Kinetics of Allopregnanolone Formation Catalyzed by Human 3α-Hydroxysteroid Dehydrogenase Type III (AKR1C2)

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ABSTRACT: Allopregnanolone is a neurosteroid which exhibits anxiolytic and anticonvulsant activities through potentiation of the $GABA_A$ receptor. The reduction of 5α-dihydroprogesterone (5α-DHP), the last step in allopregnanolone biosynthesis, is catalyzed by 3α-hydroxysteroid dehydrogenases (3α-HSDs). While the mechanism of action of allopregnanolone and the physiological and pharmacological modulation of allopregnanolone concentrations in vivo have been extensively studied, there has been little characterization of the kinetics of human 3α-HSD catalyzed allopregnanolone formation. We report here determination of the kinetic mechanism for 5α-DHP reduction catalyzed by human 3α-HSD type III by using steady-state kinetics studies and assessment of the ability of fluoxetine and various other small molecules to activate 3α-HSD type III catalyzed allopregnanolone formation. Enzyme-catalyzed 5α-DHP reduction yielded two products, allopregnanolone and 5α,20α-tetrahydroprogesterone, as measured by using a radiometric thin-layer chromatography assay, while 5*β*-DHP reduction yielded the neurosteroid pregnanolone as the only product. 5*β*-DHP reduction proceeded with a catalytic efficiency 10 times higher than that of 5α-DHP reduction. Two-substrate kinetic analysis and dead-end inhibition studies for 5α-DHP reduction and allopregnanolone oxidation indicated that 3α-HSD type III utilized a ternary complex (sequential) kinetic mechanism, with nicotinamide adenine dinucleotide cofactor binding before steroid substrate and leaving after steroid product. Since previous reports suggested that fluoxetine and certain other small molecules increased allopregnanolone concentrations in vivo by activating 3α-HSD type III, we investigated whether these small molecules were able to activate human 3α-HSD type III. Our results showed that, at concentrations up to 50 μM, fluoxetine, paroxetine, sertraline, norfluoxetine, carbamazepine, clozapine, flurbiprofen, and sulfobromophthalein did not activate the enzyme. These results characterize the role of 3α-HSD type III in allopregnanolone formation and suggest that activation of this enzyme by fluoxetine is likely not the mechanism by which fluoxetine increases allopregnanolone concentrations.

The neurosteroid allopregnanolone (5α-pregnan-3α-ol-20-one[1]) is a potent allosteric potentiator of the $GABA_A$ receptor ($K_d \sim 1$ nM) that has anxiolytic and anticonvulsant activities (*1*−*4*). Low levels of allopregnanolone have recently been associated with premenstrual syndrome (*5*−*8*) and depression (*9*−*11*). In a study of patients with major unipolar depression, the concentration of allopregnanolone in cereberospinal fluid (CSF) was found to be about 60% lower than that of control subjects (*9*). The fact that selective serotonin reuptake inhibitors (SSRIs) are effective for treatment of both premenstrual syndrome (*12*, *13*) and depression suggested that SSRIs might, either as a result of or in addition to their effect on serotonin reuptake, increase allopregnanolone concentrations. Indeed, administration of the SSRIs fluoxetine and paroxetine to rats was found to cause an acute increase in brain allopregnanolone concentration (*14*). Furthermore, treatment of depressed patients with fluoxetine for 8−10 weeks resulted in restoration of normal CSF allopregnanolone levels (*9*).

Allopregnanolone biosynthesis, which is known to occur in brain, ovaries, and adrenal glands (*15*), occurs by a two-step reduction of progesterone (*1*−*4*). In the first step, progesterone is converted to 5α-dihydroprogesterone (5α-DHP) by a 5α-reductase, and in the second step, 5α-DHP is converted to allopregnanolone by a 3α-hydroxysteroid dehydrogenase (3α-HSD). Mammalian 3α-HSDs are soluble, monomeric enzymes that belong to the aldo-keto reductase superfamily of NADP(H) dependent oxidoreductases (*16*−*19*). 3α-HSDs have diverse functions in addition to allopregnanolone formation, including the biosynthesis of the other neurosteroids (e.g., pregnanolone and tetrahydrodeoxycorticosterone), bile acids, and reduced testosterone metabolites (e.g., 5α-DHT) (*16*). There are at least four human 3α-HSD isoforms, which have varying but often

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[1] Abbreviations: allopregnanediol, 5α-pregnan-3α,20α-diol; allopregnanolone, 5α-pregnan-3α-ol-20-one; DHP, dihydroprogesterone; GST, glutathione-S-transferase; 3α-HSD type III, 3α-hydroxysteroid dehydrogenase type III (AKR1C2, dihydrodiol dehydrogenase 2); pregnanolone, 5*β*-pregnan-3α-ol-20-one; SSRI, selective serotonin reuptake inhibitor; 20α-THP, 5α-pregnan-20α-ol-3-one; UDC, ursodeoxycholate (3α,7*β*-dihydroxy-5*β*-cholanic acid); TLC, thin-layer chromatography.

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overlapping substrate specificity and tissue distribution (*16*−*19*). Steady-state kinetic studies have indicated that 3α-HSD enzymes utilize an ordered sequential kinetic mechanism, with the cofactor NADP(H) binding first and leaving last (*20*−*23*).

A potentially key role for 3α-HSD in SSRI modulation of allopregnanolone concentrations was suggested by a report that fluoxetine and paroxetine can allosterically activate 3α-HSD type III (also referred to as AKR1C2) (*19*). For example, fluoxetine (50 μM) was reported to increase the catalytic efficiency of 5α-DHP reduction to allopregnanolone by rat 3α-HSD and human 3α-HSD type III by 45- and 15-fold, respectively, by reducing the K_M for 5α-DHP (*19*). Precedent for this finding was provided by a previous study showing allosteric activation of human 3α-HSD type I by bromosulfophthalein (BSP) (*24*, *25*). However, BSP was reported to activate 3α-HSD type I by increasing the rate of substrate turnover (i.e., k_{cat}) rather than by reducing the K_M for the steroid substrate (*24*). In fact, K_M values for both substrate and cofactor increased in the presence of BSP (*24*). In addition, it was reported that BSP does not activate 3α-HSD type III (*24*).

The results described above led us to undertake a detailed kinetic characterization of allopregnanolone formation catalyzed by 3α-HSD type III, including a reevaluation of the ability of SSRIs to activate this enzyme. In initial experiments we found that enzyme-catalyzed 5α-DHP reduction produced two products, allopregnanolone and 5α-pregnan-20α-ol-3-one (20α-THP), at comparable rates, while 5β-DHP reduction produced pregnanolone as the sole product. We determined the kinetic mechanism for 5α-DHP reduction catalyzed by the enzyme by using steady-state kinetics methods. Results from two-substrate and inhibition experiments showed that the enzyme utilized an ordered ternary complex kinetic mechanism, with nicotinamide adenine dinucleotide cofactor binding before the steroid substrate and dissociating after release of the steroid product. We also tested the ability of the SSRIs fluoxetine, norfluoxetine, paroxetine, and sertraline and several other compounds that had been reported to increase allopregnanolone levels in vivo, to activate 3α-HSD type III. None of the tested compounds were found to activate the enzyme, indicating that allosteric activation of 3α-HSD type III is not the mechanism by which SSRIs modulate allopregnanolone concentrations.

