

Change in brain neurosteroid level of rats in morphine addiction and stress-induced addiction relapse condition

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Abstract: **AIM** To investigate if morphine addiction and relapse to morphine-seeking is related to the change in neurosteroid levels in the brain of rats.

METHODS Rats were injected ip morphine ($5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, 18:00–20:00) and trained in conditioned place preference (CPP) box, once daily for 10 d. CPP test was performed 24 h after the last training. After discontinuation of training for 7 d for CPP extinction, then intermittent and inescapable foot-shock (FS, 0.5 mA, 0.5 s on, 40 s off, 15 min) was applied to rats as the priming stimuli for relapse. CPP test was performed 2 h after FS. When CPP test finished, rats were decapitated and the levels of neurosteroids were analyzed using gas chromatography/mass spectrometry.

RESULTS CPP was established when rats were injected morphine and trained for 10 d. At the same time, the levels of pregnenolone and allopregnanolone in the brain tissues of rats were significantly increased. When CPP was reinstated in morphine-treated rats by FS-stress after 7 d CPP extinction, the levels of dehydroepiandrosterone and dehydroepiandrosterone sulfate were significantly increased. **CONCLUSION** The development of morphine addiction and relapse may be related to endogenous neurosteroids in rat brain tissues.

Key words: morphine; addiction relapse; stress; neurosteroids

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Drug addiction is characterized by motiva-

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tional disturbances such as compulsive drug taking and episodes of intense drug craving. Recently the animal models of conditioned place preference (CPP) reinstatement have been shown that drug-seeking behavior can be triggered by foot-shock (FS) stress^[1,2]. The mechanism underlying stress-induced relapse, however, has not been elucidated. Current evidences suggested that all stimuli induce relapse, at least in part, by activation of the mesolimbic dopamine (DA) pathway^[3]. In addition, two main neurotransmitter systems including the corticotrophin-releasing factor system and the noradrenergic system appear to contribute to stress-induced reinstatement of heroin and cocaine seeking^[4].

The term neurosteroids applies to those steroids that are synthesized in the nervous system, from cholesterol or other blood-borne steroidal precursors, and accumulate in the nervous system that are at least in part independent of steroidogenic gland secretion^[5]. Several lines of evidence suggest that neurosteroids have anti-convulsant, myorelaxant, anesthetic and anxiolytic effects when administered to animals^[6,7]. Neurosteroid sulfate esters, pregnenolone sulfate and dehydroepiandrosterone sulfate (DHEAS), have an excitatory cellular action, they are able to improve memory and learning, increase DA release in the rat nucleus accumbens and enhance the dopaminergic response to morphine^[8,9].

Recent studies demonstrated that the development of tolerance to and dependence on morphine could be inhibited by concomitant

chronic administration of neurosteroids such as allopregnanolone (AP), pregnenolone sulfate or progesterone^[10]. Moreover, some neurosteroids can influence the form of CPP and have place preference or aversion actions^[11,12]. Our previous studies also demonstrated that the levels of neurosteroids could be changed by morphine dependence and withdrawal^[13]. But whether morphine addiction and relapse is related to endogenous neurosteroids is unknown. The present study was carried out to investigate the endogenous neurosteroids levels following morphine addiction and stress-induced relapse to morphine-seeking in rat brain by gas chromatography/mass spectrometry.

1 MATERIALS AND METHODS

1.1 Drugs and reagents

Pregnenolone, progesterone, AP, dehydroepiandrosterone (DHEA), pregnenolone sulfate and DHEAS were purchased from Sigma-Aldrich Co. (USA). Heptafluorobutyric acid anhydride (HFBA) was purchased from Pierce (USA). MeOH (HPLC grade) was from Kangkede (Tianjin, China). All other organic solvents were analysis grade and further purified before use by distillation. Morphine hydrochloride injection was purchased from Shenyang First Pharmaceutical Factory (China).

1.2 Experimental animals

Adult male Sprague-Dawley rats weighing 180–200 g, provided by Experimental Animal Center of Hebei Province (Grade II, Certificate No 04084). The animals were divided into 4 groups randomly ($n = 10$ in each group): ① normal saline (NS) group: rats were administered with NS, $1 \text{ mL} \cdot \text{kg}^{-1}$, ip, daily and trained for 10 d. ② Morphine addiction group: rats were administered with morphine ($5 \text{ mg} \cdot \text{kg}^{-1}$, ip) daily and trained for 10 d. ③ NS + FS group: rats were treated with NS ($1 \text{ mL} \cdot \text{kg}^{-1}$, ip) daily and trained for 10 d, then foot-shocked after discontinuation of training for 7 d. ④ Morphine relapse group: rats were treated

with morphine ($5 \text{ mg} \cdot \text{kg}^{-1}$, ip) daily and trained for 10 d, then foot-shocked after discontinuation of training for 7 d. The animals were housed under a standard light/dark cycle with free access to food and water and handled on a daily basis for the first week before the beginning of experimental procedures. In each experiment, the rats were decapitated 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.

