R-fluoxetine Increases Extracellular DA, NE, As Well As 5-HT in Rat Prefrontal Cortex and Hypothalamus: An in vivo Microdialysis and Receptor Binding Study

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The selective serotonin reuptake inhibitor fluoxetine consists of equal amounts of R and S stereoisomers. In this study, we investigated the pharmacologic properties of the stereoisomers using transporter and receptor binding assays and in vivo microdialysis in freely moving rats. Binding to the transporter confirmed selectivity of R- and S-fluoxetine for the 5-HT transporter versus the dopamine (DA) and norepinephrine (NE) human transporters. Receptor binding studies demonstrated significant affinity of R-fluoxetine, but not S-fluoxetine, for human $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptor subtypes. Functional GTPγS binding studies indicated that R-fluoxetine is an antagonist at $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors. In microdialysis studies, acute R- and S-fluoxetine increased extracellular levels of 5-HT, DA, and

NE in prefrontal cortex (PFC), but R-fluoxetine caused significantly greater increases of catecholamines. R-fluoxetine increased extracellular levels of 5-HT and NE in PFC, nucleus accumbens, and hypothalamus, whereas it increased dopamine in PFC and hypothalamus, but not in DA-rich nucleus accumbens and striatum, thus indicating a regionally selective effect. The unexpected increases of extracellular catecholamines by a selective 5-HT uptake inhibitor like R-fluoxetine may be due to its antagonism of $5\text{-}HT_{2C}$ receptors.
[Neuropsychopharmacology 27:949–959, 2002]
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Most drug treatments for depression elevate monoaminergic neurotransmission, in particular serotonin (5-HT), norepinephrine (NE) and/or dopamine (DA). Initial agents to treat depression, like tricyclics (TCAs), have many bothersome side effects including cardiotoxicity that are mediated by their interaction with adrenergic, cholinergic and histaminergic systems (Cusack et al. 1994; Bourin and Baker 1996). The selective serotonin reuptake inhibitors (SSRI) represented a new generation of compounds for the treatment of depression (for review see Wong et al. 1995). Unlike TCAs, SSRIs are devoid of adrenergic, cholinergic, and histaminic activity and show a much better side effect profile and higher compliance in patients (Stahl 1998; Westenberg 1999). The SSRIs selectively block the serotonin transporter, thus inhibiting uptake of released serotonin back into

0893-133X/02/$–see front matter
PII S0893-133X(02)00377-9

the nerve terminal and enhancing serotonergic neu-rotransmission. The clinical success of the antidepres-sant SSRI fluoxetine (FLX, Prozac, Wong et al. 1995) has strengthened the initial serotonin hypothesis of depres-sion postulated by Van Praag and Korf (1970). Increas-ing serotonin levels alone, however, is not the full an-swer to the diverse clinical symptoms of depression. There is also a role for NE, as seen with TCAs and the more recent dual 5-HT/NE reuptake inhibitors (Dulox-etine, Berk et al. 1997; Venlafaxine, Kent 2000) and se-lective NE reuptake inhibitors (Atomoxetine (Tomoxet-ine), Wong et al. 1982; Reboxetine, Wong et al. 2000). The involvement of different neurotransmitter systems also underlines the complexity of depression. In pa-tients successfully treated with either a TCA or NE re-uptake inhibitor but not with a SSRI, a subsequent de-pletion of NE levels will precipitate a relapse into the depressive state (Miller et al. 1996a,b).

Besides the involvement of the 5-HT and NE systems, the DA system is affected as well. Anecdotal drug combi-nations, bupropion therapy and electroconvulsive ther-apy (ECT) are relevant treatment strategies, all having in common the elevation of DA levels. An abundance of lit-erature is emerging on the role of DA in depression (Brown and Gershon 1993; Fibiger 1995; Ebert and Lam-mers 1997). Successful treatment with ECT in Parkinson's Disease (Atre-Vaidya and Jampala 1988) and severe cases of depression (DasGupta 1998; Strober et al. 1998) indi-cates a DA imbalance, and indeed, ECT elevates extracel-lular DA levels (Zis et al. 1991; Yoshida et al. 1997, 1998). Additionally, anhedonia is one of the hallmarks of de-pression and is linked to DA deficiency (for review, see Pani and Gessa 1997; Heinz 1999). Thus, modulation of a single monoamine system is not the only approach to treating depression, and enhanced interaction among monoaminergic systems may increase clinical efficacy or decrease the time to onset of activity. Clinically, treat-ments elevating one or more of the monoamines, DA, 5-HT, and NE have been successful (Seth et al. 1992; Rich-elson 1996; Stahl 1998; Corrigan et al. 2000).

The 5-HT$_{2A}$ receptor is also implicated in the treatment of depression (Stefanski and Goldberg 1997) and activa-tion thereof has been linked to undesired side-effects such as altered sleep, anxiogenesis, and sexual dysfunc-tion (Pullar et al. 2000). Furthermore, activation of the 5-HT$_{2A}$ receptor or blockade of the 5-HT$_{2C}$ receptor in-creases extracellular DA and NE in the prefrontal cortex of the rat brain (Millan et al. 1998; Gobert and Millan 1999). Blockade of the 5-HT$_2$ receptor with mesulergine elevated extracellular DA levels in the nucleus accum-bens and selective blockade of the 5-HT$_{2C}$ receptor in-creases mesolimbic DA levels (Di Matteo et al. 1998; Di Matteo et al. 1999). In addition, Di Matteo (2000) sug-gested that the antidepressants amitryptyline and mi-anserin elevate DA in the nucleus accumbens through a 5-HT$_{2C}$ receptor-sensitive mechanism.

Considering the importance of interaction of the 5-HT system with the DA and NE systems, we investigated the effects of R-FLX on these systems. We evaluated the interactions of R- and S-FLX with monoaminergic re-ceptors and transporters, as well as their effects on ex-tracellular levels of 5-HT, DA, and NE, using in vivo microdialysis.