MATERIALS AND METHODS

Materials. Fluoxetine, norfluoxetine, carbamazepine, clozapine, flurbiprofen, NADPH, and $NADP^+$ were purchased from Sigma, and bromosulfophthalein was purchased from Fluka. Paroxetine was synthesized by Merck & Co., Inc., and sertraline was obtained as Zoloft tablets (Pfizer, Inc.). All unlabled steroids and ursodeoxycholate (3α,7β-dihydroxy-5β-cholanic acid) were obtained from Steraloids. Adogen-464 and $Na_2S_2O_4$ were purchased from Aldrich. [4-^{14}C]-Progesterone (50 mC_i/mmol) was purchased from Perkin-Elmer Life Sciences, and [1,2,6,7,16,17-^{3}H]-progesterone (93 C_i/mmol) was purchased from Amersham Biosciences. [9,11,12-^{3}H(N)]-Allopregnanolone (64 C_i/mmol) was purchased from Perkin-Elmer Life Sciences and purified by thin-layer chromatography (TLC) prior to use. Preparative (0.25 mm thick) and analytical silica gel TLC plates were obtained from Aldrich.

Cloning, Expression, and Purification of 3α-HSD type III. Human 3α-HSD type III (Accession # NM001354) was cloned from a human brain cDNA library (Clontech) using standard PCR techniques with *Pwo* DNA polymerase (Roche). The resulting PCR product was sub-cloned into pCR4 blunt TOPO (Invitrogen), and the DNA sequence was verified by DNA sequencing. The 3α-HSD type III coding sequence was inserted into *Sma* I- and *Xho* I-digested pGEX-4T-2 (Pharmacia) for protein expression.

3α-HSD type III was expressed as a glutathione-S-transferase (GST) fusion protein in *E. coli* strain BL21(DE3) (Novagen) under the following conditions: 5 mL from an early log phase culture was grown in Luria Broth (LB) (Biosource International) supplemented with 100 μg/mL ampicillin (Gibco) at 37 °C for 2 h and was transferred to 500 mL of LB/Amp and grown at 37 °C with shaking at 240 rpm. When the A_{600} for the culture reached 0.5, the culture was equilibrated to 20 °C for 15 min and induced with 0.5 mM (final concentration) isopropyl-β-D-galactoside (IPTG) (Gibco). Cells were grown with shaking at 240 rpm at 20 °C for 18 h and harvested at 4 °C by centrifugation at 2800*g*. Cell pellets were frozen at −70 °C for subsequent purification.

A cell pellet from a 500 mL culture (∼3 mL pellet volume) was resuspended by stirring at 4 °C in 30 mL phosphate buffered saline (PBS) (Gibco) supplemented with protease complete (Roche). Cells were lysed by French Press and the lysed cells were centrifuged at 14 500*g* at 4 °C for 45 min. The supernatant was applied at 4 °C to ∼3 mL of packed glutathione sepharose resin (Pharmacia) previously equilibrated with 10 column volumes of PBS/protease complete. Protein was eluted with 50 mM Tris, pH 8.0, + 10 mM glutathione. Approximately 35 mg of >95% pure GST-3α-HSD type III was isolated from a 500 mL culture.

The GST-3α-HSD fusion protein was cleaved as follows: 2 mL of 288 μM (∼ 35 mg) GST-3α-HSD was digested with 350 U (∼430 nM) thrombin (Pharmacia) for 18 h at 20 °C. The 2 mL cleavage reaction was applied to Bio-gel P-6DG resin (Bio-Rad) that was preequilibrated with PBS/protease complete. After the sample entered the column, 1 mL of PBS/protease complete was added, and the 3 mL effluent was discarded. Protein was eluted with 3 mL PBS/protease complete and collected on 2 mL glutathione sepharose resin (Pharmacia). Following a 30 min incubation, the resin was centrifuged at 500 g for 5 min, and supernatant was saved as purified 3α-HSD type III.

Synthesis of ^{14}C- and ^{3}H-Labeled 5α- and 5β-DHP. Radiolabeled 5α- and 5β-DHP were synthesized from radiolabeled progesterone by reduction using $Na_2S_2O_4$, sodium bicarbonate and Adogen-464 (*26*). A 2 mL vial was charged with $Na_2S_2O_4$ (40 mg) and sodium bicarbonate (20 mg). Water (0.25 mL), toluene (0.15 mL), and ^{14}C- or ^{3}H-labeled progesterone in toluene (0.1 mL) were then added, followed by Adogen-464 (25 μL). The mixture was stirred vigorously at 65 °C for 4 h and then diluted with 0.75 mL water and extracted with 0.75 mL chloroform. The chloroform extract was washed with water (6 × 0.75 mL), concentrated to ∼0.3 mL by rotary evaporation, and loaded onto a preparative silica gel thin-layer chromatography (TLC) plate (0.25 mm thick × 10 cm wide × 20 cm tall). The TLC plate was developed three times with 5% ethyl acetate/chloroform and then exposed to film (Kodak X-OMAT)

overnight. Regions of the TLC plate corresponding to 5α- and 5β-DHP were excised and extracted with 0.75 mL chloroform. Following centrifugation to remove the silica gel, the chloroform extract was concentrated to dryness by rotary evaporation. The residue was dissolved in 0.5 mL dimethyl sulfoxide (DMSO) and stored frozen at −10 °C until use. 5α- and 5β-DHP were produced in a 2:1 ratio, with an overall yield of 10−30%.

Substrate Kinetics. All reactions were carried out in a total volume of 500 μL in presiliconized 1.5 mL polypropylene microcentrifuge tubes. For the forward reaction (5α-DHP reduction), initial velocity experiments were carried out with the following final concentrations: 100 mM potassium phosphate, pH 7.0, 0.5 mg/mL BSA, 0.2−10 μM 5α-DHP, 50 000 dpm [^{3}H]-5α-DHP, 0.25−5 μM NADPH, 35 nM 3α-HSD type III, and 5% DMSO (v/v). Reactions were incubated at 25 °C for 30 min, then quenched by adding 0.7 mL chloroform and vortexing. For the reverse reaction (allopregnanolone oxidation), initial velocity experiments were carried out with the following final concentrations: 100 mM potassium phosphate, pH 7.0, 0.2 mg/mL BSA, 0.5−10 μM allopregnanolone, 50 000 dpm [^{3}H]-allopregnanolone, 0.2−10 μM $NADP^+$, 50 nM 3α-HSD, and 5% DMSO (v/v). Reactions were incubated at 25 °C for 3 h and then quenched by adding 0.7 mL chloroform and vortexing.

All reactions were initiated by addition of enzyme and were carried out such that less than 15% of either substrate was converted to product. Time course experiments showed that product formation for the forward and reverse reactions was linear with time under the stated conditions. The solubility of the substrates was measured by spinning blank reaction mixtures (no enzyme) in a microcentrifuge at 14 000 rpm for 5 min and measuring the percentage of radioactivity remaining in solution by liquid scintillation counting. Under the stated reaction conditions, 5α-DHP was >80% soluble at 0.5−10 μM (limit of solubility = 10 μM), and allopregnanolone was >90% soluble at 0.5−10 μM. We note that this method for assessing substrate solubility does not rule out the formation of steroid micelles. The identities of the reaction products allopregnanolone and 20α-THP were confirmed by TLC comigration with standards and by mass spectrometry (MS). For MS analysis, enzymatic products were isolated by TLC and exact masses determined using a Micromass ZQ (APCI+) mass spectrometer (calculated for allopregnanolone 318.3, observed 318.2; calculated for 20α-THP 318.3, observed 318.3).

To determine the percentage of steroid substrate converted to products, chloroform extracts were washed once with 0.7 mL water to remove residual DMSO and concentrated to dryness by rotary evaporation. The residue was dissolved in 10 μL chloroform and applied to a polyester-backed silica gel TLC plate. Nonradiolabeled standards were also applied, and the TLC plate was developed twice with 5% ethyl acetate/chloroform. The locations of 5α-DHP and allopregnanolone on the plate were determined by visualizing the unlabeled standards using 50% sulfuric acid/water followed by heating, and two regions of the plate corresponding to substrate (5α-DHP) and products (allopregnanolone and 20α-THP) were excised and placed in a scintillation vial. Scintillation fluid (12 mL) (Ultima Gold, Packard) was added, and the amount of radioactivity was determined by scintillation counting (Beckman-Coulter LS 6500).

