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Buspirone Ameliorates the Morphine Withdrawal-Induced Anxiety through Synaptic Ultrastructural Changes in Hippocampus of Rat[*]

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Morphine administration causes long-lasting neural changes in the brain that underpin the behavioral abnormalities, and the relationship between structural changes and behavioral symptoms is obscure. In present study, the elevated plus-maze and transmission electron microscope were applied to validate the anxiety-like behaviors and synaptic ultrastructural changes in the hippocampi of rats among the morphine group (morphine administration only), the buspirone group (morphine plus buspirone administration) and the vehicle (saline treated only). As compared with the vehicle group, lower values of OE (times of entering into the open arms), OE% (percentage of entries into the open arms), OT (time spent in the open arms), OT% (percentage of time stayed in the open arms), *Ns* (surface density (Sv)/numerical density (Nv)) and S (surface area) of synapses were observed in the morphine group , but significantly, behavior higher scores of RR (rearing), HD (head-dipping), FBA (flat back approach), and higher *Nv*, *Sv*, PSD (postsynaptic density), LPT (length of postsynaptic thickening), WCJ (widths in synaptic cleft on junctions) and CCR (curvature of the cleft region) of synapses appeared in the morphine group. However, no significant differences in values of most of those parameters above were detected between the vehicle group and the buspirone group. These results supported that anxiety-like symptoms of rats significantly occurred to the rats after acute morphine withdrawal, but buspirone administration could reverse these indexes. It also proved that the appearance/disappearance of anxiety-related symptoms was related to the ultrastructural changes/reversibility of synapses in the hippocampus with morphine and buspirone administrations. So, it suggested that anxiety-related symptoms were modified in rats subjected to the synaptic ultrastructural changes in hippocampus by morphine acute withdrawal and were further rehabilitated by buspirone administration. It is helpful to pursue the effective therapeutic methods of morphine addiction.

Keywords: Buspirone; Morphine; Anxiety; Synaptic Ultrastructure; Hippocampus

Introduction

Chronic morphine administration can induce the changes of hippocampal CA1 neurocircuitry which modulate the synaptic plasticity through the regulation of long-term potentiation (LTP) and long-term depression (LTD) (Salmanzadeh et al., 2003). The synaptic plasticity is widely studied in hippocampal CA1 and CA3 fields, which is believed to be the mechanism underlying certain types of learning and memory (Moron et al., 2007). It is a commonly accepted assumption that an increase in neuropil volume reflects an increase in the number of synapses present (Devoogd et al., 1985; Turner & Greenough, 1985; Black et al., 1990; Withers et al., 1993; Klintsova et al., 2000), but, in many regions of the brain, this has not yet to be clearly estab-

lished (Brown et al., 2002).

Buspirone, a potent anxiolytic compound in animal models (Riblet, et al., 1986), displays reversibility of synaptic activation of pyramidal cells in the hippocampus (Trulson et al.,1986; Mauk et al., 1988). It showed that buspirone bind selectively to presynaptic (dorsal raphe) and postsynaptic (hippocampus, cortex) 5-hydroxytryptamine 1A (5-HT1A) receptor binding sites, and the clinical trials of anxiety and depression may be related to the buspirone's interactions with gepirone at pre-synaptic and postsynaptic 5-HT1A receptor (Yocca, 1990). Buspirone had no effect on the immediate rewarding properties of cocaine, but the attenuation of 5-HT neurotransmission (via the autoreceptor agonist properties of buspirone) could reverse the negative impact of cocaine (Ettenberg & Bernardi, 2007).

So, the correlating ethological indexes of anxious rats with synaptic ultrastructural changes of the hippocampal CA1 and CA3 fields should enable us to evaluate the mechanism of

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morphine addiction and pursue the effective therapeutic methods. We hypothesized that anxiety-related symptoms were modified in rats subjected to the synaptic ultrastructural changes in hippocampal CA1 and CA3 fields by morphine acute withdrawal and were further rehabilitated by buspirone administration. Here, we should provide an evidence in which the appearance/disappearance of anxiety-related symptoms are related to the synaptic ultrastructural change/reversibility of the hippocampal CA1 and CA3 fields with morphine and buspirone administrations.

