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# Testosterone is required for corticosteroid-binding globulin upregulation by morphine to be fully manifested

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**Abstract**

We previously reported that morphine increases the concentration of corticosteroid-binding globulin (CBG) in blood of male, but not female, rats. This pronounced sexual dimorphism suggested that CBG upregulation by morphine might be androgen-dependent. In the current studies, we found that castration, whether performed just before or just after puberty or in adulthood, increased the concentration of CBG in adult male rats. Naltrexone did not prevent this increase and, therefore, it does not appear to be attributable to the release of endogenous opioids. Exposure to morphine for 1 week in adulthood increased (≈100%) the concentration of CBG in intact, i.e., sham-castrated, males. The CBG levels of castrated rats treated with morphine did not differ from those of intact rats treated with morphine. However, because castration increased the concentration of CBG, the difference between the placebo and morphine groups decreased with time after castration. At 4 weeks after castration, the difference between the morphine and placebo groups (19%) was no longer statistically significant. Testosterone replacement prevented the rise in CBG levels following castration and maintained the magnitude of the difference between placebo and morphine-treated rats within the normal range. Thus, testosterone appears necessary for morphine effects on CBG to be fully manifested. 

*Keywords:* Morphine; Corticosterone; CBG; Corticosteroid-binding globulin; Testosterone; Castration

## 1. Introduction

Recently, we suggested that chronic exposure to morphine might suppress glucocorticoid activity [9,10]. That conclusion was based, in part, on the discovery that, although corticosterone levels are normal after chronic exposure to morphine, the concentration of corticosteroid-binding globulin (CBG) in blood of male rats is markedly elevated [9,10]. Elevated CBG levels occur by 3 days and appear to be maximal by 7 days after morphine exposure. Thereafter, CBG levels remain elevated while morphine is detectable in blood, returning toward normal only as morphine clears from the general circulation [10].

This morphine-induced increase in CBG is significant because CBG appears to be important in regulating the amount of glucocorticoid reaching target tissues, including brain, the immune system and wounds [3,6,11–14]. Accordingly, an increase in CBG would bind a greater than normal proportion of hormone in blood and, thereby, reduce the amount available to target tissues. Thus, even though chronic exposure to morphine does not affect corticosterone secretion, we calculated that it may decrease the amount of hormone available to target tissues by as much as 95% under conditions that are unstressful and by as much as 90% after exposure to a mild stressor [9,10].

At present, little is known about the physiological parameters that determine how severely morphine affects CBG. However, we recently found that CBG is much more susceptible to upregulation in males than in females [9]. In fact, in contrast to males, chronic exposure to morphine has no effect on CBG in adult female rats. This pronounced sexual dimorphism suggests that gonadal hormones might play a role in determining the impact of morphine on CBG. Testosterone, in particular, is known to have potent effects on CBG and has been reported to masculinize the CBG phenotype during puberty [8]; adult females have CBG

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levels that are more than twice as high as those in males [4,8,9]. Thus, we carried out studies to examine the effects of castration just prior to or just after puberty or in adulthood on the ability of morphine to upregulate CBG in adult male rats. We found that castration causes a testosterone-preventable increase in CBG levels in blood. Furthermore, testosterone appears to be necessary for CBG upregulation by morphine to be fully manifested.

## 2. Materials and methods

### 2.1. Animals

Male rats from Harlan Sprague–Dawley, (Cumberland, IN) were used for all experiments. The rats were housed in groups of two to three per cage with lights in the colony room on from 6 AM to 6 PM. Prepubertal rats were raised in our colony from purchased pregnant females and were weaned at 20–22 days of age. Otherwise, rats were purchased and allowed to acclimate to our colony for at least 1 week before treatment. Animal use was in accordance with the *Washington University Animal Care and Use Committee* guidelines.

Castration and sham operations were performed under Brevital® anesthesia (40 mg/kg for adults and 20 mg/kg for prepubertal rats).