Steady-state kinetic parameters were determined by fitting the initial velocity data to the equation for a ternary complex (i.e., sequential) kinetic mechanism (*27*):

$$v/[E]_o = \frac{k_{cat}[A][B]}{K_{ia}K_B + K_A[B] + K_B[A] + [A][B]} \quad (1)$$

where k_{cat} is the turnover number, $[E]_o$, [A], and [B] are the concentrations of the enzyme, steroid substrate, and nicotinamide adenine dinucleotide cofactor, respectively, K_{ia} is the dissociation constant for the steroid substrate, and K_A and K_B are the K_M values for steroid substrate and cofactor, respectively. Nonlinear fitting was carried out using GraFit software version 5.0 (Erithacus Software). Experiments were carried out twice for the forward reaction and once for the reverse reaction.

Inhibition Kinetics. Enzyme inhibition experiments were carried out for the forward and reverse reactions as described above with the addition of ursodeoxycholate (UDC). All reactions contained 100 mM potassium phosphate, 0.2 mg/mL BSA, 50 000 dpm of the appropriate [^{3}H]-labeled steroid substrate, 5% DMSO (v/v), and, (1) for inhibition of 5α-DHP reduction versus 5α-DHP, 2 μM NADPH, 0, 0.25, and 0.5 μM UDC and 20 nM 3α-HSD III (reaction time = 60 min), (2) for inhibition of 5α-DHP reduction versus NADPH, 3 μM 5α-DHP, 0, 0.15, 0.5, and 1.5 μM UDC and 25 nM 3α-HSD III (reaction time = 30 min), (3) for inhibition of allopregnanolone oxidation versus allopregnanolone, 25 μM $NADP^+$, 0, 0.25, 0.5, and 1 μM UDC and 100 nM 3α-HSD III (reaction time = 3 h), and (4) for inhibition of allopregnanolone oxidation versus $NADP^+$, 3 μM allopregnanolone, 0, 0.25, and 0.5 μM UDC and 75 nM 3α-HSD III (reaction time = 3 h). Inhibition constants were determined by fitting the initial velocity data to the equations for competitive (eq 2), noncompetitive ("mixed-type") (eq 3), or uncompetitive (eq 4) inhibition:

$$v/[E]_o = \frac{k_{cat}[S]}{K_M(1 + [I]/K_{is}) + [S]} \quad (2)$$

$$v/[E]_o = \frac{k_{cat}[S]}{K_M(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})} \quad (3)$$

$$v/[E]_o = \frac{k_{cat}[S]}{K_M + [S](1 + [I]/K_{ii})} \quad (4)$$

where [S] and [I] are the concentrations of the varied substrate and the inhibitor, respectively, and K_{is} and K_{ii} are the dissociation constants for dissociation of the inhibitor to yield the free enzyme or the enzyme−substrate complex, respectively (*27*). Nonlinear least-squares fitting was carried out using GraFit software. Inhibition experiments were performed twice for the forward reaction and once for the reverse reaction.

Activation Kinetics and Reduction of 5β-DHP and Allopregnanolone. Experiments were carried out in a total volume of 500 μL, with final concentrations of the following: 100 mM potassium phosphate, pH 7.0, 2 mM NADPH, 0.2−10 μM [^{14}C]-5α-DHP, 0.2−3 μM [^{14}C]-5β-DHP, or 0.1 μM [^{3}H]-allopregnanolone, and 5−50 nM 3α-HSD type III. The solubility of 5α-DHP and 5β-DHP under these solution

conditions at 37 °C was determined as described above to be >80% and >90%, respectively, over the 0.5−10 μM concentration range. The identity of the 5β-DHP reduction product pregnanolone was confirmed by TLC and MS as described above (calculated for pregnanolone 318.3, observed 318.3). The identity of the allopregnanolone reduction product 5α-pregnan-3α,20α-diol was verified by TLC comigration with an authentic standard. Reactions were incubated at 37 °C for 30 min, then quenched by adding 0.7 mL chloroform and vortexing. The chloroform extracts were washed once with 0.7 mL water and concentrated to dryness by rotary evaporation. The residue was dissolved in 10 μL chloroform and applied to a polyester-backed silica gel TLC plate. The TLC plate was then developed twice with 5% ethyl acetate/chloroform, dried, and exposed to a storage phosphor screen (Kodak). The storage phosphor screen was scanned using a Storm 820 phosphorimager (Amersham Biosciences/Molecular Dynamics). Initial rates were determined by quantitating the substrates and products using ImageQuant software (Amersham Biosciences/Molecular Dynamics). Kinetic parameters were determined by fitting the initial rate data to the Michealis-Menten equation (*27*) using GraFit software.

RESULTS

Recombinant 3α-HSD type III was expressed in *E. coli* as a GST fusion protein and purified by affinity chromatography. Radiolabeled 5α- and 5β-DHP were synthesized based on a literature procedure (*26*). Initial rates for enzyme-catalyzed reactions were determined by a radio-TLC assay essentially as described previously (*18*). The reaction schemes for 3α-HSD type III catalyzed 5α-DHP and 5β-DHP reduction are shown in Figure 1A. For 5α-DHP reduction, two products identified as allopregnanolone and 5α-pregnan-20α-ol-3-one (20α-THP) were formed in comparable amounts, while 5β-DHP was converted to a single product identified as pregnanolone (Figure 1B). The identities of the products were verified by TLC comigration and by mass spectrometry following TLC purification.

Two-Substrate Kinetics. For both 5α-DHP reduction and for the reverse reaction (allopregnanolone oxidation), double-reciprocal plots of initial rate data obtained over a range of concentrations for both substrates comprised a pattern of intersecting lines (Figure 2), indicating that 3α-HSD type III catalysis follows a ternary complex (i.e., sequential) kinetic mechanism (*27*). Nonlinear fitting of the data to the equation for a ternary complex kinetic mechanism (eq 1) yielded values for the steady-state kinetic parameters k_{cat}, K_M, and K_{ia} (Table 1). We note that for 5α-DHP reduction, we determined kinetic parameters for total product formation by treating turnover of 5α-DHP to allopregnanolone and 20α-THP as a single reaction with one K_M value (consistent with our finding below that the K_M values for formation of both products are similar), one K_{ia} value, and $k_{cat} = k_{cat1} + k_{cat2}$. This approach was necessitated by the need to quantify small amounts of tritiated products in this experiment and the inability to quantify both products individually either by phosphorimaging (due to insufficient sensitivity) or by scintillation counting (due to the minimal separation of the two products by TLC).

