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Relationship between anorexia and loss of serotonin uptake sites in brain of mice and rats receiving *d*-norfenfluramine or *d*-fenfluramine

Neil E. Rowland*, Sana Rokadia, D. Joy Green, Kimberly Robertson

Department of Psychology, University of Florida, POB 112250, Gainesville, FL 32611-2250, USA

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Abstract

Previously, we have shown that repeated administration of *d*-fenfluramine (D-F) to rats is associated with development of tolerance to the initial anorexia, but that in mice no such tolerance seems to occur. In the first study, we show that chronic administration of neither *d*-norfenfluramine (D-NF; the principal metabolite of D-F) nor the serotonin (5-HT) 2C receptor agonist *m*-chlorophenyl-piperazine (mCPP) is associated with the development of anorectic tolerance tested using a dessert protocol. However, compared with mice receiving these drugs for the first time, both of these chronic treatments were associated with a significant attenuation of Fos immunoreactivity (Fos-ir) in several brain regions, including bed nucleus of the stria terminalis, paraventricular hypothalamus, and central nucleus of amygdala. This attenuation is similar to that described previously in rats. Because loss of efficacy of these agents could be related to loss of 5-HT transporter (5-HTT) sites, their presumptive primary mode of action, in the final study we determined the effect of various, low-dose regimens of D-F and D-NF on 5-HT uptake in frontal cortex of mice and rats. We show in mice that D-F causes a greater loss of 5-HT uptake than D-NF, and that at the lowest dose regimen used uptake was unaffected in rats but was reduced in mice. The data are discussed in terms of the species difference in behavioral tolerance and differences in neurochemical profile of D-F and D-NF.

Keywords: Species difference; Tolerance; Fos immunoreactivity; Synaptosome; Dessert test

1. Introduction

Dexfenfluramine (D-F) is the active *d*-enantiomer of fenfluramine, introduced as a clinical anorectic agent in the 1960s (Rowland and Carlton, 1986). Its N-dealkylated metabolite *d*-norfenfluramine (D-NF) is approximately twice as potent an anorectic as the parent molecule in short-term feeding studies in rats. Pharmacokinetic studies carried out in several species have shown that the half-life of D-NF is at least twice that of D-F, but with species differences in both the absolute half-lives and the rates of conversion of D-F to D-NF (Caccia et al., 1982; Fracasso et al., 1995; Steranka and Sanders-Bush, 1979). The conversion is relatively slow in both humans and mice compared with rats, consistent with observations that plasma levels of D-F exceed those of D-NF during chronic treatment in both mice and humans (Fracasso et al., 1995; Rowland, 1994; Rowland and Carlton, 1986).

There have been only a few studies of the anorectic effects of D-F in mice, and these have reported some as yet unexplained differences from the well-studied rat. For example, the anorectic potency of D-F is similar in food-deprived and nondeprived (dessert-fed) rats, but the responsiveness of mice increases fivefold in deprived compared with nondeprived condition (Souquet and Rowland, 1989). Furthermore, the repeated administration of D-F in a structured feeding protocol leads to rapid behavioral tolerance in nondeprived rats (Rowland et al., 1990), but mice show no such tolerance (Rowland et al., 2003). It has been suggested that tolerance in rats is related to brain serotonin (5-HT) depletion or atrophy of neuronal terminals caused by repeated administration of D-F (Kleven et al., 1988). This now appears unlikely in view of findings that intermittent administration of racemic fenfluramine to rats retains its full anorectic efficacy despite 5-HT depletion (Choi et al., 2002) and that behavioral tolerance occurs at doses of D-F below those known to produce 5-HT depletion (Rowland et al.,

* Corresponding author. Tel.: +1-352-392-0601x287; fax: +1-352-392-7985.
E-mail address: nrowland@ufl.edu (N.E. Rowland).

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1990). The depletion of 5-HT is dose and region specific and can be long lasting (Zaczek et al., 1990). On the basis of this and other evidence, a comprehensive analysis concluded that "D-NF plays an important but not exclusive role in the overall neurochemical response of the parent drug in rodents" (Fracasso et al., 1995). However, the expectation implicit in this analysis is that D-NF alone should produce a substantial neurochemical action, including 5-HT depletion (Caccia et al., 1993).