Trunk blood was collected after decapitation. All rats were sacrificed between 10 AM and 11 AM when corticosterone secretion is normally low in the males of our colony. For sacrifice, the rats were individually carried by hand to a procedural room that was isolated from the colony room. Rats were sacrificed within 15 s of being picked up, and no more than 60 s elapsed between the first and last rat in a cage.

### 2.2. Drug and hormone treatments

Placebo, morphine (75 mg) or naltrexone (30 mg) pellets were implanted subcutaneously under Brevital® anesthesia (40 mg/kg) and were allowed to remain in place for the duration of the experiment. All rats were adults at the time of morphine or naltrexone treatment. Two morphine pellets were implanted for 7 days, which is an optimal dose and time for CBG upregulation [10]. Four naltrexone pellets were used because we [5] previously found that dose to induce a maximal rate of opioid receptor upregulation in brain — an indication that the activity of EOP is completely blocked.

Testosterone was administered by implanting hormone-filled Silastic capsules s.c. [7]. The capsules were prepared using Silastic tubing (70 mm in length, 1.57 mm i.d., 3.13 mm o.d.; Dow Corning) plugged at each end with wood and sealed using Type A medical adhesive (Dow Corning). Each capsule contained 60 mm of crystalline testosterone. Empty capsules were used as the placebo. The capsules were soaked for 30 min at room temperature in phosphate-buffered saline before implantation.

### 2.3. CBG levels

CBG was measured using a protein-binding assay that we previously described in detail [9,10]. All steps were carried out at 0–4°C. In brief, endogenous steroids were removed from serum by charcoal adsorption. Aliquots of steroid-free sample were then incubated overnight with 7.0 nM [$^3$H]corticosterone with or without 16 μM corticosterone to define specific binding. Bound and free [$^3$H]corticosterone were separated using Sephadex LH-20 columns. Specific binding was expressed as picomoles specific binding per milligram serum protein. Protein content was determined by the method of Bradford [1].

### 2.4. Morphine levels in serum

Morphine was assayed in serum using a radioimmunoassay (RIA)kit purchased from Diagnostic Products (Los Angeles, CA). The antibody is highly specific for morphine with less than 0.03% cross-reactivity for morphine-3-glucuronide and less than 0.1% cross-reactivity for morphine-6-glucuronide. The lower limit of sensitivity of the assay was 5 ng/ml and the standard curve was linear over the range 5 to 1000 ng/ml. Intra- and interassay variability was less than 5%.

### 2.5. Serum testosterone levels

Testosterone was assayed using an RIA kit purchased from Diagnostic Products (Los Angeles, CA). The antibody is highly specific for testosterone with 3.3% cross-reactivity for 5α-dihydrotestosterone and 0.02% or less cross-reactivity for estradiol, progesterone and corticosterone. The lower limit of sensitivity of the assay was 0.2 ng/ml and the standard curve was linear over the range 0.2 to 16 ng/ml. Intra- and interassay variability was less than 5%.

### 2.6. Statistical analysis

All data are expressed as mean±standard error of the mean. *t*-Tests were used to compare two groups, otherwise, data were subjected to analysis of variance followed by post hoc tests. Differences between groups were considered statistically significant when the probability that they occurred by chance was less than 0.05.

## 3. Results

### 3.1. Prepubertal castration

Castration at 27 days of age, i.e., just prior to puberty, increased the concentration of CBG in adulthood (Fig. 1). Chronic exposure to morphine (two pellets)for the 7 days prior to sacrifice (i.e., in adulthood) increased CBG levels

