Dexfenfluramine and norfenfluramine: comparison of mechanism of action in feeding and brain Fos-ir studies

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Dexfenfluramine and norfenfluramine: comparison of mechanism of action in feeding and brain Fos-ir studies. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R390–R399, 2000.—Dexfenfluramine (dF) and dexnorfenfluramine (dNF), its metabolite, are anorectic agents that release serotonin (5-HT) and may have a direct postsynaptic action. The effects on the anorectic effects of dF and dNF of either acute (p-chlorophenylalanine, PCPA) or chronic (5,7-dihydroxytryptamine, 5,7-DHT) brain 5-HT depletions were studied in rats and compared with the actions of a 5-HT uptake inhibitor (fluoxetine) and 5-HT1B/2C receptor agonists [1-(3-trifluoromethyl-phenyl)-piperazine and 1-(3-chlorophenyl) piperazine]. The anorexia caused by these agonists was enhanced in rats with 5,7-DHT lesions, possibly a result of receptor supersensitivity. In contrast, fluoxetine anorexia was somewhat reduced in one study and was unchanged in a second. Both dF and dNF anorexiasts were enhanced in rats with 5,7-DHT lesions. In contrast, the anorectic effects of either dF or dNF were unchanged in PCPA-treated rats relative to controls. Compared with controls, 5,7-DHT-lesion rats showed greatly increased dF- and dNF-induced Fos-immunoreactivity (ir) in the paraventricular (PVN) and supraoptic (SON) hypothalamic nuclei, and in the median preoptic area (MnPO), but were similar to controls in most other areas. PCPA pretreatment increased dF- and dNF-induced Fos-ir in the PVN, SON, and MnPO. In controls, equianorectic doses of dF and dNF induced Fos-ir in similar brain regions, but dNF produced relatively larger effects than dF in SON, PVN, and MnPO. The data are discussed in terms of multiple pathways in the anorectic actions of dF and dNF.

Fos-immunoreactivity; paraventricular hypothalamus; serotonin; caudate nucleus; cerebral cortex; p-chloro-phenylalanine; 5,7-dihydroxytryptamine; supraoptic nucleus; median preoptic nucleus; anorexia; serotonin receptor subtypes

DEXFENFLURAMINE (dF) reduces food intake and body weight in both humans and animals. dF is absorbed rapidly after oral administration, and is dealkylated to dexnorfenfluramine (dNF), which is approximately twice as potent an anorectic as the parent dF. The rate of dealkylation varies between species. In humans, dealkylation is slow and the half-life is long, so that levels of dF exceed those of dNF for many hours after acute administration as well as during chronic dosing. In rats, dealkylation is fast and the half-life is relatively short, so that levels of dNF exceed those of dF within 1–2 h of acute dosing. In both species, however, some fraction of the long-term anorectic effects of dF will be due to dNF (34, 35).

Both dF and dNF release serotonin (5-HT) from brain synaptosomal and slice preparations and also inhibit competitively the reuptake of 5-HT (1, 3, 8, 30, 31). Broad-spectrum 5-HT receptor antagonists usually attenuate dF-dNF anorexia (6, 9, 20, 34), suggesting that the postsynaptic actions of released 5-HT underlies this effect. It also has been suggested that increased 5-HT release may be sufficient but is not necessary for the anorectic effect of dF (7, 29, 30). Based on studies with either selective antagonists or receptor gene knock-out mice it appears that the 5-HT1B/2C subtype(s) underlies much of the anorectic action of dF (5, 7, 15, 16, 20, 27, 40, 41, 43). In vitro, dNF has high (µM) affinity for 5-HT2C receptors (8). Anorexia caused by dNF was attenuated by chronic prior administration of the 5-HT2C agonist, 1-(3-chlorophenyl) piperazine (mCPP) (7, 16), and was enhanced by prior 5-HT depletion with p-chlorophenylalanine (PCPA) (7). These data suggest the development of postsynaptic 5-HT2C receptor supersensitivity. In contrast, the efficacy of dF was not affected by PCPA pretreatment (7).

