

Chronic Fenfluramine Administration Increases Plasma Serotonin (5-Hydroxytryptamine) to Nontoxic Levels

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ABSTRACT

Large elevations in blood serotonin (5-hydroxytryptamine; 5-HT) can produce valvular heart disease in humans and laboratory animals. In accordance, one prevailing hypothesis (i.e., the "5-HT hypothesis") suggests that 5-HT transporter substrates like fenfluramine increase the risk for valvular heart disease by elevating plasma 5-HT, secondary to the release of 5-HT from platelets. The main purpose of this study was to determine whether chronic administration of fenfluramine increases plasma 5-HT to concentrations that are associated with the development of valvular heart disease. To the best of our knowledge, this is the first study to address this issue using an *in vivo* microdialysis method that measures plasma 5-HT in nonhypoxic rats. We examined the effects of chronic (\pm)-fenfluramine and fluoxetine on plasma levels of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in blood samples from conscious catheterized rats. Plasma indoles

were measured by high-performance liquid chromatography with electrochemical detection in the dialysates of whole blood. The baseline plasma 5-HT level was <1.0 nM. Chronic fenfluramine (14-day minipump infusion) produced small increases in baseline plasma 5-HT (~ 2 – 4 -fold), whereas chronic fluoxetine had no effect. Chronic fenfluramine and fluoxetine markedly decreased whole-blood 5-HT and reduced the ability of acute fenfluramine to evoke 5-HT release. Elevations in baseline plasma 5-HT produced by chronic fenfluramine are far below the micromolar levels necessary to produce valvular heart disease. Furthermore, chronic fenfluramine reduces the ability of acute fenfluramine to increase plasma 5-HT, suggesting that the 5-HT hypothesis cannot explain the increased risk of valvular heart disease in patients treated with fenfluramine.

Outside of the nervous system, serotonin (5-hydroxytryptamine; 5-HT) controls a wide range of functions, including mitogenesis and vasoconstriction. More than 99% 5-HT in the bloodstream is stored in platelets, which express a 5-HT transporter protein (SERT) identical to the one found in

nervous tissue (Ni and Watts, 2006). Plasma 5-HT levels are kept exquisitely low (i.e., <1 nM) by SERT-mediated uptake of 5-HT into platelets and lung endothelial cells, in addition to metabolism of 5-HT to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase. Large elevations in circulating 5-HT are associated with significant cardiovascular pathology. For example, human patients with carcinoid syndrome display marked elevations in blood 5-HT levels (Kema et al., 1992), and valvular heart disease (VHD) develops on the right side of the heart in many of these patients (Robiolio et al., 1995). In rats, chronic administration of exogenous 5-HT at doses sufficient to increase extracellular 5-HT more than 40 to 100-fold causes VHD analogous to the condition in carcinoid patients (Gustafsson et al., 2005). An important unanswered question is whether chronic administration of anorectics like fenfluramine can persistently elevate plasma 5-HT to the levels previously reported to produce VHD. (\pm)-Fenfluramine (fenfluramine) and (+)-fenfluramine (dexfenfluramine) are anorectic drugs that were withdrawn from clinical use due to the occurrence of VHD in some patients (Connolly and McGoon, 1999). Epidemiological evidence further indicates that fenfluramines increase the risk of

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; SERT, serotonin transporter protein; 5-HIAA, 5-hydroxyindoleacetic acid; VHD, valvular heart disease; IPAH, idiopathic primary pulmonary hypertension; HPLC-ECD, high-performance liquid chromatography with electrochemical detection; RIA, radioimmunoassay.

developing idiopathic pulmonary arterial hypertension (IPAH) (Abenheim et al., 1996; Fishman, 1999). Although the mechanisms underlying fenfluramine-associated adverse effects are unresolved, clarifying such mechanisms is important for at least two reasons: 1) to help illuminate the pathogenesis of VHD and IPAH; and 2) to aid in the development of new serotonergic medications with reduced side-effects (Rothman and Baumann, 2002).

Fenfluramines evoke the release of serotonin (5-HT) from neurons by acting as SERT substrates that reverse the normal direction of transmitter flux (Baumann et al., 2000). One prevailing hypothesis to explain fenfluramine-associated side-effects like VHD (Fishman, 1999) and IPAH (MacLean et al., 2000) is known as the "5-HT hypothesis." A key prediction of this hypothesis is that fenfluramine administration increases plasma 5-HT to concentrations sufficient to produce vasoconstriction and mitogenesis, which then leads to the development of serious side effects. Despite the importance of the 5-HT hypothesis to the current dogma regarding the etiology of fenfluramine-associated VHD and IPAH, the effects of fenfluramine and related agents on plasma 5-HT have received little attention. Studies conducted in the 1990s are inconsistent with the 5-HT hypothesis, because they show that acute fenfluramine does not increase plasma 5-HT in rats (Martín and Artigas, 1992), and chronic fenfluramine lowers blood 5-HT in humans (see Rothman et al., 2000b and references therein). It is noteworthy that most studies report that plasma 5-HT levels are at least 10 times higher than actual values (i.e., <1 nM), perhaps due to the difficulties in preparing plasma without lysing platelets and releasing stored 5-HT.