Inhibition Kinetics. To determine whether the order of substrate binding was random or ordered, we determined the

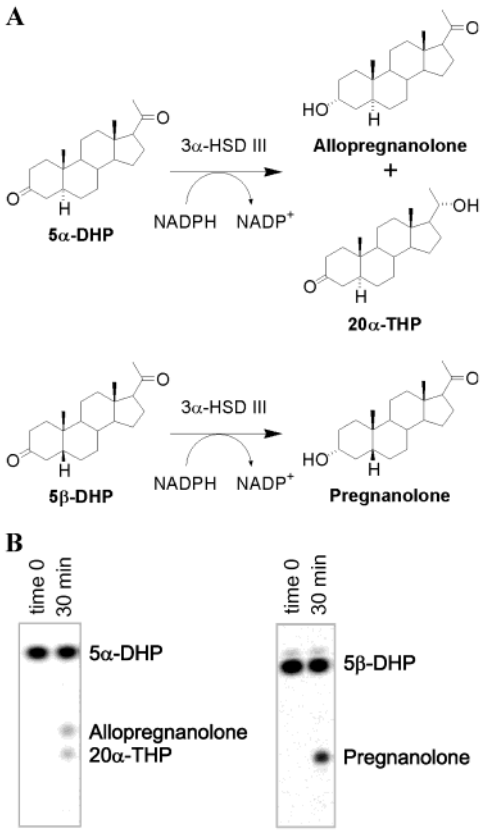

FIGURE 1: Reaction scheme and product formation for DHP reduction by human 3α-HSD type III. (A) 3α-HSD type III catalyzed reduction of (top) 5α-DHP to allopregnanolone and 20α-THP and (bottom) 5β-DHP to pregnanolone. (B) Storage phosphor autoradiograms of thin-layer chromatography plates used to separate the products obtained from 3α-HSD type III catalyzed reduction of (left) 5α-DHP and (right) 5β-DHP. Reactions contained 100 mM potassium phosphate, 0.5 mg/mL BSA, 0.2 μM ^{14}C-labeled 5α-or 5β-DHP, 2 mM NADPH, and 50 nM 3α-HSD, at pH 7.0 and 37 °C (reaction time = 30 min).

modes of inhibition of the forward and reverse reactions by ursodeoxycholate. Analysis of kinetic data for dead-end inhibitors is a useful approach to distinguishing kinetic mechanisms (*27*−*30*). Modes of inhibition and inhibition constants were determined by fitting initial rate data obtained using various concentrations of one substrate and a fixed concentration of the second substrate to the equations for competitive (eq 2), noncompetitive ("mixed-type") (eq 3), and uncompetitive (eq 4) inhibition. For 5α-DHP reduction, UDC was a competitive inhibitor versus 5α-DHP and an uncompetitive inhibitor versus NADPH (Figure 3A, Table 2). Similarly, for allopregnanolone oxidation, UDC was a competitive inhibitor versus allopregnanolone and an uncompetitive inhibitor versus $NADP^+$ (Figure 3B, Table 2).

Activation Kinetics and Reduction of 5β-DHP and Allopregnanolone. Kinetic parameters (k_{cat} and K_M) were determined for 5α-DHP reduction in the presence and absence of 50 μM fluoxetine and for 5β-DHP reduction (Figure 4A, Table 3). For 5α-DHP reduction, both products were formed with similar catalytic efficiency. Relative to 5α-DHP reduction, the catalytic efficiencies for 5β-DHP and allopregnanolone reduction were about 10-fold higher (Table 3) and >50-fold lower (data not shown), respectively. The addition of 50 μM fluoxetine to the reaction had no effect on either k_{cat} or the K_M for 5α-DHP. To probe whether paroxetine,

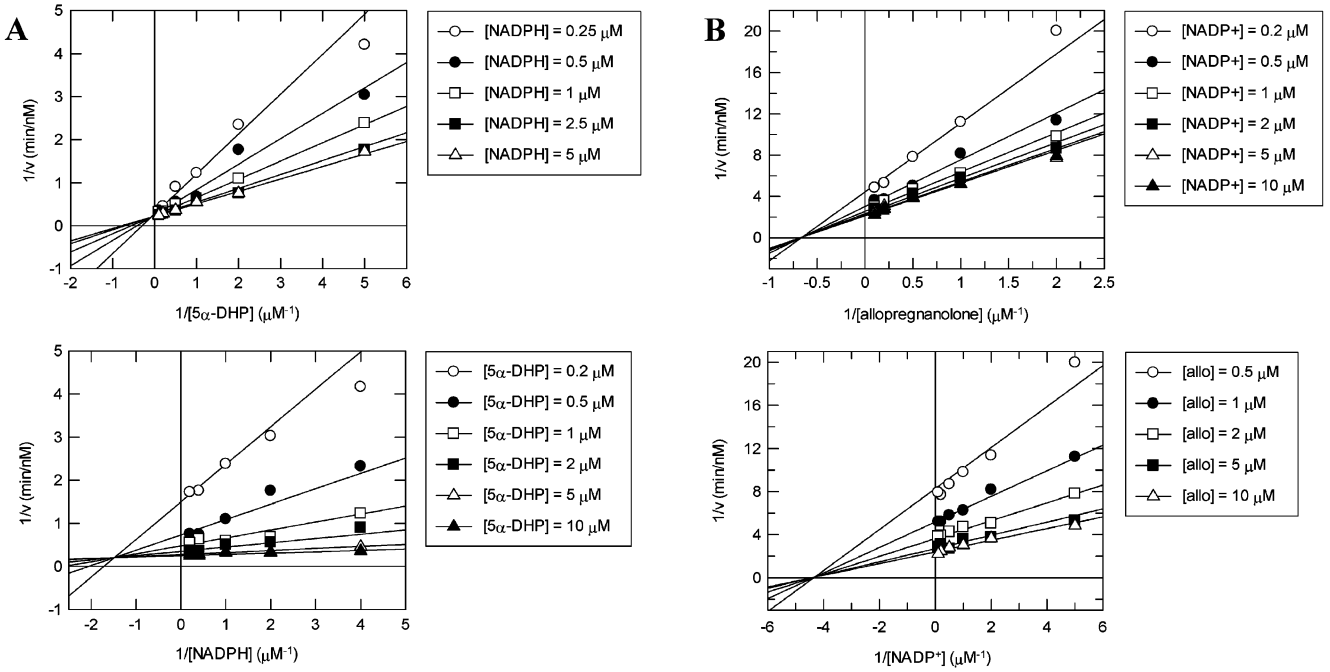

FIGURE 2: Two-substrate profiles for 3α-HSD type III catalyzed 5α-DHP reduction and allopregnanolone oxidation. (A) Double-reciprocal plots for 5α-DHP reduction: (top) $1/v$ (min/nM) vs 1/[5α-DHP] (μM^{-1}) obtained at five fixed NADPH concentrations, and (bottom) $1/v$ (min/nM) vs 1/[NADPH] (μM^{-1}) obtained at six fixed 5α-DHP concentrations. The reactions contained 100 mM potassium phosphate, 0.5 mg/mL BSA, 0.2−10 μM ^{3}H-labeled 5α-DHP, 0.25−5 μM NADPH, and 35 nM 3α-HSD, at 25 °C and pH 7.0. (B) Double-reciprocal plots for allopregnanolone oxidation: (top) $1/v$ (min/nM) vs 1/[allopregnanolone] (μM^{-1}) obtained at six fixed $NADP^+$ concentrations, and (bottom) $1/v$ (min/nM) vs 1/[$NADP^+$] (μM^{-1}) obtained at five fixed allopregnanolone concentrations. The reactions contained 100 mM potassium phosphate, 0.25 mg/mL BSA, 0.5−10 μM ^{3}H-labeled allopregnanolone, 0.2−10 μM $NADP^+$, and 50 nM 3α-HSD, at 25 °C and pH 7.0. In both panels A and B, the data were fit to the equation for a ternary complex mechanism (eq 1).