1.3 Development of conditioned place preference

CPP was monitored in four similar rectangular wooden boxes ($88 \text{ cm} \times 32 \text{ cm} \times 34 \text{ cm}$, self-made). Each box consisted of two chambers that could be blocked by the insertion of door. One of the chambers had white walls and smooth vitreous floor, while the other had black walls and wire mesh floor ($1 \text{ cm} \times 1 \text{ cm}$). Mirror was equipped under the ceiling of the box for observing the movement of rats.

Behavioral testing^[11] was consisted of three phases: preconditioning (d 1–3), conditioning (d 4–13), and testing (d 14). Preconditioning was included to habituate the rats to the conditioning boxes and provide a baseline measure before conditioning. During three 10-min sessions, rats were placed in one of the chambers (designated the start side), and free access to the entire box. The choice of start side (left or right) was counterbalanced across rats, but remained the same for each rat throughout the experiment. The chamber rat stayed less time was regarded as drug-paired one. In present experiment, the chamber of white-wall was chosen as drug-paired one for rat staying less time. During each of the ten 30-min conditioning sessions, animals were kept to one chamber by blocking the entrance. Rats in morphine addiction and morphine relapse groups were injected with NS ($1.0 \text{ mL} \cdot \text{kg}^{-1}$, ip) during the vehicle sessions (morning) and kept in black-wall chamber; while during the

drug sessions (afternoon), the rats were injected morphine ($5.0 \text{ mg} \cdot \text{kg}^{-1}$, ip) and kept in the opposite chamber (white-wall). Rats in NS and NS + FS groups were injected with NS ($1.0 \text{ mL} \cdot \text{kg}^{-1}$, ip) both in vehicle and drug sessions, then kept in black-wall chamber in the morning and white-wall chamber in the afternoon. On d 14, 24 h after the last training, the rats in a drug-free state were placed in the start chamber and free access to the entire box. During the 10-min test sessions, the time for the rats spent in the drug-paired chamber was recorded.

When behavioral studies finished, rats in morphine addiction and control groups were sacrificed by decapitation. The entire brain was quickly removed, the cerebellum was discarded and the anterior brain was divided into two hemispheres. Samples were frozen and stored at -70°C until steroid assays.

1.4 Extinction and relapse

Rats in NS + FS and morphine relapse groups were discontinued training for 7 d for CPP extinction after the last training. Then intermittent and inescapable FS (0.5 mA , 0.5 s on, 40 s off, 15 min) was used as the priming stimuli for relapse. CPP test was performed 2 h after FS. Brain samples were collected as above.

1.5 Brain steroid extraction and analysis

The method was described by our previously studies^[14]. Briefly, 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 for the unconjugated steroids. The aqueous phase was collected and repeatedly extracted three times with an equal volume of chloroform/2-butanol ($50:50$, V/V) for the sulfated steroids. A clean-up step was performed by solid-phase extraction (SPE) on C_{18} minicolumns (500 mg , 6 mL , Supelco). The sulfated steroids fraction was subjected to solvolysis. The unconjugated steroids and those obtained after solvolysis were derivatized by adding $20 \mu\text{L}$ of HFBA

and $200 \mu\text{L}$ of anhydrous acetone. The derivatized samples were analyzed by gas chromatography-mass spectrometry system (HP5973). The rate of recovery of each steroid was over 50%. The accuracy values were $(97 \pm 9)\%$, $(95 \pm 8)\%$, $(107 \pm 5)\%$ and $(98 \pm 5)\%$ for pregnenolone, DHEA, progesterone and AP, respectively. The inter-day variations for the assays were less than 15%.

1.6 Statistical analysis

All data were expressed as $\bar{x} \pm s$. Data were analyzed by one-way ANOVA and t test.

2 RESULTS

2.1 Behavioral test

Behavioral results are shown in Tab 1. There was no difference among groups during the preconditioning sessions. Morphine treatment for 10 d induced CPP, as indexed by a significant increase in the time stayed in the drug-paired side compared with control group. There was no significant difference between morphine-treated group and NS + FS group after discontinued training for 7 d ($P > 0.05$), which implicated the extinction of CPP. The time stayed on the drug-paired side was significant longer in morphine-treated group after FS, inferred the reinstatement of CPP.

