Effects of morphine dependence and withdrawal on levels of neurosteroids in rat brain

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KEY WORDS morphine dependence; withdrawal; steroids; mass fragmentography

ABSTRACT

AIM: To investigate the effects of morphine dependence and withdrawal on the concentrations of neurosteroids in rat brain. **METHODS:** A method of simultaneous quantification of neurosteroids by gas chromatography-mass spectrometry (GC-MS) had been established. **RESULTS:** The chronic morphine administration (ip) resulted in a marked decrease in the brain concentrations of pregnenolone (PREG), progesterone (PROG), and pregenenolone sulfate (PREGS) in rats killed 6 h after the last treatment. In contrast, there were no significant effects of morphine dependence on the brain concentrations of allopregnanolone (AP), dihydroepiandrosterone (DHEA), and dihydroepiandrosterone sulfate (DHEAS). Naloxone-induced withdrawal produced a significant increase in the concentrations of PREG, PROG, AP, DHEA, PREGS, and DHEAS as compared with the control group. **CONCLUSION:** Morphine dependence and withdrawal affected the concentrations of neurosteroids in rat brain, which suggests that endogenous neurosteroids in brain might be related to the development of morphine dependence and withdrawal.

INTRODUCTION

Neurosteroids were defined steroids that are synthesized from cholesterol or other early precursors in nervous system. They are still present after removal of peripheral steroidogenic glands. Neurosteroids are endogenous modulators of neuronal functions responsible for many biological and pathophysiological effects[1]. Neurosteroids, particularly 3a-hydroxy ring-A-reduced metabolites of progesterone (PROG) and allopregnanolone (AP), can act as positive modulators at the $GABA_A$ receptors. *In vivo*, effects of these neurosteroids are similar to those produced by other positive modulator of $GABA_A$ receptors such as benzodiazepines and barbiturates. Many of these neurosteroids showed anticonvulsant, myorelaxant, anesthetic, and anxiolytic effects when they were administered to animals[2,3]. Neurosteroid sulfate esters, pregnenolone sulfate (PREGS), and dihydroepiandrosterone sulfate (DHEAS), have an excitatory cellular action, since they antagonize the action of $GABA_A$ and potentiate the activation of NMDA receptors. Moreover, they can improve memory and learning, increase dopamine (DA) release in the rat nucleus accumbens, and enhance the dopaminergic response to morphine[4].

Recent studies have demonstrated that the development of tolerance and dependence of morphine could be inhibited by concomitant chronic administration of neurosteroids such as AP, PREGS, or PROG[5]. The systemic administration of these compounds prevents the development of morphine tolerance and attenuates

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Received 2003-09-16 Accepted 2004-05-31

abstinence behavior which suggesting that changes in the concentrations of endogenous neurosteroids might be related to the development of morphine dependence and withdrawal.

The present study was carried out to investigate the effects of morphine dependence and withdrawal on the concentrations of endogenous neurosteroids in male rat brain.

METERIALS AND METHODS

Chemicals and reagents Pregnenolone (PREG), PROG, AP, dihydroepiandrosterone (DHEA), PREGS, and DHEAS were purchased from Sigma-Aldrich Co (USA). Hepatafluorobutyric acid anhydride (HFBA) was purchased from Pierce (USA). MeOH was (HPLC grade) from Kangkede (Tianjin, China). All other organic solvents were analytical grade and further purified before use by distillation. Morphine hydrochloride injection was purchased from Shenyang First Pharmaceutical Factory (China).

Animals Male Sprague-Dawley rats weighing 180-200 g were provided by Experimental Animal Center of Hebei Province (Grade II, Certificate No 04084). All animals were housed at room temperature (20-25 ºC) and allowed to adapt to laboratory conditions for at least 5 d before the initiation of any experiment. The animals were housed under a standard light/dark cycle with free access to food and water.

