Fluoxetine and norfluoxetine stereospecifically facilitate pentobarbital sedation by increasing neurosteroids

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Mice housed in social isolation exhibit a decreased response to γ-aminobutyric acid-mimetic drugs [i.e., pentobarbital (PTB)] associated with a down-regulation of telencephalic allopregnanolone (Allo) levels. In these mice, the PTB-induced loss of righting reflex is greatly reduced. Fluoxetine (FLX) and norfluoxetine (NFLX) stereospecifically reverse the effect of social isolation on the PTB-induced loss of righting reflex and on the decrease of telencephalic Allo content. The S-isomers of FLX and NFLX are 2- and 7-fold more potent, respectively, than their respective R-isomers. The EC50s of FLX and NFLX required to normalize brain Allo content and PTB action are 10–50 times lower than the IC50s required for the S-isomers of FLX and NFLX are 2- and 7-fold more potent, respectively, than their respective R-isomers. The EC50s of FLX and NFLX required to normalize brain Allo content reverses the reduced PTB-induced loss of righting reflex and on the decrease of telencephalic Allo content. The S-isomers of FLX and NFLX are 2- and 7-fold more potent, respectively, than their respective R-isomers. The EC50s of FLX and NFLX required to normalize brain Allo content reverses the reduced PTB-induced loss of righting reflex and on the decrease of telencephalic Allo content. The S-isomers of FLX and NFLX are 2- and 7-fold more potent, respectively, than their respective R-isomers. The EC50s of FLX and NFLX required to normalize brain Allo content reverses the reduced PTB-induced loss of righting reflex and on the decrease of telencephalic Allo content.

Materials and Methods

Animals and Drug Treatment. Adult male Swiss–Webster mice (Harlan Breeders, Indianapolis), 22- to 25-g body weight, maintained under a 12-h dark/12-h light cycle with food and water available ad libitum, were used for all experiments. Animals were housed either in groups of five to six per cage (24 × 17 × 12 cm) or individually (SI) in a cage of the same size for a time period varying from 4 to 6 weeks preceding our behavioral and biochemical measurements (1, 4). The vivarium temperature was approximately 24°C, and the humidity was approximately 65%. Group-housed (GH) and SI male mice were subjected to i.p. injections of racemic FLX, R- or S-FLX, or imipramine. Vehicle or tested drugs were prepared in 1% DMSO solutions and given i.p. as 0.1 ml per 10 g of body weight.

Racemic FLX, R-FLX, S-FLX, R-NFLX, and S-NFLX were a generous gift of Eli Lilly. Imipramine was provided by Sigma. Heptafluorobutyric acid anhydride (HFBA) was purchased from Pierce. Unless otherwise specified, all organic solvents were of HPLC grade and were purchased from Fisher Scientific.

Measurement of PTB-Induced RRL. The duration of the PTB-induced RRL in GH and SI male mice was measured as previously reported (1) after i.p. injections of PTB sodium (50 mg/kg; 0.5 mg/0.1 ml).

Analysis of PTB Brain Content. Extraction and HPLC quantification of PTB were performed as previously reported (1, 12). In brief, cerebral cortex samples (~200 mg) were homogenized in 10 vol of 0.45 M perchloric acid in 150 mM NaCl containing 20 nmol of secobarbital sodium [5-(methylbutyl)-5-(2-propenyl)-2,4,6 (1H, 3H, 5H) pyrimidinetrione-mono-osodium salt]. The homogenate was extracted in chloroform. The organic layer was then extracted with 1 vol of 1 M NaOH in saline and subsequently with

Abbreviations: SI, social isolation; GABA, γ-aminobutyric acid; GABA_A, GABA type A; RRL, loss of righting reflex; PTB, pentobarbital; Allo, allopregnanolone; FLX, fluoxetine; NFLX, norfluoxetine; SSRI, selective serotonin reuptake inhibitor; GH, group-housed; OB, olfactory bulb; S-HT, serotonin.

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1.5 vol of 1 M HCl in diethyl ether, collected, dried, taken up in distilled water, and injected into a reverse-phase Bio-Sil ODS-SS column (250 × 4 mm, Bio-Rad). The HPLC column was equilibrated with 1% trifluoroacetic acid (TFA) in HPLC-grade distilled water and developed with a gradient of 0.1% TFA in acetonitrile. PTB and the internal standard were eluted from the column with 35% and 33% acetonitrile, respectively, and were detected by UV absorbance at 220 nm.

