Available online at www.sciencedirect.com
ScienceDirect
Pharmacology, Biochemistry and Behavior 84 (2006) 598–608
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Clozapine markedly elevates pregnenolone in rat hippocampus, cerebral cortex, and serum: Candidate mechanism for superior efficacy?

Christine E. Marx [a,b,*], Lawrence J. Shampine [a,b], Gary E. Duncan [c], Margaret J. VanDoren [c], A.Chistina Grobin [c], Mark W. Massing [b], Roger D. Madison [a,b], Daniel W. Bradford [a,b], Marian I. Butterfield [a,b], Jeffrey A. Lieberman [d], A.Leslie Morrow [c]

[a] *Duke University Medical Center, Durham NC, USA*
[b] *Durham Veterans Affairs Medical Center, Durham NC, USA*
[c] *University of North Carolina at Chapel Hill, Chapel Hill NC, USA*
[d] *Columbia University College of Physicians and Surgeons, New York NY, USA*

Received 30 March 2006; received in revised form 19 July 2006; accepted 17 August 2006
Available online 8 September 2006

Abstract

Clozapine demonstrates superior efficacy in patients with schizophrenia, but the precise mechanisms contributing to this clinical advantage are not clear. Clozapine and olanzapine increase the GABAergic neuroactive steroid (NS) allopregnanolone, and it has been hypothesized that NS induction may contribute to the therapeutic actions of these agents. Pregnenolone administration improves learning and memory in rodent models, and decreases in this NS have been associated with depressive symptoms in humans. These pregnenolone characteristics may be relevant to the actions of antipsychotics. We therefore investigated potential pregnenolone alterations in rat hippocampus and cerebral cortex following clozapine, olanzapine, and other second generation agents as a candidate NS mechanism contributing to antipsychotic efficacy. In the first set of experiments, intact, adrenalectomized, and sham-operated male rats received vehicle or clozapine (20 mg/kg) IP. In the second set, male rats received vehicle, olanzapine (5 mg/kg), quetiapine (20 mg/kg), ziprasidone (10 mg/kg) or aripiprazole (5 mg/kg) IP. Pregnenolone levels were determined by gas chromatography/mass spectrometry. Clozapine markedly elevates pregnenolone in rat hippocampus, cerebral cortex, and serum; hippocampal levels were strongly correlated with serum levels ($r=0.987$). Olanzapine also elevates pregnenolone levels, but to a lesser degree than clozapine. Pregnenolone induction may contribute to the clinical actions of clozapine and olanzapine.

Keywords: Pregnenolone; Antipsychotic; Clozapine; Neuroactive steroid; Olanzapine; Neurosteroid; Rat; Hippocampus

1. Introduction

Multiple investigations have demonstrated the superior efficacy of the antipsychotic clozapine in patients with refractory schizophrenia (Breier et al., 1994; Kane et al., 1988, 2001; Pickar et al., 1992; Conley et al., 1999; Azorin et al., 2001; Wahlbeck et al., 1999; Chakos et al., 2001; Tuunainen et al., 2002). Most recently, clozapine significantly outperformed other second generation agents in both the second phase of the multi-site Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) project (McEvoy et al., 2006) and the Cost Utility of the Latest Antipsychotics in Severe Schizophrenia (CUtLASS 2) trial (Lewis et al., in press). Despite its clear clinical advantage, the precise mechanisms contributing to clozapine's unique clinical profile remain incompletely understood. One possible mechanism contributing to its superior efficacy may involve neuroactive steroid (NS) induction. It has been demonstrated previously that both clozapine (Marx et al., 2003; Barbaccia et al., 2001) and olanzapine (Marx et al., 2000, 2003) dose-dependently increase the GABAergic NS allopregnanolone in rat cerebral cortex. In contrast, allopregnanolone was not altered following haloperidol (Marx et al., 2003; Barbaccia et al., 2001) or risperidone (Marx et al., 2003)

* Corresponding author. Durham Veterans Affairs Medical Center, 508 Fulton Street, Mental Health Service Line 116A, Durham, North Carolina 27705, USA. Tel.: +1 919 286 0411x7426; fax: +1 919 286 6811.
E-mail address: marx0001@mc.duke.edu (C.E. Marx).

0091-3057/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2006.07.026

administration. Our recent findings suggest that olanzapine administration elevates the NS pregnenolone, a precursor to allopregnanolone and other steroids, in rodent hippocampus (Marx et al., 2006-this issue). It is not known, however, if clozapine administration produces similar or more robust increases in brain pregnenolone levels. We thus investigated potential pregnenolone changes following clozapine, aripiprazole, olanzapine, quetiapine, or ziprasidone in rat hippocampus and cerebral cortex utilizing doses consistent with clinically comparable striatal dopamine D_2 receptor occupancies (Kapur et al., 1999, 2000, 2003; Natesan et al., 2006; Barth et al., 2006; Farde et al., 1988, 1992; Mamo et al., 2004; Zipursky et al., 2005; Yokoi et al., 2002; Tauscher et al., 2004).

Potential alterations in pregnenolone could theoretically be relevant to the actions of clozapine and certain other second generation antipsychotics. For example, pregnenolone enhances learning and memory in rodent models (Flood et al., 1992). Since second generation antipsychotics may have effects on cognitive outcome measures in patients with schizophrenia, pregnenolone elevations following their administration could theoretically constitute a mechanism contributing to these clinical actions. In addition, decreased pregnenolone levels have been associated with depressive symptoms in humans (George et al., 1994), and a number of second generation antipsychotics may demonstrate therapeutic actions with regard to depressive symptoms (Tollefson et al., 1998; Dollfus et al., 2005; Moller, 2005; Kasper, 2004; Simon and Nemeroff, 2005; Barbee et al., 2004; Papakostas et al., 2004) and suicidal behaviors (Meltzer et al., 2003; Modestin et al., 2005). Pregnenolone elevations following antipsychotics could thus be relevant to the antidepressant actions of these agents, and potentially represent a candidate mechanism contributing to clozapine effects on suicidality.

Additional properties of pregnenolone that may be relevant to schizophrenia include effects of this NS on the neuronal cytoskeleton (Benitez-King et al., 2004). For example, pregnenolone binds to microtubule-associated protein 2 (MAP2) and enhances microtubule polymerization in rat brain and PC12 cells (Fontaine-Lenoir et al., 2006; Murakami et al., 2000), and stabilizes microtubules during embryonic development (Hsu et al., 2006). Pregnenolone also enhances neurite outgrowth (Fontaine-Lenoir et al., 2006). Since patients with schizophrenia demonstrate decreased neuropil (Glantz and Lewis, 2000), clozapine-induced elevations in pregnenolone could potentially impact this element of schizophrenia pathophysiology.

Finally, pregnenolone may be an important molecule impacting the therapeutic efficacy of clozapine and other second generation antipsychotic agents because it can be metabolized to a sulfated derivative (pregnenolone sulfate). In addition to enhancing learning and memory in animal models (Flood et al., 1992, 1995; Akwa et al., 2001; Vallee et al., 1997, 2001), this sulfated NS also modulates both cholinergic and glutamatergic neurotransmitter systems. For example, pregnenolone sulfate enhances acetylcholine release (Darnaudery et al., 1998, 2002; Pallares et al., 1998; Mayo et al., 2003) and prevents amnesia induced by the anticholinergic scopolamine in the passive avoidance task (Vallee et al., 2001). Clozapine, olanzapine, ziprasidone, and quetiapine also increase acetylcholine release in rats (Ichikawa et al., 2002a,b; Shirazi-Southall et al., 2002). The sulfated derivative of pregnenolone is also a positive modulator of glutamatergic NMDA receptors (Wu et al., 1991; Irwin et al., 1994; Bowlby, 1993) and enhances long-term potentiation in rat CA1 hippocampal slices through NMDA receptor modulation (Sliwinski et al., 2004). In addition, pregnenolone sulfate administration prevents cognitive deficits induced by NMDA receptor antagonists (Mathis et al., 1994, 1996). Since it has been hypothesized that NMDA receptor hypofunction may be relevant to the pathophysiology of schizophrenia and that ameliorating this proposed deficit may have therapeutic potential (Javitt, 2004; Millan, 2005, reviews), pregnenolone sulfate induction following clozapine and other second generation antipsychotic agents could have significant clinical ramifications and merits future investigation.