Animal experiments Animals were randomly divided into 3 groups: control, morphine dependence, and morphine withdrawal. Morphine dependence was induced by a 7-d pretreatment (Tid, ip) with increasing doses of morphine (at 5, 10, 15, 20, 30, 40, and 50 mg/kg, respectively). Control group received an equal volume of saline. The rats in control and morphine groups were killed 6 h after the last injection. Morphine withdrawal was precipitated by naloxone (2 mg/kg). After injection of naloxone for 15 min, morphine withdrawal signs were observed and evaluated for 15 min, and then the rats in withdrawal group were killed. In each experiment, the rats were killed between 18:00-20:00, alternating the rats from each group to minimize the inter-group variability due to circadian fluctuations of circulating steroid levels. The brain was quickly removed from skull, the cerebellum was discarded and then the anterior brain was divided into two hemispheres. Brain tissues were frozen at -70 ºC until steroid assays.

Brain steroid extraction and analysis The method previously described by Liere *et al*[6] was used with some modification. In brief, the brain tissue homogenates (500 mg of tissue in 5 mL of phosphate buffered saline) were extracted three times with an equal volume of ethyl acetate and the organic phase was pooled. The aqueous phase was collected and again extracted three times with an equal volume of chloroform/2-butanol (50/50, v/v). The organic phase was pooled and dried at 60 ºC under a gentle stream of N_2, then dissolved in 1 mL MeOH-H_2O (50:50, v/v) and sonicated for 5 min, respectively. A clean-up step was performed by solid-phase extraction (SPE) on C18 minicolumns (500 mg, 6 mL, Supelco). Samples were deposited on columns previously activated successively with 5 mL of MeOH, 5 mL of water, and 5 mL of MeOH-H_2O (50:50 v/v). The fraction of steroid sulfate was eluted with 5 mL of MeOH-H_2O (50:50, v/v), the unconjugated steroid was eluted with 5 mL of MeOH-H_2O (90:10, v/v), and then evaporated. The unconjugated steroids and those obtained after solvolysis were derivatized by adding 20 μL of HFBA and 200 μL of anhydrous acetone, and then carried out at room temperature for 30 min. After evaporation, the residue was redissolved in 100 μL of hexane.

The derivatized samples (2 μL) as well as derivatized calibration solutions (1 μL) were injected with an autosampler into GC-MS system (HP5973). The silica capillary column (30 m×0.25 mm) was coated with 0.25-μm layer of cross-linked HP-5MS (5 % phengl methyl siloxane). GC was performed in the splitless mode. The temperature in the oven was initially 50 ºC for 1 min then increased to 150 ºC at a rate of 30 ºC/min and then to 280 ºC at a rate of 10 ºC/min. The temperature in injection chamber was 250 ºC and the flow-rate of helium (carrier gas) was maintained constant at 1 mL/min. The mass spectrometer was operated in the electron impact (EI) mode. The temperature in the ionization chamber and the interface were 230 ºC and 280 ºC, respectively.

The recoveries and calibration curves were prepared by spiking pooled brain tissue homogenate in two different protocols. With the first protocol, blank brain matrix samples were extracted and assayed. The second protocol involved adding steroids to the matrix, and then extracting and assaying. The data of adding fraction was calculated by subtracting the content of blank brain matrix samples. The recoveries were determined by adding the steroids (at 1, 5, 10 ng) to the blank brain matrix. The recovery of each steroid was over 50 %.

The accuracy values were 97.4 %±9 %, 94.6 %±8 %, 107 %±5 %, and 98.3 %±5 % for PREG, DHEA, PROG, and AP, respectively. The linearity of neurosteroids was evaluated by supplementing samples with progressively increasing concentrations of a mixture of standard steroids (0.5, 1, 5, 10, 20, and 50 ng in 5 mL of brain matrix), and then extracting and assaying. The inter-day variations for the assays were less then 15 %.

Statistics Data were expressed as the mean±SD. The statistical significance of differences was analyzed by independent samples *t*-test. $P<0.05$ was considered significant. Linear regression analysis using the least square method was calculated for the calibration curve of each steroid.

