Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 12599–12604, October 1996
Neurobiology

Fluoxetine-elicited changes in brain neurosteroid content measured by negative ion mass fragmentography

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ABSTRACT **Fluoxetine administered intraperitoneally to sham-operated or adrenalectomized/castrated (ADX/CX) male rats dose-dependently (2.9–58 μmol/kg i.p.) increased the brain content of the neurosteroid 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, 3α,5α-TH PROG). The increase of brain 3α,5α-TH PROG content elicited by 58 μmol/kg fluoxetine lasted more than 2 hr and the range of its extent was comparable in sham-operated (≈3-10 pmol/g) and ADX/CX rats (2–9 pmol/g) and was associated with a decrease (from 2.8 to 1.1 pmol/g) in the 5α-pregnan-3,20-dione (5α-dihydroprogesterone, 5α-DH PROG) content. The pregnenolone, progesterone, and dehydroepiandrosterone content failed to change in rats receiving fluoxetine. The extent of 3α,5α-TH PROG accumulation elicited by fluoxetine treatment differed in various brain regions, with the highest increase occurring in the olfactory bulb. Importantly, fluoxetine failed to change the 3α,5α-TH PROG levels in plasma, which in ADX/CX rats were at least two orders of magnitude lower than in the brain. Two other serotonin re-uptake inhibitors, paroxetine and imipramine, in doses equipotent to those of fluoxetine in inhibiting brain serotonin uptake, were either significantly less potent than fluoxetine (paroxetine) or failed to increase (imipramine) 3α,5α-TH PROG brain content. The addition of 10 μM of 5α-DH PROG to brain slices of ADX/CX rats preincubated with fluoxetine (10 μM, 15 min) elicited an accumulation of 3α,5α-TH PROG greater than in slices preincubated with vehicle. A fluoxetine stimulation of brain 3α,5α-TH PROG biosynthesis might be operative in the anxiolytic and antidysphoric actions of this drug.**

Neurosteroids such as 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, 3α,5α-TH PROG); pregnenolone (PREG) sulfate; and dehydroepiandrosterone (DHEA), sulfate, promptly decrease or increase brain excitability acting as potent positive (3α,5α-TH PROG) or negative (PREG sulfate, DHEA sulfate) allosteric modulators of γ-aminobutyric acid (GABA) action at $GABA_A$ receptors (1–6). These discoveries have provided a new mechanism for brain GABAergic tone modification applicable to the treatment of various neuropsychiatric disorder symptoms (7, 8). These include the anxiety and mood changes of the "late luteal dysphoria syndrome," associated with low progesterone (PROG) plasma levels (a steroid participating in 3α,5α-TH PROG biosynthesis) (8, 9).

Though the prospect that neuroactive steroids could be used in the symptomatic treatment of specific neurological and psychiatric disorders has generated some enthusiasm, substantial difficulties prevent the therapeutic use of neurosteroids. For example, the systemic administration of 3α,5α reduced derivatives of PROG or androstenedione acting as positive allosteric modulators of GABA action at $GABA_A$ receptors, indicates that the doses required to elicit a clear anxiolytic, antidysphoric, and antiepileptic activity may also produce profound sedation, motor impairment, or ataxia (6, 8, 10). Two additional pharmacological actions that may limit the protracted therapeutic use of neuroactive steroids are: (*i*) their ability to trigger complex DNA transcription modifications in neuronal (11) and glial cells (12) and (*ii*) a possible tolerance liability, which may limit the protracted therapeutic use of these compounds in sleep disorders or convulsive syndromes (13–15).

One might reduce the complications associated with the protracted administration of neuroactive steroids by developing drugs that affect selectively some rate-limiting steps of brain neurosteroid biosynthesis, which unlike that of peripheral endocrine tissues, is not under pituitary control (7).

Recently, we were intrigued by a report that fluoxetine's beneficial effects in the treatment of "late luteal dysphoria" symptoms occur after a latency time shorter than that described for the treatment of the symptoms of depression (16). Though the mechanism whereby fluoxetine relieves the symptoms of late luteal dysphoria remains uncertain, very likely it may differ from that which alleviates the symptoms of depression. Since a decrease in brain availability of PROG metabolites may contribute to the onset of late luteal dysphoria symptoms (9), we began to investigate whether fluoxetine could change rat brain levels of 3α,5α-TH PROG and its precursors, including 5α-pregnan-3,20-dione (5α-dihydroprogesterone, 5α-DH PROG) and PROG.

We measured simultaneously in the same small brain area subpicomole amounts of PROG, its 5α metabolites (3α,5α-TH PROG and 5α-DH PROG), PREG, and DHEA using gas chromatography negative ion mass fragmentography (GC/NICI-MF). In fact, steroid radioimmunoassay technology, although highly reliable for measures of PREG, PROG, and 3α,5α-TH PROG in the nanomolar range (9, 17), loses its intrinsic specificity and sensitivity in measuring neurosteroids in the picomolar range (18, 19). The present report, which documents that fluoxetine increases 3α,5α-TH PROG brain content, also shows that this action is unrelated to the inhibition of serotonin uptake (20) elicited by this drug.