MATERIALS AND METHODS

Drugs

Fluoxetine, norfluoxetine and their enantiomers, citalo-pram, fluvoxamine, paroxetine, and sertraline, were pre-pared by Lilly Laboratories. Desipramine and imi-pramine were purchased from Sigma Co. (St. Louis, MO).

Transporter Binding

Membranes from HEK 293, MDCK, and HEK 293 cell lines transfected with human 5-HT, NE, and DA trans-porters, respectively, were obtained from Receptor Bi-ology, Inc. (Beltsville, MD). All assays were performed in triplicate in a final volume of 0.8 ml of buffer contain-ing 50 mM Tris Cl pH 7.4, 150 mM NaCl, and 5 mM KCl for 5-HT and NE transporters; or 50 mM Tris Cl, pH 7.4, and 100 mM NaCl for the DA transporter); [^{3}H]-parox-etine (0.2 nM final concentration, 25 Ci/mmol, New En-gland Nuclear, Boston, MA), [^{3}H]-nisoxetine (1.0 nM fi-nal concentration, 86 Ci/mmol, New England Nuclear) or [^{3}H]-Win35,428 (1.0 nM final concentration, 86 Ci/ mmol, New England Nuclear) in buffer for the human 5-HT, NE and DA transporters, respectively. Mem-branes of 10.3 μg, 16.9 μg or 6.2 μg protein of clonal cells containing human 5-HT, NE and DA transporter, respectively, were added. After incubation, 37°C for 40 min for the 5-HT transporter and 25°C for 30 min for NE and DA transporters, the binding was terminated by rapid vacuum filtration over Whatman GF/B filters (presoaked in 0.5% polyethylenimine) and the filters were washed four times with cold 50 mM Tris Cl buffer, pH 7.4. The filters were then placed in vials containing liquid scintillation fluid and radioactivity was mea-sured by liquid scintillation spectrometry. Non-specific binding was determined by including separate samples of 1 μM duloxetine, 10 μM desipramine, or 10 μM nomifensine, for 5-HT, NE and DA transporters, re-spectively.

Determination of Binding to Neuronal Receptors

The binding to human 5-HT receptor subtypes was de-termined according to published methods (5-HT$_{1A}$ and 5-HT$_{1E}$, Zgombick et al. 1991; 5-HT$_{1B}$ and 5-HT$_{1D}$, Wein-shank et al. 1992; 5-HT$_{1F}$, Adham et al. 1993; 5-HT$_{2A}$,

5-HT$_{2B}$, 5-HT$_{2C}$, Wainscott et al. 1996; 5-HT$_6$, Boess et al. 1997; 5-HT$_7$, Bard et al. 1993). The binding to rat 5-HT$_3$ receptors was from the method of Wong et al. 1993. Inhibition of binding to 5-HT$_4$ and other neuronal receptors was provided by NovaScreen (Hanover, MD).

GTPγS Binding to 5-HT$_2$ Receptors

Cloned 5-HT$_2$ Receptors.   Human cloned 5-HT$_{2A}$, 5-HT$_{2B}$, or 5-HT$_{2C}$ receptors were expressed in AV12 cells as described earlier (Lucaites et al. 1996) and frozen until use. Pellets of cells were thawed and washed with assay buffer (excluding GDP), incubated for 10 min at 37°C to hydrolyze any residual 5-HT, washed and suspended for assay (approximately 3–4 × 10^6 cell equivalents/well). GTP-γ-[^{35}S] nucleotide exchange Immunoadsorption Scintillation Proximity Assay (ISPA) was conducted as described previously (DeLapp et al. 1999; Conway et al. 1999). Standard assay buffer consisted of 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM MgCl$_2$, 0.2 mM EDTA, and 0.1 μM GDP. GTP-γ-[^{35}S] (1250 Ci/mmol, New England Nuclear), GDP, and agonist and/or antagonist were added to each well of 96-well microtiter plates, with clear bottoms for subsequent SPA counting. Agonist stimulation of the guanyl nucleotide exchange reaction was initiated by the addition of cell membranes with cloned 5-HT$_2$ receptors. Plates were mixed and incubated for 30 min at room temperature. The reaction was quenched by the addition of IGEPAL CA-630 detergent (to final concentration 0.27%), primary anti-Gq/11 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; to about 1 μg per well), with secondary anti-rabbit SPA beads (Amersham Pharmacia Biotech, Piscataway, NJ) added to each well (about 1.25 mg/well); plates were sealed, mixed, and incubated for 3 h at room temperature. Finally plates were gently centrifuged, and counted in a Wallac TriLux MicroBeta (Perkin-Elmer, Gaithersburg, MD) scintillation counter.

Microdialysis Experiments

Animals.   Male Sprague-Dawley rats with body weight 260–300 g from Harlan Sprague Dawley (Indianapolis, IN) were used. After acclimatization for at least one week, rats were anesthetized with chloral hydrate/pentobarbital (170 mg/kg and 36 mg/kg in 30% propylene glycol and 14% ethanol, respectively) to allow the implantation of dialysis probes.

Probe Implantation.   The microdialysis technique has been previously described (Li et al. 1998). Looped dialysis probes were implanted into specific areas of the rat brain and then fixed in place with cranioplastic cement (DuraLay, ordered through BioAnalytical Systems (BAS), West Lafayette, IN). Dialysis probes of loop-type

were made using cellulose dialysis membrane (C-D Medical, Miami, FL) with a molecular weight cut-off of 5000 Da. The exposed tubing of dialysis probe was 3 mm length with an outer diameter of 0.6 mm, except for probes implanted into the nucleus accumbens which were 2 mm in length. The coordinates for specific brain areas were as follows: prefrontal cortex (PFC): A (anterior to bregma) 3.2 mm, L (lateral from the mid-sagittal suture) −0.8 mm, V (ventral from the dura surface) −5 mm; nucleus accumbens (N Acc): A 2.2 mm, L −1.5 mm, V −8 mm; striatum (STR): A 0.2 mm, L −3 mm, V −7 mm, hypothalamus (Hypothal): A −1.5 mm, L −1.3 mm, V −9 mm (all from Paxinos and Watson 1986). Probe location was determined histochemically and rats with improper placement were not included in the statistical analysis.