### Brain Neurosteroid Content

Extraction, derivatization, and GC MS analyses of neurosteroids were performed with minor modifications as previously described (3). (i) Olfactory bulbs (OBs; (4) or frontal cortices were homogenized in 10 vol of distilled water containing 2 mM Pinna and 10 mM glucose, 1 mM MgSO4, 1 mM Hepes, 10 mM NaHCO3, 2.3 mM CaCl2, 3.6 mM KCl, 10 mM MgSO4, 1 mM Hepes, 10 μM pargyline, 1 mM ascorbic acid, pH 7.4), brain slices were incubated at 37°C for 5 min in the presence of 0.3 mg of protein. The uptake was terminated by filtration through GF/B glass fiber filters. The uptake of [14C]5-HT detected in the presence of 1 μM FLX was considered to be nonspecific (because of the uptake of [14C]5-HT by other monoaminergic uptake systems) (15) and was considered a background value, which was subtracted from the total uptake of [14C]5-HT.

### Statistical Analysis

Data are given as the mean ± SEM unless otherwise indicated. Comparisons between the control group and each of the treatment groups were performed by one-way ANOVA followed by Dunnett’s test.

The IC50 was calculated from dose–response curves analyzed by the “quantal dose–response: probits test” as described by Tallarida and Murray (16) equipped with a statistical package. Statistical comparisons among the different IC50 values were performed by using the COHORT package (CoHort Software, Monterey, CA). Differences were considered significant at P < 0.05.

### Results

In the GH control mice, administration of PTB (50 mg/kg, i.p.) induced RRL that lasted for ~1 h. The duration of PTB-induced RRL in SI mice was decreased by ~50% relative to that of GH mice (Fig. 1). Administration of low doses (less than micromolar-kilogram) of S-FLX and S-NFLX 30 min before PTB treatment reversed the effect of SI on PTB-induced RRL, but it did not alter the duration of PTB-induced RRL in GH mice (Fig. 1A and B). The EC50 of the S-FLX isomer was 0.7 μmol/kg and was three to four times smaller than the EC50 of the R-FLX isomer (Table 1 and Fig. 1B). S-NFLX (EC50 = 0.25 μmol/kg) was at least 2-fold more potent than S-FLX and 7-fold more potent than R-NFLX in normalizing the PTB action in SI mice (Table 1 and Fig. 1B).
In contrast to the SSRIs, imipramine, in doses that block 5-HT uptake (Table 1), failed to reverse the decreased duration of PTB-induced RRL (Fig. 1) or to normalize the decrease of brain Allo content in SI mice (data not shown). S-FLX or S-NFLX dose ranges that normalized PTB action also normalized brain Allo content (Fig. 2 and Table 1) and were severalfold lower than the doses of R-FLX or R-NFLX that were required to obtain similar results. However, these doses of S-FLX, R-FLX, and S-NFLX or R-NFLX failed to change cortical Allo levels in GH mice (data not shown).

The measurements of Allo reported in Fig. 2 refer to the extracts from OB, an area of the brain where Allo is highly expressed. However, similar results were obtained in samples from the frontal cortex [Allo levels, means ± SEM of five mice: vehicle, 2.9 ± 0.2 pmol/g; R-FLX at 1.5 μmol/kg, 3.8 ± 0.3, nonsignificant when compared with vehicle; S-FLX at 1.5 μmol/kg, 7.2 ± 0.2 pmol/g, P < 0.05 when compared with vehicle].

To study whether the FLX-induced normalization of the action of PTB in SI mice is due to a change in the PTB degradation rate, we measured the brain content of PTB 30 min after an injection of 2.9 μmol/kg racemic FLX. At the time when vehicle-treated SI mice were reacquiring the righting reflex, the brain content of PTB (mean ± SEM, 197 ± 12 nmol/g; n = 5) was virtually identical to that of racemically FLX-treated SI mice (mean ± SEM, 210 ± 18 nmol/g; n = 5), in which the RRL induced by PTB was still present.

FLX and NFLX are known to be potent SSRIs (11). Thus, it was important to establish whether the difference between the S- and R-stereoisomers in eliciting a normalization of PTB action in SI mice was related to differences in 5-HT reuptake inhibition potency. The SSRI potency of FLX and NFLX measured \textit{ex vivo} was 1–2 orders of magnitude higher than the EC$_{50}$ doses that normalized the duration of PTB-induced RRL in SI mice (Table 1). Moreover, FLX and NFLX stereoisomers were equipotent in inhibiting 5-HT reuptake (Fig. 3), indicating a lack of stereospecificity for this action.

**Discussion**

In previous reports we have shown that racemic FLX normalizes the reduction of PTB-induced sedation in SI mice, as measured by RRL, and at the same time increases brain levels of Allo (1). The present study shows that normalization of PTB-induced sedation in SI mice is stereospecific for both FLX and NFLX and parallels the ability of FLX and NFLX to stereospecifically increase brain Allo levels. The studies on brain Allo levels confirm and extend our previous report showing that FLX and NFLX at similar dose ranges normalize brain Allo content in a stereospecific manner and have a parallel anticonflict effect in SI mice (3).

