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Olanzapine and fluoxetine administration and coadministration increase rat hippocampal pregnenolone, allopregnanolone and peripheral deoxycorticosterone: Implications for therapeutic actions

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Abstract

Olanzapine and fluoxetine elevate the GABAergic neuroactive steroid allopregnanolone to physiologically relevant concentrations in rodent cerebral cortex. It is unknown if these agents also alter pregnenolone or deoxycorticosterone. Since olanzapine and fluoxetine in combination have clinical utility and may demonstrate synergistic effects, we investigated neuroactive steroid alterations following olanzapine, fluoxetine or coadministration. Male rats received IP vehicle, olanzapine, fluoxetine or the combination of both agents in higher-dose (0, 10, 20 or 10/20 mg/kg, respectively) and lower-dose (0, 5, 10 or 5/10 mg/kg, respectively) experiments. Pregnenolone and allopregnanolone levels in hippocampus were determined by gas chromatography/mass spectrometry. Peripheral deoxycorticosterone and other steroid levels were determined by radioimmunoassay. Olanzapine, fluoxetine or the combination increased hippocampal pregnenolone and serum deoxycorticosterone in both higher- and lower-dose experiments, and elevated hippocampal allopregnanolone in higher-dose conditions. No synergistic effects on pregnenolone or allopregnanolone were observed following olanzapine and fluoxetine coadministration compared to either compound alone. Pregnenolone and its sulfate enhance learning and memory in rodent models, and therefore pregnenolone elevations may be relevant to cognitive changes in psychotic and affective disorders. Since pregnenolone decreases have been linked to depression, it is possible that olanzapine- and fluoxetine-induced pregnenolone elevations may contribute to the antidepressant actions of these agents.
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1. Introduction

Olanzapine and fluoxetine coadministration has been investigated in both preclinical models and clinical populations, yielding clues to potential synergistic effects of these agents in combination compared to monotherapy with either compound. In rodent models, olanzapine and fluoxetine coadministration results in synergistic increases in extracellular dopamine and norepinephrine concentrations in prefrontal cortex compared to either drug alone (Zhang et al., 2000). These synergistic effects on extracellular catecholamine concentrations following coadministration of both agents were also observed in hypothalamus, but not in nucleus accumbens or striatum (Koch et al., 2004). Olanzapine and fluoxetine in combination also demonstrate synergistic effects on fibroblast growth factor 2 expression (Maragnoli et al., 2004) and fluoxetine administration appears to potentiate the effects of olanzapine on locus coeruleus neuronal firing rate and burst firing (Seager et al., 2004). In contrast, olanzapine and fluoxetine in

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combination do not result in greater induction of cell proliferation in hippocampal dentate gyrus or prelimbic cortex compared to either agent alone (Kodama et al., 2004). Clinically, a randomized controlled double-blind trial ($n=833$) demonstrated that combined treatment with olanzapine and fluoxetine is more effective than olanzapine monotherapy or placebo in the treatment of bipolar depression (Tohen et al., 2003), and this olanzapine/fluoxetine combination was recently approved by the U.S. Food and Drug Administration for this indication. A randomized double-blind pilot investigation in patients with treatment-resistant depression without psychotic features ($n=28$) demonstrated that olanzapine plus fluoxetine resulted in superior efficacy compared to monotherapy with either agent (Shelton et al., 2001). In a larger subsequent clinical trial ($n=500$), however, olanzapine and fluoxetine in combination did not differ significantly from monotherapy with either agent or nortriptyline, although the combination produced a more rapid response that was sustained until treatment conclusion (Shelton et al., 2005). A recent investigation in treatment-resistant depression also demonstrated that the combination of olanzapine/fluoxetine was similarly effective at study endpoint compared to monotherapy with fluoxetine or venlafaxine, although patients receiving the combination of olanzapine/fluoxetine exhibited more rapid improvement by the first week of treatment (Corya et al., 2006). Hence, olanzapine/fluoxetine in combination appears to be clinically superior for bipolar depression compared to monotherapy with olanzapine (Tohen et al., 2003), but may not have preferred utility in other affective disorders.

The precise mechanisms mediating the synergistic effects of olanzapine and fluoxetine coadministration in specific clinical settings and rodent experimental paradigms remain to be determined. Both of these agents elevate levels of the neuroactive steroid 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) in rodent brain to physiologically relevant concentrations individually (Uzunov et al., 1996; Serra et al., 2001; Pinna et al., 2003, 2004; Marx et al., 2000, 2003). Clinically, fluoxetine also appears to elevate allopregnanolone levels in plasma (Romeo et al., 1998) and cerebrospinal fluid (Uzunova et al., 1998) in patients with major depression. We therefore hypothesize that the combination of olanzapine and fluoxetine may result in enhanced elevations of neuroactive steroids in rat brain. If these changes also occur in humans, it is possible that potential synergistic effects on neuroactive steroids following olanzapine and fluoxetine in combination could contribute to their therapeutic effects in bipolar depression (Tohen et al., 2003). We thus investigated possible synergistic effects on neuroactive steroid levels in rodent hippocampus following olanzapine, fluoxetine or the combination utilizing both higher-dose and lower-dose drug administration strategies.