RESULTS

Identification of neurosteroids The choice of the derivatization reaction was based on the presence of a hydroxyl group in the steroid structure. The formation of such steroid derivatives results in a decrease of polarity and an increase of volatility and thermal stability, which are crucial advantages for GC assay. PREG-HFB, DHEA-HFB, PROG-HFB, and AP-HFB were from unconjugated steroids by derivatization with HFBA. PREG-HFB and DHEA-HFB were from sulfated steroids after solvolysis. EI mass spectra of neurosteroids were shown in Fig 1 and Fig 2. The ions selected for DHEA-HFB, PREG-HFB, PROG-HFB and AP-HFB were *m/z* 270, *m/z* 298, *m/z* 510, and *m/z* 514, respectively, based on the relative intensity of the respective target ion in the corresponding mass spectra.

EI GC/MS selected ion chromatograms of neurosteroids in brain were shown in Fig 3. The GC conditions gave identical retention times for PREG-HFB

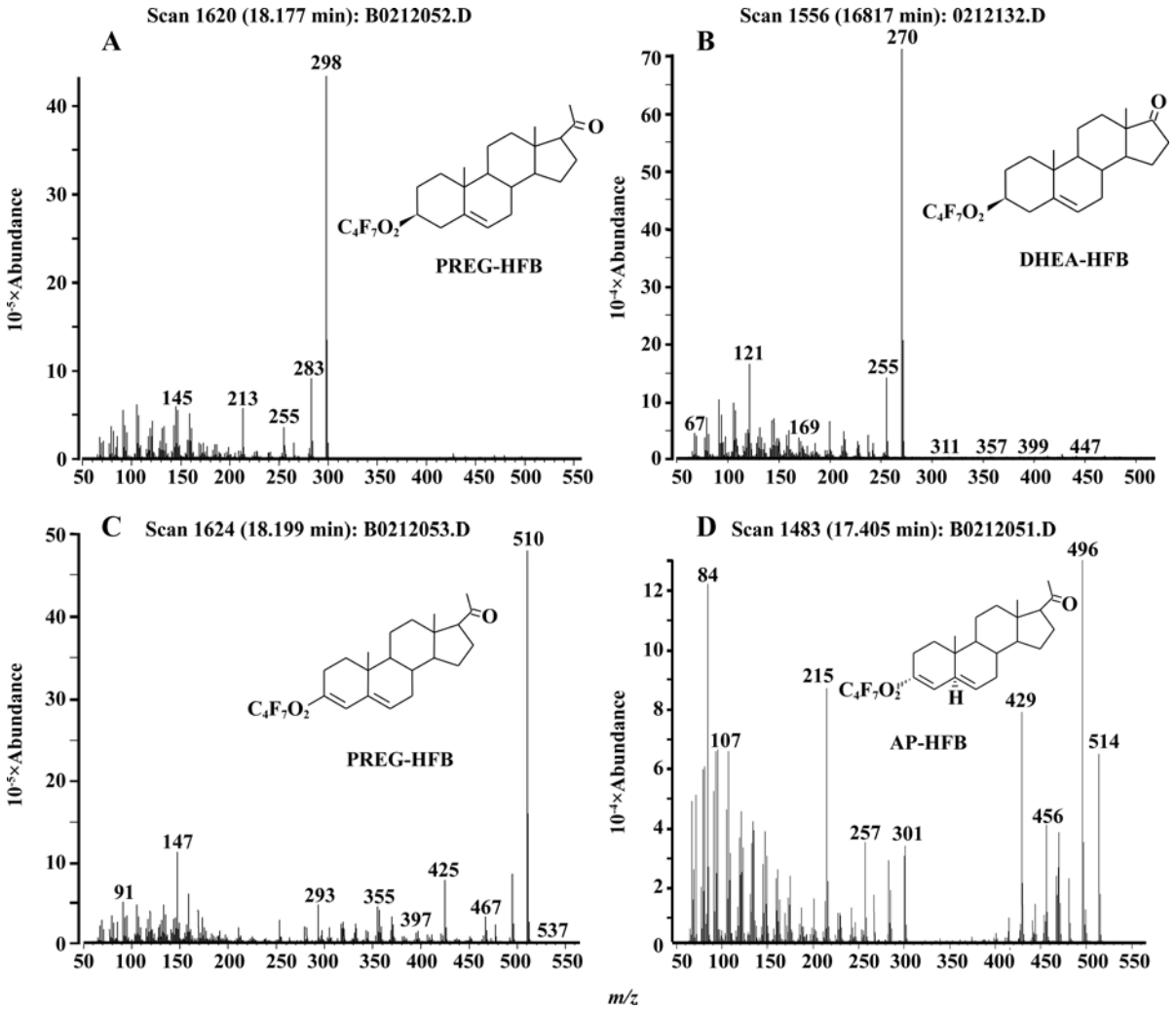

