Gas Chromatographic-Mass Fragmentographic Quantitation of 3 α -Hydroxy-5 α -pregnan-20-one (Allopregnanolone) and Its Precursors in Blood and Brain of Adrenalectomized and Castrated Rats

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Coupling high performance liquid chromatography with gas chromatography-mass fragmentography has made it possible to simultaneously measure subpicomolar concentrations of allopregnanolone and its precursors, pregnenolone, progesterone and 5 α -dihydroprogesterone (5 α -DHP) in various brain areas. Allopregnanolone was measured in the brain of adrenalectomized/castrated (ADX/CX) rats in nanomolar concentrations long after peripheral sources of allopregnanolone were removed. A partial decrease (approximately 30%) in the content of allopregnanolone was found in the brains of ADX/CX rats compared to sham-operated rats. Moreover, the content of allopregnanolone in brains of sham-operated as well as ADX/CX rats was non-uniformly distributed (olfactory bulb > striatum > cortex > hippocampus) and was one to two orders of magnitude higher than in plasma or liver. Infusion of pregnenolone sulfate in ADX/CX rats elicited a fourfold increase in 5 α -DHP and progesterone content and a seven- to eightfold increase in the content of allopregnanolone in brain but not in liver or plasma. Furthermore, the content of allopregnanolone in brain increased to the same extent in both sham-operated and ADX/CX rats following pregnenolone sulfate infusion. The 5 α -reductase inhibitor, (17 β)-17[[bis(1-methylethyl)amino]carbonyl] androstane-3,5-diene-3-carboxylic acid (SKF 105111), reduced the brain content of allopregnanolone and blocked the increased formation of allopregnanolone in brain following pregnenolone sulfate infusion. The results clearly demonstrate that the synthesis of allopregnanolone from 5 α -DHP and progesterone occurs in the brain and that a significant amount of allopregnanolone is synthesized locally in brain from its precursors. These experiments suggest that the brain, like adrenals and gonads, is a steroidogenic organ which produces allopregnanolone as one of its own most important physiologically relevant steroids.

[Key words: gas chromatography, mass fragmentography, allopregnanolone, progesterone, pregnenolone, 5 α -dihydroprogesterone, adrenalectomy, castration, neurosteroids]

The CNS has the capacity to synthesize steroids (neurosteroids) *de novo* from cholesterol (Baulieu and Robel, 1990; Guarnieri et al., 1992; Romeo et al., 1993). Among the neurosteroids, 5 α -pregnan-3 α -ol-20-one (allopregnanolone), which is synthesized in brain from 5 α -pregnane-3,20-dione (5 α -dihydroprogesterone, 5 α -DHP) by the action of 3 α -hydroxysteroid oxidoreductase (3 α -HSOR) (Karavolas and Hodges, 1991), is pharmacologically the most potent and specific modulator of neuronal activity. Allopregnanolone, in nanomolar concentrations, either increases the strength of ionotropic GABAergic synaptic transmission (Majewska et al., 1988; Mienville and Vicini, 1989; Paul and Purdy, 1992; Puia et al., 1992, 1993) or is oxidized to 5 α -DHP which can then alter gene expression via its interaction with progesterone receptors (Rupprecht et al., 1993).

These discoveries have opened an exciting new field of research on the regulation of neurosteroid synthesis in the central nervous system and have emphasized the urgent need for sensitive, selective and structure specific assays to measure the tissue concentrations of various steroids including the neurosteroids. Consequently, during the past few years numerous highly sensitive assays utilizing radioimmunoassay (RIA) have been developed (Corpechot et al., 1983; Jo et al., 1989; Purdy et al., 1990; Corpechot et al., 1993). Because of the large number of endogenous steroids that are structurally similar and because of the insufficient selectivity and specificity of the antibodies that are available for use in these assays, the RIA methods, even when combined with high performance liquid chromatography (HPLC), do not offer a reliable criterion to measure neurosteroid content with sufficient specificity for structural identification (Siekman, 1979; Corpechot et al., 1993). Consequently, literature values for the content of neurosteroids in the brain vary widely (Rohle and Breuer, 1978; Wood et al., 1985; Thienpont et al., 1991) and extensive sample purification steps with inherent product loss are necessary to avoid quantification errors due to cross reactivity or to the presence in tissue extracts of contaminating substances which interfere with the RIA (Purdy et al., 1990, 1991; Morfin et al., 1992; Corpechot et al., 1993). Korneyev et al. (1993) found it necessary to pass samples extracted from brain tissue through three different HPLC columns in order to eliminate endogenous substances which interfered with the RIA tissue analysis of pregnenolone.

There has been recent interest in the rapid and long lasting neuromodulatory actions of allopregnanolone (Corpechot et al., 1993). The entire pathway of allopregnanolone biosynthesis in brain including pregnenolone conversion to progesterone via the

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action of 3β -hydroxysteroid dehydrogenase and progesterone conversion to 5α -DHP via the action of 5α -reductase (Baulieu and Robel, 1990; Purdy et al., 1990; Karavolas and Hodges, 1991; Corpechot et al., 1993) has become an interesting subject of studies to establish the physiological relevance of allopregnenolone. Moreover, there is a need to identify drugs that can increase brain availability of this neurosteroid. These considerations prompted us to develop methodologies which are able to measure levels of neurosteroids, and their precursors unambiguously.