Since some studies suggest that olanzapine may improve cognitive symptoms in patients with schizophrenia (Keefe et al., 2004; Sharma et al., 2003), we were particularly interested in characterizing the potential effects of olanzapine administration on pregnenolone levels in hippocampus. Pregnenolone and its sulfated derivative enhance learning and memory in rodent models, and pregnenolone sulfate administration in aged rats transiently reverses age-related cognitive decline (Flood et al., 1992, 1995; Akwa et al., 2001; Vallee et al., 1997, 2001). In addition, decreased levels of pregnenolone have been linked to depressive symptoms (George et al., 1994). Potential olanzapine- and fluoxetine-induced increases in central pregnenolone levels could therefore theoretically contribute to their efficacy in the treatment of depressive symptoms, including decreased concentration. Prior reports that fluoxetine potentiates the antidepressant-like effects of the GABAergic neuroactive steroid allopregnanolone (Khisti and Chopde, 2000), and that allopregnanolone potentiates olanzapine actions on dopamine-mediated behaviors in rodents (Ugale et al., 2004) support the possibility that neuroactive steroid induction may be relevant to the therapeutic actions of these agents.

Our prior investigations have demonstrated that olanzapine dose-dependently increases the GABAergic neuroactive steroid allopregnanolone in rodent cerebral cortex (Marx et al., 2000, 2003). Allotetrahydrodeoxycorticosterone (THDOC) is a neuroactive steroid with potent modulatory $GABA_A$ receptor activity comparable to allopregnanolone (Morrow et al., 1990). We thus examined peripheral serum levels of deoxycorticosterone, a precursor molecule to THDOC. Since we have recently determined that a number of serum steroids are highly interrelated in male subjects with nicotine dependence (Marx et al., 2006a), we also examined a panel of peripheral steroids in these rodent experiments to determine if interrelationships among steroids are also present in our animal model.

2. Methods

2.1. Animals

Animals were purchased, housed and euthanized in accordance with approved IACUC protocols at the University of North Carolina at Chapel Hill. Male rats (200–250 mg/kg, Sprague–Dawley) were obtained from Harlan (Indianapolis, IN), group housed and permitted free access to food and water.

2.2. Experimental design

Male rats ($n=8$–11 per condition) were injected i.p. with vehicle, olanzapine, fluoxetine or the combination of both agents in higher-dose (0, 10, 20 or 10/20 mg/kg, respectively) and lower-dose (0, 5, 10 or 5/10 mg/kg, respectively) experiments after habituation to i.p. saline injection for 5 consecutive days. Rats were sacrificed by decapitation 1 h following i.p. drug administration. Hippocampus was rapidly dissected on ice and stored at −80 °C for pregnenolone and allopregnanolone analyses. Trunk blood was collected for serum deoxycorticosterone, progesterone, corticosterone, dehydroepiandrosterone (DHEA) and estradiol analyses, kept on ice until centrifugation for serum collection and stored at −80 °C. The neuroactive steroids pregnenolone and allopregnanolone were determined by gas chromatography/mass spectrometry, preceded by high performance liquid chromatography (HPLC). Peripheral deoxycorticosterone and other steroid levels were determined by radioimmunoassay. Statistical analyses were performed by ANOVA with post-hoc Dunnett tests. Associations between steroids were also investigated and Pearson correlation

coefficients were determined. Since multiple hypotheses were tested in the same dataset, we adopted a more conservative significance threshold of $p \leq 0.01$ for these correlational analyses. Correlational analyses yielding a p value ≤ 0.05 (but >0.01) were designated as trends.

2.3. Gas chromatography/mass spectrometry (GC/MS) preceded by high performance liquid chromatography (HPLC)

Neuroactive steroid (NS) analyses (allopregnanolone, pregnenolone) in rodent hippocampus were performed as previously described (Marx et al., 2006b; Uzunova et al., 1998) with modifications. All glassware was silanized. Brain tissue samples and standards were homogenized in distilled water containing a trace amount of tritiated neuroactive steroid (New England Nuclear) to detect the HPLC fractions of interest. Deuterated allopregnanolone and deuterated pregnenolone were utilized as the internal standards. Neuroactive steroids were extracted three times with ethyl acetate prior to HPLC purification. Each steroid was collected based upon the retention time of its radioactive analogue. The HPLC fractions containing pregnenolone and allopregnanolone were evaporated to dryness and derivatized utilizing heptafluorobutyric acid anhydride (HFBA) in ethyl acetate. Derivatized standards and samples were injected onto an Agilent 5973 GC/MS in the negative ion chemical ionization (NICI) mode utilizing methane as the reaction gas and helium as the carrier gas. In addition to the retention time of each steroid, the structural identification of each NS assayed was provided by its unique mass fragmentation pattern. Mass spectrometer single ion monitoring (SIM) mode was used to focus on the most abundant ion fragment for each steroid derivative. For NS quantification, the standard curve for the steroid of interest was prepared by combining varying known quantities of the steroid (Steraloids) with a constant amount of the respective deuterated internal standard. Only peaks with a signal to noise ratio greater or equal to 5:1 were integrated. The limit of NS detection with this method was 2 pg for allopregnanolone and 10 pg for pregnenolone.

