit feeding, however, in food-deprived rats with large electrolytic lesions of the midbrain raphe (Samanin et al., 1972; Davies et al., 1983) that probably damaged serotonergic efferents to the hindbrain (cf. Petrov et al., 1992). The brainstem therefore contains extrahypothalamic circuitry, including intrinsic 5-HT pathways, which support the hypophagic effect of these drugs. Infusion of D-fenfluramine into the fourth ventricle decreased feeding in neurologically intact rats by a mechanism that was blocked partially by the serotonergic antagonist metergoline (Grill et al., 1997). Thus, an action of D-fenfluramine in the caudal brainstem, without first acting peripherally, is adequate to reduce food intake.

The site(s) in the hindbrain that mediate ingestive actions of D-fenfluramine remain to be established. Studies using the translation of c-*fos* and other products of early immediate genes have demonstrated that systemic injection of this drug activates a pathway including the lateral parabrachial nucleus (lateral PBN; LPBN), bed nucleus of the stria terminalis (BNST) and central nucleus of the amygdala (CeNA)(Li and Rowland, 1993; Rowland et al., 2000). The PBN receives descending serotonergic afferents from the dorsal raphe nucleus (Petrov et al., 1992) and ascending serotonergic afferents from the area postrema (Lanca and van der Kooy, 1985). This region contains 5-HT reuptake sites with which D-fenfluramine could interact to elevate synaptic levels of the indoleamine (De Souza and Kuyatt, 1987). In one study (Li et al., 1994; but see also, Trifunovic and Reilly, 2001), cellular lesions of the LPBN produced by ibotenic acid attenuated both the hypophagia and expression of the c-*fos* gene in the LPBN, BNST and CeNA after systemic D-fenfluramine. Ablation of the area postrema, however, did not alter either the behavioral or the transcriptional actions of the agent in the LPBN (Rowland and Richmond, 1999). Together, the data argue that the LPBN is a primary site of action of D-fenfluramine to reduce food intake in rats. In a previous study from this laboratory, infusion of the selective 5-HT_{1B} receptor agonist, CP-93,129 (3-(1,2,5,6-tetrahydropyrid-4-yl)pyrrolo[3,2-*b*]pyrid-5-one; Macor et al., 1990) into the LPBN potently decreased food intake in a behaviorally specific manner (Lee et al., 1998). Thus, it is possible that D-fenfluramine acts presynaptically in the LPBN to enhance serotonergic neurotransmission via 5-HT_{1B} receptors to inhibit feeding.

The present study tested the hypothesis that infusing D-fenfluramine into the LPBN would decrease the consumption of food. We determined the dose-dependence and the anatomical specificity of this action. Additional experiments compared the relative potencies of D-fenfluramine and the directly acting agonist, CP-93,129, to reduce intake. Finally, we assessed whether parabrachial infusion of the selective 5-HT_{1B} antagonist, SB-216641 (*N*-[3-[3-(dimethylamino-(ethoxy]-4-methoxyphenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1′-biphenyl]-4-carboxamide; Price et al., 1997) would prevent the hypophagic effects of CP-93,129 and D-fenfluramine. SB-216641 blocked neurochemical actions of CP-93,129 in rats (Hopwood and Stamford, 2001) but this interaction has not been examined behaviorally. We report that D-fenfluramine acts via 5-HT_{1B} mechanisms in the LPBN to inhibit feeding.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats weighing 350–450 g (Taconic Farms, Germantown, NY) were housed individually in suspended wire mesh cages. Water was available freely and food was provided as described below. The AAALAC-approved animal facility was maintained on a 12-h light/dark cycle (lights on at 0600 h) at a temperature of 22–24 °C. All experiments were carried out in accordance with Federal regulations of the United States governing the use of animals for research and were approved by the Institutional Animal Care and Use Committee (IACUC) of MCP Hahnemann University.

2.2. Placement and verification of cannulae

2.2.1. Surgery

Rats were anesthetized intraperitoneally with 3.5 ml/kg equithesin, which was formulated to deliver 32 mg/kg sodium pentobarbital and 140 mg/kg chloral hydrate (both drugs from Sigma-Aldrich, St. Louis, MO). A stainless steel 26-ga guide cannula (Plastics One, Roanoke, VA) was implanted unilaterally in each rat using the flat-skull technique and a rat stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The cannula was aimed to end 4.8 mm below the surface of the skull, 1.8 mm lateral to the midline and 9.5 mm caudal to bregma. These coordinates were 1 mm above the intended infusion site in the LPBN (Paxinos and Watson, 1998). In one experiment (see below), the coordinates were varied systematically to assess the critical locus for the action of D-fenfluramine. In accordance with requirements of the IACUC, buprenorphine hydrochloride (Sigma-Aldrich) was administered (0.2 mg/ml/kg) to all animals for postoperative pain immediately after recovery from the anesthesia, twice 8 h apart on the day after surgery, and once on the next day. A stainless steel obturator (33 ga) that ended flush with the tip of the cannula remained in place except when injections were made.