Given the uncertainties regarding the validity of the 5-HT hypothesis, we developed a novel microdialysis method to measure plasma levels of 5-HT in whole-blood samples from conscious catheterized rats that involves careful blood handling to minimize the chance of platelet lysis (Zolkowska et al., 2006). Using this method, we reported that baseline dialysate levels of 5-HT in blood are ~ 0.22 nM (or ~ 0.88 nM when corrected for probe recovery), and fenfluramine and other potent SERT substrates evoke transient dose-dependent increases in plasma 5-HT ranging from 4 to 20 nM. Acute administration of fluoxetine, a SERT uptake inhibitor, produces small but significant increases in plasma 5-HT.

In the present study, we report that chronic administration of fenfluramine (14-day minipump infusion) increases baseline levels of plasma 5-HT (~ 2 – 4 -fold) in a dose-dependent manner, whereas chronic fluoxetine does not. Both chronic fenfluramine and fluoxetine decrease whole-blood levels of 5-HT and reduce the ability of acute fenfluramine to further increase plasma 5-HT. Because there is no evidence that chronic administration of fenfluramine induces either VHD (Smith et al., 2006) or IAPH in normal rats (Eddahibi et al., 2002), we do not report pathological examination of the heart or lungs. Our data indicate that elevations in baseline plasma 5-HT produced by chronic fenfluramine are well below the micromolar levels necessary to produce cardiovascular side effects, suggesting that the 5-HT hypothesis cannot explain adverse actions of fenfluramines and related drugs.

Materials and Methods

Animals. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 350 to 450 g were single-housed, and food

and water were freely available. Rats were maintained in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and procedures were approved by the Animal Care and Use Committee of the National Institute on Drug Abuse, Intramural Research Program.

Drugs and Reagents. (\pm)-Fenfluramine HCl (fenfluramine, FW 267.7) and pentobarbital sodium were obtained from the National Institute on Drug Abuse, Intramural Research Program Pharmacy. Fluoxetine HCl (fluoxetine, FW 345.8) was purchased from Spectrum Chemical Manufacturing Company (New Brunswick, NJ). Monochloroacetic acid was obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ), and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO). Drug solutions for the acute in vivo study were prepared in saline immediately before use, and doses are expressed as the salt. For filling osmotic minipumps, fenfluramine (3 and 10 mg/kg/day) was dissolved in 0.9% saline and fluoxetine (3 and 10 mg/kg/day) was dissolved in a 50% EtOH/0.9% saline vehicle immediately before filling the minipumps. A day before implantation, minipumps were filled with physiological saline, vehicle, or drug solution and placed in sterile 0.9% saline at 37°C overnight. The concentrations of drugs were adjusted according to rat body weight a day before implantation, with 10 g added to take into account the expected future weight gain.

Surgical Procedures. Rats received sodium pentobarbital (60 mg/kg i.p.) for surgical anesthesia. Indwelling jugular catheters made of Silastic Medical Grade tubing (Dow Corning, Midland, MI) were implanted into the right jugular vein and advanced to the atrium as described previously (Baumann et al., 2001). Immediately after catheter implantation, osmotic minipumps [Alzet model 2ML2 (14 days); Durect Corporation, Cupertino, CA] were inserted s.c. along the back, parallel to the spine, with the flow moderator pointing away from the incision. Rats were allowed 7 days to recover postoperatively before any other procedure was performed. On the 14th day after surgery, rats were used in microdialysis experiments as described below. On the next day (15th day after surgery), rats were euthanized, and minipumps were removed. Brains were removed, and selected brain regions were dissected out on ice and stored at -80°C for measurement of tissue biogenic amines. Minipumps were checked by visual inspection to verify that they were empty.