2. Methods

2.1. Animals

Animals were purchased, housed and euthanized in accordance with approved IACUC protocols at the Durham Veterans Affairs Medical Center and the University of North Carolina at Chapel Hill. Male rats (200–250 mg/kg, Sprague–Dawley) were obtained from Harlan (Indianapolis, IN), group housed, placed on a 12-h light–dark cycle, and permitted free access to food and water. All rats were handled and habituated to intraperitoneal (IP) vehicle injection (0.9% saline) for 5 days prior to the day of the experiment to minimize possible stress-induced steroid increases. All experiments were performed at the beginning of the light cycle between 9:00 AM and noon to minimize diurnal steroid fluctuations.

2.2. Antipsychotic administration and dosing

Clozapine (Sigma, St. Louis, MO) was dissolved in a small amount of 20% acetic acid (0.5 mL or less) and then diluted with 0.9% saline (final acetic acid concentration 0.2%). Olanzapine (Eli Lilly and Company, Indianapolis, IN) and quetiapine (AstraZeneca, Wilmington DE) were prepared in a similar manner. Aripiprazole (Bristol-Meyers Squibb, New York, NY) and ziprasidone (Pfizer, New York, NY) were dissolved in 45% w/v 2-hydroxypropyl-β-cyclodextrin (Sigma, St. Louis, MO) and then diluted with distilled water (final 2-hydroxypropyl-β-cyclodextrin concentration 22.5%). Vehicle consisted of 0.2% acetic acid in 0.9% saline.

Recognizing the importance of choosing comparable dosing strategies for each second generation antipsychotic, we reviewed the existing literature for guidelines addressing acute administration paradigms. Typical doses producing a clinical response for a number of antipsychotics have been associated with striatal dopamine D_2 receptor occupancies of approximately 60% or greater, with extrapyramidal side effects emerging when D_2 receptor occupancies exceed 80% (Farde et al., 1988, 1992; Kapur et al., 1999, 2000; Mamo et al., 2004; Zipursky et al., 2005; Tauscher et al., 2004). Aripiprazole represents a possible exception to this paradigm, demonstrating

>90% D_2 receptor occupancy at commonly utilized clinical doses with no concomitant increase in extrapyramidal symptoms (Yokoi et al., 2002). Antipsychotic doses chosen for these experiments are thus consistent with rodent striatal D_2 receptor occupancies of 60–90% following one-time acute drug administration (Kapur et al., 2003; Natesan et al., 2006; Barth et al., 2006), and also consistent with clinically comparable striatal D_2 receptor occupancies in patients with schizophrenia (Kapur et al., 1999, 2000; Mamo et al., 2004; Zipursky et al., 2005; Tauscher et al., 2004; Yokoi et al., 2002). In addition to utilizing an antipsychotic dosing strategy targeting striatal D_2 receptor occupancies that are closely aligned with clinically comparable ranges of D_2 receptor occupancies in humans, dosing decisions were also informed by literature review of antipsychotic doses producing significant effects in rodent models of psychosis (Kinkead et al., 2000; Xu et al., 2002; Wolff and Leander, 2003; Li et al., 2004; Atkins et al., 1999; Tarazi et al., 2001).

2.3. Experimental design

2.3.1. Clozapine 20 mg/kg vs. vehicle control

On the day of the experiment following 5 days of IP habituation with normal saline, male rats received either 20 mg/kg clozapine or vehicle IP, $n=6$–10 animals per treatment condition. Rats were sacrificed 1 h later by decapitation. Hippocampus and cerebral cortex were rapidly dissected on ice and trunk blood was collected for pregnenolone analyses by gas chromatography/mass spectrometry preceded by high performance liquid chromatography purification.

2.3.2. Clozapine 20 mg/kg vs. vehicle control—adrenalectomized (ADX) vs. sham-operated

Male adrenalectomized and sham-operated rats were purchased from Harlan (Indianapolis, IN). Experiments were performed 5–6 days following surgery (and 1–2 days following arrival to the animal colony). Adrenalectomized animals received 0.9% saline instead of water *ad libitum*. Sham-operated animals received water *ad libitum*. All animals had free access to rat chow. On the day of the experiment, adrenalectomized animals were injected IP with clozapine 20 mg/kg (prepared as described previously) or vehicle (0.2% acetic acid in 0.9% saline) and sham-operated animals were also injected IP with either clozapine 20 mg/kg or vehicle, $n=8$ animals per condition. Rats were decapitated 1 h following IP antipsychotic or vehicle injection, and hippocampus, cerebral cortex, and trunk blood were collected as described below.

2.3.3. Other second generation antipsychotics vs. vehicle control

On the day of the experiment following 5 days of IP habituation with normal saline, male rats received IP vehicle,

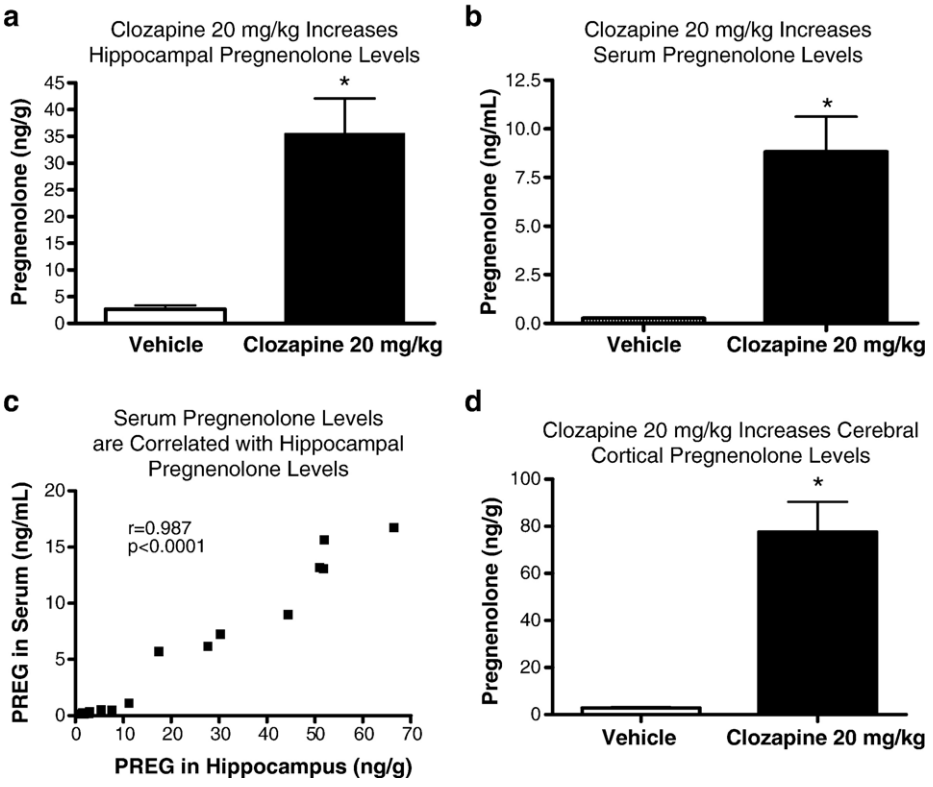

Fig. 1. (a) Clozapine 20 mg/kg IP administration increases hippocampal pregnenolone levels 13-fold to 35.50±6.56 ng/g compared to hippocampal pregnenolone levels following vehicle administration (2.70±0.66 ng/g), unpaired *t*-test, $p<0.0001$, $t=4.97$, $df=18$, $n=10$ per condition. (b) Clozapine 20 mg/kg IP elevates peripheral serum pregnenolone levels 34-fold to 8.82±1.82 ng/mL compared to serum pregnenolone levels following vehicle administration (0.26±0.04 ng/mL), unpaired *t*-test, $p=0.0002$, $t=4.71$, $df=18$, $n=10$ per condition. (c) Hippocampal pregnenolone levels and serum pregnenolone levels are strongly correlated, Pearson correlation coefficient $r=0.987$, $p<0.0001$; $n=20$ *xy* pairs. (d) Clozapine 20 mg/kg IP administration increases cerebral cortical pregnenolone levels 26-fold to 77.58±12.80 ng/g ($n=9$) compared cerebral cortical pregnenolone levels following vehicle administration (2.91±0.28 ng/g, $n=6$), unpaired *t*-test, $p=0.0004$, $t=4.70$, $df=13$.

aripiprazole 5 mg/kg, olanzapine 5 mg/kg, quetiapine 20 mg/kg, or ziprasidone 10 mg/kg, $n=9$ animals per treatment condition. Rats were sacrificed 1 h later by decapitation. Hippocampus and cerebral cortex were rapidly dissected on ice for pregnenolone analyses by gas chromatography/mass spectrometry preceded by high performance liquid chromatography purification.