MATERIALS AND METHODS

Normal, sham-operated, and ADX/CX Sprague–Dawley male rats (Zivic–Miller), weighing 220–250 g were used. Food and water or physiologic saline (ADX/CX rats) were available ad libitum. ADX/CX and sham-operated rats were used for experiments 10–15 days after surgery. To monitor the results of ADX, the plasma levels of corticosterone were measured by radioimmunoassay (ICN). All animal procedures employed were in strict accordance with the National Institutes of Health

Abbreviations: PROG, progesterone; PREG, pregnenolone; DHEA, dehydroepiandrosterone; 3α,5α-TH PROG, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone); 5α-DH PROG, 5α-pregnan-3,20-dione (5α-dihydroprogesterone); GABA, γ-aminobutyric acid; GC/NICI-MF, negative ion chemical ionization–mass fragmentographic; ADX/CX, adrenalectomized/castrated; HFBA, heptafluorobutyric acid anhydride; 5β-DH PROG, 5β-pregnane-3,20-dione; GC, gas chromatograph; SSRI, selective serotonin reuptake inhibitor.

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Guide for the *Care and Use of Laboratory Animals* (21) and were approved by the Animal Care Committee.

Studies on Brain Slices. *[^{14}C]Serotonin uptake.* The brain uptake of [^{14}C]serotonin was studied *ex vivo* with a modification of the method of Shaskan and Snyder (22) in ADX/CX rats receiving i.p. equimolar doses of fluoxetine, paroxetine, or imipramine. Brain slices (0.3 × 0.3 mm, ≈6 mg protein) were incubated in 2 ml Locke's solution (154 mM NaCl/5.6 mM KCl/3.6 mM $NaHCO_3$/2.3 mM $CaCl_2$/10 mM glucose/1 mM $MgSO_4$/1 mM Hepes/10 μM pargyline/1 mM ascorbic acid, pH 7.4) at 37°C for 5 min in the presence of 50 nM serotonin (59.7 mCi/mmol; 1 Ci = 37 GBq).

The uptake was terminated by filtration through GF/B glass fiber filters. A parallel assay at 0°C accounts for the [^{14}C]serotonin uptake by passive diffusion. The uptake of [^{14}C]serotonin detected in the presence of 1 μM to 1 mM fluoxetine (≈30%) was considered to be non-specific [due to the uptake of [^{14}C]serotonin by other monoaminergic uptake systems (22)] and was considered to be a background, which was subtracted from the total uptake of [^{14}C]serotonin.

Conversion of 5α-DH PROG into 3α,5α-TH PROG by rat brain slices. The brain slices (≈10 mg/protein in triplicate) were preincubated in 1 ml of Locke's solution at 37°C for 15 min with fluoxetine prior to adding 10 μM 5α-DH PROG. The incubation was terminated by the addition of 4 ml of ice-chilled ethyl acetate. Fluoxetine and 5α-DH PROG were dissolved in dimethyl sulfoxide (final concentration of dimethyl sulfoxide in the medium was 0.01%). Incubation without tissue was performed to establish the nonenzymatic conversion of 5α-DH PROG, PREG, PROG, and 3α,5α-TH PROG, which accounted for less than 2% of the total metabolites produced when brain slices were incubated with the above-mentioned steroid mixture.

Neurosteroid Determination. *Extraction, HPLC, and TLC purification.* Whole rat brains, dissected brain structures, brain slices, or plasma samples were homogenized in 5 vol of distilled water containing 2 fmol/ml of the [^{3}H]neurosteroid of interest. The ethyl acetate extraction and the HPLC purification were carried out as described by Cheney *et al.* (23). The recovery of tritiated steroids by means of the HPLC purification ranged from 83 to 94%.

An additional purification step on silica gel TLC was required for the fractions containing 5α-DH PROG to minimize possible contamination with cholesterol. The TLC plates were developed twice in one direction with chloroform/ethyl acetate/ethanol (30:1:0.1). The retardation factor values for 5α-DH PROG and cholesterol were 0.71 and 0.52, respectively. Standards (50 μg each) were run in parallel and were sprayed with 2% perchloric acid and heated at 120°C for 15 min to allow spot visualization of cholesterol and 5α-DH PROG. The area of the TLC plate corresponding to the 5α-DH PROG standard was extracted four times with 2 ml of methanol. The ^{3}H-5α-DH PROG recovery through this procedure was calculated and ranged between 90 and 95%.

Sample derivatization for GC/MF analysis. 3α,5α-TH PROG, PREG, DHEA, and the internal standard 3β-hydroxy-5α-pregnan-20-one (3β,5α-TH PROG) were derivatized with heptafluorobutyric acid anhydride (HFBA) as described (23). PROG, 5α-DH PROG, and the internal standard 5β-pregnane-3,20-dione (5β-DH PROG) were derivatized with *O*-(pentafluorobenzyl) hydroxylamine HCl (FLOROX reagent) according to the protocol provided by Pierce. The amount of internal standard added to the HPLC and the TLC fractions containing the individual neurosteroids extracted from the brain or plasma was of the order of magnitude of the neurosteroid concentration expected to be present in the specimen to be analyzed.