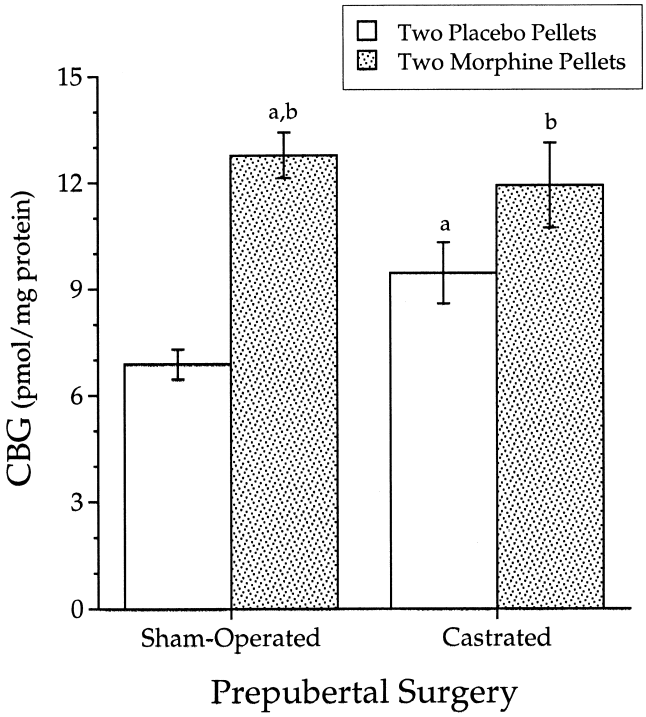


Fig. 1. Effects of castration just prior to puberty on CBG levels in adulthood and on CBG upregulation by morphine. Rats were castrated or sham-operated at 27 days of age and two placebo or morphine pellets were implanted s.c. at 60 days of age, i.e., in early adulthood. Samples were collected 7 days after pellet implantation. [a]Indicates $p<0.05$ versus the placebo sham-operated group. [b]Indicates $p<0.05$ versus placebo controls. $N=7-10$ rats/group. $F(1,32)_{surgery}=1.2$, $p=0.28$; $F(1,32)_{drug}=30$, $p=0.001$; $F(1,32)_{surgery \times drug}=4.9$, $p=0.03$.

in sham-operated rats (Fig. 1). CBG levels in the castrated rats treated with morphine did not differ significantly from those of the sham-operated rats treated with morphine (Fig. 1). That is, CBG levels were similar in morphine-treated rats regardless of whether they were castrated or not. However, because castration increased CBG levels, the magnitude of the difference between the morphine and placebo groups was smaller in castrated rats. Specifically, CBG levels in sham-operated rats were 86% higher in the morphine group than in the placebo group, whereas, in castrated rats levels were only 26% higher in the morphine group than in the placebo group. An independent replication of the experiment gave essentially the same results (sham-operated+placebo=6.6±0.9 pmol/mg protein; sham-operated+morphine=12.6±1.2 pmol/mg protein; castration+placebo=9.9±0.6 pmol/mg protein; castration+morphine=13.4±0.8 pmol/mg protein [$F_{drug}(1,35)=27.5$, $p=0.0001$; $F_{surgery}(1,35)=5.1$, $p=0.03$; $F_{drug \times surgery}(1,35)=1.3$, $p=0.19$]).

Prepubertal castration did not significantly affect the concentration of morphine in serum at the time of sacrifice (sham-operated=644±60 ng/ml versus castrated=545±52 ng/ml; $t(15)=1.5$, $p=0.15$).

### 3.2. Postpubertal castration

Castration at 38 days of age, i.e., just after puberty, had effects on CBG in adulthood (Fig. 2) that were essentially the same as those of prepubertal castration. Castration increased CBG levels in adulthood and, thereby, decreased the magnitude of CBG upregulation by morphine. Specifically, CBG levels in sham-operated rats were 135% higher in the morphine group than in the placebo group, whereas, in castrated rats levels were only 38% higher in the morphine group than in the placebo group.