Fig 1. Full scan mass spectrum for the hepafluorobutyric acid derivative of the standard neurosteroids pregnenolone (A), dihydroepiandrosterone (B), progesterone (C), and allopregnanolone (D).

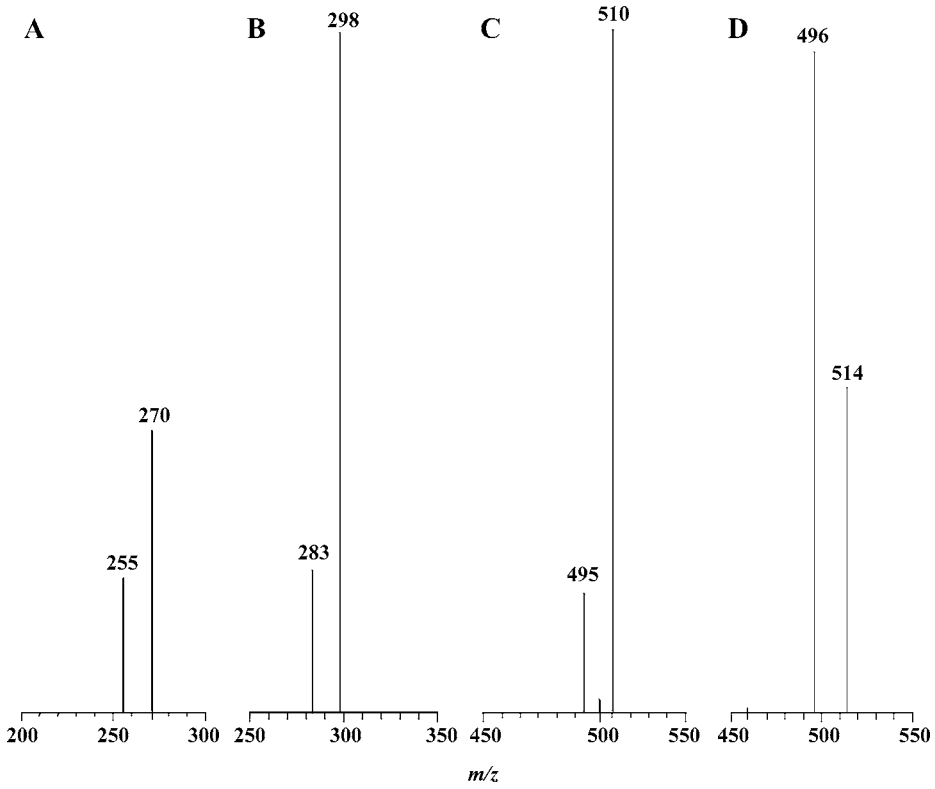

Fig 2. Selected ion mass spectra of the endogenous neurosteroids dihydroepiandrosterone (A), pregnenolone (B), progesterone (C), and allopregnanolone (D).

and PROG-HFB. However, since ions of different mass were monitored, ie *m/z* 298 for PREG-HFB and *m/z* 510 for PROG-HFB, there was no interference in the quantification of these steroids. The other steroids were clearly separated by the HP-5MS column.

Peak area of specific *m/z* for each steroid versus the concentration of steroid was fitted linearly. Correlation coefficients were obtained for PREG (y=948.89x-304.91, r=0.9976), DHEA (y=2190.77x-407.62, r=0.9991), PROG (y=603.43x+158.91, r=0.9986), and AP (y=404.43x-118.16, r=0.9988) (Fig 4).