Microdialysis experiments were performed two days after surgery to allow the rats to fully recover from the operation and resume normal feeding. The rat was placed in a plastic bowl and connected to a fraction collection system for freely moving animals (Raturn, BAS). The input tube of the dialysis probe was connected to a syringe pump (BeeHive and BabyBee, BAS) which delivered an artificial cerebrospinal fluid containing 150 mM NaCl, 3 mM KCl, 1.7 mM CaCl$_2$ and 0.9 mM MgCl$_2$ (pH 6.0) to the probe at a rate of 1.0 μl/min. The output tubes from the rats were attached to an electrically activated and refrigerated fraction collector (HoneyComb, BAS).

Dialysates were transferred to a refrigerated Gilson Model 231 Autosampler/Injector. Less than 20% variation of the basal monoamine levels was obtained 2–3 h after the start of experiments. Drugs were given by intraperitoneal (i.p.) injection after at least three stable baseline samples were obtained. Sequential doses were administered i.p. every 90 min.

Assays of Extracellular Monoamine Levels.   The sequential dose response study with R-FLX, S-FLX and R,S-FLX was conducted using an on-line system according to the method of Li et al. (1998).

The remainder of the studies were analyzed using an off-line HPLC analytical method that simultaneously detected three monoamines (DA, NE and 5-HT) and the metabolites DOPAC, 5-HIAA and HVA in the same dialysates. A BDS-Hypersil 3 μ C18 analytical column (2 × 150 mm from Keystone Scientific, Bellefonte, PA) with a 10-port HPLC valve and a 20 μl sample loop was used in configuration with a small sample clean-up column (BDS-Hypersil 3 μ C18, 2 × 10 mm) which trapped a late-eluting peak contained in the dialysate samples. The mobile phase for both columns was the same and consisted of 75 mM sodium phosphate monobasic, 350 mg/L 1-octanesulfonic acid sodium salt, 0.5 mM EDTA, 0.8% tetrahydrofuran (HPLC grade, inhibitor-free) and 8% acetonitrile at pH 3 (adjusted with phosphoric acid). The flow rate for both columns was 0.20 ml/min. The

analytical column was maintained at 40°C with a column heater, while the sample cleanup column was mounted on the 10-port valve at room temperature. An electrochemical detector (EG & G PARC, Princeton, NJ) with dual glassy carbon electrodes was used (E1 = 500 mV, E2 = −50 mV, range = 0.5 nA on both electrodes). 5-HT and metabolites were detected at E1 and NE and DA were detected at E2.

The data were collected on three channels using an EZChrom chromatography data system (Scientific Software, San Ramon, CA) running on a Compaq computer that calculated peak heights and sample concentrations. The sensitivity for DA, NE and 5-HT was 0.1 pmol/ml dialysate or 2 fmol/sample (20 μl).

Statistical Analysis

Binding studies were analyzed with GraphPad Prism software (San Diego, CA) using nonlinear regression analysis on a PC with Microsoft Windows 95 OS. K_i values were calculated from IC_{50} estimates by the method of Cheng and Prusoff (1973). All values for microdialysis studies were calculated as percentage change at each time point compared with the average of three baseline values. Significant differences for the time course of vehicle control injection on NE, DA or 5-HT were determined by a 1-way analysis of variance (ANOVA) for repeated measures with respect to time. Differences between treatment groups, including control, were determined by a 2-way ANOVA with treatment as the independent variable and time as the repeated measure. If significant, the ANOVA was followed by a post-hoc Duncan's multiple range test on the overall effect of treatment using the Statistica program (StatSoft, Tulsa, OK).

RESULTS

Transporter and Receptor Binding Studies

R-FLX inhibited binding of [³H]-paroxetine binding to membranes from HEK 293 cells transfected with human 5-HT transporters with a K_i value of 7.7 ± 1.0 nM, but did not appreciably inhibit binding to human NE or DA transporters (Table 1). R,S-FLX, S-FLX, R,S-norfluoxetine and S-norfluoxetine had similar affinity for the human 5-HT transporter as R-FLX had low affinity for the NE and DA transporters. However, R-norfluoxetine had lower affinity for the 5-HT transporter than R-FLX. Other SSRIs such as citalopram, fluvoxamine, paroxetine and sertraline also had high affinity for the 5-HT transporter and relatively low affinity for the NE and DA transporters. Of the drugs tested, paroxetine had the highest affinity for the serotonin transporter. Imipramine inhibited binding to both the 5-HT and NE transporters, whereas desipramine, a NE uptake inhibitor, had higher affinity for NE than 5-HT transporters.

The affinity of FLX, the enantiomers of FLX and R-norfluoxetine was determined for a number of serotonin receptor subtypes (Table 2). R-FLX and R-norfluoxetine had K_i values of 68 ± 1 and 115 ± 10 nM, respectively, for human 5-HT_{2A} receptors and K_i values of 70 ± 8 and 116 ± 5 nM for 5-HT_{2C} receptors, respectively. S-FLX had greater than 20-fold lower affinity for 5-HT_{2A} and 5-HT_{2C} receptors than R-fluoxetine. The compounds did not have appreciable affinity for the other 5-HT receptor subtypes investigated. In a comparison of the affinity of R-FLX on binding affinity to the human and rat 5-HT_{2A} receptors, it was found that R-FLX had significantly lower affinity for the rat than human 5-HT_{2A} receptor subtype, with K_i values of 165 nM (data not shown).