2.2 Levels of neurosteroids in rat brain when CPP development and reinstatement

Morphine treatment resulted in CPP, which was accompanied by a significant increase in the levels of pregnenolone and AP in the brain tissues of rats sacrificed 24 h after the last administration. In contrast, there was no significant difference in the levels of DHEA and DHEAS between NS and morphine addiction groups.

Compared with NS group, the levels of AP and DHEA significantly increased, while the level of pregnenolone significantly decreased in NS + FS group. When CPP was reinstated in morphine-treated rats by FS-stress (Tab 1), the levels of DHEA and DHEAS significantly

Tab 1. Time stayed in drug-paired chamber in conditioned place preference (CPP) test

Group	Time stayed in drug-paired chamber in 10 min/min			
	Before training	After training	After extinction	After FS
NS	2.3 ± 0.6	2.1 ± 1.0		
Morphine addiction	1.9 ± 0.9	5.8 ± 0.7 ^{**}		
NS + FS	2.5 ± 0.7	2.3 ± 1.0	1.7 ± 1.1	2.3 ± 1.0
Morphine relapse	2.4 ± 0.6	5.2 ± 1.1 ^{##}	2.3 ± 0.9	5.8 ± 1.5 ^{##}

Rats in morphine addiction and morphine relapse groups were injected with normal saline (NS, 1.0 mL·kg⁻¹, ip) and kept in black-wall chamber for 30 min in the morning, and were injected morphine (5.0 mg·kg⁻¹, ip) and kept in the white-wall chamber for 30 min in the afternoon. Rats in NS and NS + foot-shock (FS) groups were injected with NS (1.0 mL·kg⁻¹) both in vehicle and drug sessions, then kept in black-wall chamber for 30 min in the morning and white-wall chamber for 30 min in the afternoon, respectively. Rats were trained as above procedure for 10 d and CPP was tested 24 h after the last training. Then, after discontinuation of training for 7 d (CPP extinction), intermittent and inescapable FS, 0.5 mA, 0.5 s on, 40 s off, 15 min was applied to rats in NS + FS and morphine relapse groups as the priming stimuli for relapse. CPP test was performed 2 h after FS. $\bar{x} \pm s$, $n = 10$. ^{**} $P < 0.01$, compared with NS group; ^{##} $P < 0.01$, compared with NF + FS group.

Tab 2. Levels of neurosteroids in brain of rat when CPP development and reinstatement

Group	Neurosteroid/ng · g ⁻¹ tissue					
	PREG	PROG	AP	DHEA	PREGS	DHEAS
NS	10.0 ± 1.7	8.1 ± 2.7	1.0 ± 0.4	1.0 ± 0.6	5.5 ± 2.1	2.6 ± 1.6
Morphine addiction	16.9 ± 3.4 [*]	13.5 ± 11.1	2.4 ± 1.6 [*]	1.1 ± 0.8	3.5 ± 0.6	2.7 ± 1.6
NS + FS	7.9 ± 1.3 [*]	9.3 ± 11.9	3.8 ± 0.8 [*]	3.6 ± 0.8 [*]	4.9 ± 1.2	2.4 ± 1.5
Morphine relapse	9.9 ± 1.7	11.4 ± 3.5	3.2 ± 1.6 [*]	23.9 ± 17.9 ^{*#}	4.1 ± 0.5	4.9 ± 1.2 ^{*#}

See legend of Tab 1 for rat treatments. Rats were sacrificed by decapitation as soon as CPP tests finished on different day. PREG: pregnenolone; PROG: progesterone; AP: allopregnanolone; DHEA: dehydroepiandrosterone; PREGS: pregnenolone sulfate; DHEAS: dehydroepiandrosterone sulfate. $\bar{x} \pm s$, $n = 10$. ^{*} $P < 0.05$, compared with NS group; [#] $P < 0.05$, compared with NS + FS group.

increased compared with NS + FS group. While there was no significant difference in the levels of pregnenolone, progesterone, AP and pregnenolone sulfate between the two groups (Tab 2).

3 DISCUSSION

The results showed that morphine induced CPP, as indexed by a significant increase in the time stayed in the drug-paired side in the test phase compared with control group, and that intermittent FS could induce reinstatement of drug-seeking behavior in the former place-preferring rats. These were consistent with previous observations that stress induced relapse^[1,2].

