

# Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis

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Contributed by Erminio Costa, July 31, 2006

Allopregnanolone (ALLO) and tetrahydrodeoxycorticosterone (THDOC) are potent positive allosteric modulators of GABA action at GABA<sub>A</sub> receptors. ALLO and THDOC are synthesized in the brain from progesterone or deoxycorticosterone, respectively, by the sequential action of two enzymes: 5 $\alpha$ -reductase (5 $\alpha$ -R) type I and 3 $\alpha$ -hydroxysteroid dehydrogenase (3 $\alpha$ -HSD). This study evaluates 5 $\alpha$ -R type I and 3 $\alpha$ -HSD mRNA expression level in mouse brain by using *in situ* hybridization combined with glutamic acid decarboxylase 67/65, vesicular glutamate transporter 2, glial fibrillary acidic protein, and S100 $\beta$  immunohistochemistry. We demonstrate that 5 $\alpha$ -R type I and 3 $\alpha$ -HSD colocalize in cortical, hippocampal, and olfactory bulb glutamatergic principal neurons and in some output neurons of the amygdala and thalamus. Neither 5 $\alpha$ -R type I nor 3 $\alpha$ -HSD mRNAs are expressed in S100 $\beta$ - or glial fibrillary acidic protein-positive glial cells. Using glutamic acid decarboxylase 67/65 antibodies to mark GABAergic neurons, we failed to detect 5 $\alpha$ -R type I and 3 $\alpha$ -HSD in cortical and hippocampal GABAergic interneurons. However, 5 $\alpha$ -R type I and 3 $\alpha$ -HSD are significantly expressed in principal GABAergic output neurons, such as striatal medium spiny, reticular thalamic nucleus, and cerebellar Purkinje neurons. A similar distribution and cellular location of neurosteroidogenic enzymes was observed in rat brain. Taken together, these data suggest that ALLO and THDOC, which can be synthesized in principal output neurons, modulate GABA action at GABA<sub>A</sub> receptors, either with an autocrine or a paracrine mechanism or by reaching GABA<sub>A</sub> receptor intracellular sites through lateral membrane diffusion.

3 $\alpha$ -hydroxysteroid dehydrogenase | 5 $\alpha$ -reductase (type I) | GABAergic neurons | glutamatergic neurons

The neurosteroids 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one [allopregnanolone (ALLO)] and 3 $\alpha$ ,21-dihydroxy-5 $\alpha$ -pregnan-20-one [tetrahydrodeoxycorticosterone (THDOC)] are potent positive allosteric modulators of GABA action at GABA<sub>A</sub> receptors (1–6). These neurosteroids can be synthesized in the brain from progesterone (7) or deoxycorticosterone (8, 9), respectively, by the sequential action of two enzymes, 5 $\alpha$ -reductase (5 $\alpha$ -R) type I and 3 $\alpha$ -hydroxysteroid dehydrogenase (3 $\alpha$ -HSD) (10).

Two types (I and II) of 5 $\alpha$ -Rs, which convert progesterone into 5 $\alpha$ -dihydroprogesterone (5 $\alpha$ -DHP) or convert deoxycorticosterone into 5 $\alpha$ -dihydrodeoxycorticosterone (5 $\alpha$ -DHDHC), have been identified in tissues of rodents and humans (11). Whereas 5 $\alpha$ -R type I and II are abundantly expressed in several peripheral tissues, 5 $\alpha$ -R type I is the most abundant 5 $\alpha$ -R molecular form detected in the adult brains of rats, mice, and humans (11–17). The human brain expresses four types of 3 $\alpha$ -HSD, which, under different optimal conditions, either catalyze the reduction of 5 $\alpha$ -DHP into ALLO or reverse this reaction (18). So far, only one 3 $\alpha$ -HSD isoform has been identified in the rat or mouse brain (19–22). The mRNA sequences of 5 $\alpha$ -R type I ( $\approx$ 88%) and 3 $\alpha$ -HSD ( $\approx$ 89%) are highly homologous in mouse (5 $\alpha$ -R type I GeneBank accession number (GBAN) NM.175283.3; 3 $\alpha$ -HSD, GBAN AY730283.1) and rats (5 $\alpha$ -R type I, GBAN NM.017070.3; 3 $\alpha$ -HSD, GBAN NM.138547.1).