Table 1. Inhibition of Binding to Human Monoamine Transporters by Fluoxetine, Norfluoxetine and Other Antidepressants

Compound	Transporter		
	5-HT	**NE**	**DA**
	K_i, nM		
R-fluoxetine	7.7 ± 1.0	>10000	>10000
R-norfluoxetine	68 ± 12	>10000	>10000
S-fluoxetine	6.1 ± 0.3	>10000	>10000
S-norfluoxetine	4.8 ± 0.3	>10000	>10000
R,S-fluoxetine	6.9 ± 0.6	1021 ± 46	4752 ± 44
R,S-norfluoxetine	9.9 ± 0.5	>10000	>10000
Citalopram	9.5 ± 1.3	>10000	>10000
Fluvoxamine	10 ± 1.4	>10000	>10000
Paroxetine	0.38 ± 0.04	132 ± 6.8	393 ± 53
Sertraline	0.9 ± 0.2	715 ± 53	26 ± 1.7
Desipramine	179 ± 10	3.8 ± 0.3	>10000
Imipramine	19 ± 0.9	98 ± 4	>10000

Compounds were evaluated with 7–11 concentrations in triplicate and data are from at least three separate experiments.

Table 2. Inhibition of Radioligand Binding to Human Serotonin Receptor Subtypes by R,S-fluoxetine, S-fluoxetine, R-fluoxetine and R-norfluoxetine

Receptor	FLX	S-FLX	R-FLX	R-NFLX
	K_i, nM			
5-HT_{1A}	>5000	4045	3462	3766
5-HT_{1B}	>5000	3910	2892	4080
5-HT_{1D}	>5000	4547	4525	>5000
5-HT_{1E}	>5000	>5000	>5000	>5000
5-HT_{1F}	4955	4580	4659	4971
5-HT_{2A}	119 ± 10	1409 ± 120	68 ± 1	115 ± 10
5-HT_{2B}	2514	3189	4180	>5000
5-HT_{2C}	118 ± 11	2390 ± 310	70 ± 8	116 ± 5
5-HT_3	19000	>10000	11000	4000
5-HT_4	788	1074	2767	2088
5-HT_6	1038	2730 ± 194	591 ± 33	2450 ± 120
5-HT_7	>5000	3397	4898	>5000

Binding data is to human receptors, except for $5HT_3$ data which is from rat (Wong et al. 1993.) Data is from triplicate determination from three separate experiments with 7–11 concentrations of the drugs. (FLX = fluoxetine, NFLX = norfluoxetine)

Table 3. Agonist and Antagonist Activity of R-fluoxetine at Human $5HT_2$ Cloned Receptors Using GTPγS Binding with G_q Antibody Capture

Receptor	% Max of Full Agonist	$K_{I,}$ nM
5-HT_{2A}	-10 ± 4	193 (107–346)
5-HT_{2B}	2 ± 6	>10000
5-HT_{2C}	-1 ± 5	493 (255–953)

% max of full agonist refers to % of maximal stimulation obtained with 10 μM 5-HT. The K_i values refer to inhibition constants obtained by blocking 5-HT-stimulated GTPγS binding at 100 nM for 5-HT_{2A} and 5-HT_{2B} and 10 nM for 5-HT_{2C} receptors. (n = 4–7 separate determinations)

R-FLX did not stimulate GTPγS binding to the G protein Gq in cell lines transfected with 5-HT_{2A} and 5-HT_{2C} receptors, indicating a lack of agonist activity (Table 3). However, R-FLX antagonized 5-HT (100 nM and 10 nM) stimulated GTPγS binding in cell lines transfected with 5-HT_{2A} and 5-HT_{2C} receptors, respectively. The inhibition constants (K_i) of R-FLX for blocking 5-HT stimulated ^{35}S-GTPγS binding in cell lines were 193 nM and 493 nM for the 5-HT_{2A} and 5-HT_{2C} receptors, respectively (Table 3). In contrast, S-FLX did not block 5-HT stimulated GTPγS binding in cell lines transfected with 5-HT_{2A} and 5-HT_{2C} receptors up to concentrations of 10 μM (data not shown).

Microdialysis Experiments

Basal levels of the monoamines in different brain regions, expressed in pmol/ml are shown in Table 4. Vehicle administration did not alter monoamine levels in the PFC (Figure 1, panel A) with respect to time as shown by a 1-way ANOVA with repeated measures ($F_{8,40}$=0.399, 2.090 and 0.681 for NE, DA and 5-HT respectively). Vehicle controls in other brain regions were also not significantly changed (data not shown).

Effect of Fluoxetine Enantiomers on Extracellular Levels of Prefrontal Cortex 5-HT, NE, and DA. R-, S- or R,S-FLX were administered sequentially i.p. at 3, 10, and 20 mg/kg 90 min apart. The three compounds similarly and significantly increased 5-HT extracellular levels in prefrontal cortex at 3 mg/kg up to 211–224% of basal levels and the 10 mg/kg dose produced greater increases to 386% of basal levels. The 2-way ANOVA indicated that there was a significant effect of all compounds ($F_{9,117}$ = 26.22, $p < .001$), Figure 1, panel B. However, the 20-mg/kg dose did not produce appreciably greater increases, indicating that 10 mg/kg gives maximal 5-HT extracellular increases. There was no significant difference at any dose between the effect of FLX, R-FLX and S-FLX on extracellular 5-HT levels ($F_{18,117}$ = 0.796 with respect to dose-treatment interaction).

The extracellular levels of DA in prefrontal cortex were not significantly increased at 3 mg/kg by the compounds (Figure 1, panel C). However, the R-FLX 10-mg/kg i.p. sequential dose at 90 min significantly increased DA extracellular levels to $273 \pm 38\%$ of basal levels, whereas S-FLX and R,S-FLX increased DA extracellular levels to about 170% of basal levels . The R-FLX 20-mg/kg sequential dose further increased extracellular levels of DA to over 600% of basal levels. The 20-mg/kg sequential dose of S-fluoxetine and R,S-fluoxetine increased extracellular levels of DA up to about 254 and 372% of basal levels, respectively. The 2-way ANOVA on overall effects revealed that there was a significant effect of treatment ($F_{2,13}$ = 8.55, $p < .005$) and dose ($F_{9,117}$ = 34.23, $p < .001$) and dose-treatment interaction ($F_{18,117}$ = 3.63, $p < .001$). Thus, R-FLX produced significantly larger increases of extracellular levels of DA than S-FLX ($p < .002$) or RS-FLX ($p < .01$) according to a post-hoc Duncan's test.