Also the present experiments showed a significant increase in neurosteroids pregnenolone and AP in morphine addiction group. In contrast, there was no significant difference in the levels of DHEA and DHEAS between the two groups. Evidences suggested that neurosteroids play an important role in modulating the motivational and motive behaviors^[15], the alterations of endogenous neurosteroids may contribute to the development of motivational barrier of morphine addiction. However, the mechanisms needed further investigation.

Exposure of rats to mild FS, CO₂ inhalation or handling maneuvers that precede sacrificing elicits a selective and time-dependent increase in neurosteroids in the brain^[16-18]. The present experiments showed a significant in-

crease in AP, DHEA and a significant decrease in pregnenolone in NS + FS group after FS. The difference can be explained by the use of different experiment models and different sampling time after FS. The levels of neurosteroids were detected 2 h after FS-stress in this study, but were detected 30 or 60 min after stress in previous report^[18]. In addition, the reason for the blunted neurosteroids response to acute stress may be due to the mild stress induced by daily injection and training. It has been demonstrated that repeated exposure of rats to mild stressors decreased anxiety^[19].

It has been observed that FS-stress resulted in an increase in the levels of DHEA and DHEAS in relapse group compared with NS + FS group. This is the evidence that relapse was accompanied with changes in the endogenous neurosteroids. Accumulating evidences have indicated that there exists some correlation between opiate reward and certain kinds of learning and memory processes. Neurosteroids DHEA and DHEAS had been implicated in increasing DA release in rat nucleus accumbens and improving learning and memory^[20]. Thus, the increase in DHEA and DHEAS in relapse group might contribute to the development of pathological memory obstacle associated with drug addiction.

There are various possible interpretations about the effects of morphine on neurosteroid level. One possible explanation is that morphine may regulate directly the activity or expression of certain enzymes that catalyze the biosynthesis of neurosteroids. Thereby it selectively affected the accumulation of these compounds in the brain. Indeed, a selective action on steroidogenic enzymes by other pharmacological treatments has been recently shown^[21,22].

In conclusion, our data provided the first evidence that morphine relapse changed the levels of neurosteroids in rat brain. However, the mechanism of relapse may be much more complex than that so far demonstrated. A better

understanding of the mechanisms of relapse could lead to more effective treatment strategies for addictive disorders.

4 REFERENCES:

- [1] Wang B, Luo F, Zhang WT, Han JS. Stress or drug priming induces reinstatement of extinguished conditioned place preference [J]. *Neuroreport*, 2000, **11** (12): 2781 – 2784.
- [2] Shaham Y, Stewart J. Stress reinstates heroin-seeking in drug-free animals: an effect mimicking heroin, not withdrawal [J]. *Psychopharmacology* (Berl), 1995, **119**(3): 334 – 341.
- [3] Stewart J. Pathways to relapse: the neurobiology of drug- and stress-induced relapse to drug-taking [J]. *J Psychiatry Neurosci*, 2000, **25**(2): 125 – 136.
- [4] Shaham Y, Erb S, Stewart J. Stress-induced relapse to heroin and cocaine seeking in rats: a review [J]. *Brain Res Brain Res Rev*, 2000, **33**(1): 13 – 33.
- [5] Baulieu EE. Neurosteroids: of the nervous system, by the nervous system, for the nervous system [J]. *Recent Prog Horm Res*, 1997, **52**: 1 – 32.
- [6] Brot MD, Akwa Y, Purdy RH, Koob GF, Britton KT. The anxiolytic-like effects of the neurosteroid allopregnanolone: interactions with GABA_A receptors [J]. *Eur J Pharmacol*, 1997, **325**(1): 1 – 7.
- [7] Gasior M, Carter RB, Goldberg SR, Witkin JM. Anti-convulsant and behavioral effects of neuroactive steroids alone and in conjunction with diazepam [J]. *J Pharmacol Exp Ther*, 1997, **282**(2): 543 – 553.
- [8] Barrot M, Vallee M, Gingras MA, Le Moal M, Mayo W, Piazza PV. The neurosteroid pregnenolone sulphate increases dopamine release and the dopaminergic response to morphine in the rat nucleus accumbens [J]. *Eur J Neurosci*, 1999, **11**(10): 3757 – 3760.
- [9] Markowski M, Ungeheuer M, Bitran D, Locurto C. Memory-enhancing effects of DHEAS in aged mice on a win-shift water escape task [J]. *Physiol Behav*, 2001, **72**(4): 521 – 525.
- [10] Reddy DS, Kulkarni SK. Chronic neurosteroid treatment prevents the development of morphine tolerance and attenuates abstinence behavior in mice [J]. *Eur J Pharmacol*, 1997, **337**(1): 19 – 25.
- [11] Beauchamp MH, Ormerod BK, Jhamandas K, Boegman RJ, Beninger RJ. Neurosteroids and reward: allopregnanolone produces a conditioned place aversion in rats [J]. *Pharmacol Biochem Behav*, 2000, **67**(1): 29 – 35.
- [12] Frye CA, Park D, Tanaka M, Rosellini R, Svare B. The testosterone metabolite and neurosteroid 3 α -androstenediol may mediate the effects of testosterone on conditioned place preference [J]. *Psychoneuroendocrinology*, 2001, **26**(7): 731 – 750.
- [13] Yan CZ, Hou YN. Effects of morphine dependence and withdrawal on levels of neurosteroids in rat brain