Due to the limited availability of D-NF, there have been very few direct tests of this idea in rats (Caccia et al., 1993), and none in mice. Our laboratory has published the only two reports of reduced feeding after D-NF administration in mice (Rowland, 1994; Rowland et al., 1990) and found D-NF to be about twice as potent as D-F. Compared with effects in rats, studies of 5-HT depletion in mice have found that considerably higher doses of D-F are needed, that the maximal depletion is lower, and that recovery from the depletion is faster (Baumann et al., 1996; Caccia et al., 1993; Fracasso et al., 1995; McCann et al., 1997; O'Callaghan and Miller, 1994; Rowland, 1994; Steranka and Sanders-Bush, 1979). This difference in depletion of 5-HT between rats and mice is found with other substituted amphetamines (O'Callaghan and Miller, 1994; Stone et al., 1987) and may, in part, result from lower brain levels of these agents in mice (Fracasso et al., 1995; Steranka and Sanders-Bush, 1979).

The purpose of the present experiments is threefold. First, given that anorectic tolerance did not develop to D-F in mice using a protocol in which tolerance develops in rats (Rowland et al., 2003), we examined whether D-NF would have a sustained behavioral effect in mice. It is possible, for example, that the conversion of D-F to D-NF and a subsequent but unknown action of D-NF may underlie the development of tolerance in rats compared with mice in which the conversion is much less marked. If this were the case, then mice injected with D-NF should show behavioral tolerance. *m*-Chlorophenyl-piperazine (mCPP) was used for comparison. While this compound, like D-NF, does cause 5-HT release, its affinity for 5-HT_{2C} receptors is almost 100-fold higher than its ED_{50} for release (Gobbi et al., 2002; Rothman and Baumann, 2002). Second, we examine whether the induction in brain of Fos immunoreactivity (Fos-ir) is attenuated after repeated compared with acute injection of D-NF. This marker is attenuated after as few as two prior injections of low doses of D-F in rats (Li and Rowland, 1996; Rowland et al., 2001b) and mice (Rowland et al., 2003). Third, we compare the effect of repeated D-F and D-NF injections on 5-HT transporter (5-HTT) capacity, a sensitive metric that has been used in prior studies of D-F-induced neuronal damage in rats (McCann et al., 1997; Rowland et al., 1990; Zaczek et al., 1990). Most studies of 5-HT depletion have used doses of drugs considerably higher than those used in behavioral work. In contrast, the doses, number, and timing of the injections used in the present studies are comparable to those that employed in the present and past behavioral protocols.

2. Material and methods

2.1. Animals and housing

Sprague–Dawley rats and ICR:CD1 mice (both from Harlan Labs, Indianapolis, IN) were housed individually in separate vivaria, each with lights on 0700–1900 h and kept at 23 ± 2 °C. Rats were housed in stainless steel cages suspended over absorbent paper, and with Purina 5001 pellets available ad libitum from a hopper at the front of the cage and water from an adjacent bottle with sipper spout. Mice were housed in polycarbonate tub cages with Sani-Chips (Harlan-Teklad, Madison, WI) bedding and with Purina pellets and water available from the overhead stainless steel lid. Use of animals in this project was approved by the Institutional Animal Care and Use Committee, University of Florida.

2.2. Feeding studies (mice)

Mice with continuous access to chow pellets were additionally presented daily for 30 min with a dessert consisting of sweet milk in a gel matrix (20 g sucrose and 20 g powdered milk/100 ml mixed with 5% gelatin and solidified; approximately 1.6 kcal/g) contained in a glass beaker. This diet was presented in the home cage using a stirrup holder (Rowland et al., 2001a, 2003). This diet was developed to avoid spillage, and none was noted in these studies. After about 1 week, intakes were stable, and mean baseline was computed for each individual, averaged over the last 3 days. Three groups of eight mice were then formed, matched for baseline intake (mean 1.62 g). On six test days conducted at 2- to 3-day intervals, mice were injected with 0.15 M saline (4 ml/kg sc), mCPP (10 mg/kg) or D-NF (2.5 mg/kg for the first 3 days then 5 mg/kg for the last 3 days) about 30 min before presentation of dessert. For statistical analysis, intakes of dessert on test days were expressed as percent of individual baselines. Neither injections nor dessert was given on the intervening days.

