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Studies on neurosteroids VII. Determination of pregnenolone and its 3-stearate in rat brains using high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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Abstract

An assay method for pregnenolone and its 3-stearate in rat brains has been developed using LC–atmospheric pressure chemical ionization (APCI) isotope dilution MS. The extraction of the rat brain homogenate containing deuterated internal standards (ISs) with organic solvent followed by silica gel mini-column chromatography gave two fractions containing pregnenolone and its 3-stearate together with the respective IS. Each fraction was derivatized into 3-acetate-20-methyloxime and 20-methyloxime, respectively, followed by silica gel mini-column chromatography to remove the excess reagents, and then each derivatized fraction was applied onto reversed-phase LC–APCI-MS. The method was applied to the determination of these steroids in the gray matter and olfactory bulbs of rat brains, which were divided into control and acute stressed specimens. Although pregnenolone in both regions of the rat brains increased more than five times after stress, its 3-stearate decreased after stress.

Keywords: Steroids; Neurosteroids; Pregnenolone; Pregnenolone-3-stearate

1. Introduction

Since the discovery of dehydroepiandrosterone in rat brains, several 17- and 20-oxosteroids including pregnenolone, called ‘neurosteroids’, have been elucidated in mammalian brains [1]. The neurosteroids that exist in rat brains are reported as the free form, sulfates, lipoidal esters and sulfolipids [2]. Significant interest has thus been focused on their biological properties in this organ [3]. In a previous paper [4] of this series, we clarified the existence of pregnenolone- and dehydroepiandrosterone-3-stearate, and -3-palmitate in rat brains using LC–atmospheric pressure chemical ionization (APCI)-MS. We also developed a method for determination of pregnenolone in rat brains using fluorescent derivatization followed by reversed-phase HPLC [5]. The application of the developed method showed much lower amounts than that previously reported in rat brains [1,5]. It has been reported that stress increases the concentration of neurosteroids [6] and steroid fatty acid ester exists as a storage form in biological fluids [7]. These data gave us much interest in the

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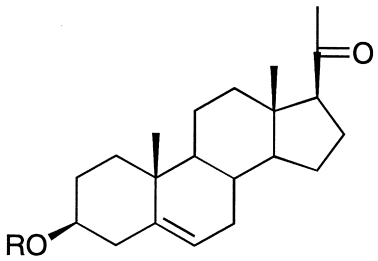

Pregnenolone: R=H

Pregnenolone-3-stearate: R=$CH_3(CH_2)_{16}CO$-

Fig. 1. Structures of pregnenolone and its 3-stearate.

correlation between pregnenolone and its 3-stearate in rat brains after acute stress (Fig. 1).

In this report, we developed the quantitative determination method of these compounds in rat brains using LC–APCI-isotope dilution MS and have applied the method.

2. Experimental

2.1. Materials, reagents and animals

Pregnenolone was obtained from Tokyo Kasei Kogyo (Tokyo, Japan), and its 3-stearate [4], its 3-palmitate [4], and [17,21,21,21-$^2H_4$]-pregnenolone ([$^2H_4$]-pregnenolone) [8] and its 3-stearate were prepared in our laboratories. Silica gel mini-column chromatography (6×0.6 cm I.D.) was performed with silica gel 60 (70–230 mesh; E. Merck, Darmstadt, Germany). All other reagents were of analytical grade and commercially available. Wistar strain rats (7 weeks old) were obtained from Japan S.L.C. (Hamamatsu, Japan). Some of these rats were stressed after fixation for 1 h before decapitation.

2.2. Apparatus

LC–APCI-MS was performed using a Hitachi M-1000H (Tokyo) quadrupole mass spectrometer connected to a Hitachi L-6200 chromatograph. The flow-rate was set to 1.0 ml/min. The multiplier and focus voltages were set at 2.5 kV and 120 V, respectively. The desolvator and vaporizer temperatures were set at 399°C and 300°C, respectively. The drift voltage was set at 50 V. The reversed-phase separations were carried out on a J'sphere ODS-L80 or -H80 (4 μm, 15×0.46 cm I.D.; YMC, Kyoto, Japan) under ambient conditions. The former and the latter columns contain ca. 9% and 22% of octadecylsilyl residue, respectively.