2.4. Tissue procurement

All rats were sacrificed by decapitation 1 h following IP drug administration. Hippocampus and cerebral cortex was rapidly dissected on ice and stored at −80 °C. Trunk blood was collected for serum neuroactive steroid analyses, kept on ice until centrifugation for serum collection, and stored at −80 °C.

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

Determinations of pregnenolone levels in rat hippocampus, cerebral cortex, and serum were performed as previously described (Marx et al., 2006; 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 pregnenolone (New England Nuclear) to detect the HPLC fraction of interest. Deuterated pregnenolone was utilized as the internal standard. Samples were extracted three times with ethyl acetate prior to HPLC. Pregnenolone was collected based upon the retention time of its radioactive analogue. The HPLC fraction containing pregnenolone was 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. Samples were injected in duplicate. 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 of the steroid derivative. For NS quantification, the standard curve 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. Mean intra-assay coefficients of variation were ≤3.8% for each

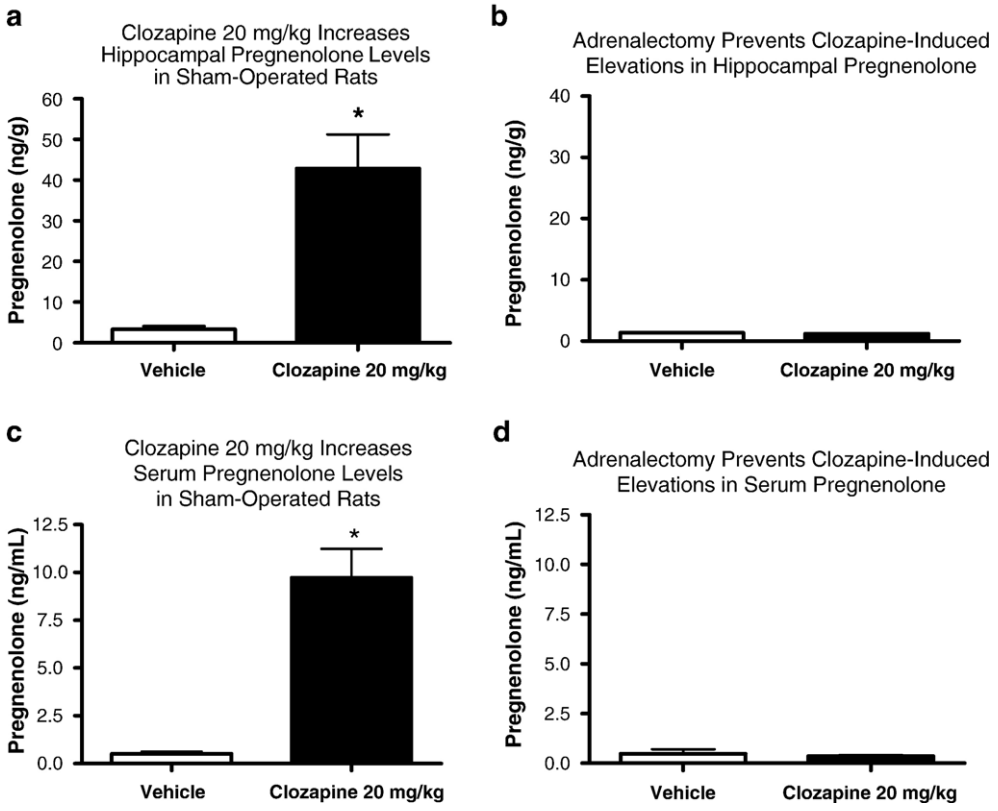

Fig. 2. (a) Clozapine 20 mg/kg IP administration increases hippocampal pregnenolone levels 13-fold to 42.89±8.33 ng/g in sham-operated rats compared to pregnenolone levels in sham-operated rats treated with vehicle (3.30±0.81 ng/g), unpaired *t*-test, $p=0.0003$, $t=4.728$, $df=14$, $n=8$ per condition. (b) Mean hippocampal pregnenolone levels in adrenalectomized (ADX) animals treated with clozapine 20 mg/kg IP (1.21±0.08 ng/g) do not differ significantly from levels in ADX animals treated with vehicle (1.33±0.10 ng/g), unpaired *t*-test, $p=0.359$, $t=0.948$, $df=14$, $n=8$ per condition. (c) Clozapine 20 mg/kg IP administration increases serum pregnenolone levels over 18-fold to 9.73±1.52 ng/mL in sham-operated rats compared to pregnenolone levels in sham-operated rats treated with vehicle (0.52±0.11 ng/mL), unpaired *t*-test, $p<0.0001$, $t=6.057$, $df=14$, $n=8$ per condition. (d) Mean serum pregnenolone levels in adrenalectomized (ADX) animals treated with clozapine 20 mg/kg IP (0.36±0.05 ng/mL) do not differ significantly from levels in ADX animals treated with vehicle (0.48±0.22 ng/mL), unpaired *t*-test, $p=0.594$, $t=0.545$, $df=14$, $n=8$ per condition.

experiment. The limit of NS detection with this method was 2 pg.

2.6. Statistics

Analyses of clozapine experiments were conducted utilizing two-tailed *t*-tests. Associations between hippocampal pregnenolone levels and peripheral serum pregnenolone levels were evaluated with the Pearson correlation coefficient. Analyses of experiments utilizing other second generation agents (aripiprazole, olanzapine, quetiapine, or ziprasidone) were conducted by one-way five-group ANOVA with post-hoc Dunnett tests comparing mean pregnenolone levels following each antipsychotic with the mean of the vehicle control group. Data are expressed as the mean ± standard error of the mean (S.E.M.).

3. Results

3.1. Clozapine 20 mg/kg vs. vehicle control: clozapine produces marked increases in hippocampal, serum, and cerebral cortical pregnenolone levels

Clozapine (20 mg/kg, IP) administration increases hippocampal pregnenolone levels 13-fold to 35.50 ± 6.56 ng/g compared to hippocampal pregnenolone levels following vehicle administration (2.70 ± 0.66 ng/g), unpaired *t*-test, $p<0.0001$, $t=4.97$, $df=18$, $n=10$ per condition, Fig. 1a. Clozapine 20 mg/kg IP elevates peripheral serum pregnenolone levels 34-fold to 8.82 ± 1.82 ng/mL compared to serum pregnenolone levels following vehicle administration (0.26 ± 0.04 ng/mL), unpaired *t*-test, $p=0.0002$, $t=4.71$, $df=18$, $n=10$ per condition, Fig. 1b. Hippocampal pregnenolone levels and serum pregnenolone levels are strongly correlated, Pearson correlation coefficient $r=0.987$, $p<0.0001$; $n=20$ *xy* pairs, Fig. 1c. Peripheral serum pregnenolone levels may thus reflect pregnenolone levels in brain, potentially providing an accessible surrogate marker for central levels of this neuroactive steroid. Clozapine 20 mg/kg IP administration increases cerebral cortical pregnenolone levels to 26-fold to 77.58 ± 12.80 ng/g ($n=9$) compared to cerebral cortical pregnenolone levels following vehicle administration (2.91 ± 0.28 ng/g, $n=6$), unpaired *t*-test, $p=0.0004$, $t=4.70$, $df=13$, Fig. 1d.