As found with DA, the 3-mg/kg dose of the compounds did not significantly alter extracellular levels of NE in prefrontal cortex (Figure 1, panel D). However, R-fluoxetine significantly increased extracellular levels of NE up to 211 and 323% after administration of the sequential doses of 10 and 20 mg/kg i.p. Administration of the sequential doses of S-FLX and R,S-FLX produced smaller peak increases in the extracellular levels of NE up to 149% and 257% (10 mg/kg) and 256% and 298% (20 mg/kg), respectively Although the 2-way ANOVA showed a significant effect of dose ($F_{9,117}$ = 41.15, $p < .001$), there was no significant difference in treatments ($F_{2,13}$ = 1.16).

Administration of R-FLX (3, 10 and 30 mg/kg i.p.) administered in single doses increased extracellular levels of 5-HT dose dependently. For comparisons, a 4-h average post drug treatment value was calculated with increases of 138%, 349% ,and 338% of basal levels, respectively (Figure 2). A post-hoc Duncan's test showed

Table 4. Basal Levels of the Monoamines in Different Brain Regions

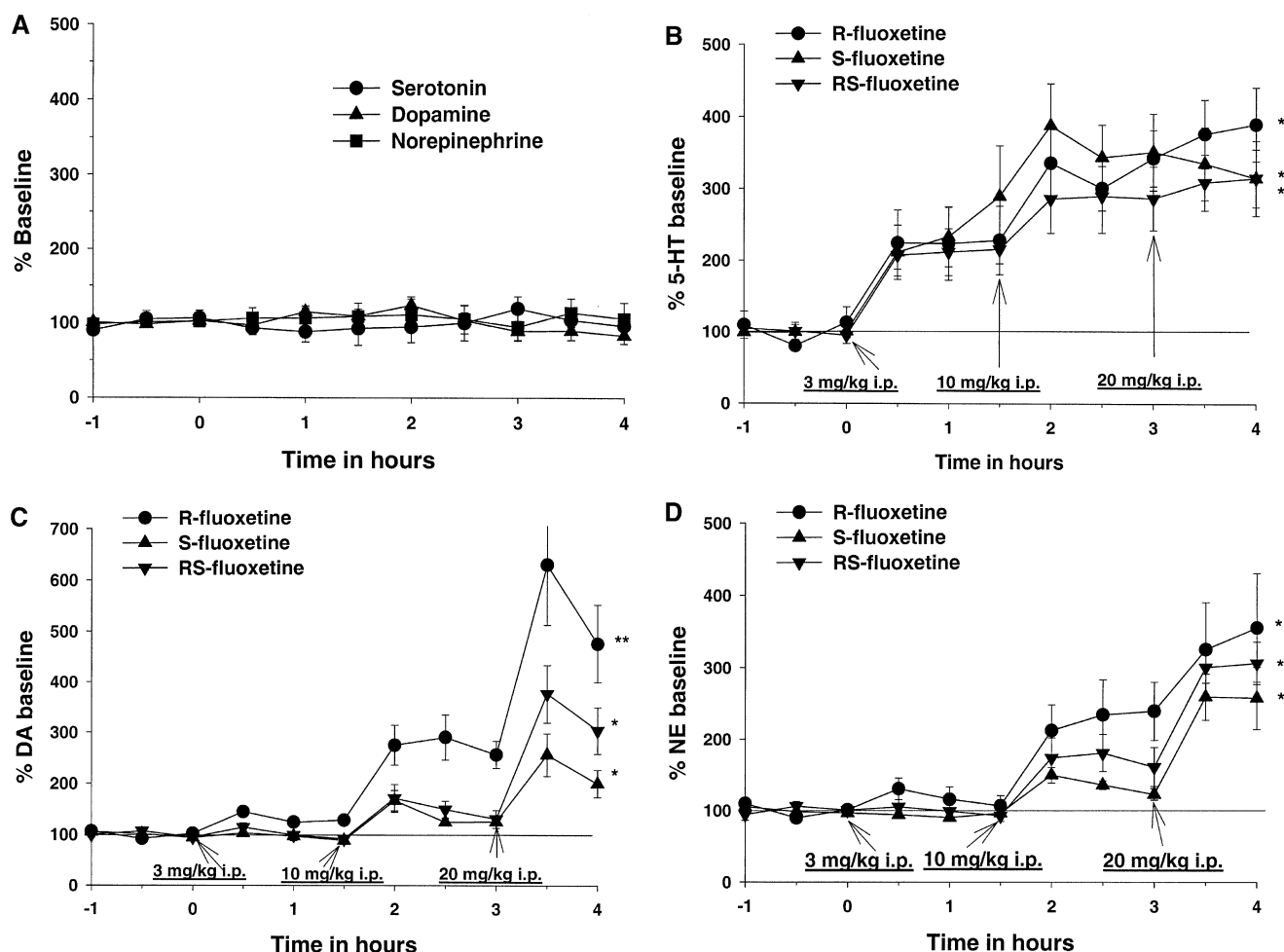| Monoamine	Prefrontal Cortex	Hypothalamus	Striatum	Nucleus Accumbens
		pmol/ml		
5-HT	0.19 ± 0.04	0.30 ± 0.04	0.25 ± 0.1	0.18 ± 0.06
NE	0.70 ± 0.25	0.80 ± 0.25	n/a	1.0 ± 0.4
DA	0.30 ± 0.1	0.26 ± 0.05	5.8 ± 0.7	4.5 ± 1.6

n/a = not available

Figure 1. Effect of vehicle injection on levels of extracellular monoamines **(A)** and R-, S-, and R,S-fluoxetine on extracellular levels of 5-HT **((B)** *p < .001 vs. control), dopamine **((C)** *p < .01 vs. control, **p < .01 vs. RS or S-fluoxetine), and norepinephrine **((D)** p < .001 vs. control) in prefrontal cortex of conscious rats. Drugs were administered sequentially at 0, 1.5, and 3 h with doses of 3, 10, and 20 mg/kg i.p., as indicated by the arrows. Values are the mean ± SEM of the % of pre-drug baseline concentration determined at −1, −0.5, and 0 h.