GC/NICI-MF analysis. GC/MF analysis of the HFBA- and FLOROX-derivatives was carried out on an HP 5988B mass spectrometer coupled to an HP 5890 gas chromatograph (GC) equipped with a J & W Scientific (Folsom, CA) capillary column (DB-5, length 30 m, i.d. 0.25 mm, film thickness 0.25 μm). Helium was used as the carrier gas. Mass fragmentation was performed with NICI using methane/ammonia (95:5) as the reaction gas. Samples were injected at a column temperature of 80°C. The oven temperature was programmed to increase at a rate of 30°C per min until it reached 240°C, and this temperature was maintained until the end of the chromatographic run. In the mass spectrometer the derivatized steroids of interest, subjected to NICI, yielded negative ions in the mass range between *m/z* 100 and *m/z* 700.

NICI/MF of PREG, PROG, 5α-DH PROG, 3α,5α-TH PROG, and DHEA. The gas chromatographic elution profile and the negative ion mass fragmentation pattern of HFBA–PREG, HFBA–3α,5α-TH PROG, FLOROX–PROG, and FLOROX–5α-DH PROG derivatives are shown in Figs. 1 and 2, respectively. In addition to the GC retention time characteristic of each steroid, the structural identification of each neurosteroid assayed was provided by its unique mass fragmentation pattern. By operating the mass spectrometer in the single ion monitoring mode, we focused on the most abundant ion fragment of each steroid derivative: *m/z* 474 for HFBA–3α,5α-TH PROG, 472 and 492 for HFBA–PREG, 464 for HFBA–DHEA, 491 for FLOROX–5α-DH PROG, 489 for FLOROX–PROG, 474 for HFBA–3β,5α-TH PROG, and 491 for FLOROX–5β-DH PROG. Despite the fact that ion fragments with the same 474 *m/z* value were selected for the single ion monitoring quantification of 3α,5α-TH PROG and its internal standard, each steroid was reliably identified by virtue of its different GC retention time.

Following derivatization with FLOROX, the 3-pentafluorobenzyl-oximes of 5α-DH PROG and PROG yielded *syn* and *anti* isomers, which can be completely separated by GC (Fig. 1), but have an identical mass fragmentation pattern (Fig. 2).

Quantitative analysis of neurosteroids by NICI/MF. Aliquots of the HPLC and the TLC fractions corresponding to 5–20 mg of brain or 10–20 μl of plasma and the respective internal standards were derivatized. The standard curve of HFBA-3α,5α-TH PROG prepared using 3β,5α-TH PROG as the internal standard is shown in Fig. 3. The area under the peak of each known quantity of 3α,5α-TH PROG was divided by the area under the peak of the internal standard. This ratio was plotted against the quantity of 3α,5α-TH PROG, which was used to generate the standard curve of Fig. 3. The detection limit of HFBA–3α,5α-TH PROG, HFBA–PREG, and HFBA–DHEA was ≈1.5 fmol, whereas that of PROG and 5α-DH PROG FLOROX derivatives was ≈3 fmol. In establishing the maximal sensitivity of the assay we considered only peaks that have a signal-to-noise ratio greater than five.

The precision of the method was estimated from the calculated concentration divided by the actual concentration percentage. The difference was less than 2% for each steroid analyzed injected in quadruplicate. For 3α,5α-TH PROG, the inter- and the intra-assay coefficients of variation were 3 and 5%, for pregnenolone 4 and 6%, for progesterone 7 and 9%, and for 5α-DH PROG 5 and 8%, respectively. The inter- or intra-assay variation of each steroid failed to reach statistical significance ($P < 0.05$) using a one-way ANOVA.

In samples without tissue, but containing trace amounts of the [^{3}H]neurosteroid of interest the neurosteroid content was below the detection limit.

Drugs and Reagents. PREG, PROG, 5α-DH PROG, 3α,5α-TH PROG, and DHEA were from Steraloids (Wilton, NH). [^{14}C]serotonin and all tritiated steroids with the exception of ^{3}H-5α-DH PROG were from New England Nuclear. Tritiated 5α-DH PROG was enzymatically synthesized by incubating ^{3}H-3α,5α-TH PROG (50 Ci/mmol) in C6-2B cells for 4 hr. ^{3}H-5α-DH PROG was purified from the cell extract by HPLC and TLC using the conditions described above for the purification of neurosteroids (see *Materials and Methods*).