2.3. Fos-ir

The mice from the above behavioral study were used to compare the effect of acute vs. chronic mCPP or D-NF on Fos-ir in brain. Either 2 or 4 days after the last feeding test, the control group that had received only saline injections received either D-NF (5 mg/kg, $n = 4$) or mCPP (10 mg/kg, $n = 4$). Another four mice from each of the previous D-NF and mCPP groups received a seventh injection of the same agent. Because not all mice could be processed on the same day, two mice in each condition were done on the first day and two more 2 days later. Exactly 1 h after D-NF or mCPP

injection, mice were anesthetized with sodium pentobarbital (200 mg/kg ip) and perfused transcardially with heparinized saline then 4% paraformaldehyde. Brains were removed, placed in paraformaldehyde overnight; then 80-μm sections were cut coronally using a Vibratome (Lancer) at various forebrain and hindbrain levels. Free-floating sections were treated to visualize Fos-ir using procedures identical to those we have reported before (Rowland et al., 2003). Briefly, sections were treated with borohydride, incubated for 72 h at 4 °C with Fos primary antibody (Santa Cruz Biotech, SC52, 1:10,000 dilution), secondary antibody (Zymed, goat anti-rabbit, 1:600 dilution) and ABC reagent. Stained sections were mounted and examined by microscope.

Fos-ir cell nuclei were counted in discrete brain regions including dorsomedial striatum (STR), dorsolateral bed nucleus of stria terminalis (BST), central nucleus of amygdala (CNA), median preoptic nucleus, supraoptic nucleus (SON), paraventricular nucleus anteromedial or parvocellular division (PVNa) and posterolateral or magnocellular division (PVNp), area postrema (AP) and nucleus of solitary tract (NST). For regions spanning several sections, the section with the greatest number of Fos-ir cells upon gross inspection was counted.

2.4. Synaptosomal 5-HT uptake

Synaptosomes were prepared from fresh frontal cortex and assayed for 5-HT uptake. Rats and mice were given four daily subcutaneous injections of either saline (control), D-F, or D-NF (2–10 mg/kg/injection). Tissue was harvested in the morning, 44–48 h after the last injection. The animals were briefly sedated in a closed chamber of isoflurane and then immediately decapitated. The brain was quickly exposed and the frontal cortical pole removed by freehand dissection. Individual tissues were used from rats; tissues from pairs of mice were pooled. Tissue was weighed and homogenized immediately in 2 ml cold 10% sucrose solution using a Teflon pestle. Homogenates were centrifuged slowly ($1000 \times g$) and the high-density debris discarded. They then were centrifuged at 13 000 rpm for 20 min to pellet the synaptosomes. These were resuspended in ~ 50 volumes of a cold buffer (Singer et al., 1999) that had been oxygenated for ~ 30 min beforehand.

Triplicate aliquots were incubated with 1 nM 5-HT spiked with [^{3}H]5-HT (New England Nuclear) either at 37 °C for 10 min or at 0 °C (nonspecific). The incubations were terminated by rapid vacuum filtration through GF/B filter disks, and washed three times with 3 ml ice cold buffer. The disks were allowed to sit for ~ 24 h in scintillation cocktail, then were counted at ~ 50% efficiency. During these studies, our environmental safety office required that we change from a toluene to an aqueous base for the scintillation fluid, but this change did not markedly affect the results. The protein concentration of each homogenate was determined using a colorimetric assay (BCA kit; Pierce). 5-HT uptake was expressed as the difference in counts per min between the mean of the triplicate 37 °C and 0 °C samples, and normalized per mg tissue. These experiments were performed in many replications. To minimize the impact of batch to batch differences in absolute counts (e.g., in the specific activity of 5-HT and in counting efficiency), at least one control animal was run in each assay. The results of the drug-treated animals run on that day were expressed and analyzed as percent of this control value.

2.5. Drugs

m-CPP and D-F, both the HCl salts, were purchased from Sigma Chemical (St. Louis, MO). D-NF HCl was a gift from Servier (Suresnes, France). All dosages are expressed as weight of the salt. All other reagents were of standard laboratory grade (Fisher Scientific, Orlando, FL).

2.6. Statistical analyses

Dessert intakes each day, as percent baseline, were analyzed by one-way ANOVA (Sigma Stat) and Newman–Keuls comparisons ($P < .05$). Fos-ir raw counts were analyzed similarly. The uptake data, expressed as percent of control, were analyzed by ANOVA with dose as main factor.