Method

Animals

Adult male Sprague Dawley rats (weighing 200 - 250 g) were obtained from the Laboratory Animal Center, the Third Military Medical University (Chongqing, China). Supplied with water and food available *ad libitum*, rats were maintained in groups on a 12-h light/dark cycle (lights on at 08:00 AM) and were allowed to acclimatize to their environment for 1 week prior to drug administration. All treatments were strictly in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Morphine and Buspirone Administration for the Elevated Plus-Maze Tests

Rats were randomly assigned to three groups (n = 12 each group): the saline treated control group (saline group), the morphine treated model group (morphine group) and the morphine plus buspirone treated experimental group (buspirone group). Morphine (morphine sulfate) was prepared in 0.9% sterile isotonic saline. In morphine group and buspirone group, three escalating doses of morphine (5, 10, and 15 mg/kg, s.c., twice per day at 12 hr intervals) were applied for 10-day injecttions as described before (Morinville et al., 2003). Briefly, a dosage of 5 mg/kg morphine was applied at the primal period of 5 days, followed by 3-day injections of 10 mg/kg, then of 15 mg/kg in the last two days. Withdrawal syndrome was precipitated with abrupt termination of morphine intake by replacement of daily injections of saline for 3 days, and was further validated by observing behaviors in a separate series of experiments. After acute morphine withdrawal, each anxious rat in the buspirone group received buspirone treatment (15 mg/kg, i.g., twice per day at 12 hr intervals) for 3 days while the anxious rats in the morphine group only received saline treatment by the same form. The rats of saline group only received saline as the program above.

All rats were tested just once after 72 h of the last treatments of saline or buspirone. To begin a test session, rats were individually placed in the center of the maze, facing towards one of the enclosed arms, and allowed 5 min of free exploration. The elevated plus-maze apparatus consisted of a center (10 × 10 cm square area), two opposing open arms (50 cm length, 10 cm width and 40 cm height), and two opposing closed arms (50 cm length, 10 cm width and 40 cm height). Each of the four arms in the maze was connected to the center at 90° relative to the adjacent arms. All sides and floor surfaces of the open and closed arms were constructed from black Plexiglas. The floor of the maze was covered with a thin layer of vulcanized black rubber to facilitate inter-trail cleaning. All trials took place in a dimly illuminated chamber and were recorded by a video camera that was linked to a video monitor, and an IBM-compatible computer positioned outside the test chamber. Behaviors of the rats were recorded with an automated system. Positions of the rats in the maze were continuously tracked by photo beam arrays embedded along the entire length of the base of each closed arm, the entry point to all arms, and in clear Plexiglas tubes that extended from the distal end of each closed arm and ran parallel to the open arms.

An auto-recorded system was applied to collect data and further these behavior measurements were analyzed using Motor Monitor Software (Kinder Scientific, Poway CA). Based on computer-recorded data, judgment of anxiety-like behaviors were validated by the following parameters for each rat: (1) times of rat entering into the open arms (OE); (2) the percentage of entries into the open arms as the total entries into both open and closed arms (OE%); (3) time spent in the open arms (OT); (4) the percentage of time stayed in the open arms as in both open and closed arms (OT%); (5) partial or total rising onto hind limbs (rearing, RR); (6) exploratory movement of head/shoulders over the sides of the maze and down toward the floor (Head-dipping, HD); and (7) locomotion when the animal stretched to its full length and cautiously moves forward (Flat-back approach, FBA).

Tissue Processing for Electron Microscopy

Hippocampal samples were harvested from rats of three separate groups (n = 6 per group). The animals were decapitated after 72 h of the last injections of saline or buspirone. According to standard procedures (Harris & Sultan, 1995; Shepherd & Harris, 1998), the brains were removed, and slices of the hippocampi were rapidly dissected free. Brains were sectioned coronally at 200 μm on a vibratome; the CA1 and CA3 fields were blocked from sections containing the dorsal hippocampus (bregma −2.3 to −4.3 mm, mediolateral 0 - 2 mm) with a tissue chopper into ice-cold phosphate buffered solution containing (in mM) 117 NaCl, 4.7 KCl, 26 NaHCO$_3$, 1 NaH$_2$PO$_4$, 2.2 CaCl$_2$, 1.2 MgSO$_4$, and 10 glucose, pH 7.4. The slices were fixed rapidly in 6% glutaraldehyde and 4% paraformaldehyde in 100 mM cacodylate buffer for 8 sec under microwave irradiation (Jensen & Harris, 1989), stored overnight in the fixative, and then rinsed in buffer. Slices were bathed with 1% osmium and 1.5% potassium ferrocyanide in 100 mM cacodylate buffer, cooled in ice bath to 12°C, and microwave for 2 min at 37°C. After several buffer rinses, they were *en bloc* stained using 2% aqueous uranyl acetate while being cooled on ice and were microwave 2 min at room temperature. Samples were dehydrated in an acetone series (50%, 70%, 90%, and 100%) for 40 sec each in the microwave at 37°C. The following treatments, involved in infiltration and embedded coffin mold, were performed as described before (Shepherd & Harris, 1998). These sections were embedded in Agar 100 resin and polymerized for 48 hr at 60°C. The block face was trimmed to include one entire antennat lobe in the ultrathin, and then a subset of six 70 nm-thin sections were selected in a random systematic manner from the entire section set for analysis using TEM. Thin sections were mounted on Pioloform-coated slot grid and counterstained with saturated ethanolic uranyl acetate, followed by Reynolds lead citrate, each for 5 min. The target areas, hippocampal CA1 and CA3 fields between strata pyramidale and lacunosum moleculare, were examined with a Hitachi H-600 transmission electron microscope.