One prototypical treatment for producing receptor supersensitivity is chronic lesion of presynaptic neurons. Several studies have examined the anorectic effect of (racemic) fenfluramine in rats with either raphe nucleus lesions or 5,7-dihydroxytryptamine (5,7-DHT)-induced 5-HT neuron degeneration, but the outcomes have been inconsistent (reviewed in Ref. 34). This confusion is perhaps understandable because a reduced presynaptic ability of these agents to release 5-HT may counterbalance any postsynaptic receptor supersensitivity. However, this lesion model should discriminate between agents with primarily postsynaptic actions and those with primarily presynaptic actions. Thus, if the foregoing mechanisms of action of dF and dNF are correct, we would expect that dF anorexia will be attenuated more than dNF anorexia in DHT-lesioned rats. However, published studies indicate that the anorectic effect of racemic fenfluramine is enhanced, not attenuated, in 5,7-DHT-lesion rats relative to controls (2, 12, 34). Thus these discrepancies need to be revisited using the 5-HT selective d-enantiomer.
Additionally, we can use the 5-HT depletion paradigm to examine potential sites of action of these agents in the brain. Studies from our own and other laboratories have used induction of the early gene c-fos through measurement of either its mRNA or translated protein Fos. This is thought to be an index of neuronal activation (33), and the induced gene products can be localized anatomically. Regions showing strong activation in rat brain after dF include the nucleus of the solitary tract (NST), lateral parabigral nucleus external division (LPBE), parvocellular hypothalamic paraventricular nucleus (PVNp), bed nucleus of the stria terminalis (BST), dorsomedial caudate-putamen (CPD), central nucleus of amygdala (CeA), and cerebral cortex (Ctx) (13, 23–25, 32). Many of these areas also show Fos-immunoreactivity (ir) after administration of the 5-HT2C agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (22, 29, 30). Excitotoxic lesions of the LPBE markedly attenuate dF-induced Fos in BST and CeA but not in CPD or PVNp, and also reduce but do not abolish acute dF anorexia (25).

In the present series of experiments, we studied the effects of 5,7-DHT lesions on the anorectic effects of the 5-HT uptake inhibitor fluoxetine, dF, dNF, and the direct 5-HT1B/C receptor agonists, 1-(3-trifluoromethylphenyl)-piperazine (TFMPP) and mCPP in food-deprived rats. In an attempt to relate these feeding data to regional actions of these agents in brain, we also examined the effects of 5,7-DHT lesions on the regional pattern of Fos-ir induced in brain by dF, dNF, and TFMPP. We compared this result to the effects of depletion of 5-HT stores by PCPA on both anorexia and the induction of Fos-ir by dF and dNF.

MATERIALS AND METHODS

Animals and housing. Sprague-Dawley rats (Harlan, Indianapolis, IN) of 3–6 mo of age and either sex were used. No systematic differences in the results were observed between males and females. All rats were housed individually in stainless steel cages suspended over absorbent, deotized animal cage board (Shepherd Specialty Papers, Kalamazoo, MI), and with Purina Rodent Chow 5001 pellets and tap water available ad libitum except as noted. The vivarium was temperature controlled (23°C) and illuminated 0600–1800 h. All studies were performed near the middle part of the daytime.

Pharmacological agents and procedures. All of the anorectic agents (HCl salts) were administered subcutaneously, and all dosages are expressed as the salt. dF, obtained from Servier (Neuilly, France), was administered at a dose of 2 mg/kg, which acutely inhibits food intake in rats by about 75% (7, 34). dNF (Servier), was administered at a dose of 1 mg/kg, which usually produces about the same degree of anorexia as 2 mg dF/kg. TFMPP, mCPP, and fluoxetine were from Research Biochemicals International (Natick, MA). dF, dNF, mCPP, and TFMPP were dissolved in 0.15 M NaCl and fluoxetine in water for injected volumes of 1 ml/kg.

Rats prepared with 5-HT-depleting brain lesions were anesthetized with Equithesin (3 ml/kg) and cotreated with desipramine HCl (Sigma Chemical, St. Louis, MO; 20 mg/kg in water ip). With use of a standard stereotaxic procedure and a flat skull, bilateral injections were made into the lateral cerebral ventricles of either 5,7-DHT (220 µg 5,7-DHT creatine sulfate, from Sigma, per side in 5-µl vehicle) or the vehicle (0.1% ascorbic acid). Desipramine prevents uptake of 5,7-DHT into noradrenergic neurons. Rats were allowed at least 2 wk after the surgery prior to testing.

Acute 5-HT depletions were produced with three daily injections of dl-p-chlorophenylalanine methyl ester HCl (Sigma, 150 mg·kg⁻¹·injection⁻¹ ip) dissolved in water (75 µg/ml); control rats received water (2 ml/kg ip). Although not measured in the present study, this PCPA regimen reliably produces ∼90% depletions of forebrain 5-HT (12, 38).

5,7-DHT studies. Three studies were performed. In the first, eight male rats (4 mo of age and average weight 350 g at the time of surgery) were prepared with 5,7-DHT lesions and eight controls received ascorbic acid vehicle. Feeding tests were started 2–3 wk after surgery. Rats were deprived of food but not water for 24 h and then received an injection of either the vehicle (1 ml/kg) or fluoxetine (10 mg/kg). Three weighed food pellets were presented in the cage 20 min after the injection, and food intake was recorded 1 h later. Spillage was collected on papers under the cages and was allowed for in the calculation of intakes. Four days later, the procedure was repeated, with the rats receiving the other treatment. Another 4 days later, the test was repeated with all rats receiving dF (2 mg/kg). Two weeks later, the rats were injected with dF (2 mg/kg) and were perfused 2 h later for determination of Fos-ir as will be described.