Naloxone-precipitated withdrawal syndrome After 15-min injection of naloxone, the rats in morphine dependence produced significantly withdrawal syndrome including wet dog shakes, writhing, defecation, tremor, and ptosis. The loss of weight was 7.8 %. Control group rats showed no withdrawal signs over the course of the study.

Effects of morphine dependence on the concentrations of neurosteroids Chronic morphine administrations resulted in a marked decrease in the brain concentrations of PREG and PROG by 62 % and 92 % (P<0.01) respectively in rats killed 6 h after the last treatment (Tab 1). The concentrations of PREGS decreased by 60 % (P<0.01). In contrast, there were no significant effects of morphine dependence on the brain concentrations of DHEA and DHEAS.

Effect of morphine withdrawal on the concentrations of neurosteroids Morphine withdrawal induced a significant increase in the concentrations of PREG by 318 %, PROG by 55 %, AP by 118 %, DHEA by 108 %, PREGS by 102 %, and DHEAS by 107 % respectively compared with control group (Tab 1).

The relationship between the PREG and its metabolites A correlation analysis between PREG values and PROG values, revealed a significant positive correlation for PREG versus PROG (r=0.894, P<0.01) and for PREG versus AP (r=0.723, P<0.01) in all animals examined. A significant correlation existed between the PREG and PREGS (r=0.756, P<0.01) in all animals (Fig 5). In contract, there was no significant correlation between PREG values and DHEA or DHEAS values.

DISCUSSION

Morphine might affect the concentrations of neurosteroids via several mechanisms, including modu-