Stereological Measurements of Synapses

Serial electron micrographs (12 photographs per section, from left top to low right) were taken on a Hitachi H-600 transmission electron microscope operating at 80 kV. Images were collected onto 35 mm plate film at a final magnification of 44,000 × and enlarged onto 7 × 12 inch photographic paper. Within each photograph, the points used for analysis were determined by placing a 9 mm^2 transparent grid over a micrograph. Stereological quantification of synapses was determined using the physical dissector method. The accurate and unbiased comparisons of numerical density (Nv), surface area (S), surface density (Sv) and the average surface density of each synapse (Ns) in the hippocampal CA1 and CA3 fields were measured as described before (Sterio, 1984). These parameters such as the total synapse number per μm^2 (Na), the mean synapses strip length (L), the sample thickness (T), the total grid length (Lt), and the cross point (Ni) between grid and synapses were recorded. Accordingly, the emendation coefficient (Ko) was calculated as follows: $Ko = 1 + 3T/2L$. Based on these sufficient parameters, stereological analysis was respectively described as the following formulas: $Nv = Na \cdot L^{-1}(Ko + T)$, $Sv = 2Ni/Lt \cdot Ko$, and $Ns = Sv/Nv$.

Further Measurements of Synaptic Junction Parameters

All identifiable synaptic junctions were marked on the micrographs above and used for further measurement. Asymmetric synapses were identified, and postsynaptic elements were classified as dendritic spines or dendritic shafts, by established morphological criteria (Scheuermann, 1991). The criterion of parallelism was waived when terminals were sectioned at an oblique angle to the cleft specialization if the presence of a thickening and synaptic vesicles was still obvious. The numbers and the areas of the synaptic junctions were recorded in order to determine postsynaptic density (PSD).

According to description before (Weibel, 1966), the membranous boundaries of terminals were outlined and a curly probe was used to determine terminal areas by permitting point counts for area estimate. More detailed measurements of terminal parameters were undertaken as described before (Dyson & Jones, 1980). A curvature-length probe (Jones & Devon, 1978) was used to measure the length of postsynaptic thickening (LPT) and the width in synaptic cleft on junctions (WCJ) at the cleft edge of the thickening, and to determine junction curvature (curvature of the cleft region, CCR) at these positions. LPT was measured directly along the arc of curvature. Adjacent junctions on the same terminal separated by membrane free of specialization were set aside.

Statistical Analysis

All data obtained from behavioral measurements of plus-maze activity, stereological detections and synaptic terminal junction parameters were subjected to one-way analysis of variance (ANOVA) as dictated by the experimental design. The mean differences were compared by least significant difference test. All analyses were performed on a completely randomized design using the Minitab software package and a value of $p < 0.05$ was considered to indicate statistical significance, $p < 0.01$ very significant.

Results

Anxiety-Like Behavioral Reactions

As shown in **Table 1**, very significant withdrawal symptoms appeared in individuals of morphine group. One-way ANOVAS between morphine group and saline group subjects revealed notably significant carry-over effects of morphine administrition on OT, OE, OT%, OE%, RR, HD, and FBA parameters. As compared with the saline group, lower values of OE, OE%, OT and OT% were observed in the morphine group ($p < 0.01$), but significantly, higher scores of RR, HD and FBA appeared in the morphine group ($p < 0.01$). However, no significant differences in values of these parameters above were detected between the saline group and the buspirone group ($p > 0.05$) except HD ($p < 0.01$). These results showed that a 10-day morphine treatment produced robust and anxiety-like behavioral response patterns in the elevated plus-maze, but the buspirone administration reversed them.