2.2.2. Histological analysis

At the conclusion of each experiment, animals were anesthetized deeply and perfused transcardially using a

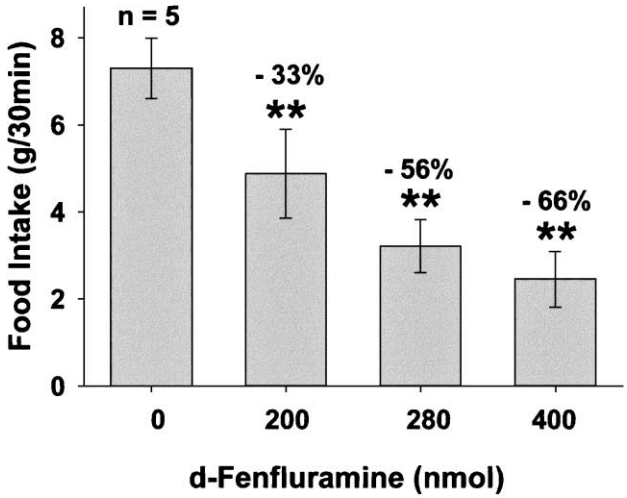

Fig. 1. Infusion of D-fenfluramine (D-FEN) into the LPBN reduces food intake in a dose-related manner. Data represent amounts of chow consumed (means ± S.E.) during 30-min period immediately after infusion of D-FEN or its vehicle (0 dose). **Differs significantly from baseline (0 dose): $P < .01$, Student's–Newman–Keuls test after significant ANOVA.

peristaltic pump (Cole Parmer Instrument, Vernon Hills, IL) with 10% phosphate-buffered formalin (pH 7.4). The brains were removed, frozen at − 16 °C in a Leica cryostat, model CM3050 (Leica, Deerfield, IL) and 40-μm sections were taken for verification of the placement after staining with Cresyl violet. To identify the sites of infusion, the stained sections were projected using a Camera Lucida (Bausch and Lomb, Rochester, NY) onto templates modified from the atlas of Paxinos and Watson (1998).

2.3. Testing procedure

Prior to surgery, animals were allowed ad libitum access to food and water. Five to seven days after surgery, the rats were adapted to a daily schedule in which food was removed from the cage at 1000 h and 26 g of standard pelleted chow (Purina, St. Louis, MO) was provided 4 h later, at 1400 h (see Lee et al., 1998). This amount was equal to 95–100% of the average daily intake of the rats during 24-h free feeding. The food consumed during the first 30 min access (i.e., 1400–1430 h) served as the measure of intake for the tests. Under these conditions, baseline 30-min intakes stabilized within 10–14 days in a range of 5–7 g corrected for minimal (< 0.5 g) spillage. After this adaptation period, some large crumbs generally remained in the cage at 1000 h the next morning. Rats gained weight on this regimen. For example, the group of five rats used for the dose–response study testing D-fenfluramine (see below) weighed 393 ± 28 g (mean ± S.E.) at the time of surgery and 413 ± 25 g 22 days later.

Drugs or saline were infused into the PBN using a stainless steel microinjector (33 ga; Plastics One), extending