Table 1: Kinetic Constants for 3α-HSD Catalyzed 5α-DHP Reduction and for Allopregnanolone Oxidation[a]

reaction	substrate	k_{cat} (min^{-1})	K_M (μM)	K_{ia} (μM)
5α-DHP reduction	5α-DHP	0.18 ± 0.01	1.8 ± 0.3	12 ± 9
	NADPH		0.07 ± 0.06	0.5 ± 0.2
allopregnanolone ox.	allopregnanolone	0.010 ± 0.001	1.5 ± 0.2	1.5 ± 0.7
	$NADP^+$		0.23 ± 0.04	0.2 ± 0.1

[a] The kinetic parameters were determined by fitting the data to the equation for a ternary complex (eq 1). Reported parameters are from a single experiment, with standard errors indicated. For experimental details, see the Materials and Methods section and the caption to Figure 2.

sertraline, norfluoxetine, carbamazepine, clozapine, flurbiprofen, or bromosulfophthalein could alter 3α-HSD type III activity, we determined the catalytic efficiency of the reaction in the presence of each of the compounds at 50 μM concentration. For this experiment, a substrate concentration of 0.25 μM, which was much lower than the K_M value (1.7 μM), was used such that the initial rate was proportional the catalytic efficiency (k_{cat}/K_M). None of the compounds tested had a significant effect on catalytic efficiency (Figure 4B, Table 4).

DISCUSSION

The 3α-HSD type III isoform attracted our interest because it was reported to be activated by the SSRIs fluoxetine and paroxetine (*19*), suggesting a key role for this enzyme in SSRI-mediated modulation of allopregnanolone concentrations (*9, 14*). The potential clinical importance of 3α-HSD in the mechanism of action of SSRIs, together with our curiosity about the different allosteric activation mechanisms that had been reported, prompted us to reevaluate the ability of SSRIs to activate 3α-HSD type III. Further, since relatively little characterization of the kinetics of neurosteroid formation by human 3α-HSD type III had been reported, we measured kinetic parameters for 5α- and 5β-DHP reduction to the neurosteroids allopregnanolone and pregnanolone, respectively, and determined the kinetic mechanism for human 3α-HSD type III catalyzed allopregnanolone formation.

In our initial characterization of 3α-HSD type III, we found that 5α-DHP was converted to two products, allopregnanolone and 20α-THP, which result from 3- and 20-ketosteroid reductase activities, respectively. The ratio of products was not changed by addition of 100−300 mM NaCl and 1 mM $MgCl_2$ (final concentrations) to the reaction (data not shown). While not previously reported for 5α-DHP reduction, previous studies have shown that human 3α-HSDs display varying degrees 20- and 17-ketosteroid reductase activities (*16*−*18*, *31*). Consistent with our observation of robust 20-ketosteroid reductase activity, 3α-HSD type III shares very high amino acid sequence identity (98%) with human 20α-HSD (AKR1C1). The 20α-HSD (considered a 3α-HSD isoform) was identified primarily as a 20α-HSD but also has 3α-HSD activity (*32*). In contrast to human 3α-HSDs, rat 3α-HSD (AKR1C9) displays no 20-ketosteroid

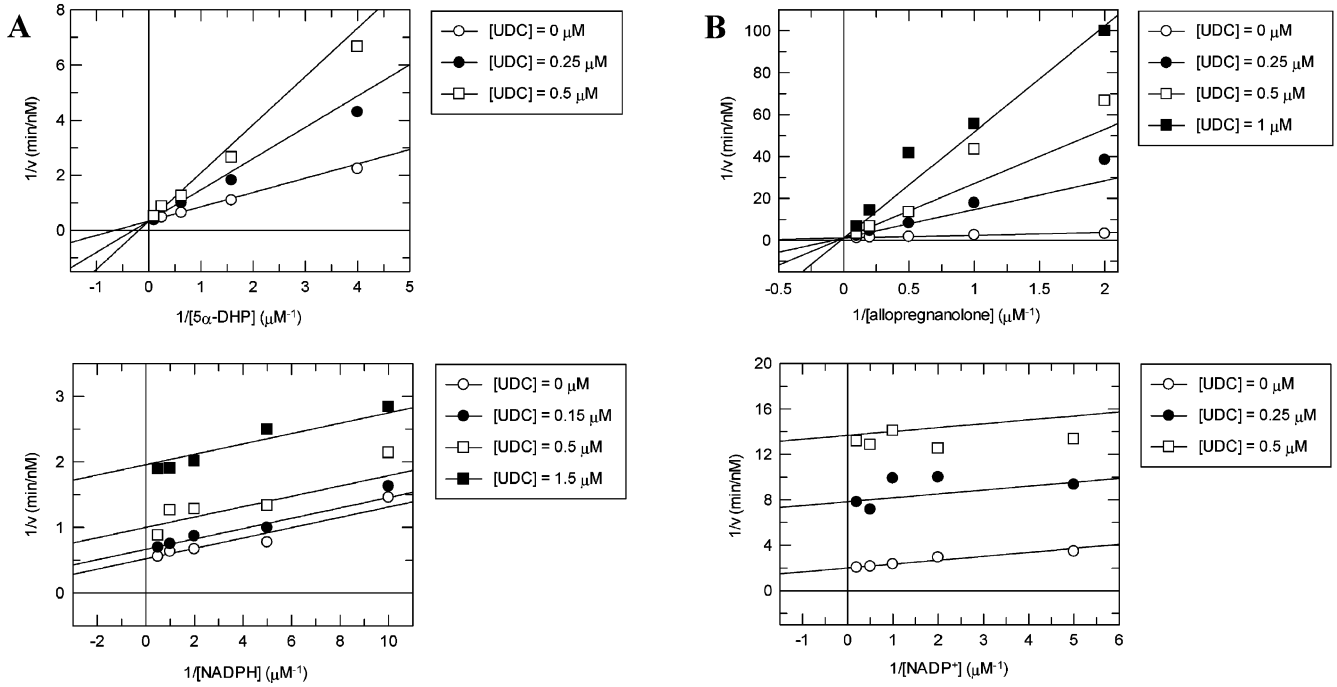

FIGURE 3: Ursodeoxycholate (UDC) inhibition of 3α-HSD type III catalyzed 5α-DHP reduction and allopregnanolone oxidation. (A) Double-reciprocal plots for inhibition of 5α-DHP reduction: (top) $1/v$ (min/nM) vs 1/[5α-DHP] (μM^{-1}) with [NADPH] fixed at 25 μM, and (bottom) $1/v$ (min/nM) vs 1/[NADPH] (μM^{-1}) with [5α-DHP] fixed at 3 μM. (B) Double-reciprocal plots for inhibition of allopregnanolone oxidation: (top) $1/v$ (min/nM) vs 1/[allopregnanolone] (μM^{-1}) with [$NADP^+$] fixed at 25 μM, and (bottom) $1/v$ (min/nM) vs 1/[$NADP^+$] (μM^{-1}) with [allopregnanolone] fixed at 3 μM. For panels A and B, the data were fit either to the equation for uncompetitive inhibition (eq 4, parallel lines) or competitive inhibition (eq 2, intersecting lines). All reactions contained 100 mM potassium phosphate and 0.5 mg/mL BSA at 25 °C and pH 7.0.

Table 2: Ursodeoxycholate Inhibition of 3α-HSD Type III Catalyzed 5α-DHP Reduction and Allopregnanolone Oxidation[a]

reaction	varied substrate	mode of inhibition	K_{is} (μM)	K_{ii} (μM)
5α-DHP reduction	5α-DHP	competitive	0.21 ± 0.07	
	NADPH	uncompetitive		0.55 ± 0.06
allopregnanolone ox.	allopregnanolone	competitive	0.027 ± 0.003	
	$NADP^+$	uncompetitive		0.086 ± 0.006

[a] Inhibition constants were determined by fitting the data to the equations for either competitive (eq 2) or uncompetitive (eq 4) inhibition. Reported values are from a single experiment, with standard errors indicated. For experimental details, see the Materials and Methods section and the caption to Figure 3.

reductase activity (*33*). Comparison of crystal structures for rat 3α-HSD (*35*−*37*) and human 3α-HSD type III (*34*, *38*) revealed that the human enzyme has a relatively large substrate binding pocket compared with the rat enzyme (*34*), consistent with its ability to productively bind steroid substrates in different orientations. Cocrystal structures of human 3α-HSD type III with testosterone or ursodeoxycholate bound in the active site provide direct evidence for multiple binding orientations (*32*, *38*). In contrast to 5α-DHP, 5β-DHP was converted only to pregnanolone, the 3α-ketosteroid reducase product. Interestingly, we noted substrate inhibition with 5β-DHP at concentrations greater than 3 μM (data not shown). This substrate inhibition suggests that 5β-DHP can bind to the enzyme in a "reversed" orientation similar to the binding mode for 5α-DHP that leads to the 20-ketosteroid reduction product, but that this binding mode does not allow catalysis.

