Studies on Neurosteroids XXV. Influence of a 5α-Reductase Inhibitor, Finasteride, on Rat Brain Neurosteroid Levels and Metabolism

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In this study, we examined the influence of finasteride (FIN), a 5α-reductase inhibitor, on the brain levels and metabolism of neurosteroids [allopregnanolone (AP), 3α-dihydroprogesterone (3α-DHP), progesterone (PROG), 20α-dihydroprogesterone and 11-deoxycorticosterone (DOC)] in rats exposed to immobilization stress. For this purpose, the sensitive, reproducible and accurate liquid chromatography-electrospray ionization-tandem mass spectrometric (LC-ESI-MS/MS) methods that enable the quantification of trace amounts of brain neurosteroids were first developed. The animal study using these methods demonstrated that FIN dose-dependently inhibits the stress-induced elevation of the brain AP, a potent positive modulator of the γ-aminobutyric acid (GABA) type A receptors, and a 10 mg/kg dose of FIN can almost completely deplete AP in the brains. The study also found that the 20α-reduction of PROG is enhanced when its 5α-reduction pathway is inhibited in the brains. No change was found in the brain levels of 3α-DHP, another GABAergic neurosteroid, and DOC by the administration of FIN.

Key words neurosteroid; finasteride; liquid chromatography-mass spectrometry; rat brain; stress; metabolism

Neurosteroids affect neurotransmission through action at the membrane ion-gated receptors and at other neurotransmitter receptors. 3α-Hydroxy-5α-pregnan-20-one (allopregnanolone, AP), a representative neurosteroid, binds to the γ-aminobutyric acid type A ($GABA_A$) receptors with a high affinity and positively modulates the action of GABA at these receptors, and hence elicits marked anticonvulsant, antidepressant and anxiolytic effects.[1] Under stressful conditions, the brain rapidly synthesizes progesterone (PROG) and subsequently converts it to AP to raise the threshold of brain excitability.[2]

The two-step metabolism of PROG produces AP through the actions of the enzymes, 5α-reductase (rate-limiting step) and 3α-hydroxysteroid dehydrogenase (3α-HSD). Two distinct 5α-reductase isozymes, types 1 (predominant form in the brain) and 2 (predominant form in the periphery), are found across mammalian species.[3] Finasteride [17α-(*N*-*t*-butyl)carbamoyl-4-aza-5α-androst-1-en-3-one, FIN] is the 5α-reductase inhibitor that has received clinical approval for the treatment of human benign prostatic hyperplasia and androgenetic alopecia. Both isozymes of the 5α-reductase in the rodent demonstrate comparable inhibition following FIN exposure; FIN can inhibit the 5α-reductase activity in the central nervous system as well as in the periphery.[4] Based on this, FIN has also been used to manipulate the brain AP level for characterization of its physiological functions and the mechanism by which it affects the brain functions, and to analyze the exact mechanisms of action of psychotropic agents, whose antidepressant and anxiolytic effects are inferred to occur through an increase in the brain AP synthesis.[5,6] Thus, FIN is an indispensable tool in the development of new psychotropic agents targeting neurosteroidogenesis as well as in basic neurosteroid research. On the contrary, the treatment with FIN may induce alternative metabolic pathways, such as 3α-reduction, 20α-reduction and 21-hydroxylation, in the PROG metabolism (Fig. 1), and the generated metabolites may consequently affect the central nervous system and modulate the emotional state. However, there is no report that examines the changes in the brain levels of the PROG and its metabolites following treatment with FIN. Even for AP, a detailed FIN-evoked change in the brain concentration has not been elucidated.

Based on this background information, the objectives of this study are to develop liquid chromatography-tandem mass spectrometric (LC-MS/MS) methods that enable the determination of trace amounts of the brain PROG and its down stream conversion products [AP, 3α-dihydroprogesterone (3α-DHP), 20α-dihydroprogesterone (20α-DHP) and 11-deoxycorticosterone (DOC)] (Fig. 1) and to determine the influence of FIN on the brain steroid levels and metabolism in rats under the stressful condition.