In the second study, eight male rats (−450 g) received 5,7-DHT lesions as previously described and eight controls received vehicle. Starting 2 wk after the lesion, the rats were tested as previously described for the anorectic effect of dNF (1 mg/kg) and TFMPP (0.5 mg/kg). After at least 1 wk of recovery from these tests, the rats were injected with either dNF (1 mg/kg) or TFMPP (0.5 mg/kg) and perfused 2 h later for determination of Fos-ir. To document the efficacy of the 5,7-DHT lesion, additional forebrain sections were immunostained for the 5-HT transporter protein (44), which allows visualization of the 5-HT-ergic axons as they ramify through the brain.

The purpose of the third study was to repeat the behavioral tests of the first two studies this time in the same animals. Nine male rats (−350 g) received 5,7-DHT lesions as previously described and 10 controls received ascorbic acid vehicle. Starting 2 wk after the lesion, and at 3–4-day intervals, the rats were given feeding tests. The first three tests, performed in counterbalanced order, were with injections of either vehicle, mCPP (2 mg/kg), or fluoxetine (10 mg/kg). In a pilot study using other rats and several dosages, we determined that 2 mg mCPP/kg reduced 1-h food intake of deprived rats from 5.0 ± 0.5 to 3.1 ± 0.3 g. The next three tests, also counterbalanced, were with injections of either vehicle, dF (2 mg/kg), or dNF (1 mg/kg). All rats then received a test with a low dose of mCPP (0.5 mg/kg), which in the pilot study we found did not significantly reduce food intake. Last, at least 1 wk after the last drug test, the rats were killed for quantification of the lesion using [3H]paroxetine binding to presynaptic uptake sites.

PCPA studies. For the behavioral study, 72 male rats (5 mo of age, ~480 g) were divided into two groups and received three daily injections of either PCPA or water. Food was removed from all rats at the time of the third injection. On the fourth day, after 24-h food deprivation, six rats from each treatment group received either vehicle, dF (0.5 or 2 mg/kg), or dNF (0.25 or 1 mg/kg), and food intake was measured after 1 h and again after 4 h.

For the immunocytochemistry study, male rats (3 mo of age, ~300 g) were divided into two groups and received three daily injections of either PCPA or water. On the fourth day,
rats from each treatment group received either dF (2 mg/kg, n = 3) or dNF (1 mg/kg, n = 6–8) and were perfused 2 h later for determination of Fos-ir. One rat from each group served as controls and received acute saline injection.

Perfusions and immunostaining procedure. The rats were anesthetized (pentobarbital sodium, 100 mg/kg) 2 h after the drug injections and were perfused through the heart with heparinized saline followed by buffered paraformaldehyde. Brains were removed, postfixed at 4°C overnight, and then were cut coronally at a thickness of 100 µm on a vibratome. In most studies, sections were incubated with a c-Fos polyclonal antibody (Santa Cruz SC52; Santa Cruz, CA; 1:20,000 dilution, 48 h at 4°C), biotinylated secondary antibody, and avidin-biotin complex (Vectastain) as previously described (23–25). In the second 5,7-DHT study, additional sections were incubated with antibody to the 5-HT transporter protein [1:10,000; a gift from Dr. F. Zhou, University of Indiana (44)]. Sections were then mounted on slides and examined using a microscope with a video attachment. Regions previously identified as responsive to dF (23) first were examined qualitatively, and then the intensity of Fos-ir staining was ranked using a qualitative scale ranging from 0 to 4 (0, none; 1, least; 2, moderate; 3, heavy; 4, heaviest), as described before (23–25). In selected regions, the number of Fos-ir cells was counted from the monitor by an observer blind to the treatment condition of the rats.

Paroxetine binding. Rats from the third 5,7-DHT study were injected with pentobarbital sodium (100 mg/kg), and as soon as they showed no reflexes they were decapitated and the brain was removed. Free-hand dissection of frontal Ctx and hypothalamus was performed, and the tissues were frozen (−70°C) immediately. The tissues were subsequently homogenized in Tris buffer, centrifuged, and washed twice, and aliquots were incubated (2 h at room temperature) with a saturating concentration (10 nM) of [3H]paroxetine (DuPont-NEN), followed by washing three times with ice-cold Tris, and the disks were immersed in Scintiverse (Fisher) for scintillation counting. Protein concentration of the homogenate was determined using the bicinchoninic acid method (Pierce Laboratories). Specific binding was the difference between total and nonspecific binding, expressed relative to total protein. Protein concentrations were determined using the bicinchoninic acid method (Pierce Laboratories). Specific binding was the difference between total and nonspecific binding, expressed relative to total protein. We have shown previously that this method yields similar results to direct measurement of synaptosomal 5-HT uptake in 5,7-DHT rats and is a useful quantifiable index of the extent of presynaptic terminal and neuronal loss (36, 39).