In various mouse and rat brain regions, the rank order of 5 $\alpha$ -R type I and 3 $\alpha$ -HSD expression matches the nonuniform distribution of ALLO and 5 $\alpha$ -DHP (7, 14). A down-regulation of brain 5 $\alpha$ -DHP and ALLO can be induced by environmental factors (i.e., social isolation) (23–26) and may be mediated by a decrease of brain 5 $\alpha$ -R type I expression (14). Taken together, this information suggests that 5 $\alpha$ -R type I and 3 $\alpha$ -HSD may act in concert to control ALLO or THDOC and 5 $\alpha$ -DHP or 5 $\alpha$ -DHDHC biosynthesis.

Neuronal cultures from neonatal or embryonic rats synthesize ALLO and express 5 $\alpha$ -R type I (27–29). However, in the adult rat brain, 5 $\alpha$ -R type I immunoreactivity is expressed primarily by glial cells that also express S100 $\beta$  (30, 31). Hence, it is not clear whether 5 $\alpha$ -R type I is selectively expressed in the neurons of the adult rodent brain and whether it coexists with 3 $\alpha$ -HSD.

Because GABA<sub>A</sub> receptors are expressed in neuronal populations of several brain regions, it is important to establish whether ALLO, which positively modulates GABAergic signal transduction, is released from GABAergic axon terminals directly on GABA<sub>A</sub> receptors or if it reaches GABA<sub>A</sub> receptors by diffusion from contiguous synapses, perhaps via a local paracrine mechanism. This article deals with our attempts to clarify these issues by using mouse brain sections and *in situ* histochemistry technologies to compare the subcellular expression of 5 $\alpha$ -R type I and 3 $\alpha$ -HSD in various brain regions. We also have combined *in situ* antisense hybridization and immunohistochemistry labeling with specific markers to verify the brain-region distribution of neurons coexpressing 5 $\alpha$ -R type I and 3 $\alpha$ -HSD with confocal fluorescence microscopy.

## Results

**Cerebral Cortex.** 5 $\alpha$ -R type I (Fig. 1*a*) and 3 $\alpha$ -HSD (Fig. 1*b*) mRNAs are expressed in somatosensory (Fig. 1*aA* and *bA*) and piriform (Fig. 1*aF* and *bF*) cortices. In the somatosensory cortex, 5 $\alpha$ -R type I and 3 $\alpha$ -HSD mRNAs are primarily expressed in layer II, III, and V pyramidal neurons. The expression of these transcripts is below detection limits in layer I neurons, which are mostly GABAergic (32, 33). The hybridization signals for 5 $\alpha$ -R type I and 3 $\alpha$ -HSD are mostly similar, but there are some differences. For example, 5 $\alpha$ -R type I mRNA expression is primarily cytosolic (Fig. 1*aA*), whereas 3 $\alpha$ -HSD mRNA expression appears to be cytosolic and nuclear (Fig. 1*bA*).

In Fig. 2*A*, the confocal fluorescence images of 3 $\alpha$ -HSD

Author contributions: R.C.A.-B., G.P., E.C., and A.G. designed research; R.C.A.-B., A.Z., E.M., and M.V. performed research; R.C.A.-B. analyzed data; and R.C.A.-B., G.P., E.C., and A.G. wrote the paper.

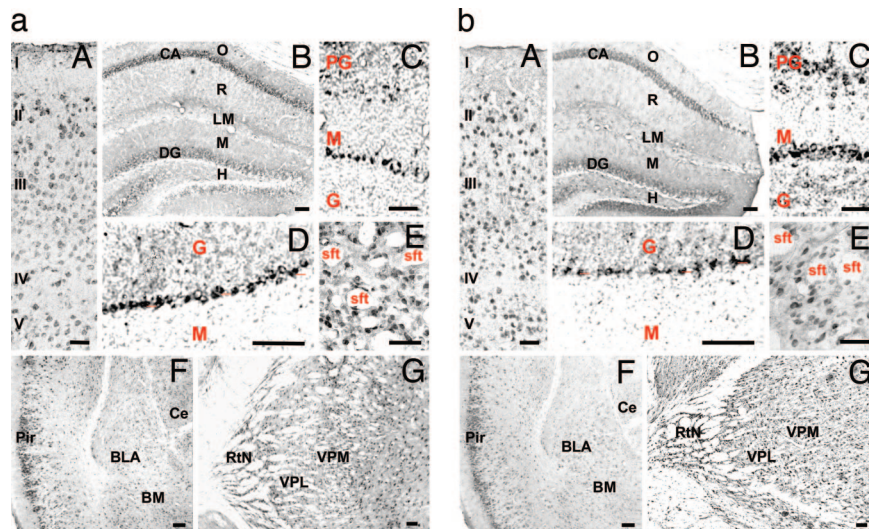
The authors declare no conflict of interest.