MATERIALS AND METHODS

Materials and Reagents AP, 3α-DHP, 3β-DHP and 20α-DHP were obtained from Steraloids (Newport, RI, U.S.A.). PROG and DOC were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), re-

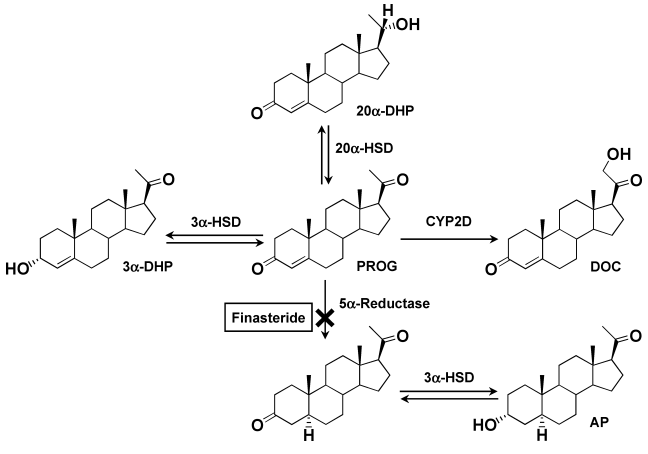

Fig. 1. Metabolic Pathways of PROG
The FIN blockade is indicated in the pathways.

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spectively. [17α,21,21,21-$^2H_4$]-AP (D_4-AP) was that used in a previous study[7] and used as the internal standard (IS) for the AP quantification. 3β-DHP was used as the IS for the 3α-DHP quantification. [2,2,4,6,6,17α,21,21,21-$^2H_9$]-PROG (D_9-PROG) was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada) and used as the IS for the PROG, 20α-DHP and DOC quantification. Each steroid was dissolved in and diluted with ethanol to prepare the standard solutions. 2-Hydroxypropyl-β-cyclodextrin (β-CD) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Strata-X cartridges (60 mg adsorbent; Phenomenex, Torrance, CA, U.S.A.) were successively washed with ethyl acetate (2 ml), methanol (2 ml) and water (2 ml) prior to use. Bond Elut Si cartridges (500 mg adsorbent; Varian, Harbor, CA, U.S.A.) were successively washed with ethyl acetate (4 ml) and hexane (4 ml) prior to use. All other reagents and solvents were of analytical grade.

Animals Male Wistar strain rats (7-weeks old, 180—220 g body weight, Japan S.L.C., Hamamatsu, Japan) were maintained under an artificial 12 h light/12 h dark cycle (light on 08:00—20:00). The rats were transferred to the laboratory and then left unrestrained in a quiet place for 2 h before the experiments. 5% β-CD in saline (w/v, 1 ml, vehicle, $n=5$) or FIN (0.5, 2.0 or 10 mg/kg, $n=5$ per group) was intraperitoneally (i.p.) administered to the rats. The solution of FIN was made in 5% β-CD in saline and 1 ml of which was administered. One hour after the administration of the vehicle or FIN, the rats were immobilized on their backs on a board for 20 min. After immobilization, the rats were unrestrained for 30 min and then euthanized. In order to examine the stress-induced increase in the brain steroids, 5 rats were euthanized by decapitation 60 min after the vehicle injection; these were labeled the unstressed rats in this study. All treatments were performed between 13:00 h and 15:00 h. All animal care and use were approved by the Institutional Animal Care and Use Committee of Kanazawa University.

Statistical Analysis The statistical analyses were performed using the Kruskal–Wallis test followed by the Steel–Dwass test. A p value of <0.05 was considered statistically significant.

Determination of AP in Rat Brains The steroids including AP were extracted from the rat brain tissue with methanol–acetic acid (99 : 1, v/v) using a previously reported method.[7] The brain extract was diluted to adjust the concentrations of 100 mg tissue/ml with methanol–acetic acid (99 : 1, v/v) and stored at -20 °C prior to use.

After the addition of D_4-AP (200 pg) and water (1 ml), 0.5 ml of the brain extract (corresponding to 50 mg of brain tissue) was passed through a Strata-X cartridge. After washing with water (2 ml) and methanol–water (3 : 2, v/v, 2 ml), the steroids were eluted with ethyl acetate (1 ml). After evaporation of the eluent from the cartridge, the residue was dissolved in hexane–ethyl acetate (3 : 1, v/v, 0.2 ml) and further purified using a Bond Elut Si cartridge. After washing with hexane (3 ml) and hexane–ethyl acetate (3 : 1, v/v, 3 ml), the steroids were eluted with ethyl acetate–hexane (3 : 2, v/v, 2 ml). After evaporation, the residue was subjected to derivatization with 2-hydrazino-1-methylpyridine (HMP) as previously reported.[7] The derivatized steroids were dissolved in methanol–water (1 : 1, v/v, 30 μl), 10 μl of which was subjected to LC-electrospray ionization (ESI)-MS/MS. The LC-MS/MS instrument and conditions were the same as those previously reported.[7] The quantification was based on the selected reaction monitoring (SRM) mode using the characteristic transition of the HMP derivatives ($[M]^+ \rightarrow m/z$ 108).