Statistical analyses. Absolute food intakes were analyzed for significance (P < 0.05) using one- or multi-way analyses of variance (ANOVA), followed by Newman-Keuls post hoc comparisons. Fos-ir ratings were compared between treatment groups using pairwise Mann-Whitney tests. Counts of Fos-ir cells were compared using ANOVA and post hoc test. Paroxetine binding in 5,7-DHT rats was examined as a group (t-test vs. controls) and on an individual basis as percentage of the control mean.

RESULTS

5,7-DHT behavioral studies. At the doses chosen, fluoxetine (10 mg/kg), dF (2 mg/kg), and dNF (1 mg/kg) produced comparable mean suppressions of food intake (40–60%) in the intact control rats. In the first batch of rats with 5,7-DHT lesions, the intakes in the dF and fluoxetine portions of the study were analyzed by separate two-way ANOVA to test for group × drug interactions against the vehicle. The data are shown in Fig. 1A. Fluoxetine had its expected anorectic effect (ANOVA P < 0.001) and the group × drug interaction approached significance (P < 0.08). The anorectic action of fluoxetine was attenuated in the rats with 5,7-DHT lesions compared with controls (mean intakes 75 and 33% of vehicle, respectively). The anorectic effect of dF was also significant (P < 0.001). The group × drug interaction did not approach significance (P > 0.2) due to relatively large within-group variability, but the suppression was nominally larger in lesion rats than in controls (mean intakes 18 and 52% of vehicle, respectively). Because these animals were used for Fos-ir, the extent of the lesion was not quantified in this study, so we cannot know whether the individual variability was related to the extent of 5-HT lesion.

In the second batch of 5,7-DHT-treated rats, intakes in the dNF and TMMP portions of the study were analyzed by separate two-way ANOVA to test for group × drug interactions against the vehicle. The data are shown in Fig. 1B. The anorectic effect of dNF treatment was highly significant (P < 0.001) but the
The two-way ANOVA was significant and with A and then by group, and the results are shown in Fig. and mCPP) intakes were analyzed by two-way ANOVA. In the first (treatments were vehicle, fluoxetine, and 5,7-DHT rats was prepared. These rats were then used in two sets of drug treatments. In the first (treatments were vehicle, fluoxetine, and 5,7-DHT rats were analyzed by two-way ANOVA. In the second set of studies with these rats (treatments were vehicle, dF, and dNF), significant main effects and interaction of group and drug were again obtained (P < 0.01), and the results are shown in Fig. 2A. The two-way ANOVA was significant and with significant main effects of group, drug, and group × drug interaction (P < 0.01). The source of this interaction was the greater suppression of intake by mCPP in lesion rats compared with controls (mean intakes 7 and 79% of vehicle, respectively). In contrast, the effect of fluoxetine was similar in both lesion and control rats (mean intakes 50 and 53% of vehicle, respectively).

In the second set of studies with these rats (treatments were vehicle, dF, and dNF), significant main effects and interaction of group and drug were again obtained (P < 0.01), and the results are shown in Fig.

2B. Whereas both dF and dNF had only modest anorectic effects in the intact rats, they were highly effective in the lesion rats. In contrast to the previous lesion study using dF, the within-group variability was quite small in the present study.

The number of 5-HT uptake sites in 5,7-DHT-treated rats compared with controls determined by paroxetine binding was 20 ± 3% (means ± SE, range 9–32%) in frontal Ctx and 17 ± 2% (range 10–32%) in hypothalamus, in both cases a highly significant (P < 0.001) reduction. There were no significant correlations between the extent of 5-HT depletion and the anorectic responses within the lesion group.

5,7-DHT Fos-ir studies. In agreement with our previous report (23), moderate but submaximal Fos-ir was induced by dF administration to control rats in frontal Ctx, CPd, BST, CeA, and PVNm (Table 1). Less consistent Fos-ir was also induced in other regions, including the supraoptic nucleus (SON), organum vasculosum laminae terminalis-median preoptic area (MnPO), and magnocellular paraventricular nucleus (PVNm). All of these regions showed dF-related Fos-ir in rats with 5,7-DHT lesions. However, the ratings were much higher in MnPO, SON, PVNm, and PVNm of the lesion rats compared with controls. Typical photomicrographs are shown in Fig. 3. By way of validation of these ratings, cell counts were made in the PVNm (235 ± 29 in the PVNm, 22 ± 10 in controls, P < 0.05) and a ~1 mm² area of CPd (98 ± 18 lesion vs. 90 ± 14 control).