2.4. Radioimmunoassays

Peripheral serum steroid levels (progesterone, corticosterone, DHEA, estradiol) were determined by radioimmunoassay (Diagnostic Systems Laboratories, ICN). Peripheral deoxycorticosterone levels were determined by radioimmunoassay as previously described (Khisti et al., 2005).

3. Results

3.1. Lower-dose conditions

3.1.1. Effects on hippocampal steroid levels—lower-dose conditions

Fluoxetine, olanzapine or the combination of both agents significantly increases hippocampal pregnenolone levels to physiologically relevant concentrations (ANOVA $p<0.0001$, $F=10.512$, df 3,28, $n=8$ per condition; post-hoc Dunnett $p=0.001$ for fluoxetine 10 mg/kg condition, $p=0.002$ for olanzapine 5 mg/kg condition and $p<0.001$ for combination, Fig. 1A). Synergistic effects on hippocampal pregnenolone levels following the combination of olanzapine and fluoxetine compared to monotherapy with either agent were not observed. Fluoxetine, olanzapine or the combination of both agents (lower-dose conditions) tend to increase hippocampal allopregnanolone levels, but ANOVA results did not achieve statistical significance, Fig. 1B. Synergistic effects on hippocampal allopregnanolone levels following the combination of olanzapine and fluoxetine compared to monotherapy with either agent were not observed.

3.2. Effects on peripheral steroid levels—lower-dose conditions

Fluoxetine, olanzapine or the combination of both agents significantly increases peripheral serum progesterone levels (ANOVA $p<0.0001$, $F=12.750$, df 3,36, $n=10$ per condition; post-hoc Dunnett $p=0.004$ for fluoxetine 10 mg/kg condition,

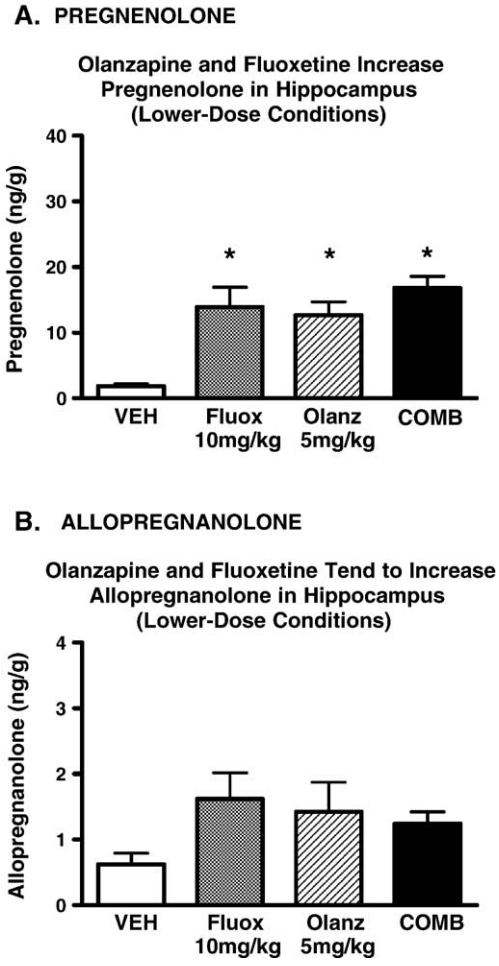

Fig. 1. (A) Pregnenolone. Fluoxetine, olanzapine or the combination of both agents significantly increases hippocampal pregnenolone levels (ANOVA $p<0.0001$, $F=10.512$, df 3,28, $n=8$ per condition; post-hoc Dunnett $p=0.001$ for fluoxetine 10 mg/kg condition, $p=0.002$ for olanzapine 5 mg/kg condition and $p<0.001$ for combination). (B) Allopregnanolone. Fluoxetine, olanzapine or the combination of both agents (lower dose condition) tend to increase hippocampal allopregnanolone levels, but results did not achieve statistical significance (ANOVA $p>0.05$).

$p<0.001$ for olanzapine 5 mg/kg condition and $p<0.001$ for combination, Fig. 2A). Fluoxetine, olanzapine or the combination of both agents significantly increases peripheral serum deoxycorticosterone levels (ANOVA $p<0.0001$, $F=18.453$, df 3,38, $n=10$–11 per condition; post-hoc Dunnett $p<0.001$ for fluoxetine 10 mg/kg condition ($n=11$), $p<0.001$ for olanzapine 5 mg/kg condition ($n=10$) and $p<0.001$ for combination ($n=11$), Fig. 2B). Fluoxetine, olanzapine or the combination of both agents significantly increases peripheral serum corticosterone levels (ANOVA $p<0.0001$, $F=29.228$, df 3,35, $n=9$–10 per condition; post-hoc Dunnett $p<0.001$ for fluoxetine 10 mg/kg condition ($n=9$), $p<0.001$ for olanzapine 5 mg/kg condition ($n=10$) and $p<0.001$ for combination ($n=10$), Fig. 2C).