2.3. Procedure for determination of pregnenolone and its 3-stearate in rat brains

A Wistar strain rat (7 weeks old, 130–155 g) was decapitated and its gray matter or olfactory bulbs (100 mg) was homogenized in isotonic saline (1.5 ml) under ice-cooling. After the addition of two internal standards {IS_1, [$^2H_4$]-pregnenolone, 10 ng/MeOH (10 μl); IS_2, [$^2H_4$]-pregnenolone-3-stearate, 10 ng/MeOH (30 μl)}, the entire mixture was extracted with $CHCl_3$–MeOH (2:1) (4.5 ml) and centrifuged at 1500 *g* for 10 min. The precipitate was further extracted twice and all the supernatants were combined and evaporated under a N_2 gas stream. The obtained residue was applied onto silica gel mini-column chromatography and washed with hexane (10 ml) and hexane–AcOEt (50:1) (10 ml). The fraction containing pregnenolone-3-stearate and IS_2 was eluted with hexane–AcOEt (20:1) (10 ml) and the solvent was evaporated under a N_2 gas stream. The column was further washed with hexane–AcOEt (10:1) (10 ml) and hexane–AcOEt (5:1) (10 ml). The fraction containing pregnenolone and IS_1 was eluted with hexane–AcOEt (3:1) (10 ml) and the solvent was evaporated under a N_2 gas stream.

The residue containing pregnenolone-3-stearate and IS_2 was treated with 2% NH_2OCH_3·HCl/pyridine (0.3 ml) at 70°C for 2 h. The entire solution was extracted with AcOEt and the organic layer was successively washed with chilled H_2O, 5% HCl and H_2O. After evaporation of the solvent, the residue was applied onto silica gel mini-column chromatography and washed with hexane (10 ml) and hexane–AcOEt (100:1) (10 ml). The fraction eluted with hexane–AcOEt (30:1) (5 ml) was evaporated under a N_2 gas stream and the obtained residue was dissolved in $CHCl_3$–MeOH (1:1) (50 μl) and an aliquot was applied onto LC–APCI-MS {column, J'sphere ODS-L80; mobile phase, MeOH containing

10 m*M* of $AcONH_4$; t_R 8.4 min; selected ion monitoring (SIM) *m*/*z* 612, 616 $[M+H]^+$}.

The residue containing pregnenolone and IS_1 was treated with Ac_2O–pyridine (1:2) (0.3 ml) at 70°C for 30 min and evaporated under a N_2 gas stream. The residue was treated with 2% $NH_2OCH_3 \cdot HCl$/pyridine (0.3 ml) as described above. The residue was applied onto silica gel mini-column chromatography and washed with hexane (10 ml), hexane–AcOEt (100:1) (10 ml) and hexane–AcOEt (50:1) (10 ml). The fraction eluted with hexane–AcOEt (20:1) (10 ml) was evaporated under a N_2 gas stream and the obtained residue was dissolved in $CHCl_3$–MeOH (1:1) (50 μl) and an aliquot was applied onto LC–APCI-MS {column, J'sphere ODS-H80; mobile phase, MeCN–H_2O (19:1) containing 5 m*M* of $AcONH_4$; t_R 12.8 min; SIM *m*/*z* 388, 392 $[M+H]^+$}.

2.4. Absolute recoveries of pregnenolone, its 3-stearate and their ISs before derivatization

The entire brains (without gray matter and olfactory bulbs, 1.79 g tissue) of Wistar strain rats (7 weeks old, 135 and 150 g) were homogenized in isotonic saline (21 ml). 3β-Hydroxy-16-methylpregna-5,16-dien-20-one (IS_3; 35 ng) in MeOH (35 μl) was added to the homogenate (7 ml) and 2 ml (170 mg tissue) of which was used as the blank sample (three samples). Pregnenolone (180 ng) in MeOH (144 μl) and IS_1 (180 ng) in MeOH (180 μl) were added to the homogenate (14 ml) and 2.046 ml (pregnenolone, IS_1, each 25.71 ng, 170 mg tissue) of which was used as the sample for measuring the absolute recoveries of pregnenolone and IS_1 before derivatization (six samples).

The samples used for measuring the recoveries of pregnenolone-3-stearate and IS_2 were prepared as described above; three blank samples [each contained pregnenolone-3-palmitate (IS_4; 10 ng); 153 mg tissue] and six samples (each contained pregnenolone-3-stearate, 25.71 ng; IS_2, 27.86 ng; 153 mg tissue).

All these samples were analysed as described above. The IS_3 and IS_4 (each 10 ng) were added to the samples before derivatization for the measurement of the recoveries of pregnenolone, IS_1, pregnenolone-3-stearate and IS_2. The peak areas of SIM of IS_3 acetate-methyloxime (*m*/*z* 400 $[M+H]^+$, t_R 11.1 min) and IS_4 methyloxime (*m*/*z* 584 $[M+H]^+$, t_R 7.2 min) were used for the calculation of the recovery.