3.2. Clozapine 20 mg/kg vs. vehicle control—adrenalectomized (ADX) vs. sham-operated: adrenalectomy prevents clozapine-induced increases in pregnenolone in hippocampus and serum

Clozapine 20 mg/kg IP administration increases hippocampal pregnenolone levels 13-fold to 42.89 ± 8.33 ng/g in sham-operated rats compared to pregnenolone levels in sham-operated rats treated with vehicle (3.30 ± 0.81 ng/g), unpaired *t*-test, $p=0.0003$, $t=4.728$, $df=14$, $n=8$ per condition, Fig. 2a. Adrenalectomy completely prevents clozapine-induced increases in hippocampal pregnenolone. Mean hippocampal pregnenolone levels in adrenalectomized (ADX) animals treated with clozapine 20 mg/kg IP (1.21 ± 0.08 ng/g) do not differ significantly from levels in ADX animals treated with vehicle (1.33 ± 0.10 ng/g), unpaired *t*-test, $p=0.359$, $t=0.948$, $df=14$, $n=8$ per condition, Fig. 2b. Clozapine 20 mg/kg IP administration increases serum pregnenolone levels over 18-fold to 9.73 ± 1.52 ng/mL in sham-operated rats compared to pregnenolone levels in sham-operated rats treated with vehicle (0.52 ± 0.11 ng/mL), unpaired *t*-test, $p<0.0001$, $t=6.057$, $df=14$, $n=8$ per condition, Fig. 2c. Adrenalectomy completely prevents clozapine-induced increases in serum pregnenolone. Mean serum pregnenolone levels in adrenalectomized (ADX) animals treated with clozapine 20 mg/kg IP (0.36 ± 0.05 ng/mL) do not differ significantly from levels in ADX animals treated with vehicle (0.48 ± 0.22 ng/mL), unpaired *t*-test, $p=0.594$, $t=0.545$, $df=14$, $n=8$ per condition, Fig. 2d.

3.3. Other second generation antipsychotics vs. vehicle control: olanzapine 5 mg/kg increases hippocampal and cerebral cortical pregnenolone, but to a lesser degree than clozapine 20 mg/kg

Olanzapine 5 mg/kg significantly increases mean hippocampal pregnenolone levels to 13.44 ± 2.60 ng/g compared to mean

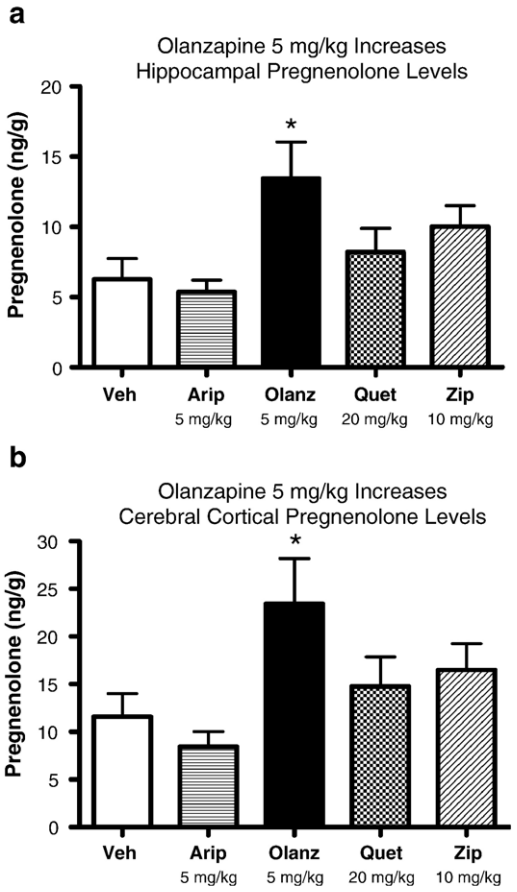

Fig. 3. (a) Olanzapine 5 mg/kg significantly increases mean hippocampal pregnenolone levels to 13.44 ± 2.60 ng/g compared to mean hippocampal pregnenolone levels following vehicle administration (6.28 ± 1.46 ng/g), one-way five-group ANOVA $p=0.015$, $F=3.514$, $df=4,40$; post-hoc Dunnett $p<0.05$ for olanzapine 5 mg/kg condition, $n=9$ per condition. (b) Olanzapine 5 mg/kg significantly increases mean cerebral cortical pregnenolone levels to 23.42 ± 4.76 ng/g compared to mean cerebral cortical pregnenolone levels following vehicle administration (11.58 ± 2.44 ng/g), one-way five-group ANOVA $p=0.020$, $F=3.296$, $df=4,40$; post-hoc Dunnett $p<0.05$ for olanzapine 5 mg/kg condition, $n=9$ per condition.

hippocampal pregnenolone levels following vehicle administration (6.28±1.46 ng/g), one-way five-group ANOVA $p=0.015$, $F=3.514$, $df=4,40$; post-hoc Dunnett $p<0.05$ for olanzapine 5 mg/kg condition, $n=9$ per condition, Fig. 3a. Of note, this elevation in hippocampal pregnenolone levels following olanzapine 5 mg/kg replicates our prior findings in which mean hippocampal pregnenolone levels increased to 12.67±2.03 ng/g following the administration of this olanzapine dose (Marx et al., 2006-this issue). Although quetiapine 20 mg/kg and ziprasidone 10 mg/kg increase mean hippocampal pregnenolone levels to 8.20±1.69 ng/g and 10.02±1.49 ng/g, respectively, these elevations did not achieve statistical significance (post-hoc Dunnett tests $p>0.05$ for both quetiapine and ziprasidone conditions, $n=9$ per condition). Mean pregnenolone levels are unaltered following aripiprazole 5 mg/kg (5.37±0.85 ng/g), post-hoc Dunnett test $p>0.05$, $n=9$.

Olanzapine 5 mg/kg significantly increases mean cerebral cortical pregnenolone levels to 23.42±4.76 ng/g compared to mean cerebral cortical pregnenolone levels following vehicle administration (11.58±2.44 ng/g), one-way five-group ANOVA $p=0.020$, $F=3.296$, $df=4,40$; post-hoc Dunnett $p<0.05$ for olanzapine 5 mg/kg condition, $n=9$ per condition, Fig. 3b. Although quetiapine 20 mg/kg and ziprasidone 10 mg/kg increase mean cerebral cortical pregnenolone levels to 14.75±3.10 ng/g and 16.48±2.80 ng/g, respectively, these elevations do not achieve statistical significance (post-hoc Dunnett tests $p>0.05$ for both quetiapine and ziprasidone conditions, $n=9$ per condition). Mean pregnenolone levels are somewhat lower following aripiprazole 5 mg/kg (8.41±1.60 ng/g), but this decrease did not achieve statistical significance (post-hoc Dunnett test $p>0.05$, $n=9$).

4. Conclusions

Clozapine administration produces the most pronounced elevations in hippocampal and cerebral cortical pregnenolone levels among the second generation antipsychotics tested in these investigations. Antipsychotic agents were studied at doses producing D_2 receptor occupancies consistent with clinically comparable ranges, and also consistent with effective doses utilized in numerous rodent models of psychosis. Individual findings and potential clinical ramifications are discussed below, as well as study limitations and future directions.

4.1. Clozapine markedly increases hippocampal, cerebral cortical, and serum pregnenolone

Clozapine increases hippocampal, cerebral cortical, and serum pregnenolone levels 13-fold, 26-fold, and 34-fold, respectively. Clozapine-induced elevations in hippocampus and cerebral cortex are considerably more robust compared to changes in this NS following other second generation agents tested in this study. Although clearly a very preliminary hypothesis that will require extensive additional testing in clinical populations, it is possible that marked pregnenolone induction following clozapine may contribute to mechanisms mediating its superior clinical efficacy. For example, clozapine-induced elevations in pregnenolone in hippocampus, cerebral cortex, and peripheral serum may be relevant to depressive symptoms and suicidality in patients with schizophrenia. Specifically, decreased cerebrospinal fluid pregnenolone levels have been associated with depressive symptoms (George et al., 1994), and therefore clozapine-induced increases in this NS could potentially impact this symptom domain. Furthermore, a 2-year prospective study of 980 patients with schizophrenia or schizoaffective disorder at high risk for suicide demonstrated that clozapine reduces suicide attempts (Meltzer et al., 2003). Clozapine is approved by the Food and Drug Administration in the United States for the treatment of suicidal behaviors in patients with schizophrenia or schizoaffective disorder, and a recent meta-analysis determined that long-term treatment with clozapine was associated with a three-fold decreased risk for suicidal behaviors (Hennen and Baldessarini, 2005). Potentially relevant to the current investigation, we recently determined that pregnenolone levels in parietal cortex and posterior cingulate were higher in patients with schizophrenia and bipolar disorder compared to control subjects utilizing postmortem brain tissue from the Stanley Foundation (Marx et al., 2006). Within the schizophrenia group, however, pregnenolone levels in parietal cortex tissue specimens are decreased in patients with schizophrenia who died by suicide compared to patients with schizophrenia who died by other causes (unpublished data). Caution should be utilized in the interpretation of these initial findings, however, given the small number of specimens analyzed in this postmortem tissue investigation. Clozapine-induced elevations in pregnenolone may thus represent a candidate mechanism contributing to its clinical actions with regard to suicidal behaviors and depressive symptoms in patients with schizophrenia or schizoaffective disorder. This possibility is clearly very preliminary, however, and extensive additional data will be required to test this hypothesis.