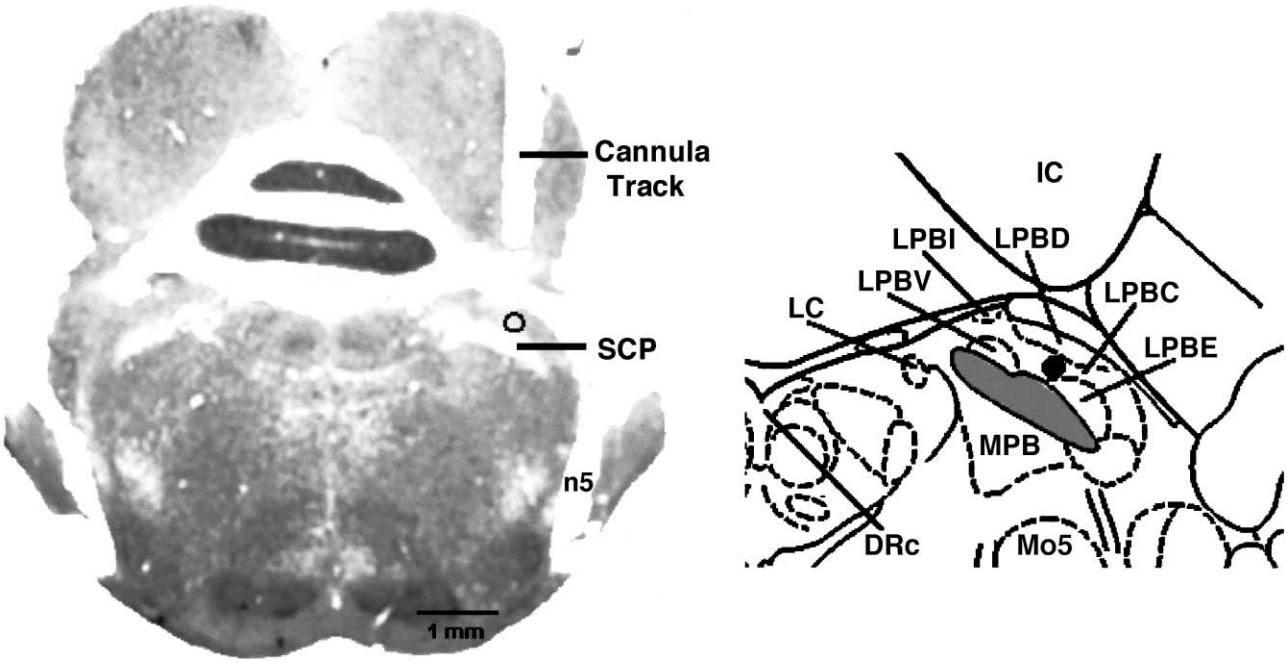

Fig. 2. Anatomical localization of the sites of infusions of D-FEN in rats used for experiment reported in Fig. 1. The photomicrograph on the left depicts the extent of damage observed from the guide cannula track and the distance from the cannula tip to the site of infusion from the injector (circle) in a representative 40 μm coronal section stained with Cresyl violet. SCP, superior cerebellar peduncle (brachium conjunctivum); n5, trigeminal (fifth) nerve. Right panel: Schematic enlarged from the parabrachial region depicting a typical site of infusion in the lateral parabrachial nucleus (LPB). The sites of infusion in each of the five rats from this group were within the ventral (LPBV), central (LPBC) or external (LPBE) subnuclei. Gray area is brachium. DRc, dorsal raphe nucleus, caudal aspect; IC, inferior colliculus; LC, locus coeruleus; LPBD, lateral dorsal subnucleus; LPBI, lateral internal subnucleus; MPB, medial parabrachial nucleus; Mo5, motor nucleus of the trigeminal nerve. Schematic modified after the atlas of Paxinos and Watson (1998).

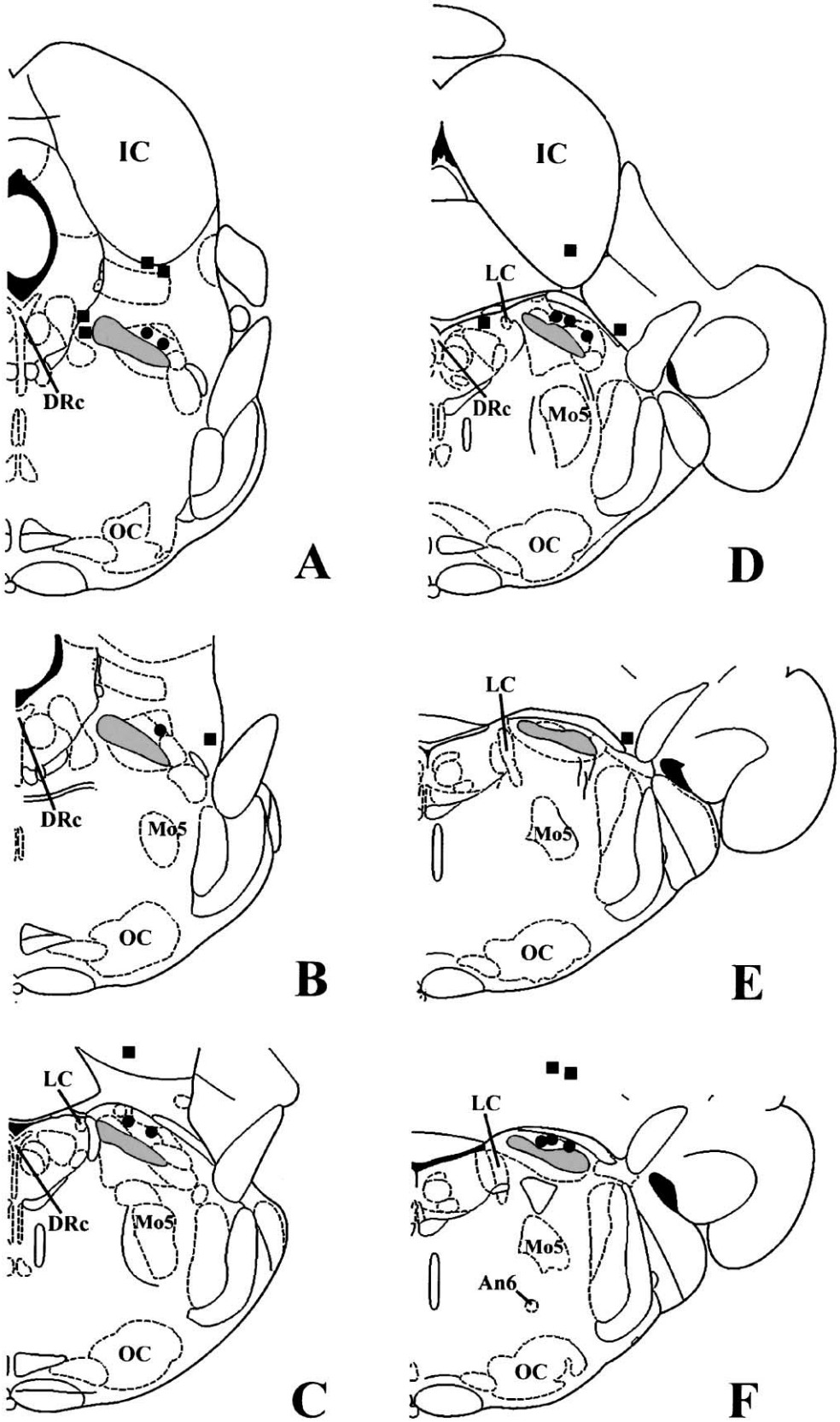
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Table 1
Anatomical specificity of the hypophagic action of parabrachial D-FEN