This goal was achieved by coupling HPLC with gas chromatography-mass fragmentography (GC-MF). The steroids may be identified, first, by their HPLC retention times, second by their GC retention times and, third, by their unique fragmentation spectra following derivatization with heptafluorobutyric anhydride (HFBA) or methoxyamine HCl. For structural identification the pattern of current intensities generated by specific ions can be compared with the pattern generated by the specific ions of an appropriate internal standard. This hierarchical approach per se yields a high degree of specificity, which is enhanced in the Selected Ion Monitoring (SIM) mode that allows for the focussing on one or a few specific ions bearing structural information. This procedure optimizes the specificity of the assay and also reduces the chemical noise of the analysis which increases its sensitivity.

Using this method we report the regional brain distribution and plasma and liver content of allopregnenolone and its parent compounds following intravenous administration of pregnenolone sulfate in adrenalectomized (ADX) and castrated (CX) rats. These studies aim to respond to the fundamental question: can allopregnenolone be synthesized in brain in quantities sufficient to modulate neuronal activity?

Materials and Methods

Animals. Male, sham operated and ADX/CX Sprague-Dawley rats (220–240 gm body weight) were supplied by Zivic-Miller (Zelionople, PA) and housed in groups of four to five with 12/12 hr dark/light cycle from 7:00 A.M. to 7:00 P.M. and were provided with food and physiologic saline ad libitum. Fifteen to 21 d after adrenalectomy and castration rats were restrained in animal holders and infused with either vehicle (dimethyl sulfoxide (DMSO), 4.5%, a solution of 15% β -hydroxycyclodextrin, 22.4%; saline, 73.1%) or pregnenolone sulfate dissolved in the vehicle (48 μ mol/kg; 0.34 ml/min; 5 min) and sacrificed by decapitation 20 min after stopping the infusion. Blood was collected in heparinized tubes and plasma samples were obtained by centrifugation at $2000 \times g$ for 15 min at 4°C . The entire brain was quickly removed and, when appropriate, brain parts were dissected and tissues were frozen on dry ice and stored at -20°C . All animal procedures were in strict accordance with the NIH guide for The Care and Use of Laboratory Animals and were approved by the Animal Care Committee.

Reagents. Progesterone, pregnenolone, allopregnenolone and 5α -DHP were purchased from Steraloids (Wilton, NH). ^3H -Pregnenolone, ^3H -progesterone, and ^3H -allopregnenolone were obtained from New England Nuclear, Boston, MA. HFBA and methoxyamine HCl were purchased from Pierce, Rockford, IL. SKF-105111 ((17 β)-17[bis(1-methylethyl)amino]carbonyl] androstane-3,5-diene-3-carboxylic acid) was a gift from Smith Kline Beecham Pharmaceuticals, King of Prussia, PA. All solvents used were HPLC grade. Tritiated- 5α -DHP was enzymatically synthesized from ^3H -allopregnenolone in C6-2B cells from which the ^3H - 5α -DHP was extracted and purified using HPLC. Pregnenolone sulfate (Sigma, St. Louis, MO) was chromatographically pure and did not contain detectable amounts of allopregnenolone (<0.6 pmol/ml), progesterone (<0.3 pmol/ml), or pregnenolone (<0.3 pmol/ml) when the solution of pregnenolone sulfate to be infused (12 μ mol/ml) into the animals was analyzed by HPLC/GC-MF.

Preparation of samples. In preparation to measure the steroids, rat plasma, liver, and brains were homogenized in 5 volumes of 0.4N formic acid using a Brinkman polytron; approximately 9000 CPM (~ 0.05

Figure 1. HPLC separation of ^3H - 5α -DHP, ^3H -progesterone, ^3H -allopregnenolone, and ^3H -pregnenolone.

pmol each) of ^3H -pregnenolone, ^3H -progesterone, ^3H - 5α -DHP, and ^3H -allopregnenolone were added to the homogenate to monitor recovery and to identify which fractions contained the steroids of interest. The homogenates were extracted with 20 ml of ethyl acetate and twice with 15 ml of ethyl acetate. The supernatants were collected, pooled, and lyophilized in preparation for HPLC.