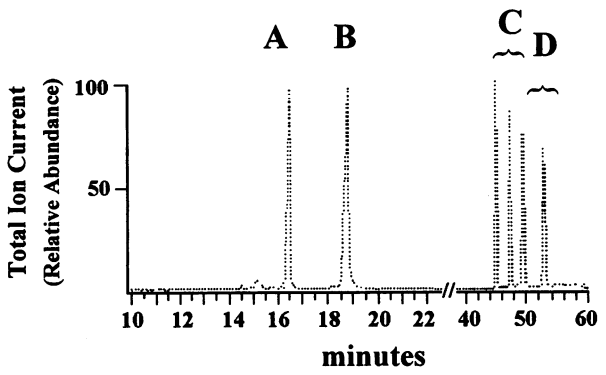


FIG. 1. Gas chromatographic retention times. A, the HFBA derivative of 3α,5α-TH PROG; B, the HFBA derivative of PREG; C, the FLOROX derivative of 5α-DH PROG; D, the FLOROX derivative of PROG. Total ion current generated by approximately 100 pmol of each derivatized steroid is recorded. The FLOROX derivative of 5α-DH PROG and PROG yielded *syn* and *anti* isomers, which were separated by the GC.

The purity of ^{3}H-5α-DH PROG was assessed by TLC, demonstrating that all the radioactivity in the sample comigrated with authentic 5α-DH PROG. Imipramine was from Sigma. Fluoxetine–HCl was kindly donated by Eli Lilly. Paroxetine–HCl (BRL-29060-A) was a gift from SmithKline Beecham. HFBA and FLOROX reagent were purchased from Pierce. Unless otherwise specified, all organic solvents were of HPLC grade and were purchased from Fisher Scientific. Drugs for *in vivo* studies were dissolved by initially mixing with 1–2 drops of Tween 80 (Sigma) and then gradually adding deionized distilled water to the volume required. The concentration of Tween 80 was less than 0.1%. If required, sonication was applied to obtain a clear solution.

Statistical Analyses. All results represent mean ± SEM. Data were subjected to ANOVA followed by Duncan multiple range post hoc comparison (24).

RESULTS

Brain and Plasma PREG, PROG, 5α-DH PROG, and 3α,5α-TH PROG Content in ADX/CX and Sham-Operated Male Rats Following Fluoxetine or Saline Treatment. Thirty minutes after fluoxetine treatment (58 μmol/kg i.p.) the brain content of 3α,5α-TH PROG increased 4-fold compared with vehicle-treated ADX/CX rats, whereas that of 5α-DH PROG decreased (Fig. 4). In contrast, the brain content of PROG, PREG, and DHEA virtually failed to change significantly (Fig. 4). Furthermore, fluoxetine failed to alter the 3α,5α-TH PROG plasma level, which consistently was less than 1/20 of the brain content (0.34 ± 0.047 SEM in saline and 0.39 ± 0.050 SEM pmol/ml in fluoxetine-treated rats, $n = 6$).

The extent of the selective brain 3α,5α-TH PROG increase elicited by fluoxetine in ADX/CX rats was comparable to that detected in sham-operated rats (Fig. 4), even though the brain content of 3α,5α-TH PROG was significantly higher in sham-operated than in ADX/CX rats (see Fig. 4 *Inset*).

As illustrated in Fig. 5, the accumulation of 3α,5α-TH PROG in the brain of ADX/CX rats receiving fluoxetine was proportional to the dose of the drug injected and peaked (about 4-fold over the control level) at 15 min following an injection of 58 μmol/kg of fluoxetine. The brain concentration of 3α,5α-TH PROG thereafter slightly decreased from the peak value but remained 2–3 times higher than that of controls at 120 min after the injection of fluoxetine.

The administration of fluoxetine to ADX/CX rats resulted in an uneven increase of 3α,5α-TH PROG and in an uneven decrease of 5α-DH PROG in the various brain areas studied (Table 1). The olfactory bulb was the brain area with the highest basal 3α,5α-TH PROG content, and the brain area with the highest increase in 3α,5α-TH PROG content following fluoxetine treatment. The brain content of 5α-DH PROG had a regional distribution similar to that of 3α,5α-TH PROG. It was highest in the olfactory bulb and lowest in the brain stem (Table 1). The frontal cortex and the cerebellum were the only structures in which fluoxetine decreased significantly the 5α-DH PROG content.

Accumulation of Brain 3α,5α-TH PROG and Inhibition of ^{14}C[Serotonin] Uptake Following Administration of Fluoxetine, Paroxetine, or Imipramine. Fluoxetine is a potent and selective serotonin reuptake inhibitor (SSRI) (20). Thus, we studied other serotonin reuptake inhibitors, such as paroxetine, which is an SSRI 10-fold more potent than fluoxetine (26), and imipramine, which is equipotent to fluoxetine in inhibiting serotonin reuptake but is less selective because in higher doses it also blocks norepinephrine reuptake (26).