3. Results

3.1. Feeding studies

The results are shown in Fig. 1. The intake of the mCPP-treated group was reduced to ~ 30% of control; this effect was significant ($P < .05$) and unchanged across the six injection days. The lower dose (2.5 mg/kg) of D-NF reduced

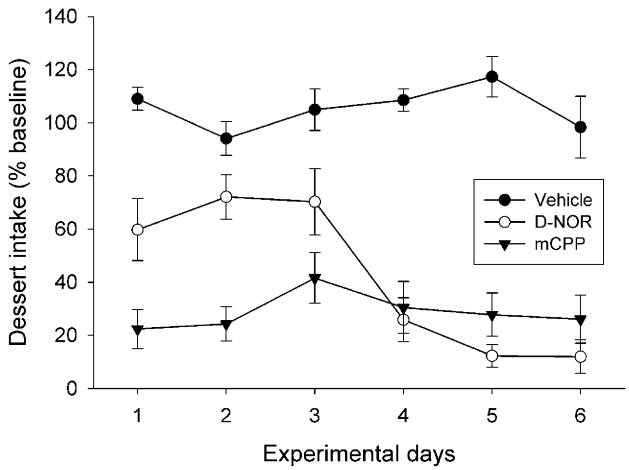

Fig. 1. Mean (± SE) 30-min intakes of sweetened milk gel dessert, expressed as % of pretreatment baseline, in groups of eight mice injected 30 min beforehand and tested every second day with either vehicle, mCPP (10 mg/kg), or *d*-norfenfluramine (D-NOR; 2.5 mg/kg first three tests; 5 mg/kg last three). Intakes of drug-treated groups are lower ($Ps < .05$) than controls at each test.

food intake to ~ 70% of control; this effect was significant on Days 1 and 3 ($P < .05$). The higher dose (5 mg/kg) of D-NF reduced food intake to ~ 15% of control; this was significant on each day and was unchanged across Treatment Days 4–6. These data thus show that there is no tolerance to either mCPP- or D-NF-induced anorexia in mice under these test conditions.

3.2. Fos-ir

The results from five regions showing marked tolerance-like effects are shown in Fig. 2. Controls that received no drug treatment were not run in this study, but in previous work (Rowland et al., 2003) mean counts in controls in these brain regions were in the range 30–60. Thus, acute injection of either mCPP or D-NF increased Fos-ir in STR, BST, PVNa, PVNp, and CNA, although the magnitude of that increase differed among these regions and between the two drugs. Further, chronic treatment significantly decreased the Fos induced in most of these regions (Ps < .05); the differences in the other regions in Fig. 2 that were not significant would most likely have been had we run larger numbers of mice. In contrast, only low to modest levels of Fos were induced in any group in the MnPO or SON. D-NF induced considerably more Fos-ir in the NTS and AP (means = 87 and 24, respectively) than mCPP (means = 36 and 10, respectively), with no difference between acute and chronic treatments.

3.3. 5-HT uptake

The results from mouse cortex are shown in the left part of Fig. 3. All of the individual values were less than the control (100%), so each of the doses represents a significant reduction from control. There was no difference as a function of dose of either D-F or D-NF; however, data across all doses for D-F (49 ± 5%) showed a greater depletion than with D-NF (77 ± 5; $P < .01$, t test).

The results from rat cortex are shown in the right part of Fig. 3. The chronic lower dose of D-F produced no reliable effect, while the higher dose produced a large and significant decrease. Not shown are the data from single acute injections: no differences from control were found.

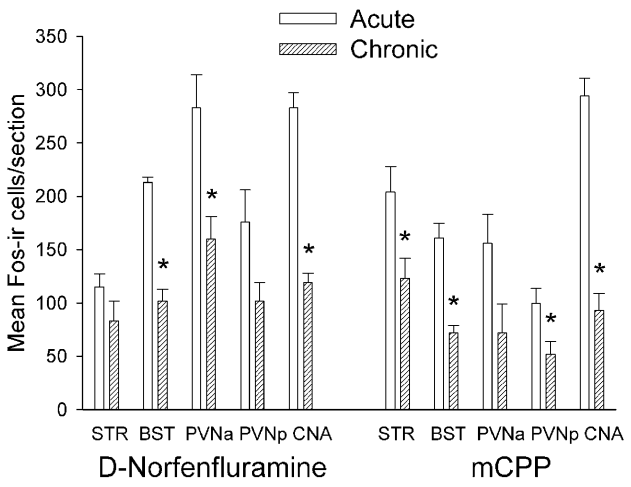

Fig. 2. Mean (± SE) numbers of Fos-ir cells per hemisection in groups of three to four mice treated either acutely or after six pretreatments (chronic) with either D-NF (5 mg/kg) or mCPP (10 mg/kg). * $P < .05$ chronic group lower than acute. Abbreviations for brain regions: medial striatum (MST), bed nucleus of stria terminalis laterodorsal part (BST), anteromedial paraventricular hypothalamic nucleus (PVNa), posterolateral paraventricular hypothalamic nucleus (PVNp), and central nucleus of amygdala (CNA).