HPLC separation of allopregnenolone, 5α -DHP, progesterone, and pregnenolone. Tissue extracts (up to 2 gm of brain, 3 gm of liver or 3 ml of plasma) were resuspended in hexane (1 ml). They were filtered through 13 mm diameter Gelman PTFE filters (0.2 μ m pore size) and were injected onto the HPLC. HPLC separation of the steroids was carried out using a 10 μ m Lichrosorb 100 Diol, 4×250 mm column (EM Science, Gibbston, NJ). The column was equilibrated with hexane at a flow rate of 1 ml/min and was developed with a tetrahydrofuran (THF) gradient using a linear increase to 8.0% in 15 min, a second linear increase to 13% in 30 min, and a final linear increase to 100% in 15 min. After running the gradient the column was washed with methanol at a flow rate of 1 ml/min for 30 min in order to remove contaminants that elute from the column. Fractions eluting from the HPLC were collected at 1 min intervals and the radioactivity was counted to determine the retention time of the individual steroids. Tritiated- 5α -DHP, ^3H -progesterone, ^3H -allopregnenolone, and ^3H -pregnenolone were eluted with retention times of 19–21 min, 28–30 min, 37–39 min, and 42–44 min, respectively (Fig. 1). Consistently, the recovery was between 87% and 93%.

GC-MF separation and analysis. To the HPLC fractions containing progesterone, 15 pmol of allopregnenolone was added as an internal standard; to the fractions containing allopregnenolone 7.5 pmol of progesterone were added as an internal standard; and to the fractions containing pregnenolone, 15 pmol of allopregnenolone were added as an internal standard. These fractions were evaporated to dryness under a stream of nitrogen, and the residues were dissolved in 100 μ l of acetone. Twenty-five microliters of HFBA were added as a derivatizing reagent and the samples were allowed to stand at room temperature for 1 hr (Björkhem et al., 1975). The reaction mixture was evaporated to dryness under a stream of nitrogen and the HFBA-derivatives were dissolved in 10–20 μ l hexane. To the HPLC containing 5α -DHP, 15 pmol of progesterone was added as an internal standard, 50 μ l of methoxyamine HCl (2%) in pyridine and the samples was reacted for 1 hr at 65°C . The derivatizing reagents were evaporated to dryness under a stream of nitrogen. The residue was taken up in 1 ml chloroform:hexane (1:1) and passed through a short column (~ 3 cm) of Sephadex LH-20. The

column was washed with 1 ml chloroform:hexane (1:1). The void volume (2 ml) containing the methoxime (MO)-derivatives was evaporated to dryness and the MO-derivatives were dissolved in 10–20 μ l hexane.

HFBA- and MO-derivatives of the steroids were analyzed by GC-MF using an HP 5971 Mass Selective detector coupled to an HP 5890A gas chromatograph. The gas chromatograph was equipped with a Hewlett Packard capillary column (HP-5 M.S.; length, 30 m; i.d., 0.25 mm; film thickness, 0.25 μ) Helium was used as the carrier gas. The temperatures of the transfer line and the ion source were 280°C and 180°C, respectively, and the temperature of the injection port was 250°C. Two to three microliters of the hexane containing the HFBA- or MO-derivatives were injected on the column equilibrated at low temperature (50°C) at a pressure of 50 psi in order to concentrate the sample and reduce the peak width. In order to concentrate the sample further, the pressure was increased to 70 psi for 1 min. After 1 min the gas pressure was programmed to maintain a 10 ml/min flow rate while the temperature was programmed to increase at a rate of 30°C/min until a temperature of 230°C was reached. A column temperature of 230°C was maintained during the remainder of the injection cycle.

Statistical analysis. All results are mean $\pm$ SEM. Data were subjected to Student's *t* test or an analysis of variance followed by Neuman-Keuls test.

Results

Identification and quantitation of allopregnanolone, 5 α -DHP, progesterone, and pregnenolone in brain

Mass spectrometric identification of neurosteroids. Samples containing tissue extracts (after HPLC purification and separation) or known quantities of allopregnanolone, 5 α -DHP, progesterone, and pregnenolone were reacted with HFBA or methoxyamine HCl in pyridine. HFBA formed 3-enol-hepta-fluorobutyric ester derivatives of allopregnanolone, progesterone, and pregnenolone (Fig. 2), but did not react with 5 α -DHP. Methoxyamine HCl formed 3, 20-methoxime derivatives of progesterone and 5 α -DHP (Fig. 3). Following derivatization and ionization of the neurosteroids in the mass spectrometer, positive ions with masses between m/z 100 and m/z 550 were recorded. The mass spectra of the neurosteroids were virtually identical in both standards and brain extracts (see Fig. 2). HFBA-progesterone had only one major ion, the molecular ion m/z 510. The relative abundance of the other ion fragments was almost negligible. The molecular ion (m/z 514) was not present in the spectrum for HFBA-pregnenolone, but the base peak, m/z 298, was of high intensity. In contrast, the mass spectrum of HFBA-allopregnanolone was complex in that there were a number of intense ion fragments which reduced the sensitivity. The molecular ion was observed at m/z 514 and the base peak was observed at m/z 496 (Fig. 2). The mass spectrum of MO-5 α -DHP showed a molecular ion at m/z 374 and the base peak at m/z 343 (Table 1).