Determination of 3α-DHP in Rat Brains The brain extract (100 mg tissue/ml) was prepared as described above. After the addition of 3β-DHP (IS, 1.0 ng), which was not detected in the brains, and water (1 ml), 1.0 ml of the extract (corresponding to 100 mg of brain tissue) was purified using Strata-X and Bond Elut Si cartridges in the same way for the determination of AP. After evaporation of the eluent from the cartridge, the residue was dissolved in methanol–water (1 : 1, v/v, 100 μl), 25 μl of which was subjected to LC-ESI-MS/MS [instrument; Applied Biosystems API 4000 Q TRAP triple quadrupole-mass spectrometer connected to an Agilent 1200 series chromatograph (Palo Alto, CA, U.S.A.), analytical column; A J'sphere ODS H-80 column (4 μm, 150×2.0 mm i.d.; YMC, Kyoto), column temperature; 40 °C, mobile phase; methanol–10 mM ammonium formate (18 : 7, v/v), flow rate; 0.2 ml/min, declustering potential; 45 V, entrance potential; 10 V, ion spray voltage; 5 kV, curtain gas; 35 psi, ion source gas 1; 90 psi, ion source gas 2; 80 psi, turbo gas temperature; 400 °C]. Nitrogen was used as the collision gas in the SRM mode with a collision energy of 34 eV and a collision cell exit potential of 5 V. The transitions (precursor and product ions) of each steroid were as follows: 3α-DHP; m/z 299.4 $[M+H-H_2O]^+ \rightarrow 81.2$ and 3β-DHP; m/z 299.4 $[M+H-H_2O]^+ \rightarrow 81.2$.

Determination of PROG, 20α-DHP and DOC in Rat Brains The brain extract (100 mg tissue/ml) was prepared as already described. After the addition of D_9-PROG (200 pg) and water (3 ml), 0.2 ml of the extract (corresponding to 20 mg of brain tissue) was passed through a Strata-X cartridge. After washing with water (3 ml), acetonitrile–water (3 : 7, v/v, 2 ml) and hexane (1 ml), the steroids were eluted with ethyl acetate (2 ml). After evaporation of the eluent from the cartridge, the residue was dissolved in methanol–water (1 : 1, v/v, 75 μl), 25 μl of which was subjected to LC-ESI-MS/MS. The LC-MS/MS instrument and conditions were the same as those for the determination of 3α-DHP except for the following parameters: declustering potential; 65 (PROG), 70 (DOC) or 80 V (20α-DHP), ion source gas 1; 65 psi, ion source gas 2; 85 psi, turbo gas temperature; 650 °C. The SRM was performed with a collision energy of 34 eV and a collision cell exit potential of 7 V. The transitions of each steroid were as follows: PROG; m/z 315.1 $[M+H]^+ \rightarrow 97.2$, 20α-DHP; m/z 317.2 $[M+H]^+ \rightarrow 97.2$, DOC; m/z 331.2 $[M+H]^+ \rightarrow 97.2$ and D_9-PROG; m/z 323.4 $[M+H]^+ \rightarrow 100.2$.

Calibration Curves The brain extract (15 ml) was stirred overnight with activated charcoal (1.5 g, Norit EXW, Nacalai Tesque) and then centrifuged at 1500 ***g*** (4 °C, 30 min). The supernatant, in which any steroids of interest were not detected by our methods, was spiked with respective steroids and ISs and then the calibration curves were constructed by plotting the peak area ratios of the respective steroids to the respective ISs *versus* the concentrations of the respective steroids.

RESULTS AND DISCUSSION

Assay Methods LC coupled with ESI-MS is currently being used for the characterization and determination of neurosteroids due to its specificity, versatility and simultaneous multi-analyte quantification capability.[7—9] The ionization efficiencies of steroids depend on their chemical structures; the 3-oxo-4-ene-steroids (PROG, 20α-DHP and DOC) are easily ionized during the ESI,[8] the 3α/β-hydroxy-4-ene-steroids (3α-DHP) are fairly ionizable, while the 3α/β-hydroxy-5α-reduced-steroids (AP) have extremely low responses for ESI-MS.[7] Therefore, these steroids were separately analyzed according to their structures in this study; the 3-oxo-4-ene-steroids and 3α-DHP were analyzed as their intact forms, while derivatization was required to determine the physiological concentration of AP.