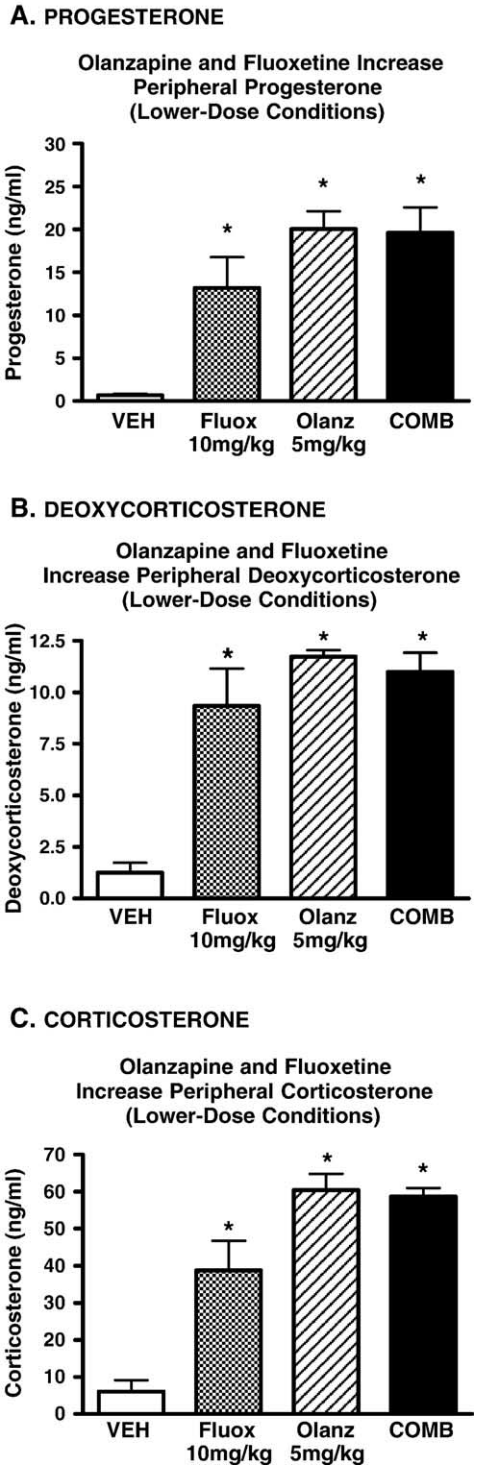

Table 1
Correlations among steroids, lower-dose conditions

		ALLO	PROG	DEOX	CORT	DHEA	ESTR
PREG	r	**0.453**	**0.848**	**0.866**	**0.865**	0.157	**−0.484**
	p	**0.009**	**<0.001**	**<0.001**	**<0.001**	0.390	**0.005**
	n	**32**	**32**	**32**	**32**	32	**32**
ALLO	r		**0.495**	0.426	0.371	−0.179	−0.069
	p		**0.004**	0.015[a]	0.036[a]	0.327	0.708
	n		**32**	32	32	32	32
PROG	r			**0.780**	**0.844**	0.141	−0.384
	p			**<0.001**	**<0.001**	0.387	0.014[a]
	n			**40**	**39**	40	40
DEOX	r				**0.879**	0.167	−0.385
	p				**<0.001**	0.304	0.014[a]
	n				**39**	40	40
CORT	r					0.150	**−0.511**
	p					0.362	**0.001**
	n					39	**39**
DHEA	r						0.032[a]
	p						0.846
	n						40

Correlation matrix of hippocampal allopregnanolone and pregnenolone levels, and peripheral serum deoxycorticosterone, corticosterone, DHEA and estradiol levels (lower-dose conditions). Pearson correlation coefficient (r). Bolded p values significant, $p<0.01$; [a]$p<0.05$, trend. *Abbreviations:* PREG=pregnenolone, ALLO=allopregnanolone, PROG=progesterone, DEOX=deoxycorticosterone, CORT=corticosterone, DHEA=dehydroepiandrosterone, ESTR=estradiol.

3.3. Steroid correlations—lower-dose conditions

There are a number of significant correlations among peripheral and central steroids in these lower-dose drug administration experiments. Hippocampal pregnenolone levels are correlated with hippocampal allopregnanolone levels, as well as peripheral serum deoxycorticosterone and progesterone levels. Numerous additional significant correlations are observed, Table 1.

3.4. Higher-dose conditions

3.4.1. Effects on hippocampal steroid levels—higher-dose conditions

Fluoxetine, olanzapine or the combination of both agents significantly increases hippocampal pregnenolone levels to

Fig. 2. (A) Progesterone. Fluoxetine, olanzapine or the combination of both agents significantly increases peripheral progesterone levels (ANOVA $p<0.0001$, $F=12.750$, df 3,36, $n=10$ per condition; post-hoc Dunnett $p=0.004$ for fluoxetine 10 mg/kg condition, $p<0.001$ for olanzapine 5 mg/kg condition and $p<0.001$ for combination). (B) Deoxycorticosterone. Fluoxetine, olanzapine or the combination of both agents significantly increases peripheral deoxycorticosterone levels (ANOVA $p<0.0001$, $F=18.453$, df 3,38, $n=10$–11 per condition; post-hoc Dunnett $p<0.001$ for fluoxetine 10 mg/kg condition ($n=11$), $p<0.001$ for olanzapine 5 mg/kg condition ($n=10$) and $p<0.001$ for combination ($n=11$)). (C) Corticosterone. Fluoxetine, olanzapine or the combination of both agents significantly increases peripheral corticosterone levels (ANOVA $p<0.0001$, $F=29.228$, df 3,35, $n=9$–10 per condition; post-hoc Dunnett $p<0.001$ for fluoxetine 10 mg/kg condition ($n=9$), $p<0.001$ for olanzapine 5 mg/kg condition ($n=10$) and $p<0.001$ for combination ($n=10$)).