Abbreviations: 3 $\alpha$ -HSD, 3 $\alpha$ -hydroxysteroid dehydrogenase; 5 $\alpha$ -DHP, 5 $\alpha$ -dihydroprogesterone; 5 $\alpha$ -R, 5 $\alpha$ -reductase; ALLO, allopregnanolone; GAD, glutamic acid decarboxylase; GBAN, GenBank accession no.; RtN, reticular thalamic nucleus; THDOC, tetrahydrodeoxycorticosterone; VGLUT, vesicular glutamate transporter.

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**Fig. 1.** Mouse brain distribution of  $5\alpha$ -R type I (a) and  $3\alpha$ -HSD (b) mRNAs. (aA and bA) Somatosensory cortex, coronal section at bregma +1.10 mm (49). I to V identify the cortical layers. (Fig. 1 aB and bB) Hippocampus, coronal section at bregma -2.46 mm (49). CA, cornus ammonis; DG, dentate gyrus; O, stratum oriens; R, stratum radiatum; LM, layer lacunosum moleculare; M, molecular layer dentate gyrus; H, hilus. (aC and bC) Olfactory bulb, coronal section at bregma +3.92 mm (49). M, mitral; PG, periglomerular; G, granular cell layers. (aD and bD) Cerebellum. The Purkinje cell layer is indicated by arrowheads ( $\blacktriangle$ ). G, granule cell layer; M, molecular layer. (aE and bE) Striatal medium spiny neurons, coronal section as in A. Shown is unstained striatal fiber tract (stf). (aF and bF) Piriform cortex and amygdala, coronal section at bregma -1.58 mm (49). BLA, basolateral anterior amygdaloid nucleus; BM, basomedial amygdaloid nucleus; Ce, central amygdaloid nucleus; Pir, piriform cortex. (aG and bG) Thalamic nuclei, coronal section as in F. VPL, ventral posterolateral thalamic nucleus; VPM, ventral posteromedial thalamic nucleus. [Scale bars, 50  $\mu$ m (aA and bA) and 100  $\mu$ m (aB and bB-aG and bG).]

mRNA and  $5\alpha$ -R type I protein show that, very frequently (>95%), both enzymes are coexpressed in the same neurons.

Fig. 2B shows that vesicular glutamate transporter (VGLUT1) mRNA, a glutamatergic neuronal marker, and  $5\alpha$ -R type I protein colocalize. In contrast, we could not detect a colocalization of  $5\alpha$ -R type I or  $3\alpha$ -HSD with glutamic acid decarboxylase (GAD)67/65 (Fig. 2D), a marker for GABAergic neurons. Finally,  $5\alpha$ -R type I mRNA fails to colocalize with S100 $\beta$ , a specific glial cell marker (Table 1).

**Hippocampus.** The detection signals for  $5\alpha$ -R type I and  $3\alpha$ -HSD mRNAs are reported in Fig. 1 aB and bB, respectively. The hybridization signal of both expressed transcripts in CA1 and CA2 is very intense, but, in CA3, the signal is faint. The cells of dentate gyrus and hilus also show a less-intense staining than that detected in CA1 and CA2, especially in the case of  $5\alpha$ -R type I (Fig. 1 aB). The stratum oriens, stratum radiatum, and layer lacunosum moleculare, and the molecular layers of the dentate gyrus show faint hybridization signal. However, a few cells are labeled for  $5\alpha$ -R type I mRNA in the stratum radiatum and, very likely, these are interneurons. A coexistence of  $5\alpha$ -R type I or  $3\alpha$ -HSD mRNAs with GAD67/65 immunoreactivity was not detected (Table 1). In the hippocampus, as in the cortex, the  $5\alpha$ -R type I signal fails to colocalize with S100 $\beta$  (Fig. 3C). As in the cortex, in CA1 pyramidal neurons, the  $5\alpha$ -R type I *in situ* hybridization signal consistently colocalizes with the VGLUT2 immunostaining (Fig. 3A).