that the 3-mg/kg dose was not significantly different from control (p < .29) but the 10 and 30-mg/kg doses were significantly different (p < .001). At the lowest dose R-FLX did not significantly alter extracellular levels of DA, but significantly increased the 4-h average at 10 and 30 mg/kg i.p. to 165% and 231% of basal, respectively (post-hoc Duncan's test, p < .006). Administration of 3 mg/kg R-FLX did not significantly increase extracellular levels of NE, but the 10 and 30-mg/kg dose increased the 4-h average to 200% and 405% of basal levels, respectively (post-hoc Duncan's test, p < .002).

Effect of R-FLX on Extracellular 5-HT, NE and DA in Prefrontal Cortex, Hypothalamus Nucleus Accumbens, and Striatum.   The effect of R-FLX (10 mg/kg i.p.) on extracellular levels of monoamine was investigated in prefrontal cortex, hypothalamus, nucleus accumbens and striatum. To determine statistical significance the

overall effect (4-h average) was compared with the appropriate control (values not shown) using a 2-way ANOVA and subsequent Duncan's test, if warranted. All p values given below are from the Duncan's test. In the prefrontal cortex, R-FLX increased 5-HT, DA and NE extracellular levels to 4-h averages of 349 ± 2% (p < .001), 165 ± 5% (p < .03) and 200 ± 8% (p < .007), respectively of basal levels (Figure 3, panel A). Extracellular 5-HT, DA and NE levels in the hypothalamus were increased by R-FLX administration with 4-h averages of 239 ± 15% (p < .002), 176 ± 17% (p < .001) and 237 ± 30% (p < .002), respectively (Figure 3, panel B). The administration of R-FLX increased the 4-h average of extracellular 5-HT and DA levels in the nucleus accumbens to 211 ± 12% (p < .003) and 108 ± 2% ($F_{1,10}$ = 0.33, non-significant) and, respectively (Figure 3, panel C). In the striatum, R-FLX increased the 4-h average of 5-HT and DA extracellular levels to 427 ± 34% (p < .001) and
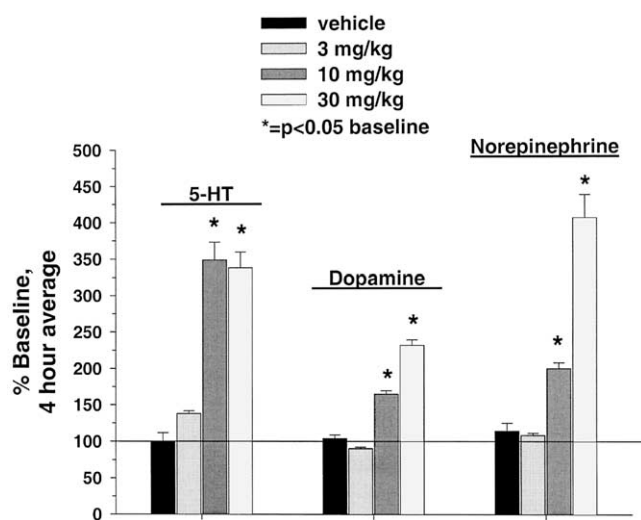
Figure 2. Dose-dependent increase of extracellular levels of serotonin (5-HT), dopamine and norepinephrine produced by R-fluoxetine in the prefrontal cortex of conscious rats. Data are expressed in % baseline for a 4-h average increase. Values are the mean ± SEM of the % of pre-drug baseline concentration determined at -1, -0.5, and 0 h ($p < .01$ vs. control).

132 ± 2% ($p < .02$), respectively (Figure 3, panel D). The NE levels were too low and variable to reliably quantitate in the nucleus accumbens or striatum.

DISCUSSION

The above studies demonstrate the unique binding properties and monoamine interactions of R-FLX, one of the two stereoisomers of FLX (Prozac). R-FLX and S-FLX have similar affinity for the human 5-HT transporter and low affinity for the DA and NE transporters. Similar affinity of the enantiomers for 5-HT uptake has been found in studies utilizing monoamine uptake in rat synaptosomes (Wong et al. 1993). However, stereoselectivity for human 5-HT_{2A} and 5-HT_{2C} receptors was found for R-FLX versus S-FLX with R-FLX having at least 20-fold higher affinity for the two receptors than S-FLX. R-FLX had K_i values for 5-HT_{2A} and 5-HT_{2C} receptors of 68 and 70 nM, respectively; this affinity was likely high enough to block these receptors in vivo, as FLX (10 mg/kg) had drug levels of 242 nM concentration in PFC (Bymaster et al. 2002). It has been previously reported that R,S-fluoxetine had moderate affinity for animal 5-HT_{2C} receptors (formerly called 5-HT1C) (Wong et al. 1995; Pälvimäki et al. 1996). Functional studies indicated that R-FLX was an antagonist at 5-HT_{2A} and 5-HT_{2C} receptors. Human monoamine transporter binding studies by Owens et al. (2001) confirmed the selectivity of R-FLX for the human serotonin transporter and 5-HT_{2C} receptors.

Stereoselective differences for R-FLX and S-FLX were also found in microdialysis studies in the rat prefrontal cortex. R-FLX and S-FLX produced similar increases in extracellular levels in 5-HT, but at the same dose R-FLX produced significantly greater increases in extracellular DA and NE levels. The racemic mixture, R,S-FLX, produced intermediate increases in the extracellular levels of DA and NE compared with R-FLX and S-FLX. Among SSRIs, fluoxetine uniquely increases extracellular levels of DA and NE as well as 5-HT (Pozzi et al. 1999; Bymaster et al. 2002). We have previously shown that R,S-FLX increases NE extracellular levels robustly in the hypothalamus (Perry and Fuller 1997). These data suggest that the elevation of DA and NE produced by R,S-FLX are predominantly mediated by R-FLX.