The pattern of Fos-ir induced in control brains by dNF was generally similar to that induced by dF (Table 1). However, at these approximately equianorectic doses, it is of note that dNF appeared to induce substantially

<table>
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<tr>
<th>Brain Structure</th>
<th>dF</th>
<th>dNF</th>
<th>TFMPP</th>
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<tr>
<td>Frontal cortex</td>
<td>2.0</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Caudate-putamen</td>
<td>2.2</td>
<td>2.3</td>
<td>2.1</td>
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<tr>
<td>Bed nucleus striatal terminus</td>
<td>2.5</td>
<td>3.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Median preoptic nucleus</td>
<td>0.5*</td>
<td>2.1*</td>
<td>1.8</td>
</tr>
<tr>
<td>Supraoptic nucleus</td>
<td>1.0*</td>
<td>2.2</td>
<td>0.2</td>
</tr>
<tr>
<td>PVN parvocellular</td>
<td>1.7</td>
<td>4.0*</td>
<td>1.8</td>
</tr>
<tr>
<td>PVN magnocellular</td>
<td>0.7</td>
<td>2.0</td>
<td>0.2</td>
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<tr>
<td>Central nucleus</td>
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<tr>
<td>Amygdala</td>
<td>2.7</td>
<td>3.0</td>
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<td>Arcuate nucleus</td>
<td>1.0</td>
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Means rounded down to 1 decimal place; n = 4/group except lesion-dF when n = 6. Not shown are data from vehicle-injected controls and 5,7-DHT rats (n = 2) in which ratings were 0–1 in all regions. dF, dexfenfluramine (2 mg/kg injections); dNF, dexnorfenfluramine (1 mg/kg injections); TFMPP, 1-(3-trifluoromethyl-phenyl)piperazine (0.5 mg/kg injections); ir, immunoreactivity; 5,7-DHT, 5,7-dihydroxytryptamine; PVN, paraventricular nucleus. Scale: 0, no Fos-ir; 1, sparse; 2, light to moderate; 3, moderate to strong; 4, very strong, most cells. *P < 0.05 (median test) lesion group differs from corresponding control group.

Table 1. Mean ratings of Fos-ir in brain regions after injection of either dF, dNF, or TFMPP in control rats and rats with serotonin-depleting (5,7-DHT) brain lesions

Fig. 2. A: 1-h intake of chow (means ± SE) after 24-h food deprivation in rats with either control surgery or 5,7-DHT lesions following acute injection of either vehicle (veh), fluoxetine (flx, 10 mg/kg), or 1-(3-chlorophenyl)piperazine (mCPP, 2 mg/kg). *P < 0.05 less than intake after veh. △P < 0.05 5,7-DHT group differs from control. B: same as A (samers rats) after either dF (2 mg/kg) or dNF (1 mg/kg).
more Fos-ir in MnPO, SON, and both PVNm and PVNp than dF. It should be cautioned that in this study the dF and dNF rats were from different batches, and therefore no statistical comparison was made between these drugs. As was the case with dF, 5,7-DHT lesions increased dNF-induced Fos-ir in MnPO, SON, and PVNm above levels seen in controls. Because these are the regions that in controls show higher Fos-ir after dNF compared with dF, and the induced Fos-ir is near maximal after either agent in lesion rats, then the fractional difference between control and lesion rats is smaller after dNF than dF.

The pattern of Fos-ir induced in control brains by TFMPP was generally similar to or less than that induced by dF, with the exception of heavier immunostaining in the MnPO (Table 1). However, there were no differences in Fos-ir between lesion and control groups treated with TFMPP.
The extent of the lesion in the second study was assessed qualitatively from the 5-HT transporter immunostaining. In regions such as Ctx and CPd, intact rats show a dense network of immunostained fine-caliber fibers, corresponding to published 5-HT fluorescence or antibody data (42, 44). In contrast, all 5,7-DHT-treated rats showed an almost complete loss of 5-HT transporter-labeled axons and terminals, consistent with extensive neurodegeneration. This is also complementary to the quantitative paroxetine binding results reported previously, but the immunocytochemical method appears to be less sensitive because it does not reveal the ~20% of remaining uptake sites.

PCPA behavioral study. PCPA-treated rats lost a mean of 41 g body wt during the 4-day treatment plus deprivation period, significantly more than the loss in water-injected controls (22 g, $P < 0.01$). The food intake data on the test day were analyzed first by two-way ANOVA. PCPA-treated rats consumed less food overall than the water controls (ANOVA main effect of pretreatment, $P < 0.001$); the pretreatment × treatment interaction term in the ANOVA was not significant ($P > 0.2$). Subsequent one-way ANOVA with post hoc tests were performed for each group. As expected in the control group both dF (2 mg/kg) and dNF (1 mg/kg) produced reliable ($P < 0.05$) reductions in food intake relative to the corresponding vehicle group (Fig. 4). dNF (72% mean suppression) was significantly ($P < 0.05$) more effective than dF (40% mean suppression) after 1 h. The fourfold lower doses of either agent did not produce significant anorexia in any group. The PCPA-pretreated groups showed similar degrees of anorexia to the water groups (77 and 38% mean suppressions for dNF and dF, respectively, but these did not differ from each other).