In Vivo Drug Administration and Microdialysis. Between 8:00 AM and 9:00 AM on the 14th day after minipump implantation, rats were moved into the testing room and allowed to acclimate to the surroundings for 1 h. Extension tubes were attached to catheters, and 0.5 ml of heparin flush (48 IU/ml in saline) was injected. Blood samples (0.3 ml) were withdrawn into 1-ml syringes and gently transferred into 300- μl polypropylene vials that were chilled on crushed ice. Vials contained 20 μl of heparin (1000 IU/ml in saline) as an anticoagulant. Heparin flush was injected into the rats after each sample to maintain volume homeostasis. A 4×0.6 -mm dialysis probe (MAB 6; SciPro, Inc., Sanborn, NY) was immediately immersed in the chilled blood sample. Ringers' solution containing 150.0 mM Na^+ , 3.0 mM K^+ , 1.4 mM Ca^{2+} , 0.8 mM Mg^{2+} , 1.0 nM phosphorus, and 155 mM Cl^- was pumped through the probe at 1 $\mu\text{l}/\text{min}$, and each blood sample was dialyzed for 15 min to generate a single dialysate sample. Serial blood samples were collected and dialyzed every 15 min. Dialysates were assayed for 5-HT and 5-HIAA using high-performance liquid chromatography with electrochemical detection (HPLC-ECD) as described below. After three to four baseline samples were obtained and dialyzed, drug treatments were administered through i.v. catheters. Fenfluramine was dissolved in saline and administered as i.v. bolus injection (1.0 mg/kg). Blood samples were collected at 15-min intervals for 90 min postinjection. Probe recoveries were performed before and after blood sampling using a 10-pg 5-HT standard prepared in Ringers' solution.

HPLC-ECD Analysis of 5-HT and 5-HIAA in Dialysates. Aliquots of the dialysate (5 μl) were injected onto a microbore HPLC column (Unijet, 100×1 mm, 5 μM ODS; Bioanalytical Systems, Inc.,

West Lafayette, IN) that was coupled to an amperometric detector (Model LC-4C; Bioanalytical Systems, Inc.). A glassy carbon electrode was set at a potential of +650 mV relative to Ag/AgCl reference. Mobile phase consisted of 180 μ M Na₂EDTA, 150 mM monochloroacetic acid, 125 mM NaOH, and 690 μ M sodium octanesulfonic acid, with 7.5% MeOH and 7.5% CH₃CN per liter of water (final pH = 3.15). Mobile phase was pumped through the column at 60 μ l/min (260D, syringe pump; Teledyne ISCO, Lincoln, NE). Chromatographic data were acquired on-line and exported to a Millennium software system (Waters Associates, Milford, MA) for peak amplification, integration, and analysis. The concentration of 5-HT and 5-HIAA in dialysate samples was compared to known standards, and the lower limit of detection was \sim 0.05 pg/5 μ l (0.047 nM) for both indoles.

Measurement of Blood 5-HT Levels. On the 7th and 13th days after minipump implantation, venous blood (1 ml) was obtained via the jugular catheters and transferred to glass tubes. The blood was allowed 30 to 45 min at 37°C to fully clot. When clotted, blood was spun at 3000 rpm for 10 min. After centrifugation, the serum was transferred to cryotubes and stored at -80° C until analysis. Serum 5-HT levels were determined using a commercially available RIA kit (ALPCO Diagnostics, Salem, NH).

Measurement of Blood Fenfluramine and Fluoxetine Levels. On the 7th and 13th days after minipump implantation, additional venous blood (1.5 ml) was obtained via the jugular catheters and put into cryotubes containing K₂EDTA as an anticoagulant, calculated to give a final concentration of 1.8 mg/ml, and stored at -80° C until analysis. Fenfluramine and norfenfluramine, or fluoxetine and norfluoxetine, were assayed in blood by NMS Labs (Willow Grove, PA) using gas chromatography. Limits of quantitation were 10 ng/ml for fenfluramine and its metabolite and 20 ng/ml for fluoxetine and its metabolite.

Measurement of Brain Monoamines. Rats were sacrificed on the 15th day after pump implantation. The brains were removed, and various brain regions were dissected on ice. Cortical monoamine concentrations were determined by HPLC-ECD as described previously (Baumann et al., 2001).

Statistical Analyses. In microdialysis studies, the first two samples collected before any treatment were considered baseline samples. 5-HT and 5-HIAA measures are the mean \pm S.E.M. expressed as picogram per 5 μ l of sample. For the RIA studies, serum 5-HT levels are the mean \pm S.E.M. expressed as nanograms per milliliter. Data were first evaluated by two-way ANOVA (drug treatment \times time). When significant *F* values were obtained, separate one-way ANOVAs were carried out at each time point, followed by Newman-Keuls post-hoc tests to compare group means. A value of *p* < 0.05 was considered as the minimal criterion for statistical significance.

Results

Acute Effects of Drugs on Plasma 5-HT. As reported in Fig. 1A, acute administration of fenfluramine significantly increased dialysate 5-HT [*F*(2,120) = 45.79; *p* < 0.0001]. This stimulatory effect was dose-dependent, with 0.3 and 1.0 mg/kg doses elevating 5-HT 9- and 14-fold, respectively. The fenfluramine-induced increase in plasma 5-HT was transient, almost returning to baseline by 90 min. Acute administration of fluoxetine (Fig. 1B) produced a smaller dose-dependent and long-lasting (>90 min) increase in plasma 5-HT [*F*(2,120) = 52.58; *p* < 0.0001], with 0.3 and 1.0 mg/kg doses increasing 5-HT levels 3- and 7-fold, respectively. Plasma 5-HIAA levels were not affected by either drug (data not shown).