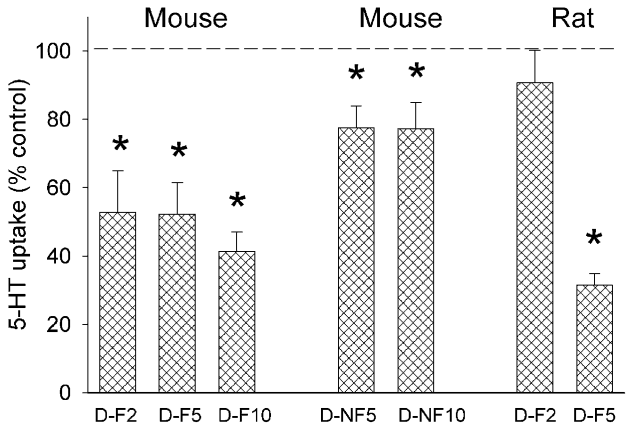

Fig. 3. Mean (± SE) 10-min uptake of [^{3}H]5-HT into synaptosomes prepared from frontal cortex of mice or rats. Shown is uptake in tissue from animals treated daily for 4 days with D-F or D-NF at the mg/kg dosage indicated. Tissues were taken 48 h after the last injection, and uptake is expressed as % of saline-injected controls sacrificed at the same time. There are four to six assays per group. All treatments except D-F2 significantly lower than control (Ps < .05).

4. Discussion

In a previous study (Rowland et al., 2003), we showed that mice tested in an identical protocol to the present showed a sustained anorectic response of about 50% when injected repeatedly with D-F (7.5 mg/kg). The present result with D-NF is similar, with a 50% inhibitory dose between 2.5 and 5 mg/kg, or about twice as potent as D-F. This ratio is consistent with previous comparisons (Rowland, 1994; Rowland and Carlton, 1986; Rowland et al., 2000, 2003) of the potencies of parent and N-dealkylated (nor-) molecules, using either the racemate or *d*-enantiomers. As we have noted before, this is unlike rats that show rapid and substantial tolerance to the anorexia in short duration tests with a familiar food (Kleven et al., 1988; Rowland et al., 2001b), but not always in free-feeding protocols (Choi et al., 2002; Rowland, 1994).

In rats, we previously identified several regions in the brain in which Fos-ir induced by D-F was greatly reduced following pretreatments (Li and Rowland, 1996). These regions included the BST and CNA, and we suggested these might be related to anorectic tolerance. However, such a loss of response could also be due to a toxic or atrophic effect of

prior D-F on 5-HT terminals. For this reason, we have often used "low" doses of D-F (2 mg/kg) that have not been thought to cause 5-HT depletion. The present demonstration in rats that a 4-day regimen of 2 mg/kg D-F does not cause loss of cortical uptake sites corroborates this assertion. By the same token, only a 2.5-fold increase in the dosage (to 5 mg/kg) produces a substantial loss of uptake sites, to levels that are as low as those attained in these respective regions using even more aggressive regimens of D-F (McCann et al., 1997; Zaczek et al., 1990). The fine-caliber 5-HT fibers that predominate in the cortex seem to be the most vulnerable to damage (Appel et al., 1989). Thus, at least in rats given 2 mg/kg, we can now assert that anorectic tolerance to D-F occurs without loss of nerve terminals but is associated with substantial loss of induced Fos-ir in select forebrain regions.

Mice seem to be substantially different from rats. The dealkylation of D-NF is slower in mice than rats, and this was a primary rationale for performing the present D-NF studies. The reasoning is that if D-NF rather than D-F is responsible for the loss of 5-HT terminal integrity and development of anorectic tolerance in rats, then because the peak D-NF concentration is lower and slower in mice than in rats (Fracasso et al., 1995) a critical threshold level of the metabolite may never be reached. Administration of D-NF would be expected to produce greater 5-HT depletions and development of more anorectic tolerance than D-F. Neither of these predictions was supported. First, although D-NF did produce decreases in cortical 5-HT uptake, these were quite small and in particular less than those achieved with the same doses of D-F. Further, the decreases after chronic D-F were not dose related. Second, as we found before with D-F (Rowland et al., 2003), there was no diminution of D-NF anorexia with repeated treatment. Also of note, that regimen of D-F used previously (5 escalating to 7.5 mg/kg) we now show is most likely associated with loss of 5-HT uptake sites in cortex. This loss of 5-HT uptake sites with chronic treatment is associated with regionally selective loss of Fos-ir response to both D-F and D-NF in mice, as well as in rats (Rowland et al., 2003), although we caution that it has not been shown whether this association is causal. We should add that given the plasma half-lives for D-F and D-NF in mice are 3.7 and 7.4 h, respectively (Fracasso et al., 1995), it is unlikely that significant accumulation of these drugs occurred with 24- to 48-h intervals between dosing used in our studies. If that had occurred it would have preferentially impacted the D-NF data, which is at variance with the observation of greater 5-HT uptake loss with D-F compared with D-NF.