The VGLUT2 antibodies predominantly label glutamatergic neuron axon terminals (34). However, under the conditions of double *in situ* hybridization and immunohistochemistry shown in Fig. 3A, the cell bodies are also labeled. This labeling is eliminated when the VGLUT2 antiserum is preabsorbed with the immunizing peptide (Fig. 3B).

**Olfactory Bulb.** The olfactory bulb is characterized by the expression of intense  $5\alpha$ -R type I (Fig. 1 aC) and  $3\alpha$ -HSD (Fig. 1 bC) hybridization signals, primarily expressed in the mitral and periglomerular cells. The granule GABAergic neurons, express

faint staining for both  $5\alpha$ -R type I and  $3\alpha$ -HSD (Fig. 4A). In the mitral cell layer,  $5\alpha$ -R type I mRNA colocalizes with VGLUT1 mRNA (data not shown) or with VGLUT2 proteins (Fig. 4A). However, we failed to observe the colocalization of  $5\alpha$ -R type I mRNA with glial fibrillary acidic protein, a specific glial cell marker (Table 1).

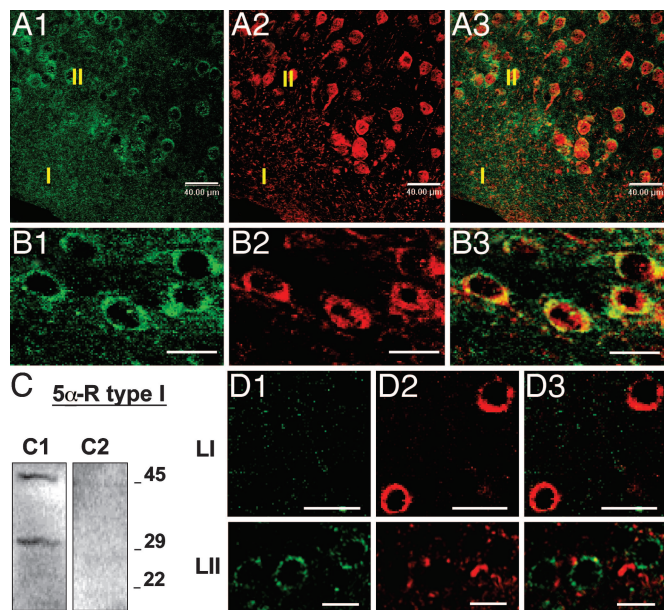
**Striatum.** The striatum includes numerous neurons that express strong  $5\alpha$ -R type I (Fig. 1 aE) and  $3\alpha$ -HSD (Fig. 1 bE) staining. Confocal fluorescence images show that  $5\alpha$ -R type I mRNA colocalizes with GAD67/65 in striatal neurons (Fig. 4B).

**Thalamus.** Sparse  $5\alpha$ -R type I (Fig. 1 aG) and  $3\alpha$ -HSD mRNA (Fig. 1 bG) positive neurons are expressed in various thalamic nuclei. An intense hybridization signal is detected in neurons of the reticular thalamic nucleus (RtN), where  $5\alpha$ -R type I is colocalized with GAD67/65 (Fig. 4C). In contrast, in the ventromedial thalamic nucleus,  $5\alpha$ -R type I colocalizes with VGLUT1 mRNA and VGLUT2 proteins (Table 1).

**Amygdala.** The central and basolateral amygdaloid nuclei are characterized by sparse neurons exhibiting a faint staining for either  $5\alpha$ -R type I (Fig. 1 aF) or  $3\alpha$ -HSD mRNAs (Fig. 1 bF). Strong staining is expressed in the basolateral anterior amygdaloid nucleus (BLA), whereas, in the central amygdaloid nucleus, staining is weak. The somata of glutamatergic principal neuron expressed in the BLA show a colocalization of  $5\alpha$ -R type I mRNA with VGLUT2 (Table 1).