Steroids with ketone groups at the position 3 were expected to form two geometric isomers of the syn and anti type upon formation of the oxime derivatives (Koshy et al., 1975). When the keto steroid progesterone was reacted with methoxyamine HCl two peaks were observed (Fig. 3) which may correspond to the syn and anti isomers. Two peaks were also observed for MO-5 α -DHP which presumably correspond to the syn and anti isomers, but only one peak was observed for its stereoisomer, 5 β -DHP (Fig. 3). By varying the temperature and pressure in the gas chromatographic column, a base line separation for the two peaks of MO-5 α -DHP and of MO-progesterone could be obtained; however, it was not possible to resolve MO-5 β -DHP into two peaks using the 30 m HP-5 M.S. capillary column. The fragmentation patterns of MO-5 β -DHP and for peaks A and B of MO-5 α -DHP were identical. The four major ion fragments were m/z 275, 288, 343 (base peak) and 374 (molecular ion)

(Table 1) and had the same relative abundance. Five- β -DHP was not observed in rat brain extracts. Calculations indicate that if this steroid were present in brain its quantity would be less than 1.6 pmol.

The neurosteroid identification was optimized further by using the mass spectrometer in the SIM mode. In this mode the derivatized steroids were identified by their GC retention time (GC-RT) and the ratio between two specific mass/charge (m/z) fragments (Table 2). A ratio differing by more than 20% indicates the possible interference by a contaminating ion fragment. Once the analyzing conditions were established, in a manner that excluded interference from unwanted ion fragments, it was possible to increase the detection sensitivity for routine analyses by focusing on only one specific m/z value for each derivatized steroid: pregnenolone, 298 m/z ; progesterone, 510 m/z ; 5 α -DHP, 343 m/z ; and allopregnanolone, 496 m/z .

Quantitation of neurosteroids. Following separation of the steroids by HPLC, fractions containing the individual steroids, as identified by trace amounts of radioactivity added to the extracts and the internal standards (see Materials and Methods), were combined. The samples were evaporated to dryness, derivatized and analyzed by GC-MF. For each tissue sample the area under the steroid peak containing an unknown quantity of steroid was divided by the area under the internal standard peak. With this ratio the content of the steroid was read from the external standard curve. The external standard curve was prepared by adding the internal standards as indicated above to varying amounts of the individual steroids. The area under the peak of a known quantity of added steroid was divided by the area under the peak of internal standard. This ratio was plotted against the quantity of added steroid. A standard curve of allopregnanolone is shown in Figure 4 prepared from data of several experiments.

Precision of the assay procedures was determined in tissue samples injected in quadruplicate. In this way the variability between different samples of neurosteroids could be accounted for in multiple assays of the same sample. The GC-MF values obtained in the absence of added steroids were subtracted from the values obtained in the presence of added steroids and the data were analyzed by a one-way ANOVA. The results of the analysis of variance are shown in Table 3. As expected the intraassay variation is lower for all four steroids than the interassay variation as indicated by the smaller values for the mean squares. Moreover, the ratio of the variances (*F* value) indicates that the variation between means of the different samples did not reach the level of significance at $p < 0.5$ (Table 3).

Accuracy was expressed as the calculated concentration divided by the actual concentration expressed as a percentage. The calculated concentration approximated the actual concentration with a difference of less than 2% for every steroid tested. The sensitivity was determined by the limit of detection wherein the mass-spectrometer recognized a peak at the appropriate retention time and the peak yielded a signal to noise ratio of at least three (Table 3). The limits of detection were lowest for progesterone and pregnenolone (about 0.32 pmol) which had simple mass fragmentation patterns and greatest for 5 α -DHP (1.6 pmol) which displayed dual peaks. Allopregnanolone had a detection limit of approximately 0.63 pmol (see also Fig. 4).

Allopregnanolone, 5 α -DHP, and progesterone in brain, plasma, and liver of normal and ADX/CX rats

Measurement of allopregnanolone in brains of normal (sham-operated) rats indicated that its content was approximately 50-

Figure 2. Mass spectra of the HFBA-derivatives of allopregnanolone, pregnenolone, and progesterone from brain and standards.

fold higher than plasma and more than 50-fold higher than liver. In fact, plasma and liver had barely detectable levels of allopregnanolone (Fig. 5). The brain content of 5α -DHP, the precursor of allopregnanolone, was comparable to that of allopregnanolone and was five- to sixfold higher in brain than in plasma. 5α -DHP was virtually nondetectable in liver (Fig. 5).

Brain and plasma content of 5α -DHP and progesterone (the precursor of 5α -DHP) decreased markedly (70 and 80%, respectively) when adrenals and gonads were removed (Fig. 5). In contrast, allopregnanolone content decreased only partially (30%) in brain and remained low but virtually unchanged in plasma and liver in spite of the fact that adrenalectomy and

castration were performed at least 15 d before the measurement (Fig. 5).