Equimolar doses of fluoxetine, paroxetine, and imipramine administered to ADX/CX rats produced in 30 min an almost complete inhibition of serotonin reuptake measured *ex vivo* in

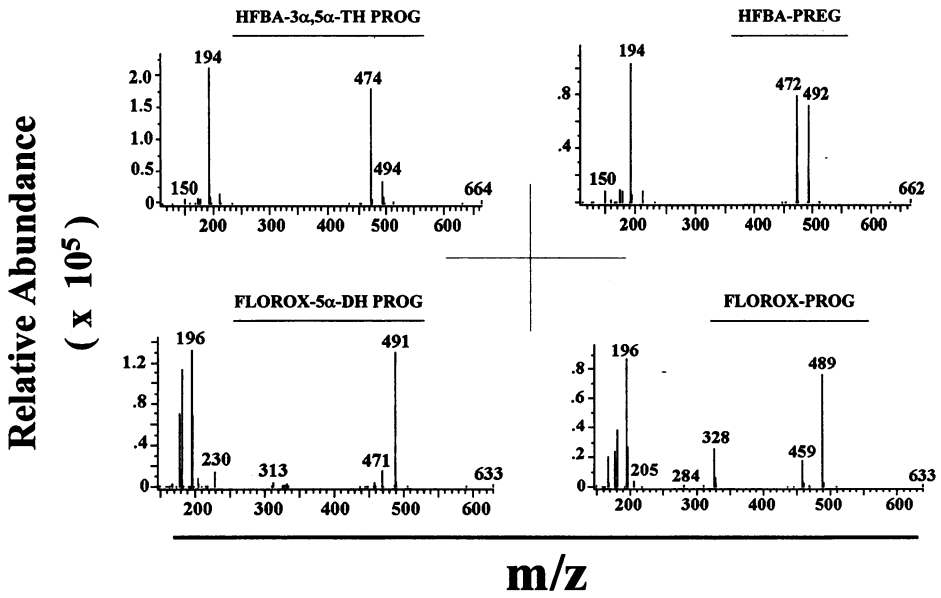


FIG. 2. Mass fragmentographic spectra of HFBA-3α,5α-TH PROG, and -PREG and FLOROX-5α-DH PROG and -PROG. Approximately 100 pmol of each steroid were used in the experiment.

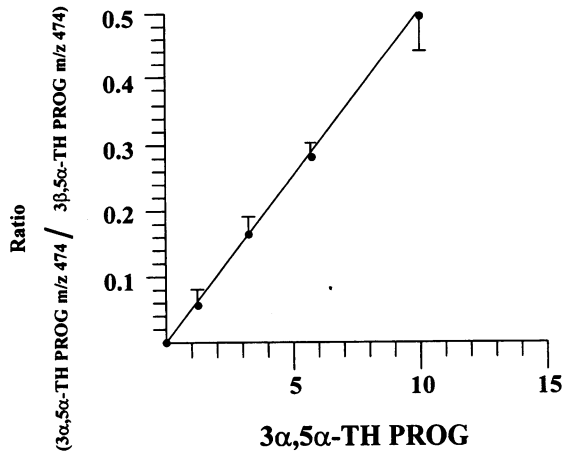


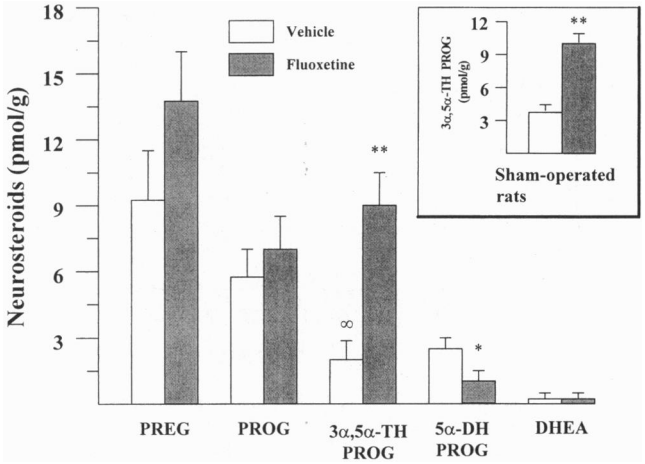
FIG. 3. 3α,5α-TH PROG standard curve. To increasing concentrations of 3α,5α-TH PROG, as indicated in the abscissa, we added before derivatization a constant 20 fm amount of 3β,5α-TH PROG as internal standard. The ratio 3α,5α-TH PROG/3β,5α-TH PROG on the ordinate represents the ratio of the area under the peak of each known quantity of 3α,5α-TH PROG (*m/z* 474) divided by the area under the peak of the internal standard (*m/z* 474). Each point represents mean ± SEM of at least five determinations; $r = 0.9998$; $P < 0.01$.

brain slices (Table 2). During this time, however, fluoxetine induced a 4-fold increase in brain 3α,5α-TH PROG content, whereas paroxetine induced a 2-fold increment of 3α,5α-TH PROG content and imipramine failed to change 3α,5α-TH PROG concentration (Table 2).

Fluoxetine Increases 3α,5α-TH PROG Accumulation in Brain Slices of ADX/CX Rats. Incubation of brain slices of ADX/CX rats with fluoxetine (10 μM; 15 min) followed by the addition of 10 μM of 5α-DH PROG, the immediate precursor of 3α,5α-TH PROG, resulted in a significant time-dependent greater accumulation of 3α,5α-TH PROG than in vehicle-pretreated slices (Fig. 6). It should be noted that rat brain slices of ADX/CX rats loaded with 5α-DH PROG extensively convert 5α-DH PROG into 3α,5α-TH PROG (≈15%/hr in vehicle-treated slices and 25%/hr in fluoxetine-treated slices), reaching in 1 hr a 10-fold 3α,5α-TH PROG accumulation over the level measured at the beginning of the incubation with 5α-DH PROG (Fig. 6).