The observation of 20-ketosteroid reductase activity for 3α-HSD type III suggested that this enzyme might catalyze the reduction of allopregnanolone to 5α-pregnan-3α,20α-diol. Indeed, we observed allopregnanolone reduction, but with a low catalytic efficiency >50-fold lower than that for 5α-DHP reduction. This result supports that the primary role for 3α-HSD type III in allopregnanolone biosynthesis is allopregnanolone formation by 5α-DHP reduction.

Determination of the kinetic mechanism for an enzyme is generally carried out by using steady-state kinetics (*27*−*30*). We investigated the kinetic mechanism of 3α-HSD type III catalyzed 5α-DHP reduction by analysis of two-substrate and inhibition kinetics. Results from two-substrate kinetics experiments for the forward (reduction) and reverse (oxidation) reactions indicated that catalysis follows a ternary complex, or sequential, kinetic mechanism. Comparison of catalytic efficiencies for the forward and reverse reactions revealed that the reaction proceeds with 15-fold higher efficiency in the reductive direction. Similar results were obtained in a comparison of the kinetics of testosterone 17-oxidation and the corresponding reverse reaction, in which case the catalytic efficiency was 5-fold higher in the reductive direction (*38*). Inhibition studies with ursodeoxycholate were used to determine the order of binding of substrates and products. For both the forward and reverse reactions, ursodeoxycholate was a competitive inhibitor versus steroid substrate and an uncompetitive inhibitor versus NADP(H).

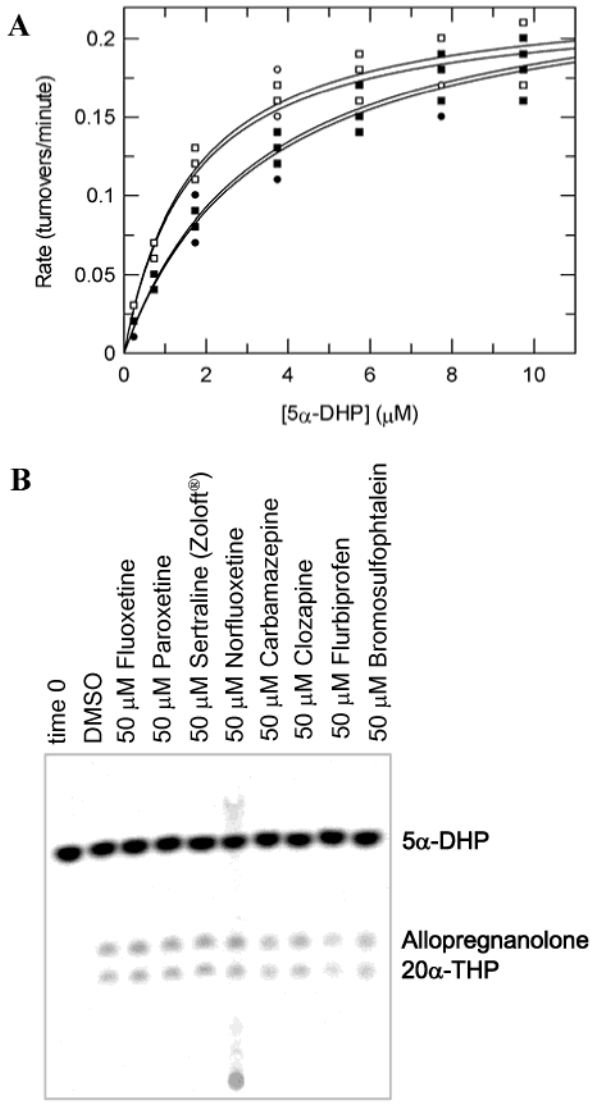

FIGURE 4: Effect of fluoxetine and other compounds on 3α-HSD type III catalyzed reduction of 5α-DHP. (A) Initial rates for allopregnanolone (open symbols) and 20α-THP (filled symbols) in the absence of fluoxetine (circles) and in the presence of 50 μM fluoxetine (squares). Reactions contained 100 mM potassium phosphate, 0.5 mg/mL BSA, 2 mM NADPH, 5% DMSO (v/v), 0.25−10 μM ^{14}C-labeled 5α-DHP, and 40−50 nM 3α-HSD III at 37 °C and pH 7.0. (B) Storage phosphor autoradiograms of a thin-layer chromatography plate used to separate the products obtained from 3α-HSD type III catalyzed reduction of 5α-DHP in the presence of various compounds (each at 50 μM). Reactions contained 100 mM potassium phosphate, 0.5 mg/mL BSA, 2 mM NADPH, 5% DMSO (v/v), 0.25 μM ^{14}C-labeled 5α-DHP, and 20 nM 3α-HSD III at 37 °C and pH 7.0.

These results are consistent with an ordered kinetic mechanism for 5α-DHP reduction with NADPH binding to the enzyme before 5α-DHP and $NADP^+$ dissociating from the enzyme after the steroid product. Indeed, it has been noted by Fromm (*30*) that this method is the simplest and most definitive for determining kinetic mechanism.

To provide additional support for this kinetic mechanism, we calculated the kinetic Haldane using eq 5 below, which assumes an ordered sequential mechanism (*39*):

$$K_{eq} = \frac{V_1 K_P K_{iq}}{V_2 K_{ia} K_B} \quad (5)$$

where V_1 and V_2 are velocities for the forward (5α-DHP reduction) and reverse (allopregnanolone oxidation) directions, respectively, K_P and K_B are the K_M values for allopregnanolone and 5α-DHP, respectively, and K_{iq} and K_{ia} are dissociation constants for $NADP^+$ and NADPH, respectively. Using values from Table 1, we obtained $K_{eq} = 5$ for the forward reaction (5α-DHP reduction). We note that, as for determination of the kinetic constants, we have modeled the reduction of 5α-DHP to allopregnanolone and 20α-THP as a single reaction with one K_{eq} value. The calculated K_{eq} value for human 3α-HSD was similar to a thermodynamically determined equilibrium constant for NADH-mediated acetone reduction ($K_{eq} = 10$ at pH 7, ionic strength = 0.1 M) (*40*), a reaction very similar to 5α -DHP reduction catalyzed by 3α-HSD, suggesting the proposed ordered sequential kinetic mechanism is correct.

Studies of the kinetic mechanisms of a number of 3α- and 20α-HSDs indicate that catalysis by these enzymes follows an ordered sequential kinetic mechanism with the cofactor binding first and dissociating last (*20*−*23*). Specifically, this kinetic mechanism has been reported for rat 3α-HSD (*20*), human 3α-HSD type I (*21*), rat 20α-HSD (*22*), and mouse 3α-HSD (*23*). Our finding that human 3α-HSD type III displays an ordered sequential mechanism supports that all 3α-HSDs of the aldo-keto reductase superfamily utilize the same kinetic mechanism. This is not unexpected and is indeed very consistent with the observation that many enzymes from the dehydrogenase family, such as alcohol dehydrogenase, lactate dehydrogenase, and malate dehydrogenase, proceed with an ordered kinetic mechanism with NAD(P)H binding first and $NAD(P)^+$ leaving last (*41*).