		Food intake (g/30 min)	
Infusion site	*N*	Vehicle	D-FEN
Within LPBN	6	5.7 ± 0.6	2.9 ± 0.4**
Dorsal (1.0 mm)	6	5.6 ± 0.6	4.9 ± 0.5
Medial (1.1 mm)	3	4.7 ± 0.4	4.4 ± 0.8
Lateral (0.6 mm)	3	6.0 ± 0.5	5.7 ± 0.5

Values are means ± S.E.; sites give distances from locus within LPBN. LPBN and dorsal injections were made in the same rats.

** Differs significantly from intake after vehicle and after dorsal injection of D-FEN (280 nmol): $P < .01$, Student's–Newman–Keuls test after significant two-factor repeated-measures ANOVA for the data from these six rats.

1 mm beyond the end of the guide cannula. The injector was connected to a 10-μl microsyringe (Hamilton, Reno, NV) via PE-20 tubing. All drugs were dissolved in 0.15 M sterile saline on the day of the experiment and infused at a rate of 0.5 μl/90 s using a Harvard Apparatus Model 975 Infusion Pump (South Natick, MA). Injectors were left in place for 30 s after infusion to minimize backflow. Food was provided immediately after each rat was returned to its home cage and intake, corrected for spillage, was measured to 0.1 g precision for the next 30 min. Experiments began after the mean baselines varied less than 10% on three successive days (i.e., 10–14 days after surgery).

2.4. Experimental design and statistical analysis

2.4.1. Dose–response for the hypophagic action of D-fenfluramine

Five rats were infused into the LPBN with 200, 280 and 400 nmol of D-fenfluramine hydrochloride (MW = 268; 200 nmol = 53 μg; RBI, Natick, MA). Saline was administered on the day before each infusion of drug; no infusion was made on the day after D-fenfluramine although all other conditions remained the same. Thus, 2 days intervened between successive tests with D-fenfluramine. For each rat, the mean of the three pretest days was taken as the baseline for statistical analysis. Data were analyzed by one-way repeated-measures ANOVA followed by Student's–Newman–Keuls tests for pairwise comparisons of means using the Sigma Stat v2.0 software program (Jandel Scientific, San Rafael, CA). An alpha level of $P < .05$ was the threshold for statistical significance.

2.4.2. Anatomical locus for the hypophagic action of D-fenfluramine

The placements of guide cannulae were varied systematically in 12 rats. In six (LPBN/dorsal group), the cannulae were implanted 1 mm dorsal to the reference coordinates given above for the dose–response study. Thus, these ended 2 mm above the usual site of infusion in the LPBN. In three (medial group), cannulae were aimed 1.1 mm medial to the reference and in three other rats (lateral group) implants ended 0.6 mm laterally. Once baselines stabilized, medial and lateral rats were infused with vehicle on 2 days followed the next day by the 280-nmol dose of D-fenfluramine. Three of the other six rats (LPBN/dorsal group) were infused with this dose into the usual site in the LPBN. Drug was infused 1 mm dorsal to this site in the remaining three rats. The sites of infusion were counterbalanced such that D-fenfluramine was administered into the LPBN target and also into the locus 1 mm dorsal to the target, in each of the six rats in this group. Data for this group were analyzed by two-factor repeated-measures ANOVA followed by Student's–Newman–Keuls tests for pairwise post hoc comparisons.

2.4.3. Hypophagic action of CP-93,129

We replicated the dose–response function for this 5-HT_{1B} agonist to reduce food intake because we changed the vendor for our rats (cf. Lee et al., 1998). Doses of 0.625, 2.5 and 10.0 nmol of CP-93,129 (3-(1,2,5,6-tetrahydropyrid-4-yl)pyrrolo[3,2-*b*]pyrid-5-one; MW = 215; a gift of Pfizer Central Research, Groton, CT) were infused into 12 rats. The testing conditions were identical to those used for the D-fenfluramine dose–response study.

2.4.4. Antagonism of the hypophagic actions of CP-93,129 and D-fenfluramine by SB-216641

Eight rats were implanted unilaterally in the LPBN and their baselines stabilized (5 days). A series of tests were then conducted in which we assessed whether 2.5 nmol of the 5-HT_{1B} partial agonist/antagonist SB-216641 (*N*-[3-[3-(dimethylamino(ethoxy]-4-methoxyphenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1′-biphenyl]-4-carboxamide; MW = 523; Tocris, Ballwin, MO) would antagonize the hypophagic actions of CP-93,129 (2.5 nmol) and D-fenfluramine (280 nmol). The doses of CP-93,129 and D-fenfluramine were those estimated to reduce food intake by 50% (i.e., the ED_{50}s) in the dose–response studies. The dose of the antagonist was chosen to be equipotent with CP-93,129; in radioligand binding, SB-216641 displayed 1–8 nM affinity (Price et al., 1997) and CP-93,129 displayed 15 nM affinity (Macor et al., 1990) for 5-HT_{1B} sites.