- [J]. *Acta Pharmacol Sin* (中国药理学报), 2004, **25** (10):1285-1291.
- [14] Yan C, Hou Y. Determination of neurosteroids in rat brain by gas chromatography/mass spectrometry [J]. *Chin J Chromatogr* (色谱), 2004, **22**(1):12-15.
- [15] van Broekhoven F, Verkes RJ. Neurosteroids in depression: a review [J]. *Psychopharmacology* (Berl), 2003, **165**(2):97-110.
- [16] Barbaccia ML, Roscetti G, Trabucchi M, Purdy RH, Mostallino MC, Concas A, *et al.* The effects of inhibitors of GABAergic transmission and stress on brain and plasma allopregnanolone concentrations [J]. *Br J Pharmacol*, 1997, **120**(8):1582-1588.
- [17] Barbaccia ML, Roscetti G, Bolacchi F, Concas A, Mostallino MC, Purdy RH, *et al.* Stress-induced increase in brain neuroactive steroids: antagonism by abecarnil [J]. *Pharmacol Biochem Behav*, 1996, **54**(1):205-210.
- [18] Barbaccia ML, Roscetti G, Trabucchi M, Mostallino MC, Concas A, Purdy RH, *et al.* Time-dependent changes in rat brain neuroactive steroid concentrations and GABA_A receptor function after acute stress [J]. *Neuroendocrinology*, 1996, **63**(2):166-172.
- [19] Haller J, Halasz J. Mild social stress abolishes the effects of isolation on anxiety and chlordiazepoxide reactivity [J]. *Psychopharmacology* (Berl), 1999, **144**(4):311-315.
- [20] Jaworska-Feil L, Budziszewska B, Leskiewicz M, Lason W. Opposite effects of inhibitory and excitatory neurosteroids on [³H]dopamine release from rat nucleus accumbens [J]. *Pol J Pharmacol*, 1998, **50**(6):449-452.
- [21] Kim HJ, Ha M, Park CH, Park SJ, Youn SM, Kang SS, *et al.* StAR and steroidogenic enzyme transcriptional regulation in the rat brain: effects of acute alcohol administration [J]. *Brain Res Mol Brain Res*, 2003, **115**(1):39-49.
- [22] Griffin LD, Mellon SH. Selective serotonin reuptake inhibitors directly alter activity of neurosteroidogenic enzymes [J]. *Proc Natl Acad Sci USA*, 1999, **96**(23):13512-13517.

吗啡成瘾和应激诱导成瘾复发的大鼠脑内神经甾体水平的变化

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摘要: **目的** 观察大鼠吗啡成瘾及应激诱导成瘾复发是否与脑组织神经甾体水平的变化有关。**方法** 给大鼠注射吗啡 ($5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, ip, 18:00 ~ 20:00) 并在条件性位置偏爱 (CPP) 箱中训练, 每日 1 次, 连续 10 d, 最后 1 次给药后 24 h 测试大鼠是否产生 CPP。再经过 7 d 的自然消退期后, 给予足底电击 (0.5 mA, 0.5 s, 间隔 40 s, 15 min) 诱发大鼠 CPP 复发, 电击后 2 h 进行 CPP 测试。测试后立即取样, 取样时间 18:00 ~ 20:00, 气相色谱-质谱联用技术测定大鼠脑组织神经甾体。**结果** 给予吗啡并训练 10 d, 大鼠形成明显的 CPP, 同时脑组织内神经

甾体孕烯醇酮和别孕烷醇酮水平显著升高。经过 7 d 的自然消退后再给予足底电击可诱发大鼠 CPP 复发, 脑组织神经甾体脱氢表雄酮和硫酸脱氢表雄酮水平显著升高。**结论** 大鼠吗啡成瘾及应激诱导成瘾复发过程可能与脑组织内源性神经甾体水平有关。

关键词: 吗啡; 成瘾复发; 应激; 神经甾体

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