DISCUSSION

Prompted by the recent report that fluoxetine is beneficial for the treatment of the late luteal dysphoria symptoms (14), using the GC/NICI-MF methodology we examined whether fluoxetine can change the content of PROG, 5α-DH PROG, 3α,5α-TH PROG, or DHEA in the whole brain, in discrete



ADX/CX RATS

FIG. 4. PREG, PROG, 3α,5α-TH PROG, 5α-DH PROG, and DHEA content in the brain of ADX/CX rats treated with vehicle or fluoxetine (58 μmol/kg i.p., 30 min). (*Inset*) The 3α,5α-TH PROG brain content of sham-operated rats treated with vehicle or fluoxetine. Data represent the mean ± SEM of 5 to 6 rats; *, $P < 0.05$; **, $P < 0.01$ when vehicle-treated rats are compared with fluoxetine-treated rats; ∞, $P < 0.05$ when the value of 3α,5α-TH PROG in ADX/CX rats is compared with that of sham-operated rats. To avoid circadian variations of the neurosteroid content, the experiments were consistently conducted between 2 and 4 p.m.

brain regions, and in plasma of ADX/CX or sham-operated rats.

The extraordinary sensitivity and reliability, plus the structural information achieved with GC/NICI-MF analysis of the neurosteroids has allowed us to measure femtomole quantities of 3α,5α-TH PROG and 5α-DH PROG and their precursors or metabolites in 5–10 mg of brain tissue and 0.01 ml of plasma for the first time ever with a precise structural identification. This absolute identification of steroids, even when they differ by small substitutions in the chemical structure, such as PREG, PROG, 5α-DH PROG, 3α,5α-TH PROG, and DHEA can be obtained at concentrations that are at least two orders of magnitude lower than those detected by radioimmunoassay or by GC/MF using the mass spectrometer in the electron impact mode [compare the present results with those of Cheney *et al.* (23)].

In rats, a single i.p. injection of fluoxetine produces a dose-related conspicuous and protracted increase of brain 3α,5α-TH PROG content. This action of fluoxetine does not require the presence of adrenals and gonads, it is not related

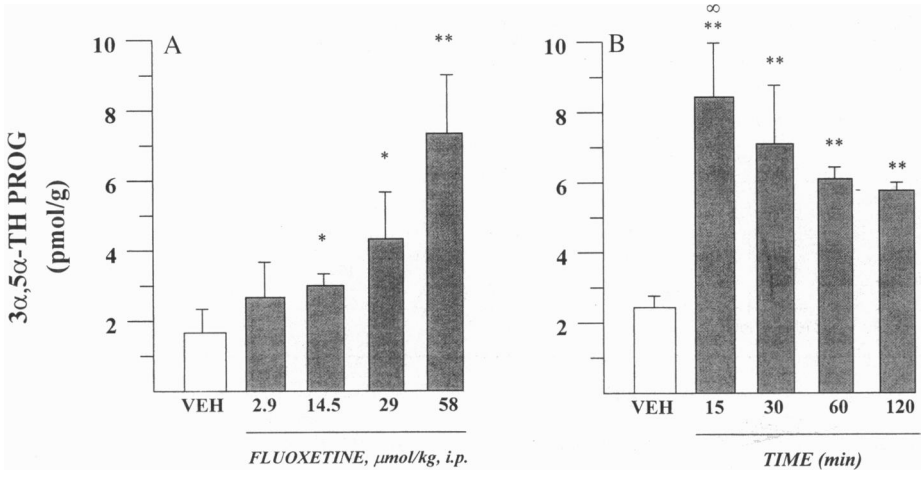


FIG. 5. 3α,5α-TH PROG content in the whole brain of ADX/CX rats receiving i.p. injections of fluoxetine. (*A*) Dose response at 30 min following fluoxetine. (*B*) Time course following administration of 58 μmol/kg of fluoxetine. Data represent the mean ± SEM of 5 to 6 rats; *, $P < 0.05$; **, $P < 0.01$ when fluoxetine-treated rats are compared with vehicle-treated rats; ∞, $P < 0.05$ when 3α,5α-TH PROG content at 15 min following fluoxetine injection (58 μmol/kg i.p.) is compared with the brain 3α,5α-TH PROG content at other time points.

to an increase in the circulating levels of 3α,5α-TH PROG, and as shown in Fig. 3, is independent from an increase in brain content of PREG, PROG, 5α-DH PROG, or DHEA.

Thus, it is presumed that the effect of fluoxetine on the brain levels of 3α,5α-TH PROG derives from a direct action of fluoxetine on brain steroidogenesis rather than being the consequence of a generalized increase of steroid biosynthesis in the endocrine tissues. Because the fluoxetine-induced increase of brain 3α,5α-TH PROG content differs in various brain structures and appears related to the rank order of the endogenous 3α,5α-TH PROG concentration of each structure, the possibility that fluoxetine influences either 3α,5α-TH PROG biosynthesis or degradation rates in different brain areas must be entertained.