Quantitatively Morphological Effects of Synapses

As shown in **Table 2** and **Figure 1**, it could be seen that significant differences occurred in hippocampal CA1 and CA3 fields in the morphine group, mainly in the profiles of presynapse and postsynapse membranes. As compared with the saline group, higher Nv and Sv (approximately 2 folds) were observed in the morphine group ($p < 0.01$ or $p < 0.05$) in the hippocampal CA1 and CA3 fields; but significantly, lower values of S and Ns were detected in the hippocampal CA1 and CA3 fields ($p < 0.01$ or $p < 0.05$). However, no significant differences in values of these parameters above were detected between the saline group and the buspirone group ($p > 0.05$) except S at CA1 ($p < 0.01$). These results showed that a 10-day morphine administration changed the synaptic quantitatively morphological effects in hippocampal CA1 and CA3 fields, but the buspirone treatment reversed them.

Table 1.
Scores of ethological measures in three independent groups.

	OT	OE	OT%	OE%	RR	HD	FBA
Saline group	68.11 ± 2.08	6.80 ± 1.16	0.25 ± 0.04	0.41 ± 0.06	1.40 ± 0.66	0.16 ± 0.09	2.35 ± 0.25
Morphine group	44.03 ± 3.04[*]	4.70 ± 0.34[*]	0.11 ± 0.07[*]	0.24 ± 0.02[*]	6.82 ± 1.07[*]	3.92 ± 0.95[*]	33.56 ± 1.28[*]
Buspirone group	65.01 ± 7.02	7.00 ± 1.60	0.25 ± 0.06	0.42 ± 0.07	1.55 ± 0.81	2.11 ± 0.23[#]	1.56 ± 0.07

Values represent means ± SEM (n = 12 in each group). Significant differences: *from the respective saline group and buspirone group ($P < 0.01$). # from the respective saline group and morphine group ($P < 0.01$). OT, time spent in the open arms; OE, times of entering into the open arms; OT%, percentage of time stayed in the open arms; OE%, percentage of entries into the open arms; RR, rearing; HD, head-dipping; FBA, flat back approach.

Table 2.
Quantitatively morphological effects of synapses in hippocampal CA1 and CA3 fields.

	CA1			CA3		
	Saline group	Morphine group	Buspirone group	Saline group	Morphine group	Buspirone group
Nv(number/mm^3)	0.723 ± 0.386	$1.513 \pm 0.817^*\Delta$	0.743 ± 0.567	0.667 ± 0.282	$1.146 \pm 0.490^*\Delta$	0.801 ± 0.361
Sv(um^2/um^3)	0.077 ± 0.037	$0.103 \pm 0.052^*\Delta$	0.088 ± 0.060	0.044 ± 0.018	0.052 ± 0.026#	0.051 ± 0.012
S (um^2)	0.174 ± 0.245	$0.079 \pm 0.046^*\blacktriangle$	0.348 ± 0.179	0.079 ± 0.059	0.059 ± 0.055#	0.062 ± 0.041
$Ns(=Sv/Nv)$	0.107	0.068	0.118	0.066	0.045	0.064