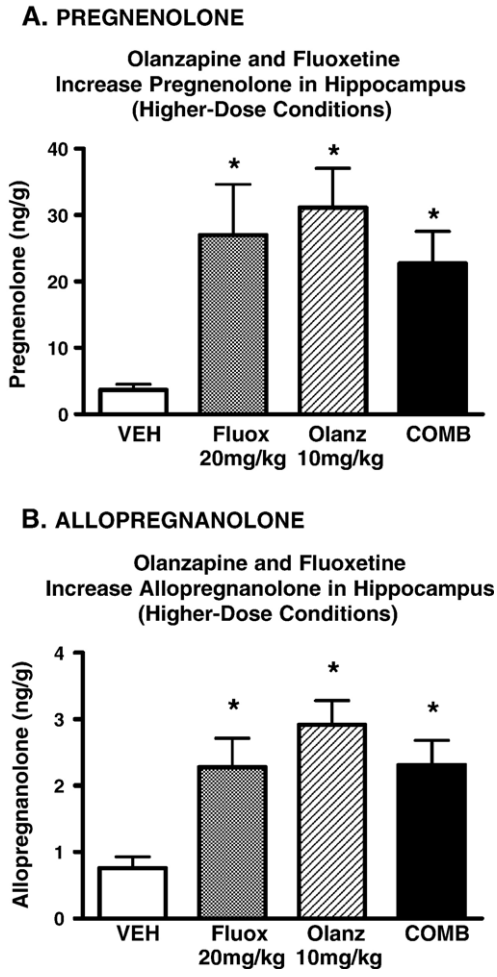

Fig. 3. (A) Pregnenolone. Fluoxetine, olanzapine or the combination of both agents significantly increases hippocampal pregnenolone levels (ANOVA $p=0.0065$, $F=5.031$, df 3,28, $n=8$ per condition; post-hoc Dunnett $p=0.014$ for fluoxetine 20 mg/kg condition, $p=0.003$ for olanzapine 10 mg/kg condition and $p=0.049$ for combination). (B) Allopregnanolone. Fluoxetine, olanzapine or the combination of both agents significantly increases hippocampal allopregnanolone levels (ANOVA $p=0.001$, $F=6.913$, df 3,28, $n=8$ per condition; post-hoc Dunnett $p=0.013$ for fluoxetine 20 mg/kg condition, $p<0.001$ for olanzapine 10 mg/kg condition, $p=0.011$ for combination).

physiologically relevant concentrations (ANOVA $p=0.0065$, $F=5.031$, df 3,28, $n=8$ per condition; post-hoc Dunnett $p=0.014$ for fluoxetine 20 mg/kg condition, $p=0.003$ for olanzapine 10 mg/kg condition, $p=0.049$ for combination, Fig. 3A). Synergistic effects on hippocampal pregnenolone levels following the combination of olanzapine and fluoxetine compared to monotherapy with either agent were not observed. Fluoxetine, olanzapine or the combination of both agents significantly increases hippocampal allopregnanolone levels to low nanomolar concentrations known to potentiate $GABA_A$ receptor response (ANOVA $p=0.001$, $F=6.913$, df 3,28, $n=8$ per