2.5. Calibration graph for pregnenolone and its 3-stearate

2.5.1. Pregnenolone

Each tube containing pregnenolone (2.5, 5, 10, 20, 40 or 80 ng) and IS_1 (10 ng) was derivatized with Ac_2O–pyridine and then 2% $NH_2OCH_3 \cdot HCl$/pyridine followed by purification including silica gel mini-column chromatography as already described.

2.5.2. Pregnenolone-3-stearate

Each tube containing pregnenolone-3-stearate (2.5, 5, 10, 20, 40 or 80 ng) and IS_2 (10 ng) was derivatized with 2% $NH_2OCH_3 \cdot HCl$/pyridine as already described. The resulting solution was treated as above without the silica gel mini-column chromatography.

The calibration graphs (2.5–80 ng/tube) were constructed by the peak area ratio method and the obtained linear relationships were as follows: pregnenolone ($y=0.132x-0.059$, $r=0.998$), pregnenolone-3-stearate ($y=0.130x+0.064$, $r=0.997$).

2.6. Method validation of determination of pregnenolone and its 3-stearate

The known amounts of pregnenolone and its 3-stearate were added to the rat brain homogenate, and each of the resulting solutions was assayed with the proposed method.

2.7. Application of the method

The developed method was applied to the determination of pregnenolone and its 3-stearate in the gray matter and olfactory bulbs of Wistar strain rat brains. The rats were divided into two groups, that is, the control rats under normal conditions and the stressed ones after fixation for 1 h.

3. Results

3.1. Determination of pregnenolone and its 3-stearate in rat brains

We developed a method for the determination of pregnenolone and its 3-stearate in rat brains using reversed-phase LC–APCI-isotope dilution MS. [$^2H_4$]-Pregnenolone and its 3-stearate were used as the IS for the determination of pregnenolone and its 3-stearate, respectively. Although these compounds showed complicated APCI-MS spectra, the respective methyloxime showed a satisfactory one in which the $[M+H]^+$ ion corresponding to pregnenolone or its 3-stearate was not observed (Fig. 2). In a previous paper of this series, we clarified that the derivatization of oxosteroids into acetate and/or methyloxime strongly increased their sensitivity in LC–APCI-MS [9]. These data prompted us to use the derivatization method for the determination of pregnenolone and its 3-stearate in rat brains using LC–APCI-isotope dilution MS.

The brains of adult Wistar strain rats were homogenized in isotonic saline, two ISs were added to the homogenate and then extracted with organic solvent. The extract was purified with silica gel mini-column chromatography to give the pregnenolone-3-stearate and pregnenolone fractions. The former fraction was derivatized with *O*-methylhydroxylamine and the latter with acetic anhydride–pyridine and then *O*-methylhydroxylamine. Each derivatized fraction was purified by silica gel mini-column chromatography, which were then applied onto LC–APCI-MS (Fig. 3).

Satisfactory LC–APCI-MS conditions were chosen based on the previously reported data [9] and compared with the half-band width of UV spectra from authentic samples. The chromatogram of oxime usually shows two peaks corresponding to the *E*- and *Z*-isomers, but the methyloximes of pregnenolone-3-acetate and -stearate showed single peaks, respectively. Those of deuterated $IS_{1,2}$ showed small peaks in front of the main peaks, but their complete

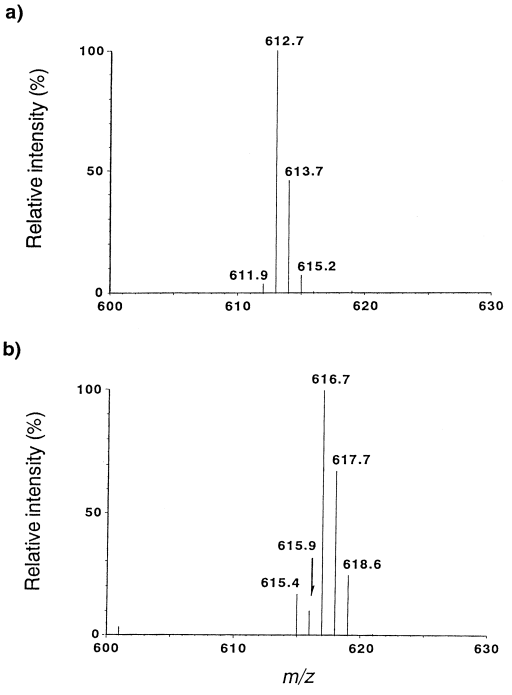

Fig. 2. Mass spectra of pregnenolone-3-stearate-20-methyloxime and its deuterated IS. (a) Pregnenolone-3-stearate-20-methyloxime; (b) [$^2H_4$]-pregnenolone-3-stearate-20-methyloxime. Conditions: column, J'sphere ODS-L80; mobile phase, MeOH containing $AcONH_4$ (10 m*M*); flow-rate, 1.0 ml/min.