All of these effects comparing the PCPA and water pretreated groups were quite similar after both 1 and 4 h (Fig. 4). Interestingly, the anorectic effect of dF was sustained after 4 h, especially in the PCPA group, but the effect of dNF was no longer present and some compensatory feeding occurred.

PCPA Fos-ir study. Rats pretreated with PCPA showed a consistent change in the dF-induced expression of Fos-ir (Fig. 5 and Table 2). First, in the CPd and Ctx there was a marked decrease in the ratio of Fos-ir intensity to Control rats. Cell counts in ~1 mm² of these regions confirmed this conclusion: CPd $207 \pm 30$ and $120 \pm 12$ (means ± SE, $P = 0.05$, Mann-Whitney test), frontal Ctx $147 \pm 3$ and $83 \pm 17$ ($P = 0.05$), in the control and PCPA groups, respectively. Second, in regions such as the BST and CeA there was no obvious difference. Finally, in the SON, PVNm, and MnPO, regions that normally show low Fos-ir after dF, there was a very robust expression of Fos-ir (means ± SE cell count in SON: $3 \pm 2$ and $250 \pm 62$, $P = 0.05$) for control and PCPA-pretreated groups, respectively. Additional rats given PCPA pretreatment only had no Fos-ir in these regions, indicating that the increase in the SON and PVNm was due to dF.

Rats pretreated with PCPA did not show corresponding changes in dNF-induced expression of Fos-ir (Table 2). However, it should be noted that this may be in part because of differences in Fos-ir induced (in control rats) by these two agents in these regions and consistent with the ratings data in Table 1 from the previous experiment. Thus, in vehicle-pretreated control rats, the cell counts after dNF were comparable to those in the PCPA-dF group.

DISCUSSION

It has been reported previously that rats with 5,7-DHT lesions are resistant to the anorectic effect of the selective 5-HT reuptake inhibitor sertraline (28). In marked contrast, the anorectic response to another reuptake inhibitor, fluoxetine, was normal in rats with 5-HT loss following either cerebroventricular (9) or raphe (4) injections of 5,7-DHT. The reason for the differing results between these two inhibitors has never been explained satisfactorily. The present 5,7-DHT lesion rats showed no consistent change in fluoxetine anorexia, supporting the previous study. That study used a lower dose of 5,7-DHT (9) than in the present work in which the 5,7-DHT regimen was the same as

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that used in the sertraline study (28). This suggests that there is a difference in the mechanisms of action of these two inhibitors. Further studies using these two agents in the same rats will be needed to advance the analysis of this discrepancy.

In the present study we showed that both dF and dNF caused a two- to threefold greater suppression of intake in rats with 5,7-DHT lesions. We and others previously found a similar effect with racemic fenfluramine (12, 34). Various other 5-HT-depleting lesions have been reported to either have no effect or attenuate fenfluramine anorexia (34) but to our knowledge the present study is the first in which both fluoxetine and dF have been tested in the same animals. The same 5-HT-depleted rats showed normal fluoxetine but enhanced dF anorexia, strongly indicating a differential dependence of these two agents on the integrity of presynaptic 5-HT processes. Ideally, a range of doses

Fig. 5. Representative photomicrographs of Fos-ir in rats pretreated with either PCPA or its vehicle and treated acutely with either dF (2 mg/kg) or dNF (1 mg/kg). Shown are MnPO on midline and ventral to anterior commissure, PVN, and supraoptic nucleus (SON) at approximately its mid rostrocaudal extent. Approximate actual widths of tissue section in each photomicrograph are 1 mm. Note higher levels of staining by dNF compared with dF in all 3 regions and in PCPA-pretreated group after dF.
should be compared in control and lesion rats to make a quantitative statement concerning altered sensitivity. Such a protocol was not used in the present study because of the complication of tolerance with repeated injections of these agents (24) or, at higher doses, 5-HT depletion. Instead, we limited each rat to only one or two treatments with dF or dNF.