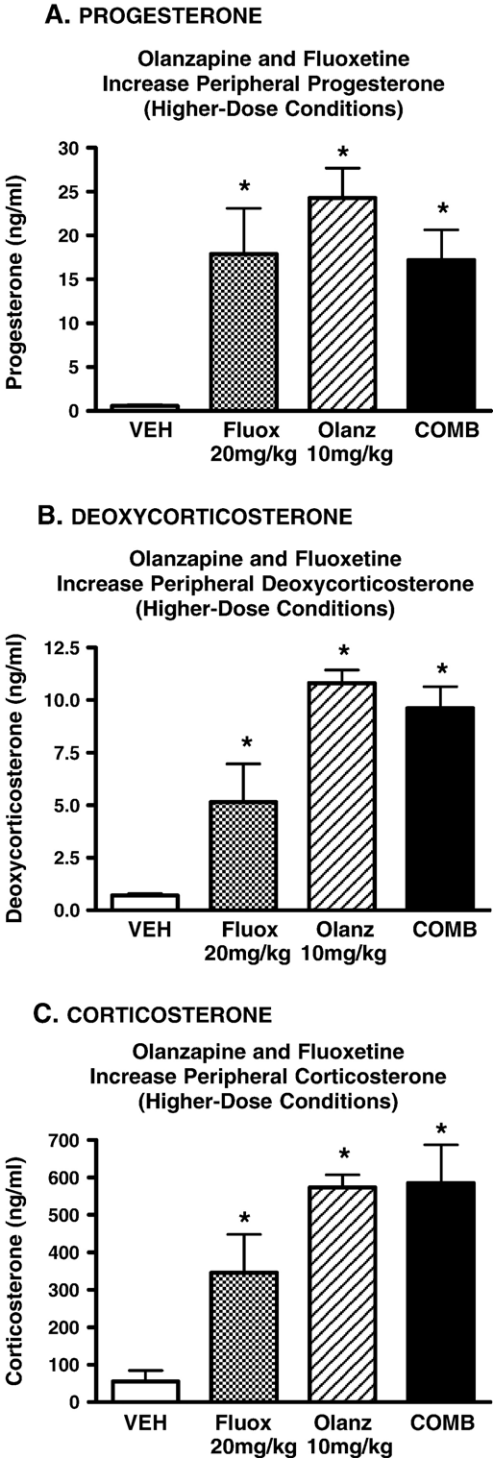

Fig. 4. (A) Progesterone. Fluoxetine, olanzapine or the combination of both agents significantly increases peripheral progesterone levels (ANOVA $p=0.003$, $F=8.109$, df 3,36, $n=10$ per condition; post-hoc Dunnett $p=0.004$ for fluoxetine 20 mg/kg condition, $p<0.001$ for olanzapine 10 mg/kg condition and $p=0.006$ for combination). (B) Deoxycorticosterone. Fluoxetine, olanzapine or the combination of both agents significantly increases peripheral deoxycorticosterone levels (ANOVA $p<0.0001$, $F=23.322$, df 3,32, $n=7$–10 per condition; post-hoc Dunnett $p=0.013$ for fluoxetine 20 mg/kg condition ($n=7$), $p<0.001$ for olanzapine 10 mg/kg condition ($n=10$) and $p<0.001$ for combination ($n=10$)). (C) Corticosterone. Fluoxetine, olanzapine or the combination of both agents significantly increases peripheral corticosterone levels (ANOVA $p<0.0001$, $F=13.173$, df 3,33, $n=7$–10 per condition; post-hoc Dunnett $p=0.028$ for fluoxetine 20 mg/kg condition ($n=7$), $p<0.001$ for olanzapine 10 mg/kg condition ($n=10$) and $p<0.001$ for combination ($n=10$)).

Table 2
Correlations among steroids, higher-dose conditions

		ALLO	PROG	DEOX	CORT	DHEA	ESTR
PREG	*r*	**0.733**	**0.891**	**0.825**	**0.830**	0.103	−0.281
	p	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.575	0.120
	n	**32**	**32**	**31**	**31**	32	32
ALLO	*r*		**0.816**	**0.754**	**0.758**	−0.124	−0.326
	p		**<0.001**	**<0.001**	**<0.001**	0.500	0.068
	n		**32**	**31**	**31**	32	32
PROG	*r*			**0.869**	**0.880**	0.242	−0.011
	p			**<0.001**	**<0.001**	0.138	0.946
	n			**35**	**37**	39	38
DEOX	*r*				**0.900**	0.097	−0.362
	p				**<0.001**	0.580	0.032[a]
	n				**34**	35	35
CORT	*r*					0.224	−0.043
	p					0.182	0.803
	n					37	36
DHEA	*r*						0.157
	p						0.345
	n						38

Correlation matrix of hippocampal allopregnanolone and pregnenolone levels, and peripheral serum deoxycorticosterone, corticosterone, DHEA and estradiol levels (higher-dose conditions). Pearson correlation coefficient (*r*). Bolded *p* values significant, $p<0.01$; [a]$p<0.05$, trend. *Abbreviations:* PREG=pregnenolone, ALLO=allopregnanolone, PROG=progesterone, DEOX=deoxycorticosterone, CORT=corticosterone, DHEA=dehydroepiandrosterone, ESTR=estradiol.

condition; post-hoc Dunnett $p=0.013$ for fluoxetine 20 mg/kg condition, $p<0.001$ for olanzapine 10 mg/kg condition, $p=0.011$ for combination, Fig. 3B). Synergistic effects on hippocampal allopregnanolone levels following the combination of olanzapine and fluoxetine compared to monotherapy with either agent are not observed.

3.4.2. Effects on peripheral steroid levels—higher-dose conditions

Fluoxetine, olanzapine or the combination of both agents significantly increases peripheral serum progesterone levels (ANOVA $p=0.003$, $F=8.109$, *df* 3,36, $n=10$ per condition; post-hoc Dunnett $p=0.004$ for fluoxetine 20 mg/kg condition, $p<0.001$ for olanzapine 10 mg/kg condition, $p=0.006$ for combination, Fig. 4A). Fluoxetine, olanzapine or the combination of both agents significantly increases peripheral serum deoxycorticosterone levels (ANOVA $p<0.0001$, $F=23.322$, *df* 3,32, $n=7$–10 per condition; post-hoc Dunnett $p=0.013$ for fluoxetine 20 mg/kg condition ($n=7$), $p<0.001$ for olanzapine 10 mg/kg condition ($n=10$), $p<0.001$ for combination ($n=10$), Fig. 4B). Fluoxetine, olanzapine or the combination of both agents significantly increases peripheral serum corticosterone levels (ANOVA $p<0.0001$, $F=13.173$, *df* 3,33, $n=7$–10 per condition; post-hoc Dunnett $p=0.028$ for fluoxetine 20 mg/kg condition ($n=7$), $p<0.001$ for olanzapine 10 mg/kg condition ($n=10$), $p<0.001$ for combination ($n=10$), Fig. 4C).