We next evaluated the ability of fluoxetine and other small molecules to allosterically activate 3α-HSD type III. These experiments were carried out at 37 °C and with 2 mM NADPH to replicate the conditions for which activation of the enzyme by fluoxetine were reported (*19*). Under these conditions, the kinetic parameters we obtained for 5α-DHP reduction in the absence of fluoxetine were consistent with values determined previously for 3α-HSD type III and other 3α-HSDs. For example, the steady-state parameters for 5α-DHP reduction we observed ($k_{cat} = 0.2$ min^{-1} for both allopregnanolone and 20α-THP formation, $K_M = 1.7$ and 3.4 μM for allopregnanolone and 20α-THP formation, respectively) were similar to values reported for reduction of 5α-DHT and 5α-androstane-3,17-dione, as well as for other steroid substrates under similar experimental conditions ($k_{cat} \sim 1$ min^{-1}, low micromolar K_M) (*16*−*18*, *21*, *42*). In addition, the parameters we determined for 5β-DHP reduction were in good agreement with those determined previously for human 3α-HSD type I (*21*). Previous experiments have shown that the catalytic efficiency of human 3α-HSD type III is lower than that of rat 3α-HSD (*16*). Consistent with this result, the kinetic efficiency we determined for 5α-DHP reduction catalyzed by 3α-HSD type III was about 10 times lower than that reported previously for rat 3α-HSD under similar conditions (*42*). Finally, for rat 3α-HSD, 5β-DHP reduction was previously found to proceed with about 10-fold higher efficiency than 5α-DHP reduction, in good agreement with our results with human 3α-HSD type III (*42*).

We found that the kinetic parameters for 5α-DHP reduction by 3α-HSD type III were not affected by the SSRIs fluoxetine, paroxetine, or sertraline. We tested each of these

Table 3: Kinetic Parameters for 3α-HSD III Catalyzed Reduction of 5α-DHP and 5β-DHP[a]

substrate	added compound	product	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (mM^{-1} min^{-1})
5α-DHP	none	allopregnanolone	0.22 ± 0.01	1.7 ± 0.3	135 ± 21
		20α-THP	0.24 ± 0.02	3.4 ± 0.3	72 ± 10
	50 μM fluoxetine	allopregnanolone	0.23 ± 0.02	1.7 ± 0.3	139 ± 17
		20α-THP	0.24 ± 0.03	3.2 ± 0.3	76 ± 2
5β-DHP	none	pregnanolone	1.0 ± 0.1	0.51 ± 0.03	2020 ± 180

[a] Reactions contained 100 mM potassium phosphate, 0.5 mg/mL BSA, 2 mM NADPH, 5% DMSO (v/v), 0.25−8 μM 5α-DHP or 0.2−3 μM 5β-DHP, and 5−50 nM 3α-HSD type III (37 °C, pH 7.0). Reported values are the averages from four experiments, with the standard deviations indicated.

Table 4: Relative k_{Cat}/K_M Values for 5α-DHP Reduction in the Presence of Various Compounds[a]

compound	relative k_{cat}/K_M: allopregnanolone	relative k_{cat}/K_M: 20α-THP
DMSO	1.0	1.0
50 μM fluoxetine	1.0 ± 0.05	1.1 ± 0.02
50 μM paroxetine	1.0 ± 0.07	1.1 ± 0.05
50 μM sertraline (Zoloft)	0.9 ± 0.09	1.0 ± 0.05
50 μM norfluoxetine	1.1 ± 0.11	1.1 ± 0.07
50 μM carbamazepine	0.7 ± 0.04	0.8 ± 0.03
50 μM clozapine	1.0 ± 0.05	1.0 ± 0.06
50 μM flurbiprofen	0.5 ± 0.03	0.5 ± 0.04
50 μM bromosulfophthalein	0.8 ± 0.05	0.8 ± 0.03

[a] Reactions contained 100 mM potassium phosphate, 0.5 mg/mL BSA, 2 mM NADPH, 5% DMSO (v/v), 0.25 μM 5α-DHP and 20 nM 3α-HSD type III (37 °C, pH 7.0). The reported values are the averages from three experiments, with the standard deviations indicated.

compounds at a concentration of 50 μM, the concentration at which fluoxetine was previously reported to produce maximal activation (*19*). Lower concentrations of fluoxetine (5−20 μM) also failed to activate the enzyme (data not shown). Given that our experiments were carried out under very similar conditions, the reason for the discrepancy between our results and the previously reported results is not clear. We note that our experimental conditions differed slightly in that we used a soluble, homogeneous enzyme preparation and pure fluoxetine, whereas in the prior study, the enzyme was obtained as insoluble inclusion bodies and fluoxetine was prepared from Prozac tablets. It is possible that polymers present in Prozac tablets might act as macromolecular crowding agents, resulting in a higher effective substrate concentration and apparent activation, although we observed no apparent activation using sertraline from Zoloft tablets. In addition, the K_M value reported in the previous study for 5α-DHP was surprisingly low (7 nM) compared with the value we measured (1.7 μM).

We found that several additional compounds reported to increase allopregnanolone levels in vivo or activate 3α-HSD also failed to activate 3α-HSD type III. Norfluoxetine, an active metabolite of fluoxetine, showed no activation at 50 μM. Similarly, carbamazepine and clozapine, which have been reported to increase brain allopregnanolone levels following adminsistration to rats (*43*, *44*), did not activate the enzyme when tested at 50 μM. Flurbiprofen and other nonsteroidal antiinflammatory drugs, which are known 3α-HSD inhibitors (*45*), have been reported to modestly activate (1.5−2.5-fold) a 3α-HSD from human liver at sub-inhibitory concentrations (*46*). We found that flurbiprofen inhibited 3α-HSD type III at 50 μM, and did not activate the enzyme at lower concentrations (data not shown). Consistent with an earlier report, we saw no activation of 3α-HSD type III by 50 μM bromosulfophthalein, a compound that activates 3α-HSD type I (*24*). Thus, we find no evidence that 3α-HSD type III has an allosteric activator binding site.

Our failure to observe activation of 3α-HSD type III by fluoxetine and other SSRIs suggests that a different mechanism underlies fluoxetine-mediated increases in allopregnanolone concentrations. Similar to depressed patients, mice subjected to social isolation display a 2-fold drop in brain allopregnanolone concentration that is normalized by fluoxetine (*47*). In the isolated mice, a ∼2-fold decrease in mRNA level for 5α-reductase was observed, suggesting that this genomic mechanism may be involved the effect of fluoxetine on allopregnanolone levels (*47*). Another possible mechanism stems from the observation that levels of the neurosteroid tetrahydrodeoxycorticosterone (THDOC) increase in depressed patients (*10*, *11*). Since THDOC and allopregnanolone biosynthesis both involve 5α-reductase and 3α-HSD, increased concentrations of the THDOC precursor 5α-dihydrodeoxycorticosterone (*10*, *11*) (which may be related to increased cortisol production, which is frequently associated with depression) might compete with the allopregnanolone precursor 5α-DHP, resulting in a shift of the biosynthetic flux toward THDOC and away from allopregnanolone. It remains intriguing that certain CNS drugs, including fluoxetine, increase levels of the neurosteroid allopregnanolone, although the mechanism for this effect remains unclear. Furthermore, allopregnanolone mimetic compounds show promise of effectiveness for treatment of epilepsy (*48*) and anxiety (*49*). Thus, elucidation of the mechanism by which fluoxetine and other drugs increase allopregnanolone levels may suggest potential sites for therapeutic intervention.