Marked elevations in hippocampal, cerebral cortical, and serum pregnenolone levels following clozapine administration could also impact the neuronal cytoskeleton, since pregnenolone stabilizes microtubules (Hsu et al., 2006), stimulates microtubule polymerization (Fontaine-Lenoir et al., 2006; Murakami et al., 2000), and enhances neuritic outgrowth (Fontaine-Lenoir et al., 2006). Furthermore, a recent report suggests that antipsychotics may upregulate mRNA coding for microtubule-associated protein 2 (MacDonald et al., 2005). In addition, it has been proposed that the neuronal cytoskeleton may represent a potential therapeutic target in schizophrenia (Benitez-King et al., 2004). Pregnenolone induction may thus be relevant to this dimension of schizophrenia pathophysiology.

Clozapine-induced elevations in central nervous system pregnenolone levels could potentially lead to increased concentrations of its sulfated derivative (pregnenolone sulfate), a positive modulator of glutamatergic NMDA receptors (Wu et al., 1991; Irwin et al., 1994; Bowlby, 1993). We did not determine pregnenolone sulfate levels in the current investigation, however, and therefore this possibility remains very speculative. Potentially supporting this hypothesis, however, pregnenolone administration in humans has been demonstrated to increase pregnenolone sulfate levels by several-fold in serum

(Morley et al., 1997). Clozapine could thus theoretically modulate the glutamatergic neurotransmitter system indirectly via pregnenolone induction, potentially leading to elevated pregnenolone sulfate levels. Although clozapine does not demonstrate direct agonist activity at NMDA receptors (K_i > 10,000 nM, suggesting very low clozapine affinity, PDSP *Ki* Database; Schoemaker et al., 1997), clozapine does appear to demonstrate indirect effects involving this neurotransmitter system. For example, clozapine reverses behaviors induced by the NMDA receptor antagonist phencyclidine (PCP) in monkeys (Linn et al., 2003), prevents neurotoxicity following the NMDA receptor antagonist MK-801 (Farber et al., 1996), and prevents alterations in brain metabolism induced by the NMDA receptor antagonist ketamine (Duncan et al., 1998, 2000). Olanzapine also prevents brain metabolism alterations following ketamine, but higher doses are required to produce this effect compared to clozapine (Duncan et al., 2000). It is therefore possible that clozapine and olanzapine may interact with the NMDA neurotransmitter system by inducing elevations in pregnenolone resulting in downstream increases in its sulfated derivative. Since we did not test pregnenolone sulfate levels directly in the current investigation, however, this hypothesis remains to be tested.

In addition to actions at glutamatergic NMDA receptors, pregnenolone sulfate also appears to modulate the cholinergic neurotransmitter system. For example, pregnenolone sulfate administration increases acetylcholine release in rat hippocampus (Vallee et al., 1997; Darnaudery et al., 2002) and frontal cortex (Darnaudery et al., 1998). Pregnenolone sulfate infused into the nucleus basalis also increases acetylcholine release in the frontal cortex and amygdala (Pallares et al., 1998). Pregnenolone sulfate reverses the retention deficit induced by the anticholinergic drug scopolamine (Vallee et al., 2001). Similar to pregnenolone sulfate, clozapine also increases acetylcholine release in rodent hippocampus, and it has been hypothesized that this clozapine effect contributes to its positive actions on cognition in patients with schizophrenia (Shirazi-Southall et al., 2002). It is currently not known, however, if clozapine also elevates pregnenolone sulfate in rodent brain, and future experiments will be required to test this possibility.

Along these lines, it should be noted that the presence of pregnenolone sulfate in rodent and human brain is the subject of ongoing inquiry. Data are currently very limited, but it is possible that elevations in peripheral serum and central nervous system pregnenolone may produce increases in pregnenolone sulfate levels in these tissues. In rodents, for example, intravenous pregnenolone administration appears to increase pregnenolone sulfate levels in multiple brain regions (Wang et al., 1997). In humans, a single oral dose of pregnenolone 175 mg results in several-fold increases in serum pregnenolone sulfate concentrations (Morley et al., 1997). Arguing against this possibility, however, are recent reports suggesting that prior assessments of pregnenolone sulfate levels in brain may have been confounded by methodological challenges (Liere et al., 2004). Recent investigations did not find pregnenolone sulfate to be present in adult male rat brain utilizing methods with limits of detection of 0.3 ng/g tissue (Liu et al., 2003) and 0.05 ng/g tissue, uncorrected for procedural losses (Ebner et al., 2006). Another study reported pregnenolone sulfate levels as 0.05–0.43 ng/g (Higashi et al., 2003). It is possible, however, that pregnenolone sulfate may be present at considerably higher concentrations in human brain compared to rat brain. An investigation in postmortem brain tissue from subjects with Alzheimer's disease and control subjects supports this possibility, reporting pregnenolone sulfate levels in the 1–8 ng/g range utilizing a mass spectrometry-based method (Weill-Engerer et al., 2002). An earlier radioimmunoassay study in human postmortem brain also detected pregnenolone sulfate at similar concentrations (Lanthier and Patwardhan, 1986). Further research will be required to definitively clarify this issue.

4.2. Adrenalectomy prevents clozapine-induced elevations in hippocampal and serum pregnenolone

To determine if clozapine-induced pregnenolone elevations require intact adrenal function, we also tested sham-operated vs. adrenalectomized animals following clozapine or vehicle administration. It has been demonstrated previously that adrenalectomy prevents clozapine-induced increases in rat cerebral cortical allopregnanolone levels (Marx et al., 2003; Barbaccia et al., 2001). In the current investigation, we demonstrate that adrenalectomy completely prevents clozapine-induced increases in pregnenolone in both hippocampus and serum. Pronounced elevations in hippocampal and serum pregnenolone levels thus appear to depend upon intact adrenal function, and pregnenolone synthesized in the adrenal apparently provides a critical contribution to markedly enhanced pregnenolone levels in brain following treatment with clozapine. Potential clozapine actions at the adrenal peripheral benzodiazepine receptor may thus merit further investigation. Since intravenous pregnenolone administration in rats results in elevated pregnenolone and pregnenolone sulfate levels in the brain (Wang et al., 1997), our findings in adrenalectomized animals may not be inconsistent with prior efforts.

4.3. Hippocampal pregnenolone levels are correlated with peripheral serum levels

It is currently unknown if brain NS levels are correlated with serum NS levels in humans, and the compartmentalization of these molecules has been very challenging to establish (Baulieu et al., 2001). Rat hippocampal pregnenolone levels were strongly correlated with peripheral serum pregnenolone levels in this investigation (r = 0.987), and it is possible that this relationship may also be present in humans. Should this be determined to be the case, serum pregnenolone levels may constitute a reliable surrogate marker for brain pregnenolone concentrations. In addition, hippocampal pregnenolone levels in male rats were approximately 10-fold higher than peripheral serum levels in vehicle-treated intact rats in this investigation. This 10-fold difference between central and peripheral levels closely resembles our recent findings in control human postmortem brain tissue, in which pregnenolone levels in both posterior cingulate and parietal cortex were determined to be

over 10-fold higher than typical serum pregnenolone levels observed in males, follicular phase females, or post-menopausal females (Marx et al., 2006). Future efforts will be required to determine the precise relationships between central and peripheral steroids, but is possible that NS levels in clinically accessible tissues such as serum may be directly related to NS brain concentrations in certain circumstances.