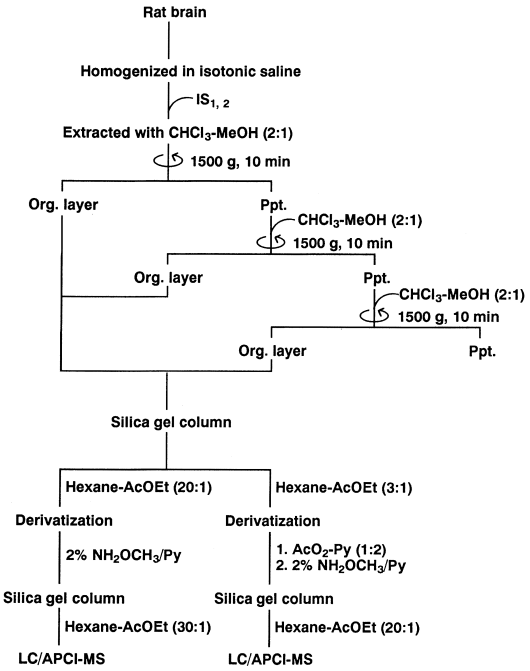

Fig. 3. Procedure for determination of pregnenolone and its 3-stearate in rat brains.

identification has not yet been achieved (Figs. 4 and 5).

3.2. Absolute recoveries before derivatization reaction

The absolute recoveries of pregnenolone, its 3-stearate and their ISs before the derivatization reactions were examined according to the cleanup procedure and the results are summarized in Table 1. Although the recoveries of pregnenolone-3-stearate is lower than that of pregnenolone, a significant difference has not been observed based on the results obtained from the determining target and its IS (pregnenolone vs IS_1, pregnenolone-3-stearate vs IS_2).

3.3. Method validation

After spiking the standard sample into the rat brain homogenate, the concentrations of pregnenolone and its 3-stearate were determined using the proposed method. The obtained data are summarized in Table 2. Satisfactory recoveries were obtained with a relative standard deviation (R.S.D.) of less than 11%.

The limits of quantitation [signal-to-noise ratio (S/N)=10] of pregnenolone and its 3-stearate were 750 pg and 500 pg, respectively.

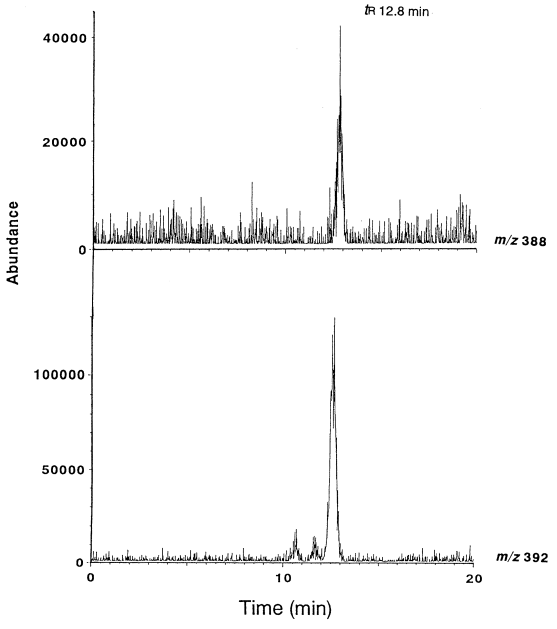

Fig. 4. Chromatograms of derivatives of pregnenolone and IS_1 obtained from rat brains. m/z 388 $[M+H]^+$; pregnenolone-3-acetate-20-methyloxime (ca. 0.8 ng); m/z 392 $[M+H]^+$; 3-acetate-20-methyloxime of IS_1 (ca. 2 ng). Conditions: column, J'sphere ODS-H80; mobile phase, MeCN–H_2O (19:1) containing $AcONH_4$ (5 mM); flow-rate, 1.0 ml/min; SIM.