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REFERENCES

1. Paul, S. M., and Purdy, R. H. (1992) *FASEB J. 6*, 2311−2322.
2. Robel, P., and Baulieu, E. E. (1994) *Trends Endocrin. Metab. 5*, 1−8.
3. Majewska, M. D. (1992) *Prog. Neurobiol. 38*, 379−395.
4. Rupprecht, R., and Holsboer, F. (1999) *Trends Neurosci. 22*, 410−416.
5. Wang, M., Seippel, L, Purdy, R. H., and Backstrom, T. (1996) *J. Clin. Endocrinol. Metab. 81*, 1076−1082.
6. Bicikova, M., Dibbelt, L., Hill, M., Hampl, R., and Starka, L. (1998) *Hormone Metab. Res. 30*, 227−230.
7. Rapkin, A. J., Morgan, M., Goldman, L., Brann, D. W., Simone, D., and Mahesh, V. B. (1997) *Obstet. Gynecol. 90*, 709−714.
8. Monteleone, P., Luisi, S., Tonetti, A., Bernardi, F., Genazzani, A. D., Luisi, M., Petraglia, F., and Genzzani, A. R. (2000) *Eur. J. Endocrinol. 142*, 269−273.

9. Uzunova, V., Sheline, Y., Davis, J. M., Rasmusson, A., Uzunov, D. P., Costa, E., and Guidotti, A. (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95, 3239−3244.
10. Ströhle, A., Romeo, E., Hermann, B., Pasini, A., Spalletta, G., di Michele, F., Holsboer, F., and Rupprecht, R. (1999) *Biol. Psych. 45*, 274−277.
11. Ströhle, A., Pasini, A., Romeo, E., Hermann, B., Spalletta, G., di Michele, F., Holsboer, F., and Rupprecht, R. (2000) *J. Psychiatr. Res. 34*, 183−186.
12. Steiner, M., Steinberg, S., Stewart, D., Carter, D., Berger, C., Reid, R., Grover, D., and Streiner, D. (1995) *N. Engl. J. Med. 332*, 1529−1534.
13. Su, T.-P., Schmidt, P. J., Danaceau, M. A., Tobin, M. B., Rosenstein, D. L., Murphy, D. L., and Rubinow, D. R. (1997) *Neuropsychopharmacology 16*, 346−356.
14. Uzunov, D. P., Cooper, T. B., Costa, E., and Guidotti, A. (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93, 12599−12604.
15. Corpechot, C., Young, J., Calvel, M., Wehrey, C., Veltz, J. N., Touyer, G., Mouren, M., Prasad, V. V., Banner, C., Sjovall, J., et al. (1993) *Endocrinology 133*, 1003−1009.
16. Penning, T. M., Pawlowski, J. E., Schlegel, B. P., Jez, J. M., Lin, H.-K., Hoog, S. S., Bennet, M. J., and Lewis, M. (1996) *Steroids 61*, 508−523.
17. Burczynski, M. E., Harvey, R. G., and Penning, T. M. (1998) *Biochemistry 37*, 6781−6790.
18. Penning, T. M., Burczynski, M. E., Jez, J. M., Hung, C.-F., Lin, H.-K., Ma, H., Moore, M., Palackal, N., and Ratnam, K. (2000) *Biochem. J. 351*, 67−77.
19. Griffin, L. D., and Mellon, S. H. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 13512−13517.
20. Askonas, L. J., Ricigliano, J. W., and Penning, T. M. (1991) *Biochem. J. 278*, 835−841.
21. Deyashiki, Y., Tamada, Y., Miyabe, Y., Nakanishi, M., Matsuura, K., and Hara, A. (1995) *J. Biochem. 118*, 285−290.
22. Pongsawasdi, P., and Anderson, B. M. (1984) *Biochim. Biophys. Acta 799*, 51−58.
23. Hara, A., Inoue, Y., Nakagawa, M., Naganeo, F., and Sawada, H. (1988) *J. Biochem. (Tokyo) 103*, 1027−1034.
24. Matsuura, K., Tamada, Y., Deyashiki, Y., Miyabe, Y., Nakanishi, Y., Ohya, I., and Hara, A. (1996) *Biochem. J. 313*, 179−184.
25. Matsuura, K., Tamada, Y., Sato, K., Iwasa, H., Miwa, G., Deyashiki, Y., and Hara, A. (1997) *Biochem. J. 322*, 89−93.
26. Akamanchi, K. G., Patel, H. C., and Meenakshi, R. (1992) *Synth. Commun. 22*, 1655−1660.
27. Segal, I. H. (1975) *Enzyme Kinetics*, John Wiley & Sons, Inc., New York.
28. Cleland, W. W. (1979) *Methods Enzymol. 63*, 103−138.
29. Cleland, W. W. (1982) *Methods Enzymol. 87*, 159−179.
30. Fromm, H. J. (1979) *Methods Enzymol. 63*, 467−486.
31. Lin, H. K., Jez, J. M., Schlegel, B. P., Peehl, D. M., Pachter, J. A., and Penning, T. M. (1997) *Mol. Endocrinol. 11*, 1971−1984.
32. Deyashiki, Y., Ogasawara, A., Nakayama, T., Nakanishi, M., Miiyabe, Y., Sato, K., and Hara, A. (1994) *Biochem. J. 299*, 545−552.
33. Ma, H., and Penning, T. M. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 11161−11166.
34. Jin, Y., Stayrook, S. E., Albert, R. H., Palackal, N. T., Penning, T. M., and Lewis, M. (2001) *Biochemistry 40*, 10161−10168.
35. Hoog, S. S., Pawlowski, J. E., Alzari, P. M., Penning, T. M., and Lewis, M. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 2517−2521.
36. Bennet, M. J., Schlegel, B. P., Jez, J. M., Penning, T. M., and Lewis, M. (1996) *Biochemistry 35*, 10702−10711.
37. Bennet, M. J., Albert, R. H., Jez, J. M., Ma, H., Penning, T. M., and Lewis, M. (1997) *Structure 5*, 799−812.
38. Nahoum, V., Gangloff, A., Legrand, P., Zhu, D.-W., Cantin, L., Zhorov, B. S., Luu-The, V., Labrie, F., Breton, R., and Lin, S.-X. (2001) *J. Biol. Chem. 276*, 42091−42098.
39. Cleland, W. W. (1982) *Methods Enzymol. 87*, 366−369.
40. Alberty, R. A. (2001) *Arch. Biochem. Biophys. 389*, 94−109.
41. Fersht, A. (1985) *Enzyme Structure and Mechanism*, W. H. Freeman and Co., New York.
42. Jez, J. M., and Penning, T. M. (1998) *Biochemistry 37*, 9695−9703.
43. Serra, M., Littera, M., Pisu, M. G., Muggironi, M., Purdy, R. H., and Biggio, G. (2000) *Neuropharmacology 39*, 2448−2456.
44. Barbaccia, M. L., Affricano, D., Purdy, R. H., Maciocco, E., Spiga, F., and Biggio, G. (2001) *Neuropsychopharmacology 25*, 489−497.
45. Penning, T. M., Mukharji, I., Barrows, S., and Talalay, P. (1984) *Biochem. J. 222*, 601−611.
46. Yamamoto, T., Matsuura, K., Shintani, S., Hara, A., Miyabe, Y., Sugiyama, T., Katagiri, Y. (1998) *Biol. Pharm. Bull. 21*, 1148−1153.
47. Guidotti, A., Dong, E., Matsumoto, K., Pinna, G., Rasmusson, A. M., and Costa, E. (2001) *Brain Res. Rev. 37*, 110−115.
48. Monaghan, E. P., McAuley, J. W., and Data, J. L. (1999) *Expert Opin. Invest. Drugs 8*, 1663−1671.
49. Vanover, K. E. et al. (2000) *J. Pharmacol. Exp. Ther. 295*, 337−345.

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