These data contrast with our observation of enhanced anorectic efficacy of 5-HT$_{1B/2C}$ receptor agonists TFMPP and mCPP, a finding consistent with the development of postsynaptic receptor supersensitivity. It has been shown that the 5-HT-releasing action of dF (and presumably of dNF) is greatly diminished in 5,7-DHT-lesion rats, but that basal levels are comparable (17). If release of 5-HT underlies the anorectic action of dF and dNF in lesion rats, then functional postsynaptic 5-HT$_{1B/2C}$ receptor supersensitivity must outweigh the diminished release. Another possibility is that both dF and dNF have direct postsynaptic actions, a possibility that has been raised by others for dNF but not for dF (7, 13, 26, 42), producing total loss of 5-HT content in fibers in some regions of the brain (42) and overall at least 90% loss of 5-HT content. The only apparent differences between our protocol and that of Gibson et al. (7) are the heavier weight of our rats (480 g vs. 220 g at the start of the studies) and the route of administration of the anorectic agents (subcutaneous in our study vs. intraperitoneal). We do not believe that these are likely to be critical differences because other studies, including those from our laboratory, have used rats of varying weights and both subcutaneous and intraperitoneal routes of administration of dF without major differences. Finally, it has been shown that this regimen of PCPA did not affect fluoxetine (per os)-induced anorexia (26).

One rationale for using PCPA rather than 5,7-DHT for presynaptic depletion of 5-HT is that the shorter time course of the PCPA protocol might minimize any time-dependent compensatory changes, such as receptor supersensitivity. An analogous situation occurs in the much more extensively studied dopamine depletion paradigm with regard to supersensitivity to agonists that occurs both after reserpine (short term) and 6-hydroxydopamine lesion (long term) dopamine depletion and using both behavior and induced Fos-ir as measures (18, 19). In these models, two classes of dopamine receptor apparently can rapidly change their functional coupling in the face of reduced transmitter release; we know of no analogous investigations in 5-HT systems.

We studied the induction of Fos-ir as a potential marker of cellular activation in an attempt to identify crucial sites of action. Based on our behavioral data with 5,7-DHT, we hypothesized first that TFMPP might induce Fos-ir in similar regions to dF and dNF and second that there would be clear evidence of an enhanced response in the lesion rats. The first of these predictions was borne out in most brain regions, with the exception of the absence of activation by TFMPP in the SON and PVNm. The second prediction was not supported because the intensity of TFMPP-induced Fos-ir was not increased in the lesion rats. It could be argued that we did not examine the correct regions, but we did inspect all of the regions that have been previously implicated in dF anorexia (13, 23, 25, 32). It could also be argued that c-fos may not be the critical early response gene induced at the receptor subtype engaged by TFMPP. Although it is possible that additional transcription factor(s) are induced by TFMPP, the fact that Fos-ir was induced in many regions demonstrates the adequacy of this agent to activate this marker in those regions.

Given the lack of effect of 5,7-DHT lesions on TFMPP-induced Fos-ir and only modest effects of the lesions on dF and dNF anorexia, we did not expect to see marked differences between control and lesioned animals in dF and dNF-induced Fos-ir. This was true in most regions of the brain, with the notable exception of large increases in the MnPO, SON, and PVNm of lesioned rats compared with control. This is a drug effect rather than

### Table 2. Mean ratings of Fos-ir in brain regions after injection of either dF or dNF in control and PCPA-pretreated rats

<table>
<thead>
<tr>
<th>Brain Structure</th>
<th>Control</th>
<th>PCPA</th>
<th>Control</th>
<th>PCPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal cortex</td>
<td>2.0</td>
<td>1.0*</td>
<td>2.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Caudate-putamen</td>
<td>1.5</td>
<td>0.8*</td>
<td>2.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Bed nucleus stria terminalis</td>
<td>3.0</td>
<td>2.3</td>
<td>3.7</td>
<td>3.2</td>
</tr>
<tr>
<td>Median preoptic nucleus</td>
<td>1.0</td>
<td>2.0*</td>
<td>2.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Supraoptic nucleus</td>
<td>0.3</td>
<td>3.0*</td>
<td>3.5</td>
<td>2.9</td>
</tr>
<tr>
<td>PVN parvocellular</td>
<td>1.2</td>
<td>3.7*</td>
<td>3.0</td>
<td>3.2</td>
</tr>
<tr>
<td>PVN magnocellular</td>
<td>0.5</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Central nucleus amygdala</td>
<td>2.5</td>
<td>3.0</td>
<td>3.7</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Means rounded down to 1 decimal place; n = 3 or 4/group for dF study and n = 6–8 for dNF study. Scale: 0, no Fos-ir; 1, sparse; 2, light to moderate; 3, moderate to strong; 4, very strong, most cells. *P < 0.05 (median test) p-chlorophenylalanine (PCPA) group differs from corresponding control group.

### References

1. Gibson et al. (7) are the heavier weight of our rats (220 g at the start of the studies) and the route of administration of the anorectic agents (subcutaneous in our study vs. intraperitoneal). We do not believe that these are likely to be critical differences because other studies, including those from our laboratory, have used rats of varying weights and both subcutaneous and intraperitoneal routes of administration of dF without major differences. Finally, it has been shown that this regimen of PCPA did not affect fluoxetine (per os)-induced anorexia (26). The authors of this latter work hypothesized, but did not test directly, that fluoxetine might also act directly at 5-HT$_{2C}$ receptors.