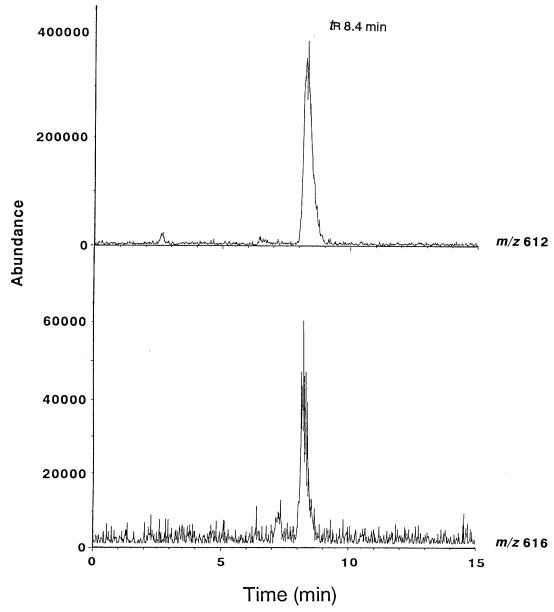

Fig. 5. Chromatograms of methyloximes of pregnenolone-3-stearate and IS_2 obtained from rat brains. m/z 612 $[M+H]^+$; pregnenolone-3-stearate-20-methyloxime (ca. 8 ng); m/z 616 $[M+H]^+$; 20-methyloxime of IS_2 (ca. 1 ng). Conditions: column, J'sphere ODS-L80; mobile phase, MeOH containing $AcONH_4$ (10 mM); flow-rate, 1.0 ml/min; SIM.

3.4. Application of the method

The developed method was applied to the determination of pregnenolone and its 3-stearate in the gray matter and olfactory bulbs of Wistar strain rat brains (Fig. 6). The method was first applied to the control rats including both male and female rats, but a clear sex difference has not been observed. Based on these data, the following experiments have been done using female rats after stress. Although pregnenolone in both regions of the rat brains increased more than five times after stress (Fig. 6a), its 3-stearate decreased after stress and some of these

Table 1
Recovery of pregnenolone, its 3-stearate and ISs

Compound	Blank	Added	Found[a]	Net recovery (%)[b]	R.S.D. (%)
	(ng/tube)				
Pregnenolone	11.85[c]	25.71	33.51	84.2±5.2	6.1
IS_1	0[c]	25.71	22.77	88.6±4.5	5.0
Pregnenolone-3-stearate	1.21[d]	25.71	15.90	57.2±3.9	6.9
IS_2	0[d]	27.86	16.57	59.5±4.6	7.7

[a] Mean.
[b] Mean±S.D., $n=5$.
[c] Mean, $n=3$; 170 mg tissue/tube.
[d] Mean, $n=3$; 153 mg tissue/tube.

could not be determined with the proposed method due to its low sensitivity (Fig. 6b).

4. Discussion

In a previous paper of this series, we developed a method for determination of pregnenolone in rat brains using fluorescent derivatization followed by reversed-phase HPLC [5]. However the method required a complicated pretreatment method including ion-exchange chromatography followed by fluorescent derivatization. The developed method using LC–APCI-MS is much more convenient than the previous one. A method for determining pregnenolone-3-stearate in rat brains has also been developed using LC–APCI-MS and this method was satisfactory in its accuracy and precision. The derivatization with *O*-methylhydroxylamine not only increased the sensitivity of these metabolites but also stabilized the deuterated IS in LC–APCI-MS. That is, the derivatization protected the hydrogen–deuterium exchange reaction at the 17 and 21 positions of the steroids under APCI. The quantitative limits of these steroids were 750 pg and 500 pg, respectively, which meant less than 7.5 and 5 ng/g tissue of these steroids could not be determined when 100 mg of rat-brain tissue was used for the determination. Although these data are not satisfactory in terms of sensitivity, LC–MS–MS will overcome this problem.