3.4.3. Steroid correlations—higher-dose conditions

Similar to lower-dose conditions, a number of steroids are significantly correlated, underscoring the importance of investigating multiple biosynthetic steroid pathways, Table 2.

4. Discussion

Olanzapine or fluoxetine administration singly or in combination produces elevations in hippocampal pregnenolone levels in both higher- and lower-dose experimental paradigms. To our knowledge, this is one of the first reports characterizing pregnenolone levels in rodent hippocampus following antipsychotic drug administration (Marx et al., 2006). It has been reported previously that fluoxetine increases pregnenolone levels in rat cerebral cortex (Serra et al., 2001). Since decreased pregnenolone levels have been linked to depressive symptoms in humans (George et al., 1994), pregnenolone induction following fluoxetine or olanzapine may potentially contribute to the antidepressant actions of these agents. This possibility currently remains very speculative, however, and additional data in humans will be required to test this hypothesis. Pregnenolone and its sulfated derivative also positively impact learning and memory in animal models (Flood et al., 1992, 1995; Akwa et al., 2001; Vallee et al., 1997). If olanzapine and fluoxetine also induce pregnenolone elevations in clinical populations, these alterations could potentially contribute to the possible attenuation of cognitive deficits in schizophrenia and impaired concentration in depression, respectively, following treatment with these compounds. We have also recently determined that olanzapine 5 mg/kg i.p. significantly increases pregnenolone levels in rat cerebral cortex (Marx et al., 2006), a finding that may be relevant to the pathophysiology of schizophrenia since cognitive impairment in this disorder involves the prefrontal cortex (Lewis et al., 2004a,b). Although clearly a very preliminary hypothesis, it is possible that hippocampal pregnenolone elevations may be relevant to the therapeutic mechanisms of these agents.

Contrary to our initial hypothesis, the combination of olanzapine and fluoxetine did not produce synergistic effects on pregnenolone levels in rodent hippocampus in either higher-dose or lower-dose experimental paradigms. The precise reasons for the absence of synergistic effects following the administration of both agents remain to be clarified. It is possible that both drugs are acting via the same mechanism to increase neuroactive steroid biosynthesis. This mechanism could potentially involve saturation of the P450scc enzyme leading to pregnenolone synthesis from cholesterol, resulting in a possible ceiling effect. Saturation of the P450scc enzyme could also explain the absence of synergistic effects on hippocampal allopregnanolone levels following the combination of olanzapine and fluoxetine, since allopregnanolone is a pregnenolone metabolite and a plateau in pregnenolone precursor levels could thus potentially limit downstream allopregnanolone formation. This hypothesis would not necessarily explain the absence of synergistic effects following the combination of olanzapine and fluoxetine in the lower-dose experiment, however, in which neuroactive steroid elevations are not as pronounced as neuroactive steroid changes in the higher-dose conditions. It is possible that olanzapine and fluoxetine administered in combination may interact in a manner that modifies the effect of either compound alone on enzyme activity. Future efforts will be required to test these possibilities.

It has also been reported that fluoxetine enhances the activity of the 3α-hydroxysteroid dehydrogenase enzyme (3α-HSD) in the reductive direction, leading to increased allopregnanolone formation (Griffin and Mellon, 2001), although enhanced activity of the 3α-HSD enzyme following fluoxetine was not demonstrated in a subsequent study (Trauger et al., 2002). The enhancement of 3α-HSD activity would not necessarily explain hippocampal pregnenolone increases following fluoxetine or olanzapine, however, although it remains a possible candidate target for antidepressant action. For example, the antidepressant mirtazapine appears to influence 3α-HSD by dose-dependently inhibiting activity in the oxidative direction, and also appears to elevate plasma allopregnanolone levels in patients with major depression (Schule et al., 2006). With regard to olanzapine, potential effects on 3α-HSD activity are currently unknown and will require future study. It is also conceivable that fluoxetine and olanzapine may act on multiple enzymes simultaneously. For example, the study demonstrating a mirtazapine effect on 3α-HSD activity also suggested that mirtazapine may impact 5α-reductase to a modest degree (Schule et al., 2006). Olanzapine or fluoxetine could therefore enhance the activity of 3α-HSD and/or upstream neurosteroidogenic enzymes such as 5α-reductase or P450scc concurrently. Future research investigating the effects of olanzapine and fluoxetine on the kinetics of P450scc, 3α-HSD, 5α-reductase and other enzymes in these steroid pathways will be required to clarify the actions of these agents on neuroactive steroid synthesis and metabolism.

In addition, it is possible that acute administration of olanzapine and fluoxetine in combination represents a suboptimal rodent model for therapeutically synergistic effects in certain clinical settings, which require chronic treatment with these agents. Findings from a recent rodent study underscore the importance of investigating neuroactive steroid changes following the chronic administration of these compounds. Specifically, cerebral cortical and plasma allopregnanolone and pregnenolone levels were decreased 48 h after the last dose of a chronic regimen of fluoxetine 10 mg/kg (daily i.p. administration for 15 days); subsequent challenge with fluoxetine 20 mg/kg, however, continued to result in significant increases in these neuroactive steroids in both plasma and brain (Serra et al., 2001). Future studies utilizing chronic drug administration paradigms will be required to explore this potential explanation for the absence of synergistic effects on hippocampal pregnenolone and allopregnanolone levels following the acute administration of olanzapine and fluoxetine in combination.