Chronic Effects of Drugs on Plasma 5-HT. Chronic administration of fenfluramine via 14 days of osmotic minipump infusion significantly increased baseline dialysate 5-HT levels

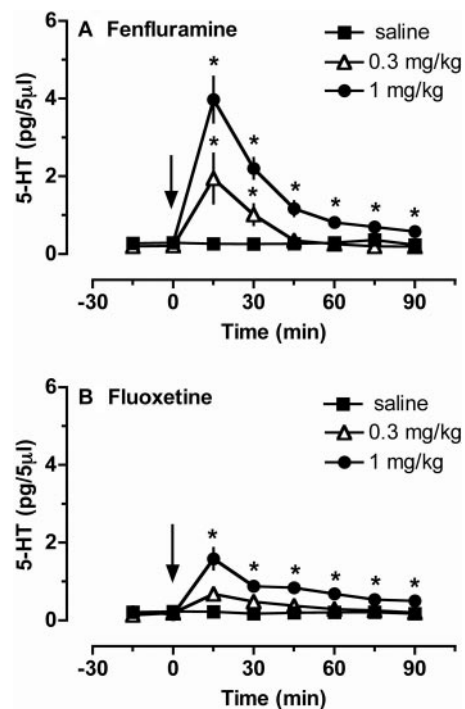


Fig. 1. Acute in vivo effects of (\pm)-fenfluramine (A) or fluoxetine (B) on dialysate 5-HT levels measured in blood from drug-naive conscious rats. Drugs were dissolved in sterile saline and administered i.v. at 0 min. Serial blood samples were withdrawn at 15-min intervals and immediately dialyzed as described under *Materials and Methods*. Data shown are the mean \pm S.E.M. for *n* = 6/group. *, *p* < 0.05 compared to saline controls at the corresponding time points.

from 0.24 ± 0.07 to 0.45 ± 0.08 and 0.86 ± 0.15 pg/5 μ l, at 3.0 and 10.0 mg/kg doses, respectively. As shown in Fig. 2A, exposure to chronic fenfluramine significantly and dose-dependently reduced the ability of acute i.v. fenfluramine (1 mg/kg) to increase plasma 5-HT [*F*(2,192) = 3.696; *p* < 0.0001]. Acute administration of fenfluramine elevated extracellular 5-HT by 19-fold in saline-pretreated rats, compared with a 6- and 3-fold increase after the chronic administration of 3.0 and 10.0 mg/kg (\pm)-fenfluramine, respectively.

Chronic administration of fluoxetine via 14 days of osmotic minipump infusion (Fig. 2B) did not change baseline dialysate 5-HT levels in blood. However, chronic fluoxetine significantly and dose-dependently reduced the effects of acute fenfluramine on plasma 5-HT [*F*(2,192) = 100.3; *p* < 0.0001]. An acute challenge dose of i.v. fenfluramine (1 mg/kg) elevated extracellular 5-HT by 18-fold in the saline condition, compared to a 4- and 2-fold increase after the chronic administration of 3.0 and 10.0 mg/kg fluoxetine, respectively.

Chronic Effects of Drugs on Whole-Blood 5-HT. Figure 3 shows the effect of chronic administration of fenfluramine or fluoxetine on 5-HT levels in whole blood. Chronic administration of fenfluramine significantly reduced whole-blood 5-HT [*F*(2,48) = 23.63; *p* < 0.0001] by \sim 30 (3 mg/kg) and \sim 40% (10 mg/kg) compared with saline control when measured 7 days after minipump implantation (Fig. 3A). Similar reductions were observed at day 13. The fenfluramine-induced reduction in blood 5-HT was not dose-dependent, indicating that the ability of fenfluramine to deplete platelet 5-HT had already reached a maximal effect at the 3 mg/kg dose. Chronic fluoxetine (Fig. 3B) dose-dependently and significantly reduced whole-blood 5-HT [*F*(2,48) = 37.97;