It has been reported that a high concentration of pregnenolone is observed in the gray matter [1] and olfactory bulbs of rat brains [10] and a synthetic enzyme of the steroid from cholesterol has been found in the olfactory bulb [11]. It was also demonstrated that pregnenolone increases in rat brains after acute stress [6]. These data prompted us to apply the

Table 2
Accuracy and precision of determination of pregnenolone and its 3-stearate in rat brains

Blank	Added	Found[a]	Net recovery (%)[b]	R.S.D. (%)
(ng/tube)				
4.16[c]	4.17	8.11	94.5±10.2	10.7
4.64[d]	8.57	12.70	94.1±6.5	6.9
3.86[c]	25.71	31.73	108.5±1.5	1.4
2.26[e]	10.00	11.46	91.9±5.6	6.1
0.56[f]	23.23	23.60	99.2±3.2	3.2

[a] Mean.
[b] Mean±S.D., $n=5$.
[c] Pregnenolone, mean, $n=3$; 182 mg tissue/tube.
[d] Pregnenolone, mean, $n=3$; 179 mg tissue/tube.
[e] Pregnenolone-3-stearate, mean, $n=3$; 180 mg tissue/tube.
[f] Pregnenolone-3-stearate, mean, $n=3$; 192 mg tissue/tube.

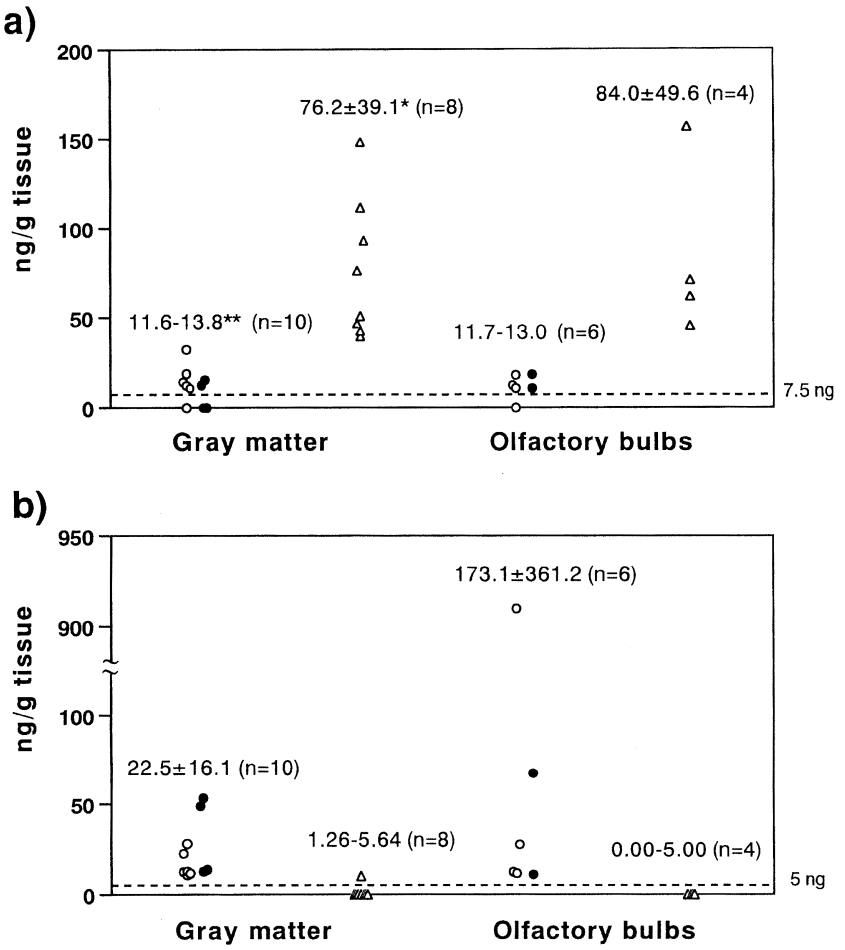

Fig. 6. Pregnenolone and its 3-stearate in rat brains. (a) Pregnenolone; (b) pregnenolone-3-stearate. *, mean±S.D.; **, range of mean [The value less than the quantitative limit was taken as 0 or 7.5 ng/g (pregnenolone) and 5.0 ng/g tissue (pregnenolone-3-stearate), respectively]. (- - - -) limit of quantitation; (○) ♀, (●) ♂ control; (△) ♀ stress-induced.

developed method to the determination of pregnenolone and its 3-stearate in the gray matter and olfactory bulbs of Wistar strain rat brains. The rats were divided into control and acute stressed groups. A clear difference has not been observed in the data obtained from each region of the rat brains, and the pregnenolone data in normal and after-stressed rat brains are compatible with those previously reported [5,6]. Although pregnenolone increased after stress as already reported [6], its 3-stearate decreased after stress. These data suggested that the fatty acid ester is a storage form of the free steroid in rat brains, which is excreted after acute stress.

The physiological significance of these steroids is under investigation in our laboratory and the results will be reported in the near future.

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