### 3.3. Castration in adulthood

Castration at 60 days of age, i.e., in early adulthood, had effects on CBG that were essentially the same as those of castration during development. Castration resulted in significantly higher CBG levels 2, 3 and 4 weeks later (Table 1). Chronic exposure to morphine (two pellets) for the 7 days prior to sacrifice increased CBG levels in sham-operated rats at all three time points after surgery. CBG levels in the castrated rats treated with morphine did not differ significantly from those in the sham-operated rats

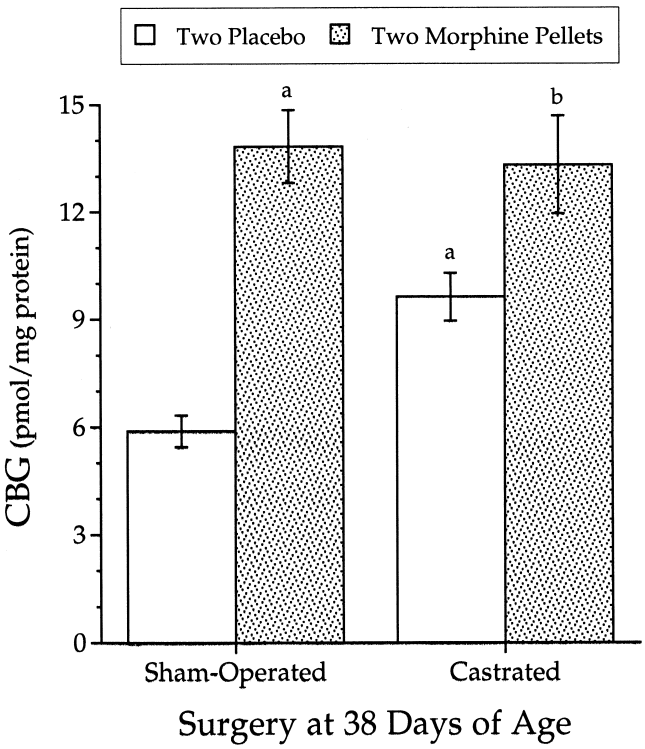


Fig. 2. Effects of castration just after puberty on CBG levels in adulthood and on CBG upregulation by morphine. Rats were castrated or sham-operated at 38 days of age and two placebo or morphine pellets were implanted s.c. at 60 days of age, i.e., in early adulthood. Samples were collected 7 days after pellet implantation. [a]Indicates $p<0.05$ versus the placebo sham-operated group. [b]Indicates $p<0.05$ versus placebo castrated controls. $N=11-13$ rats/group. $F(1,43)_{surgery}=2.7$, $p=0.11$; $F(1,43)_{drug}=35$, $p=0.0001$; $F(1,43)_{surgery \times drug}=4.6$, $p=0.04$.

Table 1
Effects of castration in adulthood on CBG levels and on CBG upregulation by morphine

| Weeks Postsurgery | Endocrine/drug status[a] | | | | | |
|---|---|---|---|---|---|---|
| | Sham-operated | | Castrated | | Castrated+testosterone | |
| | Placebo | Morphine | Placebo | Morphine | Placebo | Morphine |
| 2 | 6.3 ± 0.3 | 13.0 ± 1.1[b] | 9.5 ± 0.7[c] | 14.3 ± 0.9[b,c] | – | – |
| 3 | 7.7 ± 0.7 | 16.0 ± 1.1[b] | 10.7 ± 1.0[d] | 14.9 ± 0.7[b] | – | – |
| 4 | 8.7 ± 0.2 | 16.8 ± 0.8[e] | 12.6 ± 1.1[d] | 15.0 ± 1.0 | 6.2 ± 0.7[d] | 12.5 ± 1.0[e] |

[a] Rats were castrated or sham-operated at approximately 60 days of age and were sacrificed 2, 3, or 4 weeks later. Two placebo or morphine pellets were implanted s.c. 7 days prior to sacrifice. For the 4-week interval, rats were implanted with an empty silastic capsule (data shown under heading "Castrated") or a silastic capsule containing testosterone at the time of castration. Each time interval was carried out as an independent experiment due to practical considerations. Thus, no statistical comparisons were made across time intervals.
[b] Indicates $p<0.05$ for the main effect of morphine versus placebo.
[c] Indicates $p<0.05$ for the main effect of castration versus sham operation.
[d] Indicates $p<0.05$ versus the sham-operated placebo group.
[e] Indicates $p<0.05$ versus placebo group of the same endocrine status.

treated with morphine. However, because castrated rats had higher CBG levels than sham-operated rats, the magnitude of the difference between the morphine and placebo groups was smaller in the castrated rats. Specifically, in sham-operated rats CBG levels were 106%, 108% and 93% higher in the morphine groups than in the placebo groups at 2, 3 and 4 weeks after surgery, respectively. In castrated rats, CBG levels were only 51%, 39% and 19% higher in the morphine groups than in the placebo groups at 2, 3 and 4 weeks after surgery, respectively, and the difference at 4 weeks was not statistically significant (Table 1).