In the central nervous system, PROG is rapidly metabolized by the action of 5α-reductases into 5α-DH PROG, which in turn is transformed into 3α,5α-TH PROG by the action of 3α-hydroxysteroid oxidoreductases (3α-HSORs) (28, 29). Both enzymes are nonuniformly distributed in the brain; the highest content of these enzymes (27, 30) and of 5α-DH PROG and 3α,5α-TH PROG (see Table 1) is found in the olfactory bulb. Thus, the nonuniform increase in brain 3α,5α-TH PROG following fluoxetine (Table 1), which in ADX/CX rats occurs without concomitant changes of PREG or PROG content (Fig. 4), could be due to an action of fluoxetine on the activities of either 5α-reductases or 3α-HSORs expressed in the various brain areas. To elucidate whether either 5α-reductases or 3α-HSORs are the target of fluoxetine action, we measured the content of 5α-DH PROG in different brain areas, including the olfactory bulb. Interestingly, not only did the 5α-DH PROG content fail to increase in most brain regions, but also in many brain areas it may even decrease (see in Table 1, 5α-DH PROG content in cortex and cerebellum). This suggests that the mechanism by which fluoxetine increases brain 3α,5α-TH PROG content is not via the activation of 5α-reductases but rather via a complex interaction with the activity of the 3α-HSORs.

In partial support for this mechanism of action of fluoxetine is the demonstration reported in Fig. 6 that in brain slices of ADX/CX rats preincubated with fluoxetine and then loaded with 5α-DH PROG, the rapid accumulation of 3α,5α-TH PROG is accelerated when 10 μM of fluoxetine is present. Importantly, 10 μM of fluoxetine is approximately the concentration of this drug found in the the brain 5 min after the administration of 34 μmol/kg i.p. of fluoxetine to rats (31). Since in brain slices the degradation of 3α,5α-TH PROG to metabolites different from 5α-DH PROG is very slow (see legend of Fig. 6 and ref. 27) the only mechanism by which, in brain slices, fluoxetine can increase 3α,5α-TH PROG content at the rate shown in Fig. 5 is either by accelerating the reduction of 5α-DH PROG to 3α,5α-TH PROG or by inhibiting the oxidation of 3α,5α-TH PROG to 5α-DH PROG. We have obtained preliminary evidence that the rate of conversion of ^{3}H-3α,5α-TH PROG to ^{3}H-5α-DH PROG in rat cortical brain slices is greatly reduced if the slices are pretreated with fluoxetine (10 μM or higher).

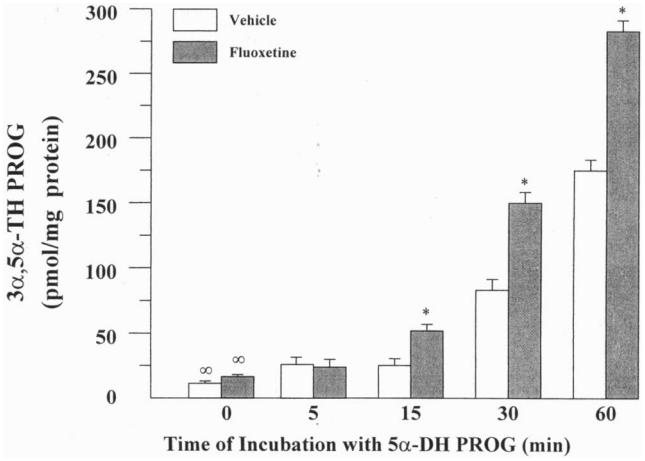


FIG. 6. Fluoxetine-induced facilitation of 5α-DH PROG conversion into 3α,5α-TH PROG in brain slices of ADX/CX rats. Fluoxetine (10 μM) was preincubated for 15 min with slices before the addition of 10 μM of 5α-DH PROG. The amount of 5α-DH PROG taken up from the brain slices in 5 min of incubation was ≈90% of the amount added in the medium (10 nmol) and was identical in the presence and absence of fluoxetine. Vehicle was 100 μl of Locke's solution containing 0.1% dimethyl sulfoxide. At time 0 are represented the levels of 3α,5α-TH PROG in slices in which 10 μM of 5α-DH PROG was added and the reaction was immediately stopped by the addition of 4 ml of cold (0–2°C) ethyl acetate. Each bar is the mean ± SEM of 3 experiments. *, $P < 0.01$ when fluoxetine-treated slices were compared with vehicle-treated slices; ∞, $P < 0.01$ when 3α,5α-TH PROG content at time 0 is compared with the 3α,5α-TH PROG content at 15, 30, and 60 min. In experiments in which ^{3}H-5α-DH PROG (0.5 μCi/10 nmol) was added to brain slices, the HPLC column chromatography (for conditions, see ref. 27) of the extract revealed two major radioactive peaks, one corresponding to 5α-DH PROG and one corresponding to 3α,5α-TH PROG. No other major radioactive peaks were eluted from the HPLC column under the experimental conditions described.