For the quantification of AP, the method that we had previously developed[7] was used with some modifications, *i.e.*, a larger sample volume and an additional purification step using a normal-phase solid-phase extraction (SPE) cartridge. Due to these modifications, the limit of quantification (LOQ) was improved to 0.1 ng/g tissue from 0.25 ng/g tissue,[7] and any endogenous components, such as other steroids and lipoidal compounds, did not interfere with the AP quantification.

The method for the determination of 3α-DHP was newly developed. For the ESI-MS operating in the positive-ion mode, 3α-DHP and IS (3β-DHP) provided their $[M+H-H_2O]^+$ as the base peak ions (Fig. 2a). The product ion mass spectra of the steroids employing the respective $[M+H-H_2O]^+$ as the precursor ions and a 34-eV collision energy are shown in Fig. 2b, in which a characteristic product ion with a satisfactory intensity was observed at *m*/*z* 81.2. The putative fragmentation that causes the product ion at *m*/*z* 81 is also illustrated in Fig. 2b. Based on these results, the SRM mode using the $[M+H-H_2O]^+$ and the ion at *m*/*z* 81.2 as the precursor and product ions, respectively, was employed in the following studies. A satisfactory separation of 3α-DHP and IS was achieved within 16 min using a J'sphere ODS H-80 column with the mobile phase of methanol–10 mM ammonium formate (18 : 7, v/v); 3α-DHP, retention time (t_R) 13.9 min and IS, t_R 11.6 min (Fig. 2c). Under these LC conditions, 3α-DHP and IS were well separated from pregnenolone (t_R 12.4 min), which is the predominant brain steroid under stressful conditions. The pretreatment procedure that had been developed for the AP assay was adopted for the 3α-DHP quantification. This procedure provided satisfactory recovery rates (>70%) of the target steroid and IS. Furthermore, in the chromatograms obtained from the brains of unstressed rats, there was no peak at the elution positions of 3α-DHP and IS (chromatograms not shown), demonstrating that the pretreatment procedure was effective for removing the inferring factors derived from the brain matrix. The relative standard deviation (RSD) and relative error of the back-calculated concentration at the minimum point (0.1 ng/g tissue) on the calibration curve were 4.0% and 6.0%, respectively. The peak of 3α-DHP at this concentration was clearly observed with a signal to noise ratio of more than 5. Based on these results, the LOQ of the brain 3α-DHP was determined to be 0.1 ng/g tissue, when a 100-mg brain tissue was used. Thus, our method can precisely and accurately quantify 0.1 ng/g tissue of the brain 3α-DHP, though no LC-MS method has heretofore been reported for the brain 3α-DHP due to its low concentration.