### 3.4. Castration in adulthood with testosterone replacement

To determine whether the effects of castration (4 weeks) were testosterone-preventable, groups of castrated rats were implanted with testosterone-filled silastic capsules at the time of surgery. These groups were run as part of the 4-week castration experiment described in the preceding section. Testosterone levels at the time of sacrifice (i.e., at 4 weeks following castration) were undetectable in castrated rats implanted with empty silastic capsules and above normal in castrated rats implanted with testosterone-filled capsules. Morphine did not affect the concentration of testosterone at the time of sacrifice. Testosterone levels at sacrifice were: sham-operated with no silastic capsule+morphine placebo=1.6±0.3 ng/ml; sham-operated with no silastic capsule+morphine=1.5±0.3 ng/ml; castrated with empty capsule+morphine placebo=not detectable; castrated with empty capsule+morphine=not detectable; castrated with testosterone capsule+morphine placebo=2.9±0.2 ng/ml; castrated with testosterone capsule+morphine=3.2±0.1 ng/ml ([$F_{\text{hormonal state}}(2,66)=123$, $p=0.0001$; $F_{\text{drug}}(1,66)=0.1$, $p=0.80$; $F_{\text{hormonal state}\times\text{drug}}(2,66)=0.6$, $p=0.53$]).

Testosterone replacement prevented the castration-induced increase in CBG. More precisely, the testosterone treatment, which produced above normal testosterone titers (see data in preceding paragraph), resulted in CBG levels that were ≈30% lower than normal (Table 1). For castrated rats that received testosterone replacement, CBG levels were 102% higher in the morphine group than in the placebo group at the time of sacrifice. That difference is somewhat greater than occurred in the sham-operated animals where CBG levels were 93% higher in the morphine group than in the placebo group and it is much greater than the 19% difference between the morphine and placebo groups of castrated rats which did not receive testosterone replacement (Table 1).

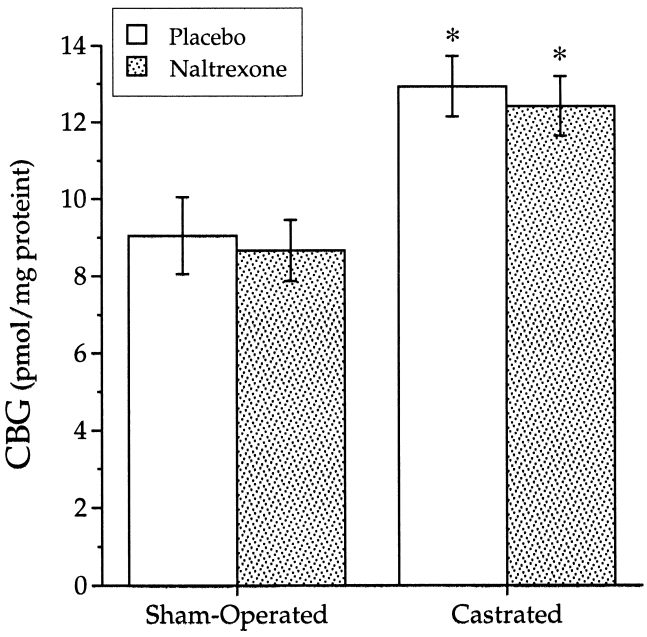


Fig. 3. Effects of the opiate antagonist naltrexone on CBG in sham-operated and castrated adult males. Rats were castrated or sham-operated at approximately 60 days of age and simultaneously four placebo or naltrexone pellets were implanted s.c. Samples were collected 2 weeks later. *Indicates $p<0.05$ for main effect of surgery. $N=8-12$ rats/group. $F(1,38)_{\text{surgery}}=21$, $p=0.001$; $F(1,38)_{\text{drug}}=0.3$, $p=0.59$; $F(1,38)_{\text{surgery}\times\text{drug}}=0.005$, $p=0.94$.