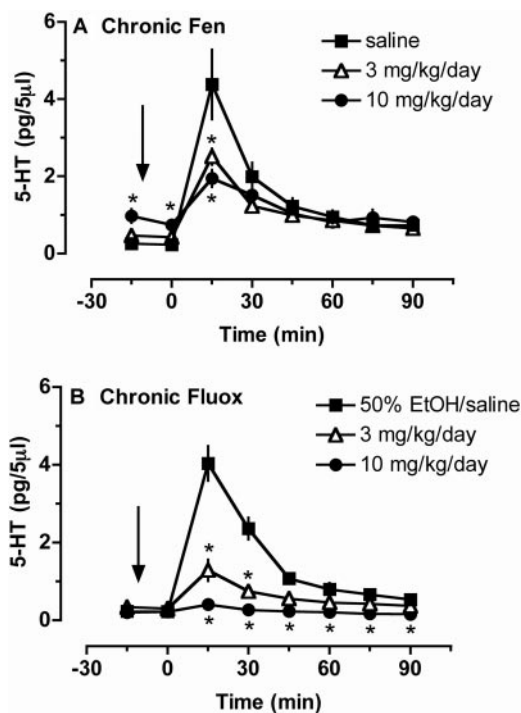


Fig. 2. Acute in vivo effects of (\pm)-fenfluramine on dialysate 5-HT levels measured in blood from conscious rats previously treated with chronic administration of (\pm)-fenfluramine (A) or fluoxetine (B). For chronic treatments, drugs were dissolved in sterile saline (A) or 50% EtOH/saline (B) and administered s.c. via osmotic minipumps. On the day of the test, fenfluramine was dissolved in saline and administered i.v. at 0 min. Serial blood samples were withdrawn at 15-min intervals and immediately dialyzed as described under *Materials and Methods*. Data shown are mean \pm S.E.M. for $n = 9$ /group. *, $p < 0.05$ compared to saline controls at the corresponding time points.

$p < 0.0001$] by $\sim 37\%$ (3 mg/kg) and $\sim 77\%$ (10 mg/kg) compared with saline control when measured 7 days after minipump implantation, and this reduction was sustained up to day 13.

Chronic Effects of Drugs on Brain Tissue Biogenic Amines. Table 1 shows that chronic administration of fenfluramine caused a dose-dependent decrease in 5-HT levels in the rat cortex. The 3 mg/kg dose decreased cortical 5-HT by 15%, whereas the 10 mg/kg dose decreased 5-HT by 87%. Chronic fenfluramine administration did not alter cortical levels of dopamine. It is interesting to note that the 10 mg/kg fenfluramine dose decreased cortical norepinephrine by 27%. Chronic fluoxetine did not significantly change cortical biogenic amines.

Blood Levels of Drugs and Metabolites. We measured the blood levels of fenfluramine and fluoxetine after 13 days of chronic administration. As reported in Fig. 4A, the blood level of fenfluramine significantly increased ($p < 0.0001$) from 55 ng/ml observed with the 3 mg/kg dose to 212 ng/ml with the 10 mg/kg dose. The blood level of norfenfluramine also increased ($p < 0.0001$) from 19 ng/ml produced by the 3 mg/kg dose to 115 ng/ml observed with the 10 mg/kg dose. The plasma levels of fenfluramine and norfenfluramine observed with the 3 mg/kg dose are similar to those reported in humans taking 60 mg of fenfluramine per day (Rothman et al., 2000b).

After 13 days of chronic administration of fluoxetine, the fluoxetine blood concentration was significantly increased

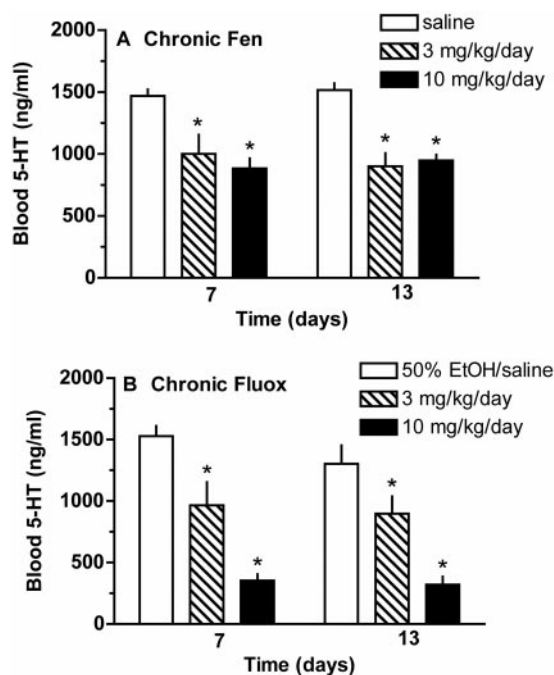


Fig. 3. Effects of chronic administration of (\pm)-fenfluramine (A) and fluoxetine (B) on whole-blood 5-HT levels measured 7 and 13 days after minimal implantation. Drugs were dissolved in saline (A) or 50% EtOH/saline (B) and administered s.c. via osmotic minipumps. Blood samples were withdrawn via indwelling jugular catheters, and serum 5-HT was measured using double-antibody RIA methods. Data shown are the mean \pm S.E.M. for $n = 9$ /group. *, $p < 0.05$ compared to saline or ethanol/saline controls at the corresponding time points.

TABLE 1

Effects of chronic fenfluramine and fluoxetine on monoamines in rat frontal cortex

Monoamine levels were measured as described under *Materials and Methods*. Each value is the mean \pm S.E.M. for $n = 9$ /group.