The potency of the S-FLX or S-NFLX isomers to normalize the duration of PTB-induced RRL was 2- to 7-fold higher, respectively, than that of their respective R-isomers and appeared not to be caused by an alteration in the pharmacokinetic properties of PTB. Furthermore, the EC$_{50}$ of S-FLX and S-
NFLX required to reverse the reduced PTB-elicited RRL in SI mice was virtually identical to the EC50 required to normalize the reduced brain (OB or frontal cortex) Allo levels in such mice.

The present study further demonstrates that S-FLX and S-NFLX actions on neither PTB-induced RRL nor brain Allo content are related to their intrinsic SSRI activity. In fact, we show that the PTB and Allo effects of FLX and NFLX are stereospecific, whereas their SSRI activity lacks stereospecificity (Table 1 and Fig. 3). Additionally, the EC50 of S-FLX or S-NFLX required to normalize the PTB-induced RRL and brain Allo levels in SI mice is at least 1 order of magnitude lower than the IC50 required to inhibit 5-HT reuptake (Table 1). Indirect evidence that SSRI activity is not part of the mechanism of action of the S-FLX and S-NFLX on PTB and Allo was obtained in experiments with imipramine. In doses that block 5-HT reuptake (Table 1), imipramine fails to correct the reduced PTB action or to normalize brain Allo level down-regulation in SI mice. These data are consistent with a previous report (1) showing that p-chlorophenylalanine, in doses that reduce brain levels of 5-HT by >80%, does not prevent FLX action on PTB-induced sedation and on the increase of brain Allo levels in SI mice (1).

FLX and NFLX have been reported to increase GABA action at most GABA_A receptor subtypes, presumably via an allosteric modulatory mechanism (17). This effect occurs for FLX and NFLX doses in the high micromolar range. Based on pharmacokinetic studies (18), it can be expected that the content of FLX or NFLX present in brain 30 min after the systemic administration of micromolar/kilogram doses of these compounds is only in the low nanomolar range. Thus, it is likely that at these concentrations S-FLX and S-NFLX do not have a direct action on GABA_A receptors. Instead they may exert an indirect positive GABA_A receptor modulatory action by increasing brain Allo levels.

We have recently demonstrated that variations in the physiological (nanomolar) concentrations of brain Allo play a significant role in regulating the responses of GABA_A receptors to GABA or GABA mimetic drugs (4, 10). Moreover, administration of Allo in doses that per se fail to change the gross behavior of GH mice corrects the reduced response to PTB and muscimol observed in SI and SKF 105,111-treated mice, which express reduced levels of telencephalic Allo (1, 4).

Thus, these data provide important pharmacological evidence suggesting that the action of S-FLX and S-NFLX on PTB-induced RRL in SI mice is indirectly mediated at GABA_A receptors by the ability of S-FLX and S-NFLX to increase brain Allo content. They also encourage an in-depth study of the mechanisms of action of S-FLX and S-NFLX on neurosteroid biosynthesis in normal and SI mice.

In mouse neurons, Allo is synthesized from progesterone (pyramidal neurons in the cortex and the hippocampus, mitral cells in OBs, and Purkinje cells in the cerebellum) (unpublished data) via the action of 5α-reductase type I, which transforms progesterone into 5α-dihydropregesterone (5α-DHP), and 3α-hydroxysteroidoxydo-reductase (3α-HSOR), which reduces 5α-DHP into Allo (5, 19, 20). Because the decrease of brain Allo content in SI mice is associated with a decrease of 5α-reductase type I expression (5) and not with a decrease of 3α-HSOR, an attractive potential mechanism to explain the normalization of brain Allo content by S-FLX or S-NFLX may be through a direct action on 5α-reductase activity. In a previous study, we have shown that racemic FLX facilitates the conversion of 5α-DHP into Allo (21) in rat cortical brain slices. This effect has not been studied in brain slices from SI mice. Thus, whether the effect of FLX occurs at the levels of 3α-HSOR in SI mice, either by accelerating the reduction of 5α-DHP into Allo (22) or by inhibiting the oxidation of Allo into 5α-DHP, remains to be elucidated.

In conclusion, based on the data reported in this study, FLX could be considered a prototypic molecule for a new class of anxiolytic and antiaggressive drugs. Although acting with high potency and stereoselectivity on neurosteroid brain biosynthesis, this new drug class would not require significant action on brain 5-HT reuptake mechanisms.

FLX analogs that are weak inhibitors or fail to inhibit 5-HT transporter are available (23). Identification of derivatives of FLX or NFLX with weak or no SSRI activity that stimulate neurosteroidogenesis may ameliorate signs of anxiety and the response of GABA_A receptors to GABA_A receptor agonists in SI mice. These drugs may represent new pharmacological tools that could be beneficial in the treatment of anxiety, impulsive behavior, and premenstrual dysphoria in the absence of an inhibitory action on 5-HT uptake.

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