On the first test day, the rats were infused twice, 10 min apart, with saline vehicle (Veh/Veh treatment combination). The 30-min intake after this treatment was used as the

Fig. 3. The lateral subnuclei contain the critical site for the hypophagic action of D-FEN infused into the parabrachial region. Twelve naïve rats were tested with the 280-nmol dose of D-FEN. Six rats were infused at two sites: one within the LPBN (circles) and another site 1 mm dorsal to the LPBN (squares above circles). Three other rats were infused more medially (panels A and D) and the last three rats more laterally (panels B, D and E). Only the infusions into the LPBN reduced food intake. Abbreviations as in Fig. 2 and: OC, olivary complex; An6, accessory abducens (sixth nerve) nucleus. Schematics modified after the atlas of Paxinos and Watson (1998).

baseline. On the second day, an appropriate combination of treatments including drug(s) was delivered (e.g., an infusion of saline vehicle followed by CP-93,129; the Veh/CP combination). On the day after this test, rats were handled without receiving infusions and fed on the usual schedule. This 3-day testing cycle (double vehicle infusion; treatment combination including drug; handling but no infusion) was continued until each rat received each treatment combination. The order of drug combinations for four of the rats was: Veh/CP; SB-216641 plus vehicle (SB/Veh); SB/CP; vehicle plus D-fenfluramine (Veh/Fen); and SB/Fen. The order of combinations for the other four rats was: SB/Veh; SB/CP; Veh/CP; SB/Fen; and Veh/Fen. Data were analyzed by repeated-measures ANOVA followed by Student's–Newman–Keuls test.

3. Results

3.1. Parabrachial infusion of D-fenfluramine decreases food intake

Infusion of D-fenfluramine into the lateral parabrachial region reduced 30 min food intake in a dose-related manner (Fig. 1) [$F(3,12) = 61.88$, $P < .01$]. The ED_{50} for this action was approximately 280 nmol. All five placements of the injector tips were localized within the lateral subnuclei of the PBN (Fig. 2) and within coronal planes ranging from the level at which the motor nucleus of the trigeminal nerve appeared rostrally to the level of the accessory abducens nucleus caudally (Fig. 3).

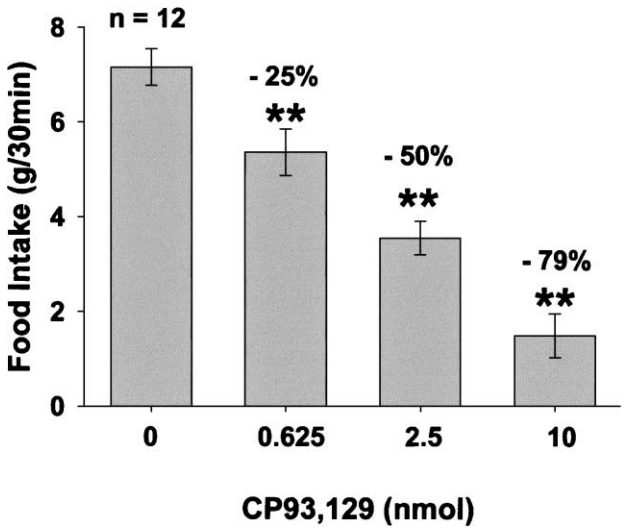

Fig. 4. Infusion of the directly acting 5-HT_{1B} receptor agonist, CP-93,129 into the LPBN reduces food intake in a dose-related manner. Infusion loci were verified at the coronal levels depicted in panels B/C ($N = 10$) and D/E ($N = 2$) of Fig. 2. Values are mean ± S.E. intakes for 30-min tests. **Differs significantly from baseline (0 dose): $P < .01$, Student's–Newman–Keuls test after significant ANOVA.

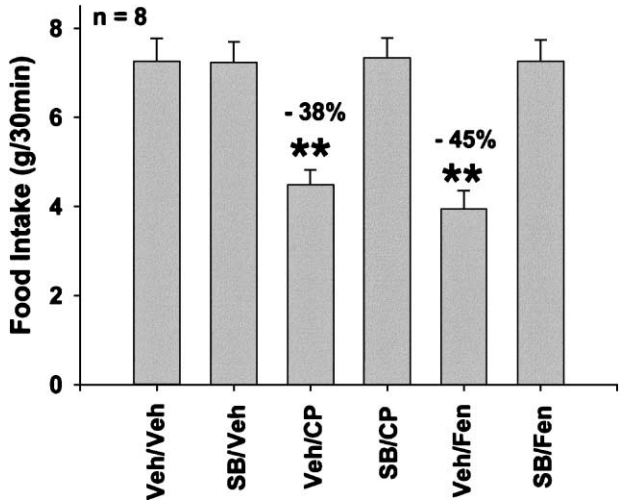

Fig. 5. Parabrachial pretreatment with the 5-HT_{1B} antagonist SB-216641 (2.5 nmol) prevents the hypophagic actions of CP-93,129 (2.5 nmol) and D-FEN (280 nmol). Infusion loci were in the LPBN at Levels C ($N = 1$), E ($N = 1$) and F ($N = 6$). **Differs significantly from intake after Veh/Veh and SB/drug: $P < .01$, Student's–Newman–Keuls test after significant ANOVA.