	Cortical Amine		
	NE	DA	5-HT
	pg/mg tissue		
Chronic fenfluramine			
Saline	599 \pm 25	45 \pm 3	691 \pm 33
3 mg/kg/day	550 \pm 28	54 \pm 8	589 \pm 36*
10 mg/kg/day	435 \pm 16*	40 \pm 4	89 \pm 13*
Chronic fluoxetine			
50% EtOH/saline	581 \pm 27	55 \pm 6	604 \pm 27
3 mg/kg/day	565 \pm 31	48 \pm 4	595 \pm 26
10 mg/kg/day	535 \pm 21	46 \pm 2	556 \pm 54

NE, norepinephrine; DA, dopamine.

* $p < 0.05$ when compared to the vehicle control (ANOVA with post-hoc Newman-Keuls multiple comparison test).

($p < 0.016$) from 11 ng/ml at the 3 mg/kg dose to 113 ng/ml at the 10 mg/kg dose (Fig. 4B). Likewise, the blood level of norfluoxetine was also significantly increased ($p < 0.0006$) from 74 ng/ml with the 3 mg/kg dose to 795 ng/ml with the 10 mg/kg dose. The plasma levels of fluoxetine and norfluoxetine observed with the 10 mg/kg dose in rats are similar to those reported in humans taking 20 mg of fluoxetine per day (Lundmark et al., 2001).

Correlations. Our previous work established that acute fenfluramine increases plasma 5-HT, most probably via SERT-mediated release of platelet 5-HT (Zolkowska et al., 2006). As reported in Fig. 3, chronic fenfluramine and fluoxetine decrease blood 5-HT content, suggesting that both

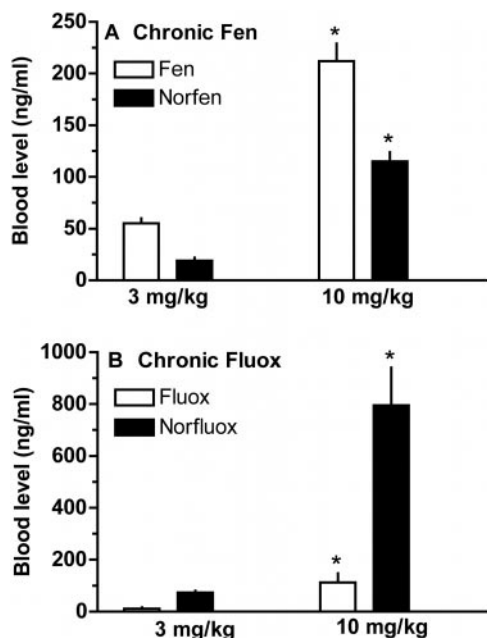


Fig. 4. Effects of chronic administration of (\pm)-fenfluramine on blood levels of (\pm)-fenfluramine and (\pm)-norfenfluramine (A) and chronic administration of fluoxetine on blood levels of fluoxetine and norfluoxetine (B) measured 13 days after chronic administration. Blood samples were withdrawn via indwelling jugular catheters and assayed as described under *Materials and Methods*. Data shown are the mean \pm S.E.M. for $n = 6$ /group. *, $p < 0.05$ compared to the 3 mg/kg dose group (two-tailed Student's *t* test).

drugs deplete 5-HT stored in platelets. In accordance, our data predict that peak increases in dialysate 5-HT produced by acute fenfluramine should be correlated with blood 5-HT content. As reported in Fig. 5A for the rats chronically treated with fenfluramine, the positive correlation between peak dialysate 5-HT and blood 5-HT just achieved statistical significance ($p < 0.055$). For the rats chronically treated with fluoxetine, this positive correlation was highly significant ($p < 0.004$). Such correlative relationships indicate that the magnitude of fenfluramine-evoked increases in plasma 5-HT are at least partially dependent on platelet 5-HT levels available for release.

Discussion

Experimental and pathological elevations of blood 5-HT can produce VHD with characteristic plaque-like encasement of valve leaflets (Robiolio et al., 1995; Gustafsson et al., 2005), but there are no reports demonstrating that chronic administration of fenfluramine induces either VHD (Smith et al., 2006) or IAPH in rats (Eddahibi et al., 2002). The main purpose of the present study was to determine whether chronic fenfluramine administration at pharmacologically and clinically relevant doses would increase blood and plasma 5-HT to the levels needed to produce VHD. To our knowledge, this is the first study to address this question using an *in vivo* microdialysis method for measuring plasma 5-HT (Zolkowska et al., 2006) in rats not exposed to hypoxic conditions. Due to the problem of platelet lysis during sample preparation, accurate measurement of plasma 5-HT is technically challenging (for review, see Zolkowska et al., 2006). When reviewing studies that report plasma concentrations of 5-HT, "normal" 5-HT levels greater than 1 nM indicate that

