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Research Report

Morphine withdrawal affects both delayed-escape behaviour in Morris water maze and hippocampal NR2A/2B expression ratio

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ABSTRACT

Repeated low-dose morphine treatment facilitates delayed-escape behaviour of hippocampus-dependent Morris water maze and morphine withdrawal influences hippocampal NMDA receptor-dependent synaptic plasticity. Here, we examined whether and how morphine withdrawal influenced delayed-escape behaviour and NR2A/2B expression ratio of hippocampal synaptosomes. We found that both delayed-escape behaviour and NR2A/2B expression ratio showed an inverted-*U* curve and peaked on 4-day withdrawal during a 20-day withdrawal period. Furthermore, treatment of the glucocorticoid receptor antagonist RU38486 for 3 days reduced delayed-escape behaviour and NR2A/2B ratio on 4-day withdrawal to a level similar to those of 18-h withdrawal. In contrast, elevated-platform stress enabled delayed-escape behaviour of 18-h withdrawal to a higher level similar to that of 4-day withdrawal, but had no significant effect on the NR2A/2B ratio. Similar behavioural effects were also found after intrahippocampal infusions of the NMDAR antagonist AP-5 or NR2B-containing NMDAR antagonist Ro25-6981 for 3 days. These findings suggest that delayed-escape behaviour enabled by repeated low-dose morphine treatment may be a useful and simple rat model for studying addictive memories to be retrieved by stress exposure.

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Abbreviations: LTP, Long-term potentiation; NR2A and NR2B, *N*-methyl-D-aspartate receptor subunits NR2A and NR2B
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1. Introduction

Drug addiction is marked by persistent drug craving and compulsive relapse, leading to the withdrawal/drug use cycles perhaps for a life-time (Hyman and Malenka, 2001; Koob and Le, 1997; Robinson and Berridge, 2000; White, 1996; Wise, 1998). Regarding the withdrawal state, opiate addicts first show aversive withdrawal symptoms, followed by drug craving and relapse even if withdrawal symptoms have long diminished (Hyman and Malenka, 2001; Nestler, 2001). It is suggested that the relapse is experienced to avoid aversive withdrawal symptoms and to enhance mood (Khantzian, 1985; Koob and Le, 1997). Alternatively, drug withdrawal may increase incentive values of drugs by learning mechanisms, thereby increasing the risk for later relapse (Robinson and Berridge, 2000). Withdrawal state-associated learning may be attributed to either aversive opiate withdrawal symptoms or drug craving without significant withdrawal symptoms. Thus, it is of interest to test withdrawal effects from repeated low-dose morphine treatment on whether learning mechanisms can be triggered without significant withdrawal symptoms.

The hippocampus is well-known to be critical for certain types of memory (Eichenbaum, 2000). The underlying mechanism is