**Cerebellum.** The staining of  $5\alpha$ -R type I and  $3\alpha$ -HSD mRNA is selectively localized in the somata of Purkinje neurons (Fig. 1 aD and bD). A fainter staining is also present in the granule cell layer but virtually absent in the molecular layer. As expected,  $5\alpha$ -R type I mRNA is colocalized with GAD67/65 proteins in Purkinje cells (Table 1).

**Overview of  $5\alpha$ -R Type I and  $3\alpha$ -HSD mRNA Expression in the Mouse Brain.** Table 1 shows that, in the mouse brain,  $5\alpha$ -R type I and  $3\alpha$ -HSD are expressed in principal output neurons, for in-



**Fig. 2.**  $5\alpha$ -R type I and  $3\alpha$ -HSD colocalize in glutamatergic (pyramidal) neurons but not in GABAergic interneurons of somatosensory cortex. (A)  $5\alpha$ -R type I protein colocalizes with  $3\alpha$ -HSD mRNA in somatosensory cortical layer II.  $3\alpha$ -HSD mRNA, green. (A2)  $5\alpha$ -R type I protein, red. (A3) Merge of A1 and A2. Note that  $3\alpha$ -HSD mRNA and  $5\alpha$ -R type I protein cellular staining is absent in cortical layer I. (B)  $5\alpha$ -R type I colocalizes with VGLUT1 mRNA. (B1) VGLUT1 mRNA, green. (B2)  $5\alpha$ -R type I protein, red. (B3) Merge of B1 and B2. (C)  $5\alpha$ -R type I antiserum specificity. Western blot of mouse brain extract incubated with  $5\alpha$ -R type I antibody (C1), and  $5\alpha$ -R type I antibody preabsorbed with the  $5\alpha$ -R type I immunizing antigen (see *Materials and Methods*). Molecular markers are indicated at the right. (D) The  $3\alpha$ -HSD mRNA is not expressed with GAD67/65 in somatosensory layer I (LI) and layer II (LII) cortical interneurons. (D1)  $3\alpha$ -HSD mRNA, green. (D2) GAD67/65 protein, red. (D3) Merge of D1 and D2. [Scale bars, 40  $\mu$ m (A) and 20  $\mu$ m (B–D).]

stance, by the glutamatergic principal output neurons in the cortex, hippocampus, olfactory bulb, thalamus, and amygdala and also by GABAergic principal output neurons from the striatum, RtN, and cerebellum. However,  $5\alpha$ -R type I and  $3\alpha$ -HSD have not been detected in cortical and hippocampal GABAergic interneurons. The expression of  $5\alpha$ -R type I and  $3\alpha$ -HSD could not be detected in S100 $\beta$ - or glial fibrillary acidic protein-positive cells in various brain areas, including the corpus callosum.

## Discussion

**Brain Regional and Cellular Distribution of Enzymes Regulating Neurosteroid Biosynthesis.** The expression of  $5\alpha$ -R type I and  $3\alpha$ -HSD transcripts was greater in the mitral cell layer of the olfactory bulb than in layer II, III, or V of the neocortex or CA1–3 and dentate gyrus of the hippocampus. In these structures, the transcript expression was stronger than in the striatum, thalamus, amygdala, or cerebellum. This brain-region distribution of  $5\alpha$ -R type I and  $3\alpha$ -HSD is consistent with that of the distribution of their respective mRNAs quantified with RT-PCR in homogenates of various mouse brain regions (14).

The identification of cell types expressing  $5\alpha$ -R type I and  $3\alpha$ -HSD was carried out by using specific neuronal or glial markers, such as S100 $\beta$  and glial fibrillary acidic protein (for glial cells), VGLUT1 and VGLUT2 (for glutamatergic neurons), and GAD67/65 (for GABAergic neurons).