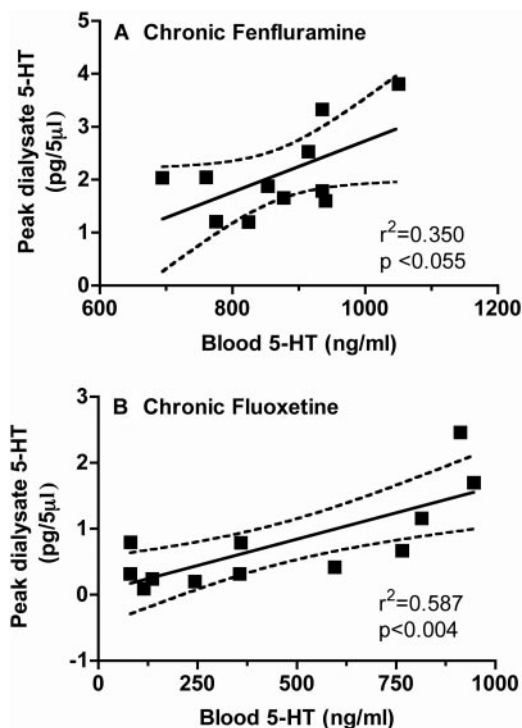


Fig. 5. Correlations between whole-blood 5-HT levels and peak-dialysate 5-HT response to acute *i.v.* (\pm)-fenfluramine in rats pretreated with chronic administration of (\pm)-fenfluramine (A) or fluoxetine (B). Data represent the individual values for $n = 12$ rats/drug.

platelet lysis probably occurred, and we will not consider those particular results in the forthcoming discussion.

Chronic high-dose administration of 5-HT produces VHD (Gustafsson et al., 2005) in rats. In the study of Gustafsson et al. (2005), "free" 5-HT was measured using *in vivo* microdialysis in the femoral muscle rather than in a peripheral vein. The interstitial concentration of 5-HT was assumed to reflect and probably underestimated plasma 5-HT. Free 5-HT levels ranged from 580 to 974 nM (corrected for the 31% probe recovery), depending on the sampling time. These values are at least 100-fold higher than the 5-HT levels (~ 5 nM) observed after chronic fenfluramine (10 mg/kg) treatment in our study. Gustafsson et al. (2005) also reported that chronic administration of 5-HT increased whole-blood 5-HT 3-fold from 6986 to 21,236 nM. In our study, as expected on the basis of previous work (see Rothman et al., 2000b and references therein), chronic fenfluramine decreased whole-blood 5-HT by ~ 30 and $\sim 40\%$ at the 3 and 10 mg/kg doses (Fig. 3). These considerations support the hypothesis that chronic fenfluramine does not increase plasma 5-HT (or blood 5-HT) to a level reported to cause VHD in the rat. It is important to note, however, that the minimal dose of exogenous 5-HT required to produce VHD has not been determined, and this issue warrants further experimentation.

Several studies report that carcinoid syndrome, a disease associated with VHD, causes marked increases in platelet 5-HT. Kema et al. (1992) reported that patients with midgut-carcinoid tumors had median platelet 5-HT levels of 31.45 (nmol/ 10^9 platelets) compared to 4.4 in normal controls (7.1-fold increase). In a later study, the same group reported that patients with midgut-carcinoid tumors had median platelet 5-HT levels of 23.8 (nmol/ 10^9 platelets) compared to 3.4 in

normal controls (7.0-fold increase) (Kema et al., 2001). A similar 8.6-fold increase in platelet 5-HT was reported by others (Meijer et al., 2000). Robiolio et al. (1995) reported that carcinoid patients with VHD had higher serum and platelet 5-HT levels than carcinoid patients without VHD. The serum and platelet 5-HT levels in carcinoid patients with VHD were elevated 11.5- and 6.6-fold, respectively, compared to normal controls. Because the increased levels of 5-HT in blood and platelets observed in carcinoid syndrome and in rat models (Gustafsson et al., 2005) are driven by indirect administration of 5-HT into the blood stream, it is reasonable to conclude that such elevations in blood and platelet 5-HT will also result in large increases (>20-fold) in plasma 5-HT. In our study, chronic fenfluramine at a dose (3 mg/kg) similar to that prescribed for humans decreases blood 5-HT levels and increases plasma 5-HT only 2 to 4-fold. These data support the hypothesis that chronic fenfluramine does not increase plasma 5-HT (or blood 5-HT) to a level sufficient to cause VHD in humans. These results are similar to those observed in rats chronically exposed to hypoxic conditions (Eddahibi et al., 1998).