Values represent means ± SEM ($n = 6$ in each group). Significant differences: *from the respective saline group ($P < 0.01$); # from the respective saline group ($P < 0.05$); ▲ from the respective buspirone group ($P < 0.01$); Δ from the respective buspirone group ($P < 0.05$). Nv, numerical density; Sv, surface density; S, surface area; Ns, = Sv/Nv.
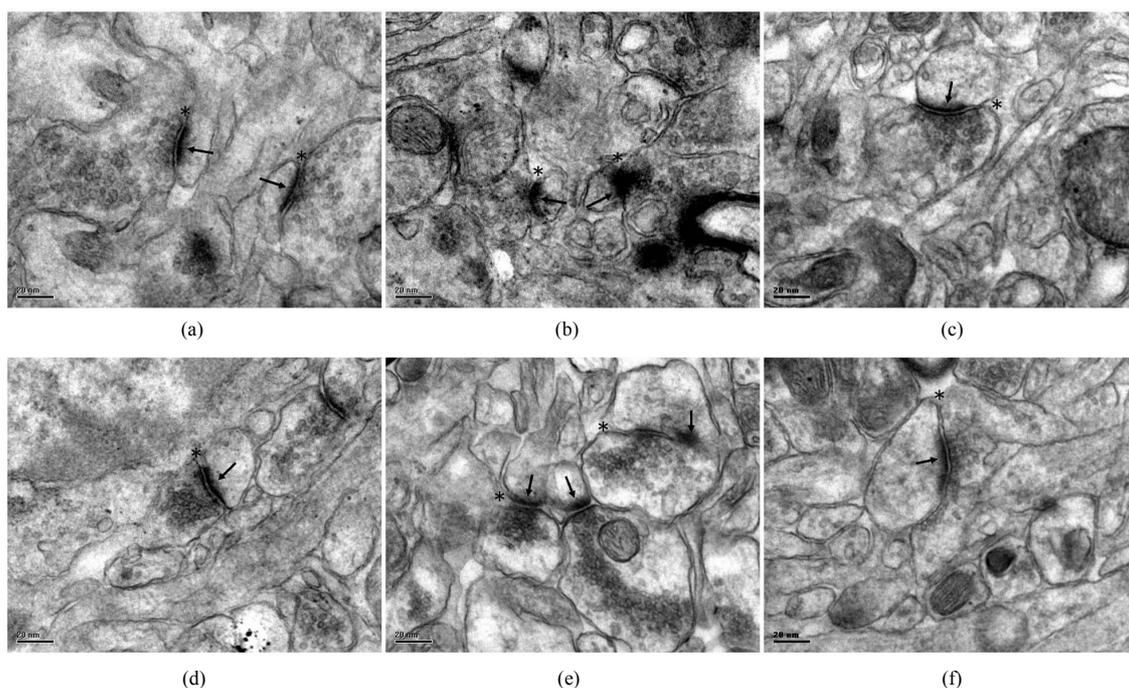

Figure 1.
Electron micrographs of the hippocampal CA1 and CA3 fields, showing the morphological differences of synapses in three independent groups (n = 6 in each group, bar = 20 nm). Thin synaptic profiles (*arrows*) and low values of PSD, LPT, WCJ, and CCR on the synaptic junctions (*asterisk*) at CA1 (A) and CA3 (D) fields in the saline group; Thick synaptic profiles (*arrows*) and high values of PSD, LPT, WCJ, and CCR on the synaptic junctions (*asterisk*) at CA1 (B) and CA3 (E) fields in the morphine group; Thin synaptic profiles (*arrows*) and low values of PSD, LPT, WCJ, and CCR on the synaptic junctions (*asterisk*) at CA1(C) and CA3 (F) fields in the buspirone group. PSD, postsynaptic density; LPT, length of postsynaptic thickening; WCJ, widths in synaptic cleft on junctions; CCR, curvature of the cleft region.

synaptic junction regions in any way reflect their functional states, more detailed measurements of terminal synaptic parameters were undertaken in the hippocampal CA1 and CA3 fields from three independent groups above (**Table 3** and **Figure 1**). Significantly, higher values of PSD, LPT, WCJ and CCR from hippocampal CA1 and CA3 fields were observed in the morphine group than that in the saline group ($p < 0.01$ or $p < 0.05$). To some extent, buspirone administration to the acute morphine withdrawal animals rehabilitated synaptic profile, the scores of most of these indexes above were only slightly increased in contrast with the saline group ($P > 0.05$), and they still were significant lower than that in the morphine group ($p < 0.01$ or $p < 0.05$).

Discussion

Concerning the ethological measurements, anxiety related symptoms significantly occurred to the rats suffered from the escalating doses of morphine administration while they were exposed to the elevated plus-maze after 72 h acute morphine withdrawal, but buspirone administration can reverse these indexes. Morphine withdrawal increased ethological indexes related to exploration and locomotor behavior such as end-exploration, head-dipping, rearing, and so on. These evidences indicated that morphine withdrawal increased the general motor activity and decreased aversion to the open spaces of the maze in rats. It has been reported that systemic injections of morphine

Table 3.
Measurements of terminal synapse junctions in hippocampal CA1 and CA3 fields.