One rationale for using PCPA rather than 5,7-DHT for presynaptic depletion of 5-HT is that the shorter time course of the PCPA protocol might minimize any time-dependent compensatory changes, such as receptor supersensitivity. An analogous situation occurs in the much more extensively studied dopamine depletion paradigm with regard to supersensitivity to agonists that occurs both after reserpine (short term) and 6-hydroxydopamine lesion (long term) dopamine depletion and using both behavior and induced Fos-ir as measures (18, 19). In these models, two classes of dopamine receptor apparently can rapidly change their functional coupling in the face of reduced transmitter release; we know of no analogous investigations in 5-HT systems.

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a sequel of the lesion because rats bearing these lesions and injected with vehicle showed no Fos-ir in the brain except in regions such as pyriform cortex in which Fos-ir is constitutive (data not shown). The MnPO, SON, and PVNm have been especially implicated in body fluid regulation and show Fos-ir during dehydration (33). At these doses, neither dF nor dNF has known effects on fluid intake of intact rats (34, 35), although we have found that the low dose of 1 mg dF/kg greatly reduced sodium appetite following acute sodium depletion (37), a behavior in which the MnPO is critically involved (33). Water intake following drug injections was not measured in the lesion rats in this study. It is of considerable interest that the SON and PVNm were regions that were not at all activated by the direct agonist TFMPP. Thus the Fos-ir in these regions of dF and/or dNF-treated lesion rats either is due to 5-HT released onto another type of receptor that does show supersensitivity or to an action of these drugs on other transmitter system(s). Lesion rats treated with dF also showed increased Fos-ir in the PVNm.

The PCPA Fos-ir data generally complement the 5,7-DHT results. While these studies were in progress, another laboratory reported that treatment with PCPA in the same regimen produced no change in most regions but an increase in dF-induced Fos-ir in SON and PVNm (13). We ran a small group of animals to confirm this observation and larger series to examine the effect with dNF. In contrast to the increase in these regions after dF, as well as a decrease in CPd and frontal Ctx, PCPA pretreatment was without effect on Fos-ir induced by dNF. This may be in part because dNF itself induced higher levels of Fos-ir in the SON and PVNm than dF and further increases may have been obscured using this method. We do not believe that such a ceiling effect is likely to be the case because, in our unrelated fluid balance work (33), we have often seen more cells and more intensely stained with Fos-ir in these regions.

In additional studies not reported, we examined the effect of the 5-HT2C receptor antagonist ritanserin on dF and dNF-induced Fos-ir. Prior work suggests that dNF has more of a 5-HT2C agonist component than dF (7), and therefore if we assume ritanserin is a highly selective antagonist at this receptor, we predicted a greater inhibition of dNF-induced Fos-ir by ritanserin compared with dF. In general, that proved to be the case, with seven or eight forebrain regions showing less dNF-induced Fos-ir after ritanserin (0.4 or 1 mg/kg) compared with dNF alone. In contrast, only two of eight regions (CPd and PVNm) showed significant ritanserin inhibition after dF. A recent report concluded that ritanserin (0.4 mg/kg) did not attenuate dF (5 mg/kg)-induced Fos-ir (14), but we note that a higher antagonist/agonist ratio was used in our study.

**Perspectives**

On the basis of these and other observations, we currently believe that at least three neural systems are activated by dF and/or dNF, the first including PVNm, CPd, and frontal Ctx; the second including LPBE, BST, and CeA; and the third including MnPO and SON. Recruitment of additional regions in 5,7-DHT and PCPA-treated rats adds a new level of complexity to interpretation of their behavioral data. Ibotenate lesions of the LPBE abolished dF-induced Fos in BST and CeA but not in other regions such as PVNm and CPd, and also partly attenuated the anorectic effect of dF (25). The recent observations that 5-HT1B receptors in the LPBE can support anorexia (21) and that mice with targeted deletion of the 5-HT1B receptor gene are resistant to the dF-induced anorexia and Fos-ir in the PVNm and CeA (27), both support and extend our LPBE lesion interpretation. Other evidence suggests that the hindbrain is involved in the action of 5-HTergic agents on food intake. Injection of dF into the fourth cerebral ventricle produced anorexia (10), and administration of the 5-HT2C receptor antagonist mesulergine into the fourth cerebral ventricle completely reversed the anorexia caused by either central or peripheral administration of the 5-HT2C agonist mCPP (15). Mice with deletion of the 5-HT2C receptor gene (41) also are resistant to the anorectic effects of dF (5). The possible contribution of other regions such as CPd and Ctx that both have the 5-HT2C receptors and show agonist-induced Fos-ir (11, 22, 29) to the anorectic effects of dF is not well understood. These agents may have direct action at these sites and have interactions with dopaminergic and glutamatergic systems (11), among others.

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