Viewed collectively, these findings are not compatible with the so-called 5-HT hypothesis of fenfluramine-associated VHD. This hypothesis postulates that fenfluramine increases the risk of developing VHD by increasing plasma levels of 5-HT (Fishman, 1999; MacLean et al., 2000). Although the 5-HT hypothesis may have been tenable as an explanation of fenfluramine-associated VHD at one point, recent studies implicate the 5-HT_{2B} receptor, rather than plasma 5-HT per se, as the culprit in the pathogenesis of this disease (Rothman et al., 2000a). Medications that are known to induce VHD such as fenfluramine, dexfenfluramine, methysergide, ergotamine, pergolide, and cabergoline share a common mechanism—these drugs or their major metabolites are potent and efficacious 5-HT_{2B} agonists. 5-HT_{2B} receptors are localized on cells expressed in heart valves. When stimulated, these cells undergo mitogenesis that leads to excessive growth culminating in valvulopathy (for review, see Roth, 2007).

The present findings also have implications for hypotheses concerning the etiology of IPAH, including an analogous 5-HT hypothesis (Fishman, 1999; MacLean et al., 2000; Eddahibi et al., 2006). Our data indicate that fenfluramine does not increase plasma 5-HT to a high enough concentration to contract pulmonary arteries or produce adverse pulmonary effects (for an in-depth discussion of this point, see Zolkowska et al., 2006). We cannot exclude the possibility that 2 to 4-fold increases in plasma 5-HT produced by chronic fenfluramine could be enough to stimulate mitogenic responses in susceptible individuals and increase the risk of developing IPAH. However, this scenario seems unlikely because treatment with lithium (Artigas et al., 1989) and monoamine oxidase inhibitors (Celada et al., 1992) produces comparable increases in plasma 5-HT without increasing the risk for IPAH.

The effects of chronic fenfluramine were compared to those of the SERT uptake inhibitor, fluoxetine, a drug that is not associated with either IPAH or VHD. The doses we chose are both pharmacologically and clinically relevant. Measurement of circulating drug levels (Fig. 4) demonstrated that chronic administration of 3 mg/kg fenfluramine and 10 mg/kg fluoxetine produced blood levels in rats similar to those in humans treated with prescribed doses of these medications (Rothman

et al., 2000b; Lundmark et al., 2001). In addition, as observed in this study, clinically relevant doses of fenfluramine (Rothman et al., 2000b) and fluoxetine both produce substantial reductions in blood 5-HT levels (Alvarez et al., 1999). In conclusion, the fact that chronic fenfluramine produced dose-dependent reductions in brain 5-HT levels (Table 1) indicates that the fenfluramine doses used in this study are pharmacologically relevant.

The mechanisms underlying fenfluramine-associated IPAH in humans remain enigmatic. One significant problem is that widely used animal models of IPAH require the induction of hypoxia, which is not a major contributing factor to the disease in human patients. Furthermore, the etiology of fenfluramine-associated IPAH in humans may substantially differ from that of nondrug-related IPAH. Despite these caveats, recent research has provided alternative hypotheses to the 5-HT hypothesis to explain how fenfluramine might increase the risk of IPAH. For example, recent studies by Eddahibi et al. (2006) did not focus attention on plasma 5-HT as a critical player in pathogenesis of pulmonary smooth muscle cell hyperplasia but rather on 5-HT produced in the lung by microvascular endothelial cells. In addition, Launay et al. (2002) provided evidence that the activation of 5-HT_{2B} receptors in the lung is critical to the development of IPAH in a hypoxic mouse model. Because the fenfluramine metabolite norfenfluramine is a potent and selective 5-HT_{2B} agonist (Rothman et al., 2000a), these findings provide a reasonable mechanism by which fenfluramine could increase the risk of IPAH in humans. However, an argument against this hypothesis is the fact that other medications that produce VHD via activation of 5-HT_{2B} receptors are not known to increase the risk of IPAH (methysergide, ergotamine, pergolide, and cabergoline). Moreover, recent findings from our laboratory show that the anorectic drug aminorex, which is associated with IPAH, does not display appreciable affinity for 5-HT_{2B} receptors. More investigation is required to determine the role of 5-HT_{2B} receptors in the pathology of IPAH.

In summary, our data provide additional evidence that acute administration of SERT substrates can elevate plasma 5-HT into the low nanomolar range. However, chronic administration of fenfluramine at therapeutically relevant doses produces only small elevations in plasma 5-HT and reduces the ability of acute fenfluramine to increase plasma 5-HT. These data suggest that daily administration of fenfluramine in humans would actually blunt fenfluramine-induced increases in plasma 5-HT, providing a “protective” effect from fenfluramine-induced spikes in plasma 5-HT. Our findings do not provide support for the 5-HT hypothesis because elevations in baseline plasma 5-HT produced by chronic fenfluramine are much too low to induce adverse effects. Our data strongly suggest that mechanisms other than changes in plasma 5-HT must be invoked to explain the ability of fenfluramine and other SERT substrates to increase the risk of VHD and IPAH.

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