Allopregnanolone, pregnenolone, progesterone, and 5α -DHP in brains of ADX/CX rats infused with pregnenolone sulfate

To further demonstrate that in ADX/CX rats the persistent, high content of brain allopregnanolone is derived from a brain biosynthetic pathway, ADX/CX rats were infused with pregnenolone sulfate. Pregnenolone sulfate was used because it is a more soluble precursor than pregnenolone for intravenous studies. Twenty min following infusion of pregnenolone sulfate (48 μ mol/kg; 0.34 ml/min; 5 min) in ADX/CX rats the content of

Figure 3. Gas chromatographic separation of MO-derivatives of 5 β -DHP, 5 α -DHP, and progesterone. MO- β -DHP is seen as a single peak. MO-5 α -DHP and MO-progesterone are both seen as double peaks which are designated in the text as MO 5 α DHP (peaks A and B) and MO-progesterone (peaks A and B) which presumably correspond to the syn and anti isomers formed during derivatization with methoxyamine HCl.

pregnenolone in whole brain (540 ± 73 pmol/gm brain) increased 47-fold (Table 4). In the same animals the brain content of allopregnanolone increased by seven- to eightfold, whereas the brain content of allopregnanolone in sham-operated rats increased by four- to fivefold (Fig. 6). Thus, following pregnenolone sulfate infusion the brain content of allopregnanolone was considerably elevated and was similar in brains of sham-operated and ADX/CX rats (Fig. 6). Infusion of pregnenolone sulfate also caused a significant increase in brain content of 5 α -DHP and progesterone (Table 4). The level of progesterone (25 ± 5.1 pmol/gm) and 5 α -DHP (3.8 ± 1.1 pmol/gm) following infusion of pregnenolone sulfate in ADX/CX rats reached concentrations similar to those found in brain of sham-operated rats (progesterone, 37 ± 7.6 pmol/gm; 5 α -DHP, 3.8 ± 0.8 pmol/gm) (compare Table 4 with Fig. 5). In contrast, in ADX/CX rats infused with pregnenolone sulfate, the plasma content of allopregnanolone (Fig. 6) and 5 α -DHP (0.37 ± 0.22 , $n = 5$, ADX/CX rats; 0.22 ± 0.046 , $n = 5$, ADX/CX rats infused with pregnenolone sulfate) failed to increase, although the plasma content of progesterone increased from 0.57 ± 0.21 ($n = 5$) in ADX/CX rats to 3.0 ± 1.1 pmol/ml ($n = 5$) in ADX/CX rats infused with pregnenolone sulfate. In liver, the content of allopregnanolone, 5 α -DHP, and progesterone in ADX/CX rats treated with pregnenolone sulfate did not increase and remained virtually undetectable. These data strongly suggest that following pregnenolone sulfate infusion the large increase of allopregnanolone and its immediate precursor, 5 α -DHP in brain are not due to their biosynthesis in peripheral tissues.

Allopregnanolone content in various brain regions

A strong argument in support of local brain biosynthetic pathways for allopregnanolone would be to demonstrate that allopregnanolone is distributed nonuniformly in brain. Figure 7 shows that in ADX/CX rats allopregnanolone was not uniformly distributed in olfactory bulb, cortex, hippocampus, striatum and cerebellum. The olfactory bulb had the highest concentration of allopregnanolone (8.3 pmol/gm brain). The concentrations of allopregnanolone found in cortex, hippocampus, cerebellum and

Table 1. Relative abundance of the four major ion fragments of MO-5 β -DHP, MO-5 α -DHP (peak A), and MO-5 α -DHP (peak B)

Ion fragment (m/z)	MO-5 β -DHP (% of base peak)	MO-5 α -DHP (% of base peak)	
		Peak A	Peak B
374 (molecular ion)	7.4	7.4	11.0
343 (base peak)	100	100	100
288	78	78	83
275	54	48	53

striatum ranged from 2.2 to 4.6 pmol/gm brain. Following infusion with pregnenolone sulfate in ADX/CX rats the concentration of allopregnanolone increased four fold in olfactory bulb, sixfold in cortex, and approximately three fold in hippocampus, cerebellum, and striatum (Fig. 7). To rule out the possibility that the uneven distribution of brain allopregnanolone may be the result of a nonuniform brain uptake of pregnenolone, progesterone, 5 α -DHP or allopregnanolone, 3 H-pregnenolone, 3 H-progesterone, 3 H-5 α -DHP, and 3 H-allopregnanolone (2 μ Ci/20 pmol/1.8 ml saline) were infused intravenously in rats via the tail vein. The radioactivity associated with authentic pregnenolone, progesterone, 5 α -DHP or allopregnanolone was determined in each of the brain areas after extraction with ethyl acetate and HPLC separation. All of the steroids tested produced a uniform distribution throughout the various areas of the brain.

Effect of the 5 α -reductase inhibitor SKF-105111 on allopregnanolone brain content

To determine whether 5 α -reductase, the enzyme that catalyses the conversion of progesterone to 5 α -DHP, is responsible for allopregnanolone biosynthesis, the allopregnanolone content in selected brain areas of ADX/CX rats was determined 30 min following the injection of the type 1 and type 2 5 α -reductase inhibitor, SKF-105111 (Levy et al., 1989, 1990; Mark Levy, personal communication), with and without pregnenolone sulfate infusion. In previous experiments in rats we demonstrated that SKF-105111, in doses of 11.7 μ mol/kg was capable of preventing the anti-amnesic effect of pregnenolone sulfate in a behavioral paradigm after dizocilpine treatment (Romeo et al., 1994). Following administration of this dose of SKF-105111, the content of allopregnanolone decreased significantly in olfactory bulb, cortex, hippocampus, and striatum. Moreover, the increased allopregnanolone content elicited by pregnenolone sulfate was virtually abolished in the several brain areas studied (Fig. 7).