3.2. Localization of the hypophagic action of parabrachial D-fenfluramine

Fig. 3 shows the distribution of infusion sites for the 12 rats in which the coordinates were varied systematically. In six rats, infusion of 280 nmol of this agent into the LPBN reduced intake by 49%, whereas administration 1 mm more dorsally in the same rats was ineffective (Table 1). D-Fenfluramine was ineffective also when delivered 1.1 mm medial or 0.6 mm lateral to the LPBN in two other groups of three rats each.

3.3. Inhibition of food intake by CP-93,129

Fig. 4 shows that infusion of the directly acting 5-HT_{1B} agonist CP-93,129 into the LPBN decreased food intake [$F(3,33) = 47.99$, $P < .01$], 100-fold more potently than did D-fenfluramine.

3.4. Antagonism by SB-216641

In a separate group of eight rats, pretreatment with 2.5 nmol of the 5-HT_{1B} antagonist, SB-216641, blocked completely the hypophagic effects of the ED_{50} doses of CP-93,129 and D-fenfluramine (Fig. 5).

4. Discussion

The results of the present study establish that administering D-fenfluramine into the lateral parabrachial region decreases the intake of pelleted chow by rats. The ED_{50}

($\sim$74 μg) was within the range for reducing consumption of a glucose solution that was ingested passively through an oral catheter (ED_{50} $\sim$120 μg) or actively from a sipper tube (ED_{50} $\sim$30 μg) after infusion of drug into the fourth ventricle in nondeprived rats (Grill et al., 1997). Direct intraparenchymal administration of a drug into a critical locus should be more potent than intralumenal delivery. Comparison between these two studies is problematic, however, because of the distinct difference between our testing conditions requiring chewing and swallowing of familiar hard food and theirs involving a situation-specific, palatable liquid diet. Furthermore, the fourth ventricular route provides bilateral access to the caudal site(s) at which D-fenfluramine acts to influence eating. Certainly, parabrachial administration would have been more potent if we had used bilateral rather than unilateral infusion of drug. It might be questioned whether D-fenfluramine diffused from the LPBN to an active site elsewhere in the hindbrain. The failures of infusions into sites more lateral, medial or dorsal to the effective parabrachial loci to inhibit eating militated against such a hypothesis. Diffusion along the peduncle may have contributed to the distribution of responsive sites for a considerable distance in the rostrocaudal dimension. Overall, the data argue strongly that the stimulation by D-fenfluramine of one (or several) subnuclei of the LPBN is sufficient to reduce food intake.

Systemic injection of reasonable, hypophagic doses of D-fenfluramine increases c-*fos*-like immunoreactivity (FLI) in the lateral central, lateral superior and Kölliker–Fuse nuclei, and strong FLI in the lateral external subnucleus (Li and Rowland, 1993; Li et al., 1994). Given our 0.5-μl volume, the central, lateral and external subnuclei were likely bathed by each of our successful infusions. Notably, Li et al. (1994) reported that excitotoxic cellular lesions of the lateral parabrachial region eliminated the transcriptional action on the expression of the c-*fos* gene in the external lateral subnucleus (but not the others) and attenuated the hypophagic effect of systemic D-fenfluramine. The lesions also reduced dramatically the c-*fos* activation by this agent in the major targets of the LPBN in the BNST and CeNA, but not in ingestive areas of the medial hypothalamus. These data implied that the external lateral subnucleus is critical for at least part of the response to D-fenfluramine. Recently though, Trifunovic and Reilly (2001) conducted a more extensive dose–response study and reported that larger cellular lesions of the LPBN, including the external lateral subnucleus, did not affect D-fenfluramine-induced hypophagia. Interestingly, lesions of the medial PBN enhanced the behavioral action of D-fenfluramine. One speculation, therefore, is that the wider lesion in their study produced damage that counteracted the effects of cytotoxicity in the external lateral subnucleus. Notwithstanding this controversy, our data establish that mechanisms within the lateral parabrachial region are sufficient for D-fenfluramine to initiate inhibitory processes in eating.