In rats fluoxetine metabolism is rather slow (20); thus, the ability of fluoxetine to increase and maintain for longer than 2 hr high levels of 3α,5α-TH PROG in the brain of ADX/CX rats is in keeping with a prolonged decrease of the conversion rates of 3α,5α-TH PROG into 5α-DH PROG by a direct action on brain 3α-HSOR activity by fluoxetine.

The mechanisms whereby fluoxetine alleviates depression have been linked to the ability of fluoxetine to increase

Table 1. 3α,5α-TH PROG and 5α-DH PROG content in brain structures of ADX/CX rats receiving fluoxetine or vehicle

	3α,5α-TH PROG		5α-DH PROG	
Brain region	Vehicle, pmol/g brain	Fluoxetine, pmol/g brain	Vehicle, pmol/g brain	Fluoxetine, pmol/g brain
Olfactory bulb	10 ± 1.9*	31 ± 4.1**	13 ± 2.3*	15 ± 2.4
Frontal cortex	2.9 ± 0.48†	5.4 ± 0.74**	4.1 ± 0.58†	2.2 ± 0.47‡
Striatum	4.6 ± 0.39†	12 ± 2.1**	5.8 ± 0.72†	6.4 ± 2.4
Hippocampus	3.4 ± 0.29†	7.1 ± 0.96‡	3.9 ± 0.69	3.2 ± 1.2
Cerebellum	1.1 ± 0.21	1.4 ± 0.26	2.4 ± 0.17	0.8 ± 0.09**
Brain stem	1.9 ± 0.29	1.9 ± 0.57	1.4 ± 0.13	1.4 ± 0.18

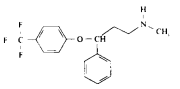
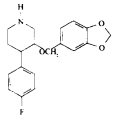
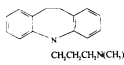
Neurosteroid content was measured 30 min after the i.p. injection of vehicle or 58 μmol/kg of fluoxetine. Brain parts were dissected as described by Glowinsky and Iversen (25). Data represent the mean ± SEM of three experiments.

*$P < 0.01$ when the olfactory bulb is compared with all the other brain parts.

‡$P < 0.05$ and **$P < 0.01$ when the neurosteroid content in brain regions of vehicle-treated rats is compared with the corresponding regions of fluoxetine-treated rats.

†$P < 0.05$ when compared to the values of cerebellum and brain stem.

Table 2. The efficacy of inhibitors of serotonin transporter does not correlate with their ability to increase 3α,5α TH PROG brain content

Structure	Compound	Brain 3α,5α-TH PROG content, pmol/g brain	Brain [^{14}C]serotonin uptake, fmol/mg protein/min
—	Vehicle	1.8 ± 0.36	720 ± 65
	Fluoxetine	7.9 ± 1.3*	42 ± 5.6*
	Paroxetine	4.4 ± 0.57**	25 ± 3.0*
	Imipramine	2.3 ± 0.49	55 ± 7.8*

Compounds were administered in doses of 58 μmol/kg i.p. 30 min before 3α,5α-TH PROG content determination and [^{14}C]serotonin uptake analysis. Note that the [^{14}C]serotonin uptake is virtually completely inhibited (3.4–7.6% of control) in the group of rats receiving equimolar amounts of fluoxetine, paroxetine, or imipramine. Data represent the mean ± SEM of 3 to 4 rats.
*$P < 0.01$ when compared to vehicle-treated rats; **$P < 0.05$.

serotonergic synaptic activity by selectively blocking its reuptake into nerve endings (26). This action may either balance the equilibrium among different monoaminergic mechanisms or cause a down-regulation of various serotonergic receptors. The question we have addressed here is whether the fluoxetine-elicited increase in brain 3α,5α-TH PROG depends on a fluoxetine-mediated increase in the amount of serotonin available to postsynaptic receptors.

The experiment in which the SSRI activity of fluoxetine was compared with that of equimolar doses of paroxetine (another potent SSRI) or imipramine (a blocker of serotonin and norepinephrine reuptake) makes it improbable that serotonin represents the (still) unknown putative signaling system responsible for the control of 3α,5α-TH PROG brain biosynthesis. In fact doses of paroxetine and imipramine that, like those of fluoxetine, completely block serotonin reuptake (Table 2) produce either a smaller increase (paroxetine) or even fail to produce an increase of 3α,5α-TH PROG brain content (imipramine). In view of the fluoxetine and paroxetine action on the brain content of 3α,5α-TH PROG, however, it seems likely that 3α,5α-TH PROG may mediate some of the broad psychopharmacological effects of these two SSRIs, even though the changes in the level of 3α,5α-TH PROG might not be correlated with the drugs' effects on the serotonin reuptake.

We thank Dr. E. E. Baulieu (Institut National de la Santé et de la Recherche Médicale, Paris) and Dr. I. Hanin (Loyola University, Chicago) for reading our manuscript, and for their helpful suggestions and constructive critiques. This work was supported by National Institute of Mental Health Grant RO1 MH49486-04.

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