Discussion

Because of the increased understanding of allopregnanolone in the modulation of neurotransmitter-gated ion channels (Baulieu

Table 2. GC-Retention time (GC-RT) and ratios for two *m/z* values for HFBA derivatives of allopregnenolone, progesterone, and pregnenolone

	HFBA-Allopregnenolone		HFBA-Progesterone		HFBA-Pregnenolone	
	GC-RT (min)	Ratio (<i>m/z</i> 496):(<i>m/z</i> 514)	GC-RT (min)	Ratio (<i>m/z</i> 510):(<i>m/z</i> 425)	GC-RT (min)	Ratio (<i>m/z</i> 298):(<i>m/z</i> 283)
Standards	18.4 ± 0.020	1.6 ± 0.011	21.5 ± 0.028	8.8 ± 0.73	24.9 ± 0.058	10.2 ± 0.27
Brain tissue	18.4 ± 0.025	1.6 ± 0.010	21.5 ± 0.085	7.5 ± 0.53	23.8 ± 0.017	9.6 ± 0.27

Data represent mean ± SEM of 9–15 values.

and Robel, 1990; Majewska, 1990; Morrow et al., 1990; Park-Chung et al., 1994) and gene expression via conversion to 5 α -DHP (Rupprecht et al., 1993), numerous antibodies have been developed for the determination of the levels of pregnenolone, progesterone, 5 α -DHP and allopregnenolone using RIA (Corpechot et al., 1983; Jo et al., 1989; Purdy et al., 1990; Corpechot et al., 1993). However, the results of several studies indicate that neurosteroid concentrations measured by RIA vary considerably from one laboratory to the other (Rohle and Breuer, 1978; Purdy et al., 1990; Morfin et al., 1992; Corpechot et al., 1993). This is probably due to the low specificity of various antibodies which were, and are currently, in use for radioimmunoassays of steroids. While using RIA as a routine assay, investigators have adopted the comparison between RIA determinations and semi-quantitative GC-MF identification of the steroids in selected samples to establish whether a steroid concentration is accurate or not (Akwa et al., 1993; Corpechot et al., 1993; Korneyev et al., 1993). However, as yet, no GC-MF method has been developed for routine simultaneous quantitation of pregnenolone, progesterone, 5 α -DHP, and allopregnenolone in brain samples.

The present work reports the development and use of highly specific, accurate and sensitive GC-MF methodology utilizing electron ionization that can be used to quantitate these neurosteroids in discrete brain areas of the rat. Neurosteroids, separated

on an HPLC column and identified by their retention times, were derivatized and injected onto a GC-capillary column where they were further separated from coeluting contaminants and identified by their GC-retention times.

The HFBA-derivatives of pregnenolone, progesterone, and allopregnenolone and the MO-derivative of 5 α -DHP were less polar, more volatile and had higher molecular weights than the underivatized steroids. The moiety derived from HFBA added 196 mass units to the respective molecule whereas the moiety derived from methoxyamine HCl added 58 mass units. This increase in molecular weight allowed the ion fragments to be monitored in a region of the mass spectrum that had less interference from the biological material or from the bleed of the stationary phase of the column.

5 α -DHP was derivatized with methoxyamine HCl because 5 α -DHP does not react with HFBA. Steroids, with a keto group as position 3, such as 5 α -DHP and progesterone, form geometric isomers of the syn and anti type (Koshy et al., 1975) when derivatized with methoxyamine HCl were expressed as dual peaks. Although, on one hand, the formation of two peaks instead of one caused a loss of detection sensitivity (see Table 3), on the other hand, the dual peaks for MO-5 α -DHP and the internal standard, MO-progesterone, actually aided in the identification of the steroids. The characteristics of the ion pattern emerging from the mass spectrometer and the constant ratio between peaks A and B increased the confidence that there were no interfering ions.

An estimate of the precision with which the quantitative measurements of the neurosteroids could be reproduced by our method was obtained from an ANOVA. Both within- and between-assay variations were determined and no significant differences were found between the ratio of the variances indicating an acceptable level of precision. For these methods internal standards were added at the beginning of the procedures. Because of the cost of custom synthesis of stable isotope-labeled analogs (Giovannini et al., 1991) of each of the steroids of interest, we used internal standards which were close homologs of the neurosteroids of interest. They differed by a double bond, a substituted hydroxyl or ketone group and had different gas chromatographic retention times and different *m/z* values. Because of their similar chemical characteristics, they provided the steroid quantitation with an acceptable degree of precision (Table 3).