Since R-fluoxetine produced robust increases in extracellular 5-HT levels as well as DA and NE, the dose-dependent increases in extracellular monoamines produced by R-fluoxetine in prefrontal cortex were investigated in single dose studies. At 3 mg/kg i.p., R-FLX only increased 5-HT extracellular levels, but at 10 and 30 mg/kg i.p., the extracellular levels of DA and NE were also robustly increased. In microdialysis studies in various brain regions, R-FLX at 10 mg/kg i.p. was effective in increasing extracellular levels of 5-HT and NE in all brain areas examined, except the striatum and nucleus accumbens, where we were unable to measure NE levels. The elevation of 5-HT levels was greatest in the prefrontal cortex and striatum. The extracellular levels of NE in the hypothalamus increased to a greater extent than 5-HT for 1.5 h after drug administration. Extracellular levels of DA were significantly elevated in the prefrontal cortex and hypothalamus, but were not appreciably elevated in the dopamine-rich nucleus accumbens and striatum.

The mechanism by which R-FLX increases extracellular levels of DA and NE is not clear. Acute administration of SSRIs such as citalopram, paroxetine and fluvoxamine increases extracellular levels of 5-HT, but not DA and NE (Pozzi et al. 1999; Jordan et al. 1994; Bymaster et al. 2002), thus suggesting that the catecholamine increases are not mediated by increased extracellular levels of 5-HT. Furthermore, R-FLX was shown in the present study to have low affinity for NE and DA transporters, suggesting the increases are not due to nonselective blockade of catecholamine transporters. However, the demonstrated antagonism with 5-HT_{2A} and 5-HT_{2C} receptors by R-FLX may be involved in the increased levels of catecholamines, particularly in cortical areas. The other major SSRIs, citalopram, fluvoxamine, paroxetine and citalopram, do not have significant affinity for human 5-HT_{2A} and 5-HT_{2C} receptors, (Jenck et al. 1993; Pälvimäki et al. 1996, Owens et al. 1997). The interactions at these receptors may be responsible for the robust elevations of both DA and NE in the prefrontal cortex produced by R-FLX. This finding is in agreement
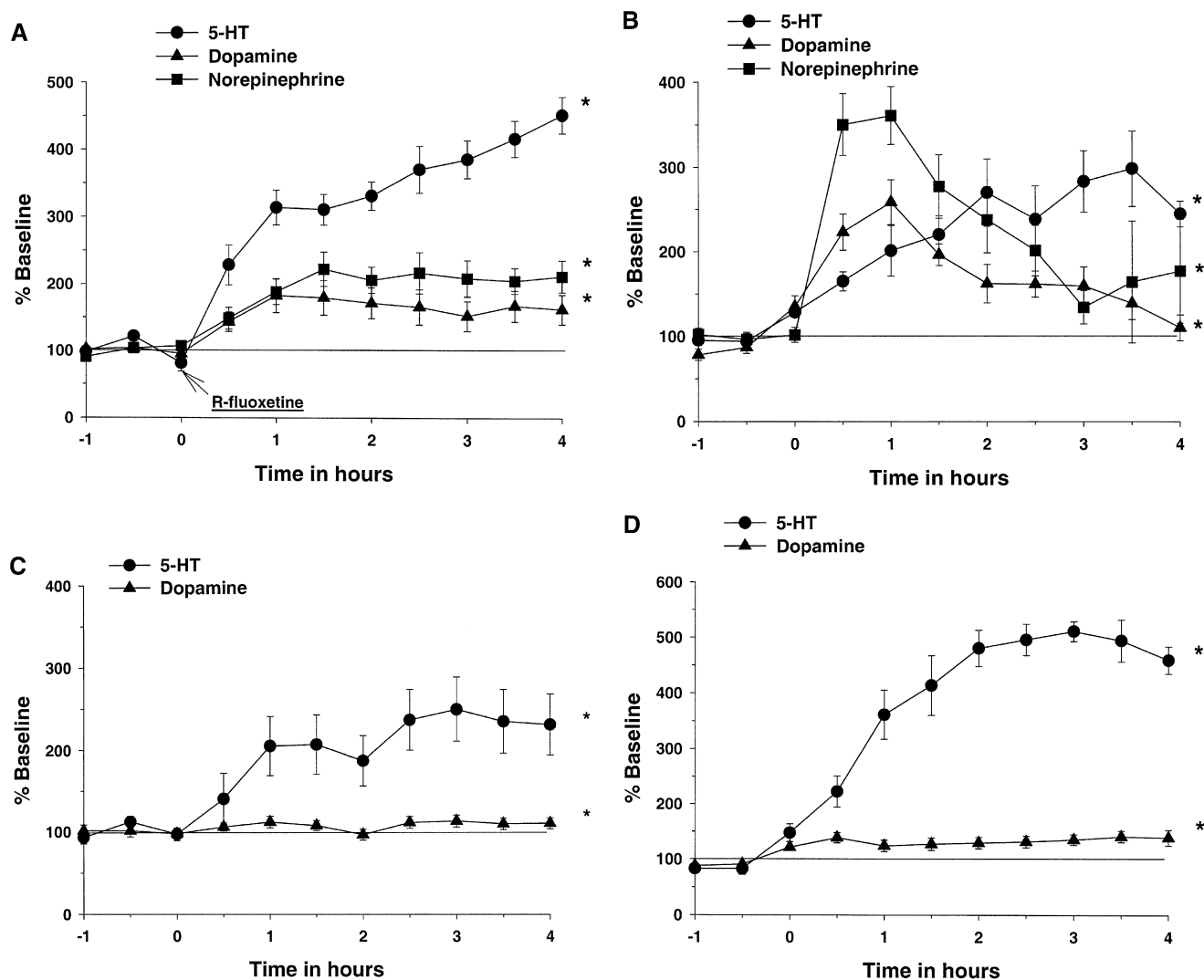
Figure 3. Effect of R-FLX on extracellular monoamine levels in the prefrontal cortex, **((A)** *$p < .03$), hypothalamus **((B)** *$p < .002$), nucleus accumbens **((C)** *$p < .003$), and striatum of conscious rats **((D)** *$p < .02$). Data are expressed as % baseline concentrations and drug was administered at time zero. Statistical significance was determined by calculating the overall effect (4-h average) of the drug.