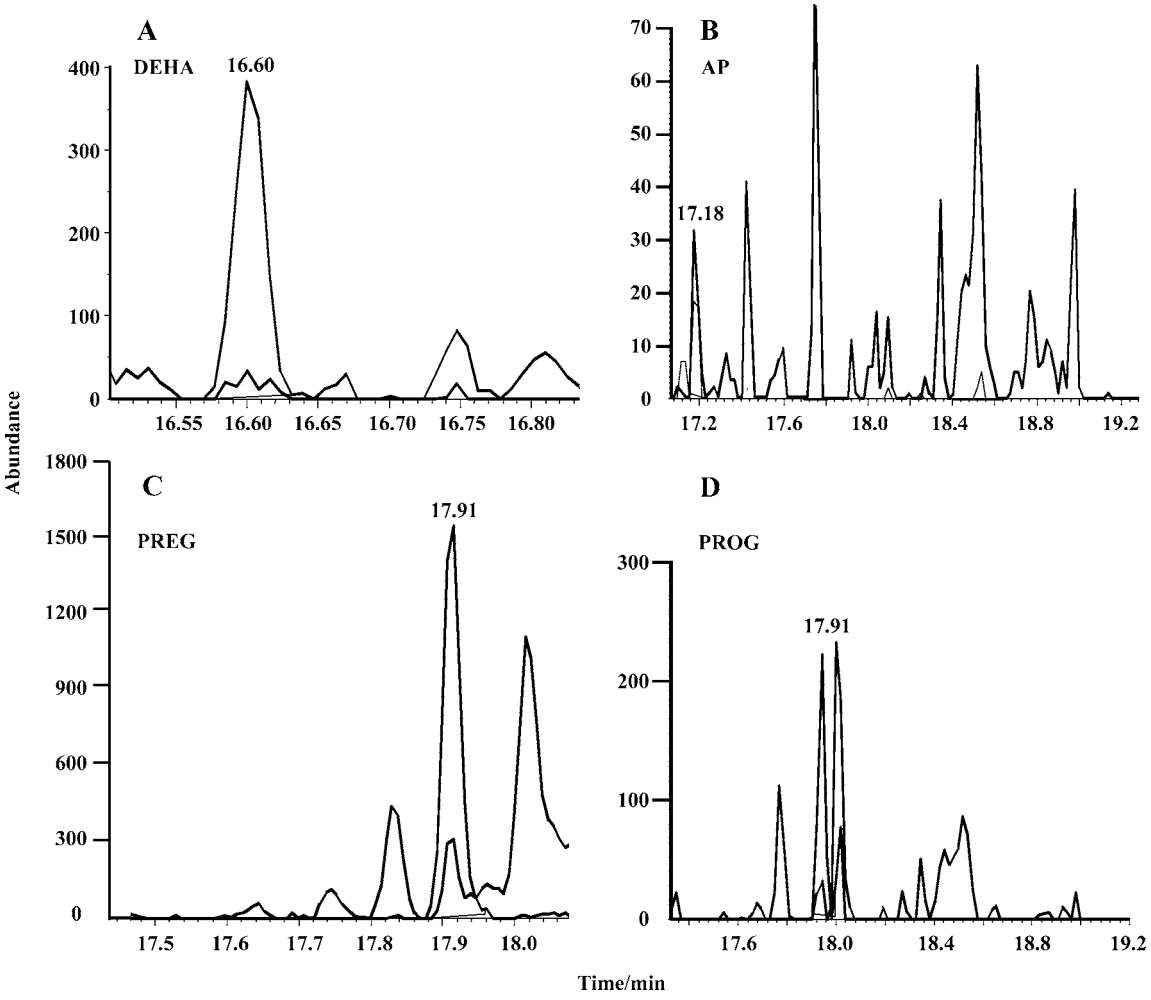

Fig 3. EI GC/MS ion chromatograms of neurosteroids dihydroepiandrosterone (A), allopregnanolone (B), pregnenolone (C), and progesterone (D) in brain samples of male rats.

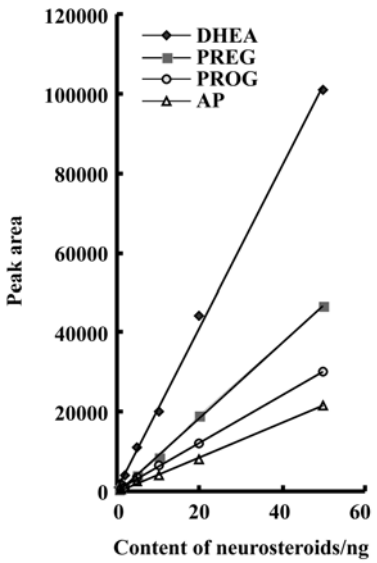

Fig 4. Calibration curves for neurosteroids.

lation of biosynthesis, catabolism or release of endogenous steroid pools. Chronic morphine administration was found to inhibit the hypothalamic-pituitary-adrenal gland (HPA) axis and the hypothalamic-pituitary-gonad (HPG) axis, reduced the levels of plasma corticosterone, testosterone, ACTH, and LH in rats[7,8]. The inhibitory effect would then decrease the production and release of neurosteroids.

We observed that chronic administration of morphine resulted in a significant decrease in the concentrations of PREG, PROG, and PREGS in the brain tissue of male rats. However, chronic morphine administration did not alter concentrations of DHEA and DHEAS. One possible explanation is that rate of synthesis/metabolism of these steroids is differently affected by morphine in brain. Meanwhile, our results showed that there was a significant positive correlation between PREG and its metabolites PROG or AP in rat brain.

Tab 1. Effects of morphine dependence and withdrawal on the levels of neurosteroids in male rat brain. The rats were treated with saline (control), morphine hydrochloride (dependence), or naloxone (withdrawal). *n*=10. Mean±SD. $^cP<0.01$ *vs* control.