### 3.5. Castration in adulthood with naltrexone treatment

To determine whether castration increases CBG levels by releasing EOP, adult rats were sham-operated or castrated and simultaneously implanted with four placebo or naltrexone pellets. Samples were collected 2 weeks later. As in the experiments described above, castration caused a significant increase in CBG. Naltrexone did not affect CBG levels in either sham-operated or castrated rats (Fig. 3).

## 4. Discussion

Castration, whether performed before or after puberty, increased the concentration of CBG in adult male rats. Similar results have been reported previously by others (e.g., Ref. [4]). Although no direct comparisons were made, we could discern no substantive difference between the effects of castration just prior to puberty, just after puberty or in adulthood on CBG. In adults, castration caused a significant increase in CBG levels within 2 weeks and the increase was prevented by testosterone replacement.

Because castration, like morphine [9,10], increased CBG, it seemed plausible that castration might affect CBG by releasing EOP. If so, we predicted that the castration-induced increase would be inhibited by the opioid antagonist naltrexone. However, we did not find that to be the case. Implantation of naltrexone pellets at the time of surgery had no effect on CBG levels in castrated or sham-operated rats. It is likely that the dose of naltrexone used was sufficient to antagonize the actions of EOP since we [5] previously found it to induce a maximal rate of opioid receptor upregulation in brain — an indication that EOP activity was completely blocked. EOP, therefore, do not appear to mediate the castration-induced increase in CBG levels, and it follows that testosterone probably does not decrease CBG levels by suppressing EOP release.

As in previously reported studies [9,10], exposure to morphine for 1 week in adulthood increased (≈100%) the concentration of CBG in serum of intact, i.e., sham-castrated, rats. Morphine-treated castrated rats had CBG levels that did not differ significantly from those of morphine-treated intact rats. In other words, morphine-treated rats, whether castrated or intact, had similar levels of CBG. However, because castration itself increased the concentration of CBG, the difference between the placebo and morphine groups was much greater in sham-operated rats than in castrated rats. At 4 weeks after castration in adulthood, the morphine treated rats had CBG levels that were only 19% higher than those of the placebo treated rats and the difference was not statistically significant.

Subcutaneous implantation of testosterone-filled silastic capsules at the time of castration prevented the castration-induced increase in CBG. Furthermore, testosterone replacement restored the magnitude of the difference between the placebo and morphine groups to approximately that occurring in intact rats. Thus, testosterone appears necessary for morphine effects on CBG to be fully manifested. Whether this is attributable, solely or partly, to the effects of testosterone on CBG levels per se is uncertain. That is, testosterone may simply maintain CBG at a submaximal level from which upregulation by morphine is possible. Accordingly, in the absence of the suppressive influence of testosterone, CBG levels may rise toward a maximal level where further upregulation by morphine may not be possible.

Our previously reported findings with female rats are consistent with this possibility. Due to the absence of androgen during development, adult females have CBG levels that are more than twice as high as in males [4,8,9]. Furthermore, in contrast to males, chronic exposure to morphine has no apparent effect on CBG in adult females [9]. In view of our current findings, it seems that the lack of testosterone and the resulting high levels of CBG in females might be a contributing factor in this marked gender difference in the effects of morphine on CBG.