	CA1			CA3		
	Saline group	Morphine group	Buspirone group	Saline group	Morphine group	Buspirone group
PSD	7.83 ± 0.80	10.86 ± 0.86#Δ	8.37 ± 1.58	7.73 ± 1.02	12.86 ± 1.15[*]Δ	10.31 ± 1.02
LPT	37.35 ± 2.30	44.93 ± 3.48*Δ	39.78 ± 3.26	45.66 ± 3.81	52.99 ± 7.81#▲	41.41 ± 3.55
WCJ	2.34 ± 0.29	3.80 ± 0.30*Δ	3.07 ± 0.22	2.38 ± 0.73	3.81 ± 0.59*Δ	2.91 ± 0.15
CCR	1.21 ± 0.11	1.37 ± 0.12*Δ	1.29 ± 0.14	1.23 ± 0.18	1.39 ± 0.30*Δ	1.30 ± 0.14

Values represent means ± SEM (n = 6 in each group). Significant differences: *from the respective saline group ($P < 0.01$); # from the respective saline group ($P < 0.05$); ▲ from the respective buspirone group ($P < 0.01$); Δ from the respective buspirone group ($P < 0.05$). PSD, postsynaptic density; LPT, length of postsynaptic thickening; WCJ, widths in synaptic cleft on junctions; CCR, curvature of the cleft region.

may produce euphoric or dysphoric effects (Ebert et al., 1998). It was commenced establishment of anxiety symptoms in rats by opiate administration because of the similarities in time course and intensities of craving and anxiety experienced during acute opiate withdrawal (Swift & Stout, 1992). The intraperitoneal injections of morphine (0.1 and 0.3 mg/kg) in rats increased both the total entries into the arms and the percentage of entries and time spent in the open arms of the maze (Anseloni et al., 1999).

Opioid ligands may mediate reward or aversive process depending on the structures on which they are acting and on the receptor types with which they interact (Mucha & Herz, 1985; Bals-Kubik et al., 1989). It is a commonly accepted assumption that an increase in neuropil volume reflects the increase in the number of synapses (Klintsova et al., 2000), but in many regions of brain, this has yet to be clearly established (Brown et al., 2002). It has been suggested that the increase in the motor activity caused by opiate may be due to the activation of dopaminergic cells in mesolimbic system (Koob, 1992). Indeed, morphine injections into the ventral tegmental area (VTA) can increase the dopamine-dependent locomotion (Yuan et al., 1992). Previous models of dysphoria-like and anxiety-like behaviors resulting from acute opioid dependence suggested the dopaminergic pathway may also play a prominent role in the transition from casual to compulsive use (Zhang & Schulteis, 2008).

In the present study, both behavioral and synaptic data are consistent with the idea that synaptic potentiation on hippocampus is critical for the appearance in anxiety-like behavior. The collaterals of axons between cortex and the granule cells in hippocampal dentate gyrus (DG) innervate the distal parts of the apical dendrites of pyramidal neurons in hippocampal CA1 and CA3 fields (Safiulina et al., 2005). The striking differences in the structural changes of hippocampal CA1 and CA3 fields suggested that the anxiety-like symptoms may be related to morphological changes in rats, associated with morphine and buspirone administrations. It is a good confirmation with our results that anterograde and retrograde alteration related to synaptic plasticity are necessary for memory formation (Morris et al., 2003). It is also supporting our results that tianeptine administrition for several hours is able to reverse the effects of stress when the stress and anesthesia onset is remarkable (Shakesby et al., 2002). In an attempt to isolate the principal driving force(s) of structural plasticity from combined influences of age, behavior, and hormonal control, it is found that structural changes in the antennal lobes of the bee coincide not

only with shifts in the tasks being performed but also with behavioral changes associated with olfactory learning and memory. Based on correlation between thickness of the postsynaptic nucleus density and excitatory or inhibitory function of a synapse holding in the nucleus, it argued that the changes in morphology of synapses contributed some inhibitory synapses to excitatory contacts (Guldner & Ingham, 1980). Recent evidences demonstrated that synaptic plasticity in hippocampal-nucleus accumbens (NAc) was disrupted by repeated cocaine treatment (Goto & Grace, 2005). Taken together these data, we suggested that, related to functional roles of hippocampal CA1 and CA3 fields, the changes of these excitatory synapses on dopaminergic cells were a neural adaption common to all major classes of addictive substances and therefore might play a critical role in the development of addiction.

In conclusion, we showed that anxiety-like symptoms of rats significantly occurred to the rats after acute morphine withdrawal, but buspirone administration could reverse these indexes. We also proved that the appearance/disappearance of anxiety-related symptoms was related to the ultrastructural changes/reversibility of synapses in the hippocampus with morphine and buspirone administrations. So, these results are theoretically related the psychological symptoms with neural circuitry regulation. Future studies will focus on the hippocampal neuron signal pathway of the buspirone administration after the morphine acute withdrawal. It is helpful to pursue the effective therapeutic methods of morphine addiction.

Acknowledgements

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