Accuracy was determined from the ratio of the calculated concentration of steroid to the actual amount added. The error in the calculated concentrations of added neurosteroid was within 1–2%. The limit of detection was lowest (<0.32 pmol) for pregnenolone and progesterone because of their more simple fragmentation spectra and highest (<1.6 pmol) for 5 α -DHP. The sensitivity achieved by this method allowed the accurate mea-

Figure 4. Allopregnenolone standard curve. Data represent mean ± SEM of three to seven determinations taken from a number of experiments. $r = 0.98747$; $p = 1.587E-8$.

Table 3. Characteristics of the GC-MF assay for allopregnanolone and its precursors: 5 α -DHP, progesterone, and pregnenolone

	Source of variation	Sum of squares	De-grees of free-dom	Mean squares	F value*	Accuracy (%) ^a	Sensi-tivity (pmol)
Allopregnanolone	Interassay	0.0099	3	0.0033	2.74	98.6 $\pm$ 2.4	0.63
	Intraassay	0.0133	11	0.0012	$F_{(95\%)} = 3.59$ $F_{(99\%)} = 6.22$		
5 α -DHP	Interassay	0.1558	3	0.0519	0.42	100 $\pm$ 3.3	1.6
	Intraassay	1.4938	12	0.1245	$F_{(95\%)} = 3.49$ $F_{(99\%)} = 5.95$		
Progesterone	Interassay	0.3741	2	0.1870	4.09	98.4 $\pm$ 3.3	0.32
	Intraassay	0.4118	9	0.0458	$F_{(95\%)} = 4.26$ $F_{(99\%)} = 8.02$		
Pregnenolone	Interassay	0.8924	3	0.2974	1.88	101 $\pm$ 0.64	0.32
	Intraassay	1.9006	12	0.1584	$F_{(95\%)} = 3.49$ $F_{(99\%)} = 5.95$		

^a Data represent mean $\pm$ SEM of 12–16 values.* Differences between the means were not significant at $p < 0.05$. **Figure 5.** Allopregnanolone (ALLO), 5 α -DHP, and progesterone (PROG) content in brain, plasma, and liver of sham operated and ADX/CX rats at least 15 d following ADX/CX. Data represent mean $\pm$ SEM of 7–12 rats for brain and 4–5 rats for plasma and liver. *, $p < 0.05$; **, $p < 0.01$.

surement of allopregnanolone and its precursors, progesterone and pregnenolone simultaneously starting from approximately 100–300 mg of brain tissue (for 5 α -DHP, however, it was necessary to use approximately 1 gm of brain tissue). This characteristic became very desirable when we began to determine

Figure 6. Allopregnanolone content in brain and plasma of rats infused with pregnenolone sulfate (Preg Sulf). Data represent the mean $\pm$ SEM of seven or eight rats for brain and four or five rats for plasma; *, $p < 0.05$ compared to sham operated animals; **, $p < 0.01$ compared to vehicle treated controls.

Table 4. Content of 5 α -DHP, progesterone, and pregnenolone in whole brain of ADX/CX male rats following intravenous infusion of 48 μ mol/kg of pregnenolone sulfate (Preg Sulf)

	Vehicle (pmol/gm brain)	Preg Sulf (pmol/gm brain)
5 α -DHP	0.79 $\pm$ 0.22 (8)	3.8 $\pm$ 1.1 (8)*
Progesterone	6.4 $\pm$ 1.2 (11)	25 $\pm$ 5.1 (8)*
Pregnenolone	540 $\pm$ 73 (12)	26,000 $\pm$ 3500 (8)*

Rats were adrenalectomized and castrated at least 15 d before the experiment. Data represent mean $\pm$ SEM. Number of animals is shown in parentheses.

* $p < 0.01$ compared to vehicle controls.

brain concentration, distribution, and rate of biosynthesis of allopregnanolone in different brain areas.

Among putative neurosteroids allopregnanolone has been shown to facilitate GABA action in nanomolar concentrations and to open Cl^- channels in micromolar concentrations (Paul and Purdy, 1992; Puia et al., 1994). It is known that allopregnanolone is present and persists in brain of rats long after the adrenals are removed (Purdy et al., 1992), but there is very limited quantitative data available to suggest that changes in brain GABA receptor function can be related to changes in allopregnanolone or 5 α -DHP brain content.

Using the coupled HPLC/GC-MF technique we showed (Fig. 5) that brain contained nM concentrations of allopregnanolone and also demonstrated that these concentrations of allopregnanolone persisted in brain of rats from which adrenals and testis had been removed 15–21 d earlier.

To document that the allopregnanolone present in the brains of ADX/CX rats is synthesized in brain and is not transported there from blood after being synthesized in liver or some other peripheral organ, we measured the content of allopregnanolone in both plasma and liver. Since plasma levels of allopregnanolone in ADX/CX rats with or without pregnenolone sulfate infusion were virtually undetectable, we conclude that the persistent brain content of allopregnanolone is not derived from allopregnanolone synthesized in peripheral tissue and transported via blood to brain. This conclusion is confirmed by our experiments in liver. Liver contains high levels of 5 α -reductase and 3 α -HSOR and although the amount of progesterone in liver of ADX/CX rats is very small (Fig. 5), this large organ could contribute significantly to blood-borne allopregnanolone especially in animals infused with pregnenolone sulfate. However, the content of allopregnanolone in liver of ADX/CX rats with or without pregnenolone sulfate infusion was particularly low and virtually below the level of detection.