with a study by Millan et al. (1998), showing that a 5-HT$_{2C}$ receptor antagonist elevated both DA and NE levels in the prefrontal cortex, whereas an agonist decreased extracellular DA and NE. Furthermore, fluoxetine (10 and 20 mg/kg) has been shown to have in vivo occupancy of the 5-HT$_{2C}$ receptors in rat choroid plexus (Pälvimäki et al. 1999), suggesting that R-FLX may block 5-HT$_{2C}$ receptors in vivo. However, other mechanisms may be involved since S-FLX also increased NE and DA to some extent in the prefrontal cortex in spite of low affinity for 5-HT$_{2C}$ receptors. The results in the nucleus accumbens, however, are in contrast to the findings by Di Matteo at al. (2000), who found increased DA release in the nucleus accumbens after blocking 5-HT$_{2C}$ receptors. The sensitivity and distribution of 5-HT$_2$ receptors within the nucleus accumbens may account for these

differences. A report by Bowers et al. (2000) demonstrates that 5-HT$_2$ receptor stimulation by DOI elevates DA only in the posterior nucleus accumbens; however, no reference is made to the specific receptor subtype involved.

The PFC is a region of interest due to the fact that depressed patients have reduced metabolism in the PFC as well as other anterior cortical regions, indicating an abnormality in these brain regions (Baxter et al. 1989; Rubin et al. 1995). Histopathological studies of post mortem brain tissues from depressed patients showed decreased size of neurons in prefrontal cortical regions such as dorsolateral PFC (Rajkowska et al. 1999). Further, the PFC is a complex collection of neural systems with extensive afferents of serotonergic, dopamine and noradrenergic neurons (Lindvall and Bjorklund 1978,

1983; Van Bockstaele et al. 1993), suggesting the potential for interactions and synergistic effects of the monoamines. Given the success of antidepressants aimed at elevating the monoamines, elevating NE and DA as well as serotonergic neurotransmission in the PFC likely plays a role in depression therapy.

These data suggest that the novel pharmacologic effects of R-FLX may provide added benefits in aiding development of new potential antidepressant compounds. In addition to increases of extracellular NE and DA concentrations in prefrontal cortex and other brain regions, the side effect profile of R-FLX–like compounds may also be improved through its blockade of the 5-HT$_{2A}$ receptor, which mediates sleep disturbances, sexual dysfunction and anxiety (Pullar et al. 2000). Indeed, blockade of the 5-HT$_2$ receptor can reverse sexual dysfunction and antidepressants with 5-HT$_2$ affinity do not induce sexual dysfunction (Stein and Hollander 1994; Balon 1997). Two other antidepressants, nefazodone and trazodone, share this property of potent antagonism of the 5-HT$_{2A}$ receptor (Cusack et al. 1994). Interestingly, the dopaminergic activity of R-FLX like compounds may also be advantageous in this respect since bupropion does not cause sexual dysfunction, but can alleviate this side effect caused by SSRIs (Stein and Hollander 1994; Balon 1997).

Interesting to note is the fact that 5-HT neuronal firing rates in the raphe recover much faster following chronic R-FLX application compared with racemic FLX (Lodge and Webb 2000). Acute administration of SSRIs decrease the firing rate of dorsal raphe neurons, presumably due to activation of 5-HT$_{1A}$ somatodendritic inhibitory autoreceptors which results in decreased release of 5-HT (Chaput et al. 1986; Czachura and Rasmussen 2000). In vivo microdialysis studies showed different effects of acutely versus repeatedly applied SSRIs on the release of 5-HT in the cell body area versus terminal fields. It was shown that acute doses of SSRIs preferentially increased 5-HT in the raphe rather than in projection fields (fluvoxamine, Bel and Artigas 1992; citalopram, Invernizzi et al. 1992; fluoxetine, Malagie et al. 1995; paroxetine, Gartside et al. 1995). However, there are also reports that acute SSRIs do elevate monoamines, particularly 5-HT, in certain projection fields (Perry and Fuller 1997; Pozzi et al. 1999; Bymaster et al. 2002), this effect, however, is blunted by acute autoreceptor stimulation. Unlike transporter substrates that reverse the action of the transporter irrespective of impulse flow, the SSRIs are dependent on the presence of newly released neurotransmitter to have an active effect. The clinical response has been hypothesized to be delayed until gradual desensitization of the 5-HT$_{1A}$ autoreceptors takes place (Artigas 1993; Blier and de Montigny 1994); however, agents that allow faster recovery of raphe firing, like R-FLX, may have a more rapid onset.

Thus, R-FLX robustly increases extracellular levels of 5-HT, NE, and DA in the prefrontal cortex, which is unique for SSRIs (Richelson 1996). Given the diverse symptomatology of depression and the clinical success of agents acting on all three monoamine systems, pharmaceutical agents with the properties like R-FLX represent a potential major improvement for treatment strategies. Increases in extracellular DA and NE levels, in addition to elevating 5-HT levels, may be very efficient in the treatment of certain types of depression. Selectivity for certain receptor subtypes will alleviate bothersome side effects, and may also shorten the onset to a clinical recovery. More research on 5-HT receptor subtypes and the regulation of interaction between the 5-HT, NE and DA systems is warranted to improve the efficacy of currently available treatments for depression.

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