4.1. Pharmacological mechanism

The neurochemical mechanisms responsible for hypophagic effects of D-fenfluramine are uncertain. Fourteen subtypes of serotonergic receptors have been cloned from mammals (for a review, see Barnes and Sharp, 1999). Pharmacological data have implicated two of these—5-HT_{1B} and 5-HT_{2C} receptors—in the inhibitory control of eating by central serotonergic neurotransmission (for reviews, see Simansky, 1996a; Clifton, 2000; De Vry and Schreiber, 2000). In the present study, pretreatment in the LPBN with the 5-HT_{1B} receptor antagonist, SB-216641, reversed completely the hypophagic effect of the ED_{50} dose of D-fenfluramine and also of the directly acting 5-HT_{1B} agonist CP-93,129. In cortical membranes from rats, CP-93,129 displayed 15 nM affinity for 5-HT_{1B} receptors. This was 200-, 150-, >700- and 400-fold more avid than binding at 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2A} and 5-HT_{2C} receptors (Macor et al., 1990). SB-216641 displayed 1 nM affinity for human 5-HT_{1B} receptors and this was 500-, 25-, 50- and 160-fold selective (Price et al., 1997). Rodent and human 5-HT_{1B} receptors differ pharmacologically for some classes of antagonists (esp., β-adrenergic receptor antagonists; see Barnes and Sharp, 1999), but 0.2 μM SB-216641 blocked neurochemical actions of 0.3 μM CP-93,129 in brain slices from rats (Hopwood and Stamford, 2001). Thus, our data are consistent with a role for parabrachial 5-HT_{1B} receptors in the decrease in food intake produced by local administration of D-fenfluramine.

The 5-HT_{1B} receptors have been implicated in mediating fenfluramine-induced hypophagia by the failure of the racemic form of this drug to reduce food intake in mutant mice with deletion of the 5-HT_{1B} receptor gene (Lucas et al., 1998). In earlier pharmacological studies, the 5-HT_{1A}/5-HT_{1B} antagonist cyanopindolol (Neill and Cooper, 1989; Grignaschi and Samanin, 1992; Grignaschi et al., 1995), but not the 5-HT_{1A} antagonist, WAY-100,635 (Vickers et al., 1996), antagonized D-fenfluramine. These data support a role for the 5-HT_{1B} subclass. More recently, however, neither cyanopindolol nor the much more selective 5-HT_{1B} antagonists GR-127,935 and SB-224289 affected D-fenfluramine-induced hypophagia (Vickers et al., 2001). Instead, 5-HT_{2C} receptors appear to be involved. This conclusion agreed with previous studies using antagonists (e.g., Neill and Cooper, 1989; Grignaschi and Samanin, 1992), 5-HT_{2C} knockout mice (Vickers et al., 1999) and detailed behavioral comparison of D-fenfluramine and a 5-HT_{2C} direct agonist (Clifton et al., 2000). In rats, 5-HT_{2C} receptors in the LPBN do inhibit sodium and fluid consumption but without influencing sucrose intake (Menani et al., 1996, 1998). Parabrachial CP-93,129 did not alter water intake in our experiments (Lee et al., 1998). We have not analyzed systematically the relative contributions of the 1B and 2C subtypes in the LPBN to different ingestive processes. It is possible that these receptors segregate serotonergic functions in consummatory behavior in this

area of the pons. Together the data from lesions, systemic pharmacology and local intracerebral infusions suggest that 5-HT_{1B} receptor activation in the LPBN is sufficient but not necessary for D-fenfluramine to decrease consumption of food.

D-Fenfluramine was 100-fold less potent than the direct agonist CP-93,129 to inhibit feeding after infusion into the LPBN. This difference may reflect the similar ratio between the estimated 50% concentration (EC_{50}) for D-fenfluramine to release 5-HT in rat brain (1.78 μM; Mennini et al., 1991) and the EC_{50} for CP-93,129 to inhibit adenylyl cyclase (0.026 μM; Macor et al., 1990). Once released by D-fenfluramine, 5-HT has identical potency to CP-93,129 in its cellular actions at 5-HT_{1B} receptors (Mennini et al., 1991). In addition to acting on nonserotonergic neurons as inhibitory heteroreceptors, 5-HT_{1B} receptors serve an autoreceptor function to inhibit release of 5-HT (see Barnes and Sharp, 1999). Activating these autoreceptors therefore would be expected to increase rather than decrease food intake. Thus, the effects of CP-93,129 and D-fenfluramine to reduce eating in the LPBN must be due to interactions with 5-HT_{1B} sites on nonserotonergic neurons.

D-Fenfluramine is de-ethylated to an active metabolite, D-norfenfluramine, which also inhibits eating (Mennini et al., 1991; Gibson et al., 1993; Oluyomi et al., 1994; Rowland et al., 2000). Unlike its parent, which has 7–61 μM affinity for 5-HT_{1B} and 5-HT_{2C} receptors, D-norfenfluramine has an affinity for the 2C subtype (0.3 μM; 5-HT_{1B}, 13 μM) that falls within the concentration range achieved by hypophagic doses in vivo (Mennini et al., 1991). Thus, the metabolite can act directly at 5-HT_{2C} receptors to reduce intake. Fenfluramine is metabolized by brain tissue (Sherman and Gál, 1977), but it is unlikely that this occurs within the timeframe of the current experiments. Thus, after infusion into the LPBN, D-fenfluramine acted to inhibit eating by releasing neuronal 5-HT to stimulate postsynaptic 5-HT_{1B} receptors.