4.4. Olanzapine also increases hippocampal and cerebral cortical pregnenolone

Olanzapine 5 mg/kg also increases hippocampal and cerebral cortical pregnenolone levels, but to a lesser degree than clozapine at doses leading to clinically comparable D_2 receptor occupancies. Nonetheless, elevations in pregnenolone following olanzapine are statistically significant in both hippocampus and cerebral cortex, and may potentially contribute to the clinical actions of this antipsychotic. Neuroactive steroid data in clinical populations receiving olanzapine will be required to test this hypothesis. Olanzapine-induced elevations in pregnenolone may also contribute to increases in the GABAergic NS allopregnanolone, a downstream metabolite (Marx et al., 2003, 2000). Since low pregnenolone levels have been associated with depressive symptoms (George et al., 1994), it is also possible that olanzapine-induced elevations in this NS may play a role in olanzapine's antidepressant activity.

4.5. Neuroactive steroids in rodent hippocampus and cerebral cortex following aripiprazole, quetiapine, or ziprasidone administration

Significant changes in pregnenolone levels in rat hippocampus or cerebral cortex are not observed following aripiprazole, quetiapine, or ziprasidone administration (although non-significant decreases in pregnenolone following aripiprazole and non-significant increases in pregnenolone following quetiapine and ziprasidone administration appear to be present in both brain regions). Since these agents may demonstrate efficacy for depressive symptoms (Kasper, 2004; Simon and Nemeroff, 2005; Barbee et al., 2004; Papakostas et al., 2004), it is possible that their potential antidepressant effects are mediated via mechanisms that are unrelated to neuroactive steroid induction. Alternatively, our study may be underpowered to detect neuroactive steroid changes of lesser magnitudes.

4.6. Limitations

Limitations of the current investigation include the absence of a conventional antipsychotic comparator. In two prior rat studies, however, the conventional antipsychotic haloperidol did not alter central allopregnanolone levels, and changes in progesterone levels were modest or non-existent at doses resulting in clinically comparable D_2 receptor occupancies (Marx et al., 2003; Barbaccia et al., 2001). Pregnenolone alterations following the administration of a conventional antipsychotic may therefore be unlikely, but this possibility remains to be examined experimentally. The use of a single dose for each second generation antipsychotic may also constitute a potential limitation. Future investigations would also benefit from the direct assessment of pregnenolone sulfate levels, since the sulfated pregnenolone derivative demonstrates positive modulatory actions at NMDA receptors and enhances acetylcholine release. Finally, limited statistical power may have precluded the detection of pregnenolone elevations (or reductions) following the administration of certain second generation agents. Specifically, the number of animals utilized in the majority of these experiments consisted of 8–10 animals per condition (with the exception of one vehicle condition which utilized 6 animals). It is therefore possible that these numbers may be too small to detect subtle differences following the administration of aripiprazole, quetiapine, or ziprasidone. Future research will be required to test these possibilities.

4.7. Summary

Clozapine markedly elevates pregnenolone levels in rat hippocampus, cerebral cortex, and serum. Given the magnitude of clozapine effects on pregnenolone levels in two brain regions and peripheral serum in these investigations, it is possible that induction of this NS may contribute to the therapeutic actions of this antipsychotic and to its distinct clinical advantage. Substantial additional data will be required to test this possibility. It is currently not clear if clozapine or olanzapine increase peripheral or central pregnenolone levels in patients with schizophrenia, or if pregnenolone levels are related to psychiatric symptomatology at baseline or following treatment with these agents. Should changes in pregnenolone following clozapine or olanzapine be demonstrated in humans, it is possible that peripheral serum pregnenolone levels could be utilized as a proxy or surrogate marker for central pregnenolone levels in brain. If pregnenolone induction proves relevant to the antipsychotic efficacy of clozapine or other second generation antipsychotics, it may be reasonable to target pregnenolone directly as an agent for pharmacological intervention.

Acknowledgements

We thank Gillian Parke for her excellent assistance in the preparation of this manuscript. [Veterans Affairs Advanced Research Career Development Award (CEM), K23 MH 65080 (CEM), MIRECC VISN 6 (PI Gregory McCarthy, PhD), Durham VA REAP (PI Roger Madison, PhD).]

References

Akwa Y, Ladurelle N, Covey DF, Baulieu EE. The synthetic enantiomer of pregnenolone sulfate is very active on memory in rats and mice, even more so than its physiological neurosteroid counterpart: distinct mechanisms? Proc Natl Acad Sci U S A 2001;98(24):14033–7.

Atkins JB, Chlan-Fourney J, Nye HE, Hiroi N, Carlezon Jr WA, Nestler EJ. Region-specific induction of deltaFosB by repeated administration of typical versus atypical antipsychotic drugs. Synapse 1999;33(2):118–28.

Azorin JM, Spiegel R, Remington G, Vanelle JM, Pere JJ, Giguere M, et al. A double-blind comparative study of clozapine and risperidone in the management of severe chronic schizophrenia. Am J Psychiatry 2001;158 (8):1305–13.

Barbaccia ML, Affricano D, Purdy RH, Maciocco E, Spiga F, Biggio G. Clozapine, but not haloperidol, increases brain concentrations of neuroactive steroids in the rat. Neuropsychopharmacology 2001;25(4):489–97.

Barbee JG, Conrad EJ, Jamhour NJ. Aripiprazole augmentation in treatment-resistant depression. Ann Clin Psychiatry 2004;16(4):189–94.

Barth VN, Chernet E, Martin LJ, Need AB, Rash KS, Morin M, et al. Comparison of rat dopamine D_2 receptor occupancy for a series of antipsychotic drugs measured using radiolabeled or nonlabeled raclopride tracer. Life Sci 2006;78(26):3007–12.

Baulieu EE, Robel P, Schumacher M. Neurosteroids: beginning of the story. Int Rev Neurobiol 2001;46:1-32.

Benitez-King G, Ramirez-Rodriguez G, Ortiz L, Meza I. The neuronal cytoskeleton as a potential therapeutical target in neurodegenerative diseases and schizophrenia. Curr Drug Targets CNS Neurol Disord 2004;3(6):515–33.

Bowlby MR. Pregnenolone sulfate potentiation of *N*-methyl-D-aspartate receptor channels in hippocampal neurons. Mol Pharmacol 1993;43(5):813–9.

Breier A, Buchanan RW, Kirkpatrick B, Davis OR, Irish D, Summerfelt A, et al. Effects of clozapine on positive and negative symptoms in outpatients with schizophrenia. Am J Psychiatry 1994;151(1):20–6.

Chakos M, Lieberman J, Hoffman E, Bradford D, Sheitman B. Effectiveness of second-generation antipsychotics in patients with treatment-resistant schizophrenia: a review and meta-analysis of randomized trials. Am J Psychiatry 2001;158(4):518–26.

Conley RR, Tamminga CA, Kelly DL, Richardson CM. Treatment-resistant schizophrenic patients respond to clozapine after olanzapine non-response. Biol Psychiatry 1999;46(1):73–7.

Darnaudery M, Koehl M, Pallares M, Le Moal M, Mayo W. The neurosteroid pregnenolone sulfate increases cortical acetylcholine release: a microdialysis study in freely moving rats. J Neurochem 1998;71(5):2018–22.

Darnaudery M, Pallares M, Piazza PV, Le Moal M, Mayo W. The neurosteroid pregnenolone sulfate infused into the medial septum nucleus increases hippocampal acetylcholine and spatial memory in rats. Brain Res 2002;951 (2):237–42.

Dollfus S, Olivier V, Chabot B, Deal C, Perrin E. Olanzapine versus risperidone in the treatment of post-psychotic depression in schizophrenic patients. Schizophr Res 2005;78(2–3):157–9.

Duncan GE, Leipzig JN, Mailman RB, Lieberman JA. Differential effects of clozapine and haloperidol on ketamine-induced brain metabolic activation. Brain Res 1998;812(1–2):65–75.

Duncan GE, Miyamoto S, Leipzig JN, Lieberman JA. Comparison of the effects of clozapine, risperidone, and olanzapine on ketamine-induced alterations in regional brain metabolism. J Pharmacol Exp Ther 2000;293(1):8-14.

Ebner MJ, Corol DI, Havlikova H, Honour JW, Fry JP. Identification of neuroactive steroids and their precursors and metabolites in adult male rat brain. Endocrinology 2006;147(1):179–90.

Farber NB, Foster J, Duhan NL, Olney JW. Olanzapine and fluperlapine mimic clozapine in preventing MK-801 neurotoxicity. Schizophr Res 1996;21 (1):33–7.