Since allopregnanolone in brain may be synthesized from its precursors, 5 α -DHP and progesterone, we adopted the strategy of infusing ADX/CX rats with pregnenolone sulfate to ensure an adequate amount of 5 α -DHP and progesterone at the site of synthesis. When administered in large amounts pregnenolone sulfate is desulfated in peripheral tissue (i.e., liver) and significant amounts of pregnenolone can reach, and be evenly distributed throughout, the brain. The enzyme 3 β -hydroxysteroid dehydrogenase which is the rate limiting step for the formation of progesterone from pregnenolone is present in brain (Robel et al., 1991) but is not found in most peripheral tissues with the exception of adrenal and testis which were removed in our animals. Thus, in ADX/CX rats infused with pregnenolone sulfate, the progesterone levels in liver were virtually undetectable. In the

Figure 7. Inhibition by SKF-105111 of the pregnenolone sulfate (Preg Sulf) induced increase of allopregnanolone content in selected brain areas of ADX/CX rats. Brain areas from four rats were pooled and samples were assayed in duplicate. Data represent the mean $\pm$ SEM of three to five duplicate assays for noninfused animals and 5–10 animals infused with pregnenolone sulfate. *, $p < 0.05$.

absence of progesterone, liver, although rich in 5 α -reductase and 3 α -HSOR, was not able to synthesize 5 α -DHP and allopregnanolone. Therefore, we can presume that the large increase in allopregnanolone (Fig. 6), progesterone, and 5 α -DHP (Table 4) found in the brain of ADX/CX rats infused with pregnenolone sulfate reflects a local synthesis of these steroids from pregnenolone.

Importantly, in ADX/CX rats we observed a nonuniform brain distribution of allopregnanolone with the highest content occurring in the olfactory bulb, striatum, and cortex. Following infusion of pregnenolone sulfate there was a uniform seven- to eightfold increase in brain allopregnanolone and the rank order of allopregnanolone content in various brain areas was identical to that of nonperfused rats. Those areas that have the highest content of allopregnanolone are the same areas in which the highest levels of 5 α -reductase activity and the highest conversion rate of progesterone to allopregnanolone have been demonstrated in brain slices (Karavolas and Hodges, 1991; Korneyev et al., 1993). Moreover, the 5 α -reductase inhibitor, SKF-105111, reduced the brain content of allopregnanolone in the various brain areas and blocked the increase in allopregnanolone content induced by pregnenolone sulfate infusion.

In conclusion, the work presented here utilizes a highly specific, accurate and sensitive HPLC/GC-MF technology to demonstrate a brain specific synthesis of allopregnanolone in quantities that may be relevant to a physiological modulatory role of neuronal activity for this neurosteroid. These conclusions are based on the following observations.

(1) Pharmacological relevant (nanomolar) concentrations of allopregnanolone were present in the brain of ADX/CX rats long

after peripheral sources of allopregnanolone were removed. The relatively small decrease in the content of allopregnanolone (30%) in the brains of ADX/CX rats compared to sham-operated rats vis-a-vis the substantial decrease in the brain content (70%) of its precursors, 5 α -DHP and progesterone further support that allopregnanolone biosynthesis is occurring in brain from a pool of brain progesterone and 5 α -DHP which is newly synthesized in brain and is not dependent on peripheral sources of these steroids.

(2) The content of allopregnanolone in brains of sham operated as well as ADX/CX rats was one to two orders of magnitude higher than in plasma or liver.

(3) Infusion of pregnenolone sulfate in ADX/CX rats elicited a fourfold increase in 5 α -DHP and progesterone content and a seven- to eightfold increase in the content of allopregnanolone in brain but not in liver or plasma. Moreover, the content of allopregnanolone in brain increased to the same extent in both sham-operated and ADX/CX rats following pregnenolone sulfate infusion.

(4) Allopregnanolone was not uniformly distributed in brains of sham-operated and ADX/CX rats following infusion of pregnenolone sulfate. This uneven distribution cannot be due to blood borne 5 α -DHP, pregnenolone or to uptake of allopregnanolone because ^3H -pregnenolone, ^3H -progesterone, ^3H -5 α -DHP, and ^3H -allopregnanolone were uniformly distributed in various brain areas when they were infused into rats.

(5) The 5 α -reductase inhibitor, SKF 105111, reduced the brain content of allopregnanolone and blocked the increased formation of allopregnanolone in brain following pregnenolone sulfate infusion thereby reducing the uneven distribution of allopregnanolone in brain.

These results clearly demonstrate that the synthesis of allopregnanolone from 5 α -DHP and progesterone is brain specific and that a significant amount (nanomolar) of allopregnanolone is synthesized locally in brain from its precursors. Thus, these experiments confirm that the brain, like adrenals and gonads, is a steroidogenic organ which produces allopregnanolone as one of its own most important physiologically relevant steroid.

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