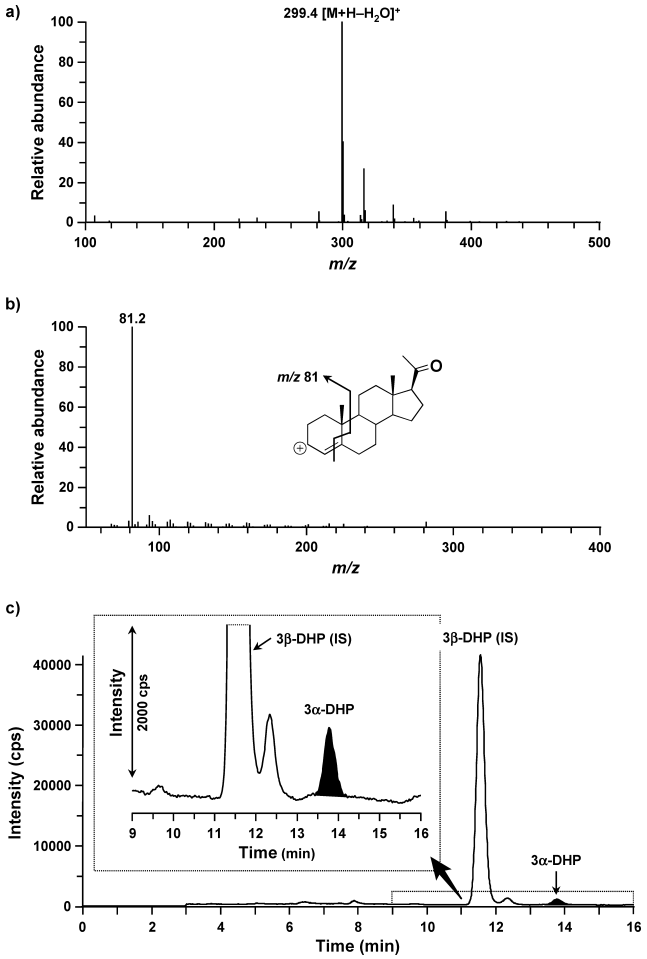

Fig. 2. LC-ESI-MS(/MS) Data for 3α-DHP

(a) ESI-MS and (b) ESI-MS/MS spectra were recorded by injection of 20 ng of 3α-DHP. (c) Chromatograms of 3α-DHP and IS in the brain of rat exposed to immobilization stress. The enlargement around the elution position of 3α-DHP is also presented. The LC-MS/MS conditions are described in Materials and Methods.

For quantification of the 3-oxo-4-ene-steroids, some modifications were made to the previous assay.[8] The improved method employed a very simple pretreatment procedure, *i.e.*, a one-step SPE, and the recovery rates of all the steroids were over 90%, whereas the previous method required two steps SPE.[8] This method enables the simultaneous quantification of PROG, 20α-DHP and DOC without interferences by any endogenous components.

The results of the validation tests for the respective assays are summarized in Table 1; as is obvious from this table, all the methods allowed the reproducible and accurate quantification of the brain neurosteroids.

Influence of FIN on Brain Neurosteroid Levels and Metabolism Although the brain AP level is extremely low (practically none) under normal conditions, it is significantly elevated by acute stress paradigms, such as immobilization,[7] forced swimming[10] and CO_2 inhalation.[11] In this study, the influence of FIN on the brain neurosteroid levels was examined using rats exposed to immobilization, in which the AP synthesis was significantly enhanced.

Rats were intraperitoneally injected with vehicle (saline)

Table 1. Validation Data of LC-MS/MS Methods for the Brain Neurosteroids

Steroids	Measurable range (ng/g tissue)	Intra-assay precision[a] (RSD, %)	Inter-assay precision[a] (RSD, %)	Accuracy[b] (%)	Recovery[c] (%)
AP	0.1—2.0 (r=0.999)[d]	2.7	3.2	103.4—106.0	75.7±2.4
3α-DHP	0.1—2.0 (r=0.999)	6.6	8.7	101.5—106.3	73.3±1.9
PROG	0.05—50 (r=0.999)	3.6	6.8	98.4—99.2	93.5±3.2
20α-DHP	0.05—50 (r=0.999)	3.2	6.3	98.0—98.4	93.7±3.3
DOC	0.05—50 (r=0.999)	2.9	4.9	94.6—100.5	94.8±1.4

a) The intra- and inter-assay precisions were evaluated by analyzing 5 replicates of a brain sample on 1 d and over 5 d, respectively. The brain sample used for the determination of the assay precisions contained 0.81 ng/g tissue of AP, 0.20 ng/g tissue of 3α-DHP, 14.8 ng/g tissue of PROG, 0.50 ng/g tissue of 20α-DHP and 14.3 ng/g tissue of DOC. *b*) The assay accuracy was evaluated by determining the analytical recovery rates, which were defined as $F/(F_0+A)\times100$ (%); F is the concentration of a steroid in the spiked sample, F_0 is the concentration of a steroid in the unspiked sample and A is the spiked concentration. Data represent ranges of analytical recoveries (n=4). *c*) Recovery rate during the pretreatment (mean±S.D., n=5). *d*) Correlation coefficient.