Farde L, Wiesel FA, Jansson P, Uppfeldt G, Wahlen A, Sedvall G. An open label trial of raclopride in acute schizophrenia. Confirmation of D_2-dopamine receptor occupancy by PET. Psychopharmacology (Berl) 1988;94(1):1–7.

Farde L, Nordstrom AL, Wiesel FA, Pauli S, Halldin C, Sedvall G. Positron emission tomographic analysis of central D_1 and D_2 dopamine receptor occupancy in patients treated with classical neuroleptics and clozapine. Relation to extrapyramidal side effects. Arch Gen Psychiatry 1992;49(7):538–44.

Flood JF, Morley JE, Roberts E. Memory-enhancing effects in male mice of pregnenolone and steroids metabolically derived from it. Proc Natl Acad Sci U S A 1992;89(5):1567–71.

Flood JF, Morley JE, Roberts E. Pregnenolone sulfate enhances post-training memory processes when injected in very low doses into limbic system structures: the amygdala is by far the most sensitive. Proc Natl Acad Sci U S A 1995;92(23):10806–10.

Fontaine-Lenoir V, Chambraud B, Fellous A, David S, Duchossoy Y, Baulieu EE, et al. Microtubule-associated protein 2 (MAP2) is a neurosteroid receptor. Proc Natl Acad Sci U S A 2006;103(12):4711–6.

George M, Guidotti A, Rubinow D, Pan B, Mikalauskas K, Post R. CSF neuroactive steroids in affective disorders: pregnenolone, progesterone, and DBI. Biol Psychiatry 1994;35:775–80.

Glantz LA, Lewis DA. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. Arch Gen Psychiatry 2000;57 (1):65–73.

Hennen J, Baldessarini RJ. Suicidal risk during treatment with clozapine: a meta-analysis. Schizophr Res 2005;73(2–3):139–45.

Higashi T, Daifu Y, Ikeshima T, Yagi T, Shimada K. Studies on neurosteroids: XV. Development of enzyme-linked immunosorbent assay for examining whether pregnenolone sulfate is a veritable neurosteroid. J Pharm Biomed Anal 2003;30(6):1907–17.

Hsu HJ, Liang MR, Chen CT, Chung BC. Pregnenolone stabilizes microtubules and promotes zebrafish embryonic cell movement. Nature 2006;439 (7075):480–3.

Ichikawa J, Dai J, O'Laughlin IA, Fowler WL, Meltzer HY. Atypical, but not typical, antipsychotic drugs increase cortical acetylcholine release without an effect in the nucleus accumbens or striatum. Neuropsychopharmacology 2002a;26(3):325–39.

Ichikawa J, Li Z, Dai J, Meltzer HY. Atypical antipsychotic drugs, quetiapine, iloperidone, and melperone, preferentially increase dopamine and acetylcholine release in rat medial prefrontal cortex: role of 5-HT1A receptor agonism. Brain Res 2002b;956(2):349–57.

Irwin RP, Lin SZ, Rogawski MA, Purdy RH, Paul SM. Steroid potentiation and inhibition of *N*-methyl-D-aspartate receptor-mediated intracellular Ca^{2+} responses: structure-activity studies. J Pharmacol Exp Ther 1994;271 (2):677–82.

Javitt DC. Glutamate as a therapeutic target in psychiatric disorders. Mol Psychiatry 2004;9(11):984–97.

Kane J, Honigfeld G, Singer J, Meltzer H. Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine. Arch Gen Psychiatry 1988;45(9):789–96.

Kane JM, Marder SR, Schooler NR, Wirshing WC, Umbricht D, Baker RW, et al. Clozapine and haloperidol in moderately refractory schizophrenia: a 6-month randomized and double-blind comparison. Arch Gen Psychiatry 2001;58 (10):965–72.

Kapur S, Zipursky RB, Remington G. Clinical and theoretical implications of 5-HT_2 and D_2 receptor occupancy of clozapine, risperidone, and olanzapine in schizophrenia. Am J Psychiatry 1999;156(2):286–93.

Kapur S, Zipursky R, Jones C, Remington G, Houle S. Relationship between dopamine D(2) occupancy, clinical response, and side effects: a double-blind PET study of first-episode schizophrenia. Am J Psychiatry 2000;157 (4):514–20.

Kapur S, VanderSpek SC, Brownlee BA, Nobrega JN. Antipsychotic dosing in preclinical models is often unrepresentative of the clinical condition: a suggested solution based on in vivo occupancy. J Pharmacol Exp Ther 2003;305(2):625–31.

Kasper S. Quetiapine is effective against anxiety and depressive symptoms in long-term treatment of patients with schizophrenia. Depress Anxiety 2004;20(1):44–7.

Kinkead B, Shahid S, Owens MJ, Nemeroff CB. Effects of acute and subchronic administration of typical and atypical antipsychotic drugs on the neurotensin system of the rat brain. J Pharmacol Exp Ther 2000;295 (1):67–73.

Lanthier A, Patwardhan VV. Sex steroids and 5-en-3 beta-hydroxysteroids in specific regions of the human brain and cranial nerves. J Steroid Biochem 1986;25(3):445–9.

Lewis S, Barnes T, Davies L, Murray R, Dunn G, Hayhurst K, et al. Randomized controlled trial of effect of prescription of clozapine versus other second-generation antipsychotic drugs in resistant schizophrenia. Schizophrenia Bulletin (in press).

Li Z, Ichikawa J, Dai J, Meltzer HY. Aripiprazole, a novel antipsychotic drug, preferentially increases dopamine release in the prefrontal cortex and hippocampus in rat brain. Eur J Pharmacol 2004;493(1–3):75–83.

Liere P, Pianos A, Eychenne B, Cambourg A, Liu S, Griffiths W, et al. Novel lipoidal derivatives of pregnenolone and dehydroepiandrosterone and absence of their sulfated counterparts in rodent brain. J Lipid Res 2004;45 (12):2287–302.

Linn GS, Negi SS, Gerum SV, Javitt DC. Reversal of phencyclidine-induced prepulse inhibition deficits by clozapine in monkeys. Psychopharmacology (Berl) 2003;169(3–4):234–9.

Liu S, Sjovall J, Griffiths WJ. Neurosteroids in rat brain: extraction, isolation, and analysis by nanoscale liquid chromatography-electrospray mass spectrometry. Anal Chem 2003;75(21):5835–46.

MacDonald ML, Eaton ME, Dudman JT, Konradi C. Antipsychotic drugs elevate mRNA levels of presynaptic proteins in the frontal cortex of the rat. Biol Psychiatry 2005;57(9):1041–51.

Mamo D, Kapur S, Shammi CM, Papatheodorou G, Mann S, Therrien F, et al. A PET study of dopamine D_2 and serotonin 5-HT_2 receptor occupancy in patients with schizophrenia treated with therapeutic doses of ziprasidone. Am J Psychiatry 2004;161(5):818–25.

Marx CE, Duncan GE, Gilmore JH, Lieberman JA, Morrow AL. Olanzapine increases allopregnanolone in the rat cerebral cortex. Biol Psychiatry 2000;47(11):1000–4.

Marx CE, VanDoren MJ, Duncan GE, Lieberman JA, Morrow AL. Olanzapine and clozapine increase the GABAergic neuroactive steroid allopregnanolone in rodents. Neuropsychopharmacology 2003;28(1):1-13.

Marx CE, Stevens RD, Shampine LJ, Uzunova V, Trost WT, Butterfield MI, et al. Neuroactive steroids are altered in schizophrenia and bipolar disorder: relevance to pathophysiology and therapeutics. Neuropsychopharmacology 2006;31(6):1249–63.

Marx CE, Trost WT, Shampine LJ, Khisti R, Grobin AC, Lieberman JA, Morrow AL. Olanzapine and fluoxetine administration and coadministration increase rat hippocampal pregnenolone, allopregnanolone, and peripheral deoxycorticosterone: Implications for therapeutic actions. Pharmacology, Biochemistry, and Behavior 2006;84(4):608–16 (this issue) doi:10.1016/j.pbb.2006.07.032.

Mathis C, Paul SM, Crawley JN. The neurosteroid pregnenolone sulfate blocks NMDA antagonist-induced deficits in a passive avoidance memory task. Psychopharmacology (Berl) 1994;116(2):201–6.

Mathis C, Vogel E, Cagniard B, Criscuolo F, Ungerer A. The neurosteroid pregnenolone sulfate blocks deficits induced by a competitive NMDA antagonist in active avoidance and lever-press learning tasks in mice. Neuropharmacology 1996;35(8):1057–64.