We have previously determined that olanzapine increases allopregnanolone in rat cerebral cortex to physiologically relevant concentrations (Marx et al., 2000, 2003), but it has not been reported if allopregnanolone levels are also increased in rat hippocampus following treatment with olanzapine. Fluoxetine-induced elevations in rat hippocampal allopregnanolone have been demonstrated previously (Uzunov et al., 1996), as have fluoxetine-induced increases in rat cerebral cortical allopregnanolone and pregnenolone (Serra et al., 2001). Fluoxetine-induced elevations in allopregnanolone have also been demonstrated in mouse brain (Pinna et al., 2003, 2004). These experiments demonstrate that olanzapine, fluoxetine, or the combination of both agents produces increases in the GABAergic neuroactive steroid allopregnanolone in rodent hippocampus to levels known to potentiate $GABA_A$ receptor response. It is therefore possible that olanzapine-induced elevations in allopregnanolone levels in hippocampus may contribute to a number of its therapeutic effects. Since allopregnanolone reductions have been linked to depression in humans (Romeo et al., 1998; Uzunova et al., 1998), it is possible that olanzapine- and fluoxetine-induced elevations in hippocampal allopregnanolone could potentially contribute to their antidepressant actions. Allopregnanolone also produces anxiolytic-like effects in animal models (Bitran et al., 1991; Wieland et al., 1991, 1995), and induction of this neuroactive steroid could therefore represent a candidate mechanism contributing to the anxiolytic actions of these compounds. Furthermore, allopregnanolone potentiates olanzapine actions on dopamine-mediated behaviors in rodents (Ugale et al., 2004); fluoxetine potentiates the antidepressant-like effects of allopregnanolone in rodent behavioral models (Khisti and Chopde, 2000); and fluoxetine-induced reversal of allopregnanolone downregulation in socially isolated mice mediates the anti-aggressive actions of fluoxetine (Pinna et al., 2003). Behavioral investigations therefore suggest potential mechanistic roles for this neuroactive steroid that may contribute to the efficacy fluoxetine or olanzapine. At the present time, however, evidence in clinical populations supporting these hypotheses remains limited.

The administration of olanzapine, fluoxetine, or the combination of the two agents significantly increased serum deoxycorticosterone, a precursor to the potent GABAergic neuroactive steroid allotetrahydrodeoxycorticosterone (THDOC), in both higher- and lower-dose experiments. Elevations in deoxycorticosterone precursor could result in downstream THDOC metabolite formation. It is therefore possible that other GABAergic neuroactive steroids in addition to allopregnanolone, including THDOC, may also be altered in rodent brain following treatment with olanzapine or fluoxetine in clinical populations. Olanzapine, fluoxetine and the combination also markedly elevated progesterone, a precursor to allopregnanolone and a number of other GABAergic neuroactive steroids. Since progesterone demonstrates neuroprotective effects in a number of rodent models (Djebaili et al., 2004, 2005; Roof et al., 1994, 1997; Roof and Hall, 2000) and also enhances myelination (Schumacher et al., 2001), it is possible that induction of this steroid (which can be synthesized *de novo* from cholesterol in the brain) may be clinically therapeutic. It is possible, however, that progesterone elevations may not accompany allopregnanolone elevations following treatment with fluoxetine. Specifically, two studies have reported elevations in plasma (Romeo et al., 1998) and cerebrospinal fluid (Uzunova et al., 1998) allopregnanolone levels in the absence of elevated progesterone levels in patients with major depression treated with fluoxetine, in contrast to our rodent findings demonstrating pronounced increases in progesterone following fluoxetine administration. Potential extrapolations from these rodent data to the clinical realm should therefore be tempered with a great deal of caution and will require extensive additional data in clinical populations.

Many steroids proved to be highly interrelated in both the higher-dose and lower-dose drug administration paradigms,

underscoring the importance of characterizing multiple steroid biosynthetic pathways simultaneously. For example, hippocampal pregnenolone levels were positively correlated with hippocampal allopregnanolone levels and peripheral deoxycorticosterone levels in both higher- and lower-dose and conditions. Pregnenolone levels were also correlated with a number of other peripheral steroids. Recent data from our laboratory suggest that rat hippocampal pregnenolone levels are highly correlated with peripheral serum levels of these neuroactive steroids (Marx et al., 2006). It is therefore possible that the determination of peripheral neuroactive steroid levels in serum may have utility as a surrogate marker for neuroactive steroid concentrations in brain.

In summary, olanzapine, fluoxetine and the combination of both compounds elevate the neuroactive steroids pregnenolone and allopregnanolone in rodent hippocampus, and it is possible that these alterations may be relevant to the clinical efficacy of these molecules. Synergistic effects on hippocampal neuroactive steroid levels were not observed following the administration of these agents in combination, potentially suggesting that both drugs may act via the same mechanism(s) to enhance neuroactive steroid biosynthesis. It is also possible that mechanisms other than neuroactive steroid induction may contribute to the enhanced therapeutic efficacy of olanzapine and fluoxetine in combination in bipolar depression. Alternatively, chronic treatment more closely resembling the use of these compounds in clinical settings may be required for potential synergistic effects to become apparent.

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