Regardless of the brain region studied,  $5\alpha$ -R type I and  $3\alpha$ -HSD appear to colocalize in the same neuronal population. A systematic cellular mapping of the transcripts for these two specific neurosteroidogenic enzymes indicates that  $5\alpha$ -R type I

**Table 1.** Expression intensity of  $5\alpha$ -R type I and  $3\alpha$ -HSD mRNAs

Brain areas	$5\alpha$ -R type I mRNA	$3\alpha$ -HSD mRNA	S100 $\beta$ /GFAP proteins
<b>Cortex</b>			
Glutamatergic (pyramidal) neurons	+++	+++	
GABAergic interneurons	–	–	
Glial cells	–	–	+++
<b>Hippocampus</b>			
Glutamatergic (pyramidal) neurons (CA1–3)	+++	+++	
Glutamatergic dentate gyrus granular cells	++	+++	
Hilus cells*	+/-	++	
GABAergic interneurons	–	–	
Glial cells	–	–	+++
<b>Olfactory bulb</b>			
Glutamatergic mitral cells	+++	+++	
Periglomerular cells*	++	++	
GABAergic granule cells	+/-	+/-	
Glial cells	–	–	+++
<b>Striatum</b>			
GABAergic (medium spiny) neurons	++	++	
<b>Thalamus</b>			
Glutamatergic dorsomedial nuclei neurons	+++	+++	
GABAergic reticular thalamic nuclei neurons	++	++	
<b>Amygdala</b>			
Glutamatergic basolateral nucleus neurons	++	++	
<b>Cerebellum</b>			
Glutamatergic granular neurons	+/-	+/-	
GABAergic Purkinje neurons	+++	+++	
<b>Corpus callosum</b>			
Glial cells	–	–	+++

The degree of immunostaining intensity is indicated by (+++), (++) , (+/-), and (–).

\*The neurotransmitters expressed by these cells were not characterized.

and  $3\alpha$ -HSD expression occurs in neurons but not in glial cells. In neurons, this expression is independent of the chemical structure of the neurotransmitter phenotype (glutamatergic or GABAergic). The neurosteroidogenic enzymes are highly expressed in “principal” output neurons (i.e., glutamatergic pyramidal, GABAergic reticulothalamic, striatal, and Purkinje neurons) and are virtually absent in GABAergic interneurons expressed in both the telencephalon or hippocampus (Table 1). The only interneurons that express weak staining for both  $5\alpha$ -R type I and  $3\alpha$ -HSD are the GABAergic granule cells of the olfactory bulb and the glutamatergic granular neurons of the cerebellum (Table 1).

The evidence that the  $5\alpha$ -R type I mRNA and protein and the  $3\alpha$ -HSD mRNA are highly expressed in neurons of the adult mouse brain confirms the Melcangi *et al.* (27, 28) finding on the expression of  $5\alpha$ -R type I activity in primary cultures of neurons obtained from various brain regions of rat embryos. Our studies, however, differ from the preceding immunohistochemical studies conducted with  $5\alpha$ -R type I antibodies (30, 31, 35) in rat brains. In fact, using a specific  $5\alpha$ -R type I antibody (see Fig. 2C for specificity) we could not confirm, in mouse brain, the studies in rats (30, 31, 35) showing that  $5\alpha$ -R type I immunoreactivity is abundantly expressed in glial cells. In preliminary experiments, we observed that, in the rat brain, the distribution and cellular



the inactive 20 $\alpha$ -hydroxyprogesterone or converts ALLO into 20 $\alpha$ -hydroxyallopregnanolone (40). Cortical and hippocampal neurons also express 3 $\beta$ -HSD, the enzyme that converts pregnanolone into progesterone (41). Thus, glutamatergic cortical and hippocampal neurons and, also, very likely, the olfactory mitral neurons, may synthesize both progesterone and 5 $\alpha$ -DHP, which may be involved in the regulation of intracellular progesterone-receptor function.

In this context, it is noteworthy to mention that neurosteroids have been reported to (i) influence prefrontal cortex structure and thalamocortical connectivity (42), (ii) increase cerebellar granule-cell neurogenesis (43), (iii) increase the proliferation of neuroprogenitor cells expressed in the rat hippocampus (44), and (iv) increase the proliferation of human embryonic neural stem cells expressed in the cerebral cortex (44). It is probable that a receptor mechanism different from a direct modulation of GABA<sub>A</sub> receptor signal transduction may be operative in these actions. We can speculate that the microtubular-associated protein 2 (MAP2) is a possible putative intracellular receptor for neurosteroids (i.e., ALLO) (45).

## Conclusion

A decrease of brain neurosteroid availability has been associated with psychiatric conditions, including anxiety, aggression, premenstrual dysphoria, and cognitive and mood disorders. Antidepressants (fluoxetine and other selective serotonin-reuptake inhibitors, "SSRIs") and antipsychotics (clozapine) may exert their beneficial effects, at least in part, by increasing the brain levels of neurosteroids (26, 46–48).