Mayo W, George O, Darbra S, Bouyer JJ, Vallee M, Darnaudery M, et al. Individual differences in cognitive aging: implication of pregnenolone sulfate. Prog Neurobiol 2003;71(1):43–8.

McEvoy JP, Lieberman JA, Stroup TS, Davis SM, Meltzer HY, Rosenheck RA, et al. Effectiveness of clozapine versus olanzapine, quetiapine, and risperidone in patients with chronic schizophrenia who did not respond to prior atypical antipsychotic treatment. Am J Psychiatry 2006;163(4):600–10.

Meltzer HY, Alphs L, Green AI, Altamura AC, Anand R, Bertoldi A, et al. Clozapine treatment for suicidality in schizophrenia: International Suicide Prevention Trial (InterSePT). Arch Gen Psychiatry 2003;60(1):82–91.

Millan MJ. *N*-methyl-D-aspartate receptors as a target for improved antipsychotic agents: novel insights and clinical perspectives. Psychopharmacology 2005;179:30–53.

Modestin J, Dal Pian D, Agarwalla P. Clozapine diminishes suicidal behavior: a retrospective evaluation of clinical records. J Clin Psychiatry 2005;66(4):534–8.

Moller HJ. Antipsychotic and antidepressive effects of second generation antipsychotics: two different pharmacological mechanisms? Eur Arch Psychiatry Clin Neurosci 2005;255(3):190–201.

Morley JE, Kaiser F, Raum WJ, Perry III HM, Flood JF, Jensen J, et al. Potentially predictive and manipulable blood serum correlates of aging in the healthy human male: progressive decreases in bioavailable testosterone, dehydroepiandrosterone sulfate, and the ratio of insulin-like growth factor 1 to growth hormone. Proc Natl Acad Sci U S A 1997;94(14): 7537-7542.

Murakami K, Fellous A, Baulieu EE, Robel P. Pregnenolone binds to microtubule-associated protein 2 and stimulates microtubule assembly. Proc Natl Acad Sci U S A 2000;97(7):3579–84.

Natesan S, Reckless GE, Nobrega JN, Fletcher PJ, Kapur S. Dissociation between in vivo occupancy and functional antagonism of dopamine D(2) receptors: comparing aripiprazole to other antipsychotics in animal models. Neuropsychopharmacology 2006;31:1854–63.

Pallares M, Darnaudery M, Day J, Le Moal M, Mayo W. The neurosteroid pregnenolone sulfate infused into the nucleus basalis increases both acetylcholine release in the frontal cortex or amygdala and spatial memory. Neuroscience 1998;87(3):551–8.

Papakostas GI, Petersen TJ, Nierenberg AA, Murakami JL, Alpert JE, Rosenbaum JF, et al. Ziprasidone augmentation of selective serotonin reuptake inhibitors (SSRIs) for SSRI-resistant major depressive disorder. J Clin Psychiatry 2004;65(2):217–21.

PDSP K_i Database, Retrieved March 29, 2006 from PubChem Compound, PubChem Substance and NCI databases, http://kipd.cwru.edu/pdsp.php.

Pickar D, Owen RR, Litman RE, Konicki E, Gutierrez R, Rapaport MH. Clinical and biologic response to clozapine in patients with schizophrenia. Crossover comparison with fluphenazine. Arch Gen Psychiatry 1992;49 (5):345–53.

Schoemaker H, Claustre Y, Fage D, Rouquier L, Chergui K, Curet O, et al. Neurochemical characteristics of amisulpride, an atypical dopamine D_2/D_3 receptor antagonist with both presynaptic and limbic selectivity. J Pharmacol Exp Ther 1997;280(1):83–97.

Shirazi-Southall S, Rodriguez DE, Nomikos GG. Effects of typical and atypical antipsychotics and receptor selective compounds on acetylcholine efflux in the hippocampus of the rat. Neuropsychopharmacology 2002;26(5):583–94.

Simon JS, Nemeroff CB. Aripiprazole augmentation of antidepressants for the treatment of partially responding and nonresponding patients with major depressive disorder. J Clin Psychiatry 2005;66(10):1216–20.

Sliwinski A, Monnet FP, Schumacher M, Morin-Surun MP. Pregnenolone sulfate enhances long-term potentiation in CA1 in rat hippocampus slices through the modulation of *N*-methyl-D-aspartate receptors. J Neurosci Res 2004;78(5):691–701.

Tarazi FI, Zhang K, Baldessarini RJ. Long-term effects of olanzapine, risperidone, and quetiapine on dopamine receptor types in regions of rat brain: implications for antipsychotic drug treatment. J Pharmacol Exp Ther 2001;297(2):711–7.

Tauscher J, Hussain T, Agid O, Verhoeff NP, Wilson AA, Houle S, et al. Equivalent occupancy of dopamine D_1 and D_2 receptors with clozapine: differentiation from other atypical antipsychotics. Am J Psychiatry 2004;161 (9):1620–5.

Tollefson GD, Sanger TM, Beasley CM, Tran PV. A double-blind, controlled comparison of the novel antipsychotic olanzapine versus haloperidol or placebo on anxious and depressive symptoms accompanying schizophrenia. Biol Psychiatry 1998;43(11):803–10.

Tuunainen A, Wahlbeck K, Gilbody S. Newer atypical antipsychotic medication in comparison to clozapine: a systematic review of randomized trials. Schizophr Res 2002;56(1–2):1-10.

Uzunova V, Sheline Y, Davis JM, Rasmusson A, Uzunov DP, Costa E, et al. Increase in the cerebrospinal fluid content of neurosteroids in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine. Proc Natl Acad Sci U S A 1998;95(6):3239–44.

Vallee M, Mayo W, Darnaudery M, Corpechot C, Young J, Koehl M, et al. Neurosteroids: deficient cognitive performance in aged rats depends on low pregnenolone sulfate levels in the hippocampus. Proc Natl Acad Sci U S A 1997;94(26):14865–70.

Vallee M, Shen W, Heinrichs SC, Zorumski CF, Covey DF, Koob GF, et al. Steroid structure and pharmacological properties determine the anti-amnesic effects of pregnenolone sulphate in the passive avoidance task in rats. Eur J Neurosci 2001;14(12):2003–10.

Wahlbeck K, Cheine M, Essali A, Adams C. Evidence of clozapine's effectiveness in schizophrenia: a systematic review and meta-analysis of randomized trials. Am J Psychiatry 1999;156(7):990–9.

Wang MD, Wahlstrom G, Backstrom T. The regional brain distribution of the neurosteroids pregnenolone and pregnenolone sulfate following intravenous infusion. J Steroid Biochem Mol Biol 1997;62(4):299–306.

Weill-Engerer S, David JP, Sazdovitch V, Liere P, Eychenne B, Pianos A, et al. Neurosteroid quantification in human brain regions: comparison between Alzheimer's and nondemented patients. J Clin Endocrinol Metab 2002;87 (11):5138–43.

Wolff MC, Leander JD. Comparison of the effects of antipsychotics on a delayed radial maze task in the rat. Psychopharmacology (Berl) 2003;168(4):410–6.

Wu FS, Gibbs TT, Farb DH. Pregnenolone sulfate: a positive allosteric modulator at the *N*-methyl-D-aspartate receptor. Mol Pharmacol 1991;40 (3):333–6.

Xu H, Qing H, Lu W, Keegan D, Richardson JS, Chlan-Fourney J, et al. Quetiapine attenuates the immobilization stress-induced decrease of brain-

derived neurotrophic factor expression in rat hippocampus. Neurosci Lett 2002;321(1–2):65–8.

Yokoi F, Grunder G, Biziere K, Stephane M, Dogan AS, Dannals RF, et al. Dopamine D_2 and D_3 receptor occupancy in normal humans treated with the antipsychotic drug aripiprazole (OPC 14597): a study using positron emission tomography and [^{11}C]raclopride. Neuropsychopharmacology 2002;27(2):248–59.

Zipursky RB, Christensen BK, Daskalakis Z, Epstein I, Roy P, Furimsky I, et al. Treatment response to olanzapine and haloperidol and its association with dopamine D receptor occupancy in first-episode psychosis. Can J Psychiatry 2005;50(8):462–9.