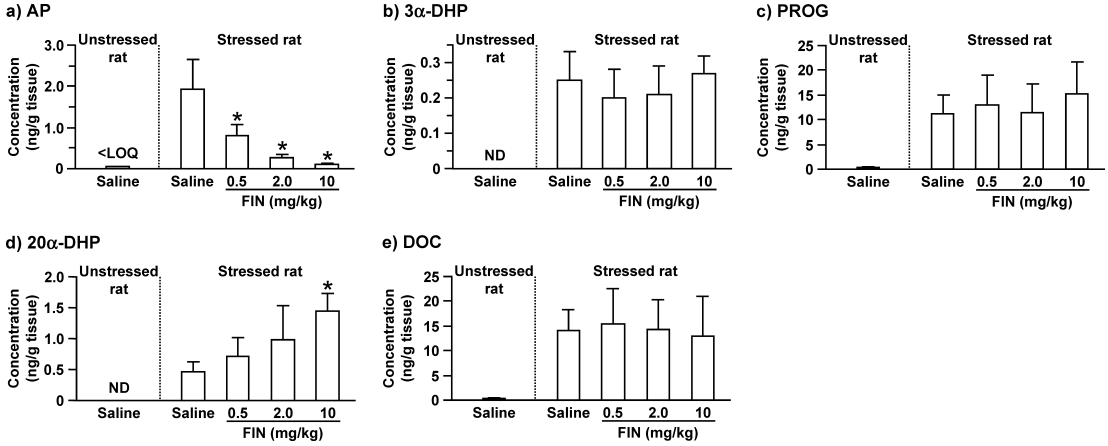

Fig. 3. Changes in Rat Brain Levels of Neurosteroids by the Intraperioneal Administration of FIN

The results represent mean±S.D (n=5 per group). $*p<0.05$ *versus* the stressed rats administered saline. ND; not detected.

or FIN 1 h prior to the immobilization, and then the brain neurosteroid levels were determined using the developed LC-MS/MS methods. The results are shown in Fig. 3. The AP level of the stressed rat dose-dependently decreased by the FIN treatment, and thus, that in the 10 mg/kg of FIN-injected rat was less than 10% of that for the vehicle-injected rat (Fig. 3a). Our previous study demonstrated that the administration of FIN (10 mg/kg, i.p.) also depleted the neuroactive androgen, 5α-androstane-3α,17β-diol (3α,5α-Adiol),[12] which is also the potent positive modulator of the $GABA_A$ receptors, in rat brains. In the previously reported animal studies, FIN at doses ranging from 25—150 mg/kg have typically been administered.[3] However, our studies showed that a 10 mg/kg dose of FIN can almost completely deplete the GABAergic neurosteroids (AP and 3α,5α-Adiol) in the rat brains.

3α-DHP has been reported to enhance the effect of GABA by potentiating the GABA-induced Cl^- conductance,[13] and this study demonstrates that the brain level of 3α-DHP is also increased by the acute stress (Fig. 3b). Based on these data, this steroid would also work as an endogenous anxiolytic agent under stressful conditions. Therefore, we expected that in the FIN-treated rats, the synthesis of 3α-DHP might be increased in order to utilize it as the alternative to AP in the defensive response to acute stress, but no change was found in its brain level by the administration of FIN (Fig. 3b). It has been known that both the rat 3α-HSD and the human type 2_{brain} 3α-HSD convert PROG to 3α-DHP.[14] As the type 2_{brain} 3α-HSD mRNA is found in different amounts in different regions of the brain (type 2_{brain} 3α-HSD mRNA is predominately expressed in the thalamus, subthalamic nuclei and amygdala),[15] the 3α-DHP production may occur in specific regions of the brain. Therefore, a further examination analyzing the regional level changes in 3α-DHP is required to arrive at a reliable conclusion.

It has been known that the brain PROG, 20α-DHP and DOC levels are also increased by acute stress[8,11] and similar results were reproduced in this study (Figs. 3c—e). Although there were no changes in the brain PROG and DOC levels by the FIN treatment (Figs. 3c, e), the 20α-DHP level in the rats administered with 10 mg/kg of FIN was significantly higher than that in the saline-administered rats (Fig. 3d). These data indicate that the 20α-reduction is promoted when the 5α-reduction pathway is inhibited during the PROG metabolism in the brain. Although the biological significance of 20α-DHP in the brain is still not well elucidated, it is thought to be a catabolic metabolite to be excreted from the target organ or tissue. In humans, aldo-keto reductase 1C1 (AKR1C1) works as the 20α-HSD for PROG and AP, and it is thought that this enzyme decreases the AP concentrations in the brain by inactivating AP and eliminating its precursor, PROG, from the synthetic pathways.[16] The 21-hydroxylation of PROG catalyzed by the cytochrome P450 2D isoforms is also considered to be involved in the regulation of the brain neurosteroid contents.[17] The present study demonstrates that the rat brain decreases the accumulated PROG by its conversion into 20α-DHP rather than into DOC.

CONCLUSION

In this study, we developed LC-MS/MS methods that enable the reproducible and accurate determination of trace amounts of brain neurosteroids. The animal studies using these methods clarified the changes in the brain neurosteroid levels due to the FIN treatment; a 10 mg/kg dose of FIN can almost completely deplete AP in the rat brains, and 20α-DHP is enhanced when the 5α-reduction pathway is inhibited during the PROG metabolism in the brains.

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