Neurosteroids	Levels of neurosteroids (ng/g brain tissue)		
	Control	Dependence	Withdrawal
Pregnenolone	12.9±2.8	4.9±1.6^c	53.9±21.4^c
Progesterone	10.7±3.3	0.7±0.3^c	23.9±9.4^c
Allopregnanolone	1.7±0.8	1.4±0.3	3.7±1.2^c
Dihydroepiandrosterone	1.3±0.5	1.2±0.2	2.7±0.8^c
Pregnenolone sulfate	5.0±2.0	2.0±0.4^c	10.1±4.1^c
Dihydroepiandrosterone sulfate	3.0±1.4	2.8±0.6	6.2±1.8^c

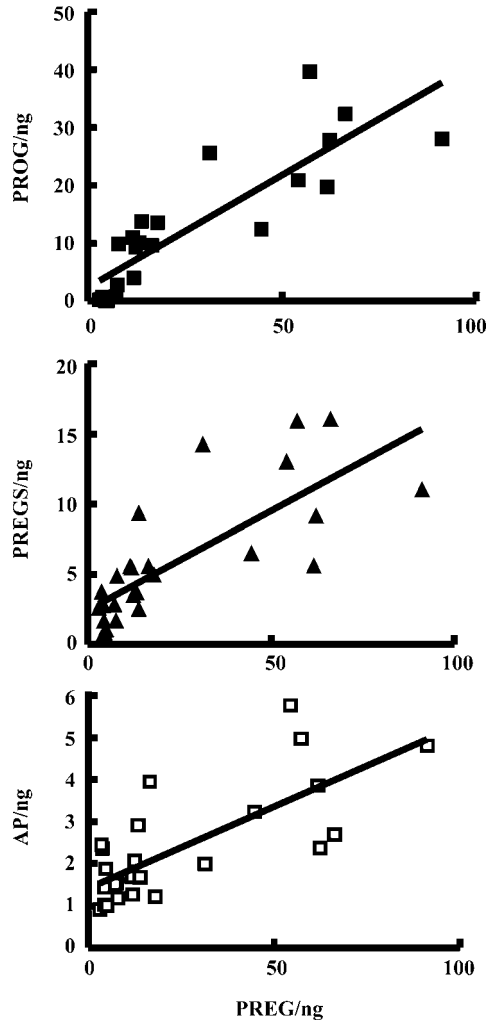

Fig 5. The relationship between the PREG and its metabolites (PROG, PREGS, AP) in rat brains from all animals examined.

These data suggest that morphine-induced decrease of rat brain neurosteroids PREG, PREGS and PROG may depend on the local expression or activity of steroidogenic enzymes in the CNS. In fact, a selective action on steroidogenic enzymes by other pharmacological treatment has been shown[9-11]. Our other experiments have also shown that the expression of steroidogenic enzymes in CNS can be inhibited by morphine treatment (data are not shown).

Our data showed that naloxone-induced withdrawal resulted in a significant increase in the brain concentrations of neurosteroids PREG, PROG, AP, DHEA, PREGS, and DHEAS. The effects are similar to those induced by various acute stress paradigms. Exposure of rats to mild foot shock, CO_2 inhalation, forced swimming, or handling maneuvers that precede killing induces rapid and marked increases in both brain and plasma concentrations of neurosteroids[12,13]. The levels of PREG, DHEA, and PREGS increased significantly in plasma and brain after CRF and ACTH (two typical stress hormones) administration in rats[14]. Our other experiments also support the idea that the effects of morphine withdrawal on the levels of brain neurosteroids might mainly depend on the activity of HPA axis, but not steroidogenic enzymes in the CNS (data are not shown). Withdrawal of morphine induces a stress-like response of the HPA axis in rats. The consequent activation of the HPA axis would then increase the secretion of neurosteroids.

The decrease in the concentration of PROG induced by morphine administration might account for the anxious behavior of rats treated with morphine. Negative mood symptoms are well-known effects of morphine dependence. Both PREG and AP injected systemically or intracerebroventricularly induce anxiolytic effects in various behavior tests[15]. Thus, the decrease in PROG (precursor of AP) concentration

induced by morphine dependence might be an important factor in the development of the mood side effects of abuser.

In conclusion, our data provide the evidence that morphine dependence markedly decreased the brain concentrations of neurosteroids PREG, PROG, and PREGS. Because both morphine and neurosteroids are implicated in the regulation of motional behavior, our data may open a new perspective to understand the molecular mechanisms underlying the dependence of morphine.

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