In part to address some of the foregoing concerns, we made selected comparisons with mCPP, a putative direct 5-HT receptor agonist with substantial selectivity for the 2C receptor (Rothman and Baumann, 2002). We have shown previously that mCPP anorexia is unaffected by prior D-F in mice (Rowland et al., 2003), but produces full tolerance to its effect in rats (Rowland et al., 2001b). However, Fos-ir induced by either mCPP or the chemically similar *m*-trifluoromethyl derivative (TFMPP) was significantly reduced in CNA and BST by two prior treatments with D-F in both rats (Rowland et al., 2001) and mice (Rowland et al., 2003). Thus, there is a dissociation between the anorectic action of these agents and their 5-HT-depleting or Fos-inducing effects (Choi et al., 2002; McCann et al., 1997; Rowland et al., 2001b), although the nature of that dissociation differs somewhat between rats and mice.

Is it possible that all of these 5-HT related measures are in fact irrelevant to the anorectic action? The strongest form of this argument seems to be untenable because 5-HT receptor antagonists are able to block D-F anorexia (Rowland and Carlton, 1986), but there are other interpretations of these data. For example, a melanocortin receptor antagonist SHU9119 has been shown to block D-F anorexia in free-feeding mice (Heisler et al., 2002), possibly via action in the arcuate nucleus of the hypothalamus, a region that has not previously been considered in D-F action. Indeed, whole hypothalamus is generally quite refractory to 5-HT depletion by repeated D-F (Zaczek et al., 1990). In the present studies, although not reported, we did take a small number of hypothalamus samples and found exactly this result: depletions were usually found but were smaller than in cortex. In rats, D-F given intravenously has been reported to induce Fos-ir in arcuate nucleus cells that also contain α-MSH; it is possible that this subset of cells might show tolerance to D-F action, but that would not show up in a whole hypothalamus assay. Thus, in an additional study to address this possibility we treated rats with D-F (3 mg/kg sc) either once or three times at 2-day intervals ($ns = 3$) then examined Fos-ir in the arcuate and PVNp. As before (Li and Rowland, 1996; Rowland et al., 2001b), counts in the PVNp were almost completely absent in the group with two antecedent treatments compared with acute groups (means: 23 vs. 383 cells). In contrast, the effect in arcuate was much smaller; the summed cell counts over medial and lateral sites at three anteroposterior levels were 158 vs. 202 (the cells were not identified chemically). No location seemed different from others suggesting that minimal tolerance to induction of Fos-ir occurs within this region in rats despite the occurrence of strong behavioral tolerance.

An extension of this idea that the behavioral actions of D-F and D-NF may reflect their action at a non-5-HT system may be illustrated by the observation that D-F is ninefold more potent in inhibiting 5-HT uptake compared with NE uptake, while D-NF is equipotent at both transporter sites in rat brain (Rothman et al., 2003). Indeed, it is possible that one of the reasons that D-NF is more potent as an anorectic than D-F relates to the co-involvement of NE, which is thought to produce an additive or supra-additive effect on food intake (Roth and Rowland, 1999; Wellman et al., 2003). However, this does not obviously explain the similar tolerance profiles to these agents in rats, and their dissimilarity from mice.

In summary, we show that dosage regimens of D-F in rats that have been associated with complete anorectic tolerance and regionally selective loss of induced brain Fos-ir do not have down-regulatory or damaging effects on cortical 5-HTTs. In contrast, regimens of D-F or D-NF that do not lose their anorectic efficacy with chronic treatment in mice are associated with loss of 5-HT uptake capacity. Unexpectedly, the uptake-decreasing effect of D-F appeared to be more pronounced than that of D-NF in mice.

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