Although the neurosteroids that act as positive allosteric modulators of GABA action at GABA<sub>A</sub> receptors were initially considered endocrine messengers that are indiscriminately synthesized and secreted from glial cells in all brain regions, the present histochemical data suggest that there is a cellular and molecular basis to imply that neurosteroids are synthesized in neurons and act locally at GABA<sub>A</sub> receptors expressed on specific corticolimbic circuitries. Understanding the contribution of local brain neurosteroid biosynthesis to the pathogenesis of neurological and psychiatric disorders may become a stimulus to develop new psychoactive drugs that act selectively by normalizing neurosteroid action on GABAergic neurotransmission.

## Materials and Methods

**Animals and Tissue Preparation.** Adult male Swiss–Webster mice (Harlan, Indianapolis, IN), 25–30 g in body weight, were perfused with 0.9% NaCl and 4% paraformaldehyde. The brains were (i) postfixed for 72 h in 4% paraformaldehyde and (ii) embedded in 30% sucrose in 0.15 M PBS, pH 7.4, at 4°C. All experiments were conducted in groups of three to five animals. For each condition, (antisense probes or antibodies) four to six sections were processed for each animal. All animal procedures were approved by the University of Illinois Animal Care Committee.

**mRNA *in Situ* Hybridization. Antisense probe design.** To visualize 5 $\alpha$ -R type I mRNA, free-floating 16- to 20- $\mu$ m coronal sections (49) were incubated for 72 h at 42°C with a mixture of 50 pmol/ml of three antisense oligonucleotide probes: R1 (nt 910–933), R2 (nt 989–1,012), and R3 (nt 1180–1203) (GBAN NM.175283). To visualize 3 $\alpha$ -HSD mRNA, adjacent sections were incubated with two antisense oligonucleotide probes, H1 (nt 526–549) and H2 (nt 804–827) (GBAN AY730283.1). To visualize VGLUT1 mRNA, we used a hybridization technique with antisense oligonucleotide probes complementary to bases 626–649 (V1) and 1499–1522 (V2) of the mouse VGLUT1 cDNA (GBAN NM.182993). The oligonucleotide 3' terminals were labeled with digoxigenin by using the Oligonucleotide Digoxigenin Tailing kit (Roche Diagnostics, Indianapolis, IN). The *in situ* hybridization protocol followed a variation of the procedure described by Rodriguez *et al.* (32) for the avidin–biotin–

peroxidase complex (ABC; Vector Laboratories, Burlingame, CA) method and by Pesold *et al.* (50, 51) and Veldic *et al.* (52) for confocal immunofluorescence.

**Antisense probes: specificity tests.** The antisense probe sequences did not match any other known mRNA sequences, as determined by multiple genome-wide BLAST comparisons. However, because genomic sequences that have not been reported as part of the intron/exon structures of transcribed genes can be transcribed (53), we performed separate *in situ* hybridization studies with the three 5 $\alpha$ -R type I antisense probes, the two 3 $\alpha$ -HSD antisense probes, and the two VGLUT1 antisense probes to establish specificity. In coronal prefrontal cortex slices, the distribution of neurons stained with the 5 $\alpha$ -R type I probe R1 is virtually identical to the distribution of neurons stained with probes R2 and R3. The distribution of neurons stained with the 3 $\alpha$ -HSD probe H1 is also virtually identical to the distribution of neurons detected with H2. The same virtually identical distribution was also found with the VGLUT1 probes V1 and V2. Oligoprobe specificity was tested by using digoxigenin-labeled scramble oligonucleotides for 5 $\alpha$ -R type I, 3 $\alpha$ -HSD, and VGLUT1. As expected, specific neuronal staining was not detected.

**Double *in Situ* Hybridization and Immunohistochemistry.** Double *in situ* hybridization and immunohistochemistry were performed by following a variation of the procedure described by Pesold *et al.* (50, 51) and Veldic *et al.* (52). After the *in situ* hybridization procedure was terminated, the following antibodies were used: (i) rabbit anti-GAD67/65 (diluted 1:2,000; Chemicon, Temecula, CA), (ii) rabbit anti-VGLUT2 (diluted 1:500; Synaptic Systems, Göttingen, Germany), (iii) rabbit anti-GFAP (diluted 1:250; Chemicon), (iv) rabbit anti-S-100 $\beta$  (diluted 1:5,000; Swant, Bellinzona, Switzerland), and (v) rabbit anti-rat 5 $\alpha$ -R type I (diluted 1:100; Acris Antibodies, Hiddenhausen, Germany).