One plausible mechanism by which morphine could increase CBG levels is by blocking CBG downregulation by testosterone. In fact, morphine is known to suppress activity of the pituitary-gonadal axis in male rats and to decrease testosterone levels in blood. Nevertheless, three lines of evidence suggest that this is an unlikely mechanism. First, we have previously shown that morphine effects on testosterone are rapid and transient with titers returning to normal by 7 days after pellet implantation [2]. Consistent with the latter observation, testosterone levels were normal at 7 days after implantation of two morphine pellets in the current study. On the other hand, morphine effects on CBG develop relatively slowly and appear to persist for at least as long as morphine is detectable in blood [10]. Thus, the time-courses for morphine effects on testosterone and on CBG are considerably different. Second, we reported previously that morphine maximally upregulates CBG within about a week [10], whereas, in the studies reported here CBG had not attained the levels caused by morphine by 4 weeks after castration. Thus, even the total elimination of testosterone by castration does not affect CBG levels as rapidly as morphine affects CBG. Third, morphine increased CBG in castrated rats that received testosterone implants. Therefore, morphine upregulated CBG even though a morphine-induced decrease in testosterone levels was prevented. Thus, the currently available evidence does not appear to be consistent with the idea that morphine increases CBG by reducing testosterone levels.

In conclusion, our findings indicate that testosterone and morphine have opposite effects on CBG levels; testosterone decreases, whereas, morphine increases CBG. The effects of testosterone/castration on CBG do not appear to be mediated by EOP. Furthermore, morphine does not appear to increase CBG through effects on testosterone. Nevertheless, testosterone appears to be necessary for CBG upregulation by morphine to be expressed.

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## References

[1] Bradford MM. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[2] Cicero TJ, O'Connor L, Nock B, Adams ML, Miller BT, Bell ER, Meyer ER. Age-related differences in the sensitivity to opiate-induced perturbations in reproductive endocrinology in the developing and adult male rat. J Pharmacol Exp Ther 1989; 248:256–61.

[3] de Kloet ER, Reul JMHM, de Ronde FSW, Bloemers M, Ratka A. Function and plasticity of brain corticosteroid receptor systems: action of neuropeptides. J Steroid Biochem 1986;25:723–31.

[4] Gala RR, Westphal U. Corticosteroid-binding globulin in the rat: studies on the sex difference. Endocrinology 1965;77:841–51.

[5] Giordano AL, Nock B, Cicero TJ. Antagonist-induced up-regulation of the putative epsilon opioid receptor in rat brain: comparison with kappa, mu and delta opioid receptors. J Pharmacol Exp Ther 1990;255:536–40.

[6] Hammond GL, Smith CL, Paterson NAM, Sibbald WJ. A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils. J Clin Endocrinol Metab 1990;71:34–9.

[7] Legan SJ, Coon GA, Karsch FJ. Role of estrogen as initiator of daily LH surges in the ovariectomized rat. Endocrinology 1975; 96:50–6.

[8] Mataradze GD, Kurabekova RM, Rozen VB. The role of sex steroids in the formation of sex-differentiated concentrations of corticosteroid-binding globulin in rats. J Endocrinol 1992;132:235–40.

[9] Nock B, Cicero TJ, Wich M. Chronic exposure to morphine decreases physiologically active corticosterone in both male and female rats but by different mechanisms. J Pharmacol Exp Ther 1998;286:875–82.

[10] Nock B, Wich M, Cicero TJ. Chronic exposure to morphine increases corticosteroid-binding globulin. J Pharmacol Exp Ther 1997; 282:1262–8.

[11] Ogawa K, Sueda K, Matsui N. The effect of cortisol, progesterone, and transcortin on phytohemagglutinin-stimulated human blood mononuclear cells and their interplay. J Clin Endocrinol Metab 1983;56:121–6.

[12] Pardridge WM, Sakiyama R, Judd HL. Protein-bound corticosteroid in human serum is selectively transported into rat brain and liver in vivo. J Clin Endocrinol Metab 1983;57:160–5.

[13] Séralini G-E. Regulation factors of corticosteroid-binding globulin: lesson from ontogenesis. Horm Res 1996;45:192–6.

[14] Viau V, Sharma S, Meaney MJ. Changes in plasma adrenocorticotropin, corticosterone, corticosteroid-binding globulin, and hippocampal glucocorticoid receptor occupancy/translocation in rat pups in response to stress. J Neuroendocrinol 1996;8:1–8.