Several reports would appear to conflict with this hypothesis. Although some evidence exists that serotonergic neurotoxins interfere with the hypophagic action of fenfluramine (Clineschmidt, 1973), most studies have demonstrated either no effect or enhanced responsiveness after such lesions (e.g., Hollister et al., 1975; Carlton and Rowland, 1984; Rowland et al., 2000). Systemic pretreatment with the 5-HT synthesis inhibitor *p*-chlorophenylalanine (pCPA) did not alter the hypophagic action of systemic D-fenfluramine (Gibson et al., 1993; Oluyomi et al., 1994; Rowland et al., 2000), although it did deplete hypothalamic 5-HT as measured by microdialysis (Oluyomi et al., 1994). It is possible, though not assessed, that neither the neurotoxins nor the synthesis inhibition eliminated 5-HT in the PBN (see Javed et al., 1997). Thus, this locus could mediate actions of systemic fenfluramine. Under some conditions, large electrolytic lesions of the midbrain raphe that probably encroached on the 5-HT cells that project to the PBN did prevent fenfluramine-induced hypophagia (Samanin et al., 1972; Davies et al., 1983). Thus, a more directed manipulation of parabrachial 5-HT should provide new information about this locus in behavioral actions of D-fenfluramine. Complementary serotonergic mechanisms may be located in the periphery (Simansky, 1996b).

4.2. Functional relevance

The PBN is a major node in the brainstem where information from numerous sensory modalities is integrated to regulate diverse autonomic and behavioral functions, including respiration, cardiovascular activity and ingestion (Chamberlin and Saper, 1994; Jhamandas et al., 1991; Saper, 1995). The LPBN, for example, has been implicated in the increased eating produced by antimetabolites (Calingasan and Ritter, 1993; Horn and Friedman, 1998), decreased eating produced by satiety factors (Trifunovic and Reilly, 2001); detection of aversive and appetitive ingestants from the oral cavity and gastrointestinal sites (Yamamoto and Sawa, 2000; Yamamoto et al., 1994); and formation of associations between taste and other feeding-related stimuli and visceral consequences (e.g., Grigson et al., 1998; Reilly and Trifunovic, 2000). To accomplish such complex tasks, afferent input to the PBN is sorted among and then projected from nine subnuclei arranged around the brachium conjunctivum (or superior cerebellar peduncle) in the dorsolateral pons and the Kölliker–Fuse subnucleus just ventrolateral to the peduncle (Fulwiler and Saper, 1984; Saper, 1995; Karimnamazi and Travers, 1998). Gustatory function is supported, for example, by a heavy projection from the nucleus tractus solitarius (NTS) to the medial PBN, by several subnuclei of the LPBN and by neurons within the waist area across the brachium. These different parabrachial subregions project qualitatively different taste information to separate rostral targets (Norgren, 1976; Norgren and Pfaffman, 1975; Karimnamazi and Travers, 1998). Also relevant for feeding, second-order gastrointestinal afferents innervate much of the lateral region (Saper, 1995). This innervation sometimes overlaps with the gustatory areas of the LPBN; to a limited extent visceral and taste-related neurotransmission converge on the same cells (Hermann and Rogers, 1985).

In a previous study (Lee et al., 1998), we demonstrated that infusion of CP-93,129 into the LPBN not only reduced food consumption but also produced behaviorally selective changes typical of enhanced satiation (Lee et al., 1998). These data mimicked those observed after systemic administration of the 5-HT_{1B} agonist, CP-94,253. This structural analogue of CP-93,129 reduced the size of meals by enhancing the satiating effect of ingested food rather than by altering the hedonics of the tastant (Lee and Simansky, 1997). The present study did not analyze the behavioral organization of feeding after parabrachial administration of D-fenfluramine. Superficial observations suggested that the rats ate normally without displaying motor deficits or disordered meals. Thus, a logical, testable

hypothesis is that CP-93,129 and D-fenfluramine promote satiation via parabrachial 5-HT_{1B} mechanisms by increasing the signal strength of negative feedback from the gastrointestinal system.

In conclusion, infusion of the indirectly acting serotonergic agonist, D-fenfluramine, into the lateral parabrachial region of the dorsal pons reduced food intake in rats. The action was anatomically specific for the LPBN and blocked by the 5-HT_{1B} antagonist, SB-216641. Together with existing data, the current evidence suggests that serotonergic innervation to the lateral subnuclei, probably of raphe origin, modulates the value of visceral feedback relevant for satiation. Activating these serotonergic mechanisms is sufficient—although possibly not necessary—for the onset of satiety. The implication of this serotonergic terminal field in regulating food intake furthers previous calls to direct attention at the hindbrain in the ingestive role of 5-HT (Menani et al., 1996; Grill et al., 1997; Lee et al., 1998).

Acknowledgments

The authors are grateful to Dr. Vincent J. Aloyo for his helpful discussion of this research and criticism of the manuscript and to Pfizer Central Research for supplying CP-93,129. U.S. Public Health Service Grant MH-41987 to K.J.S. supported this work.

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