After the double *in situ* hybridization and immunohistochemistry procedures, the slices were incubated with Cy5-labeled goat anti-rabbit IgG (diluted 1:1,000; Amersham Biosciences, Piscataway, NJ) or Cy5-labeled goat anti-guinea pig IgG (diluted 1:1,000; Abcam, Cambridge, MA) to produce red fluorescent staining or Cy2-labeled streptavidin (diluted 1:1,000; Amersham Biosciences) to produce green fluorescent staining, as indicated in the figure legends. The number of cells in which green and red fluorescence colocalize compared with the number of cells that express only green or only red fluorescence was quantified with confocal microscopy (Leica, Bannockburn, IL) at a magnification of  $\times 40$  in a counting box of 100  $\times$  100  $\times$  20  $\mu$ m.

**Western Blot Analysis to Assess 5 $\alpha$ -R Type I and VGLUT2 Antibody Specificity.** Mouse brain extracts (10 mg per 200  $\mu$ l of SDS loading buffer) were electrophorized on 10–20% SDS/PAGE gel and blotted onto a nitrocellulose membrane (Amersham Biosciences). For the study of 5 $\alpha$ -R type I antiserum specificity, the membranes were incubated with either anti-5 $\alpha$ -R type I (diluted 1:2,500; Acris Antibodies) or anti-5 $\alpha$ -R type I (diluted 1:2,500) preabsorbed for 12 h at 4°C with a 1 mM solution of the immunizing peptide (V-V-F-A-L-F-T-L-S-T-L-T-R-A-K-Q-H-H-Q-W-Y) in 0.005 M NaPHO<sub>4</sub> buffer (pH 7.2), 0.2 M NaCl, and 5% BSA. Two immunoreactive bands, one of  $\approx 45$  kDa and one of  $\approx 29$  kDa, are recognized by the nonpreabsorbed antiserum (Fig. 2C1). The immunoreactive bands disappear when the preabsorbed antiserum is used (Fig. 2C2). The size of 5 $\alpha$ -R type I in mice is  $\approx 29$  kDa, according to the GenBank accession no. AAH94503; however, as indicated by Russell and Wilson (11), the hydrophobic amino acid content of 5 $\alpha$ -R type I ( $\approx 37\%$ ) may explain the aberrant electrophoretic mobilities in SDD/PAGE that have been reported for the 5 $\alpha$ -R isoenzymes. The preabsorbed antiserum failed to immunoreact with mouse brain slices.

The membranes were also incubated with anti-VGLUT2 antiserum (diluted 1:4,000; Synaptic Systems) or anti-VGLUT2 anti-

serum (diluted 1:4,000) preabsorbed for 12 h at 4°C with 20  $\mu$ l of solution, 1 mg per 1 ml of the immunizing control peptide (amino acids 510–582 of rat VGLUT2/DNPI; Synaptic Systems) in blotting buffer (3% nonfat dry milk/0.1% Tween-20). Only one immunoreactive band of 65 kDa is recognized by the nonpreabsorbed antiserum (Fig. 3B1). This band completely disappears in the preabsorbed antiserum (Fig. 3B2). The preabsorbed antiserum failed to immunoreact with mouse brain slices.

**Digital Photomicrography.** DAB (3,3'-diaminobenzidine tetrahydrochloride) (Sigma, St. Louis, MO) staining images were

captured by Axiovision 3.1 (Zeiss) and confocal immunofluorescence by a confocal microscope (Leica Microsystems, Bannockburn, IL). The final composites were processed by using Photoshop (Adobe Systems, Mountain View, CA) and Powerpoint (Microsoft, Redmond, WA).

We thank Dr. A. L. Morrow (University of North Carolina, Chapel Hill, NC) and Dr. H. Möhler (University of Zurich, Switzerland) for the constructive criticism and suggestions in the preparation of the manuscript. This work was supported by National Institute of Mental Health Grant R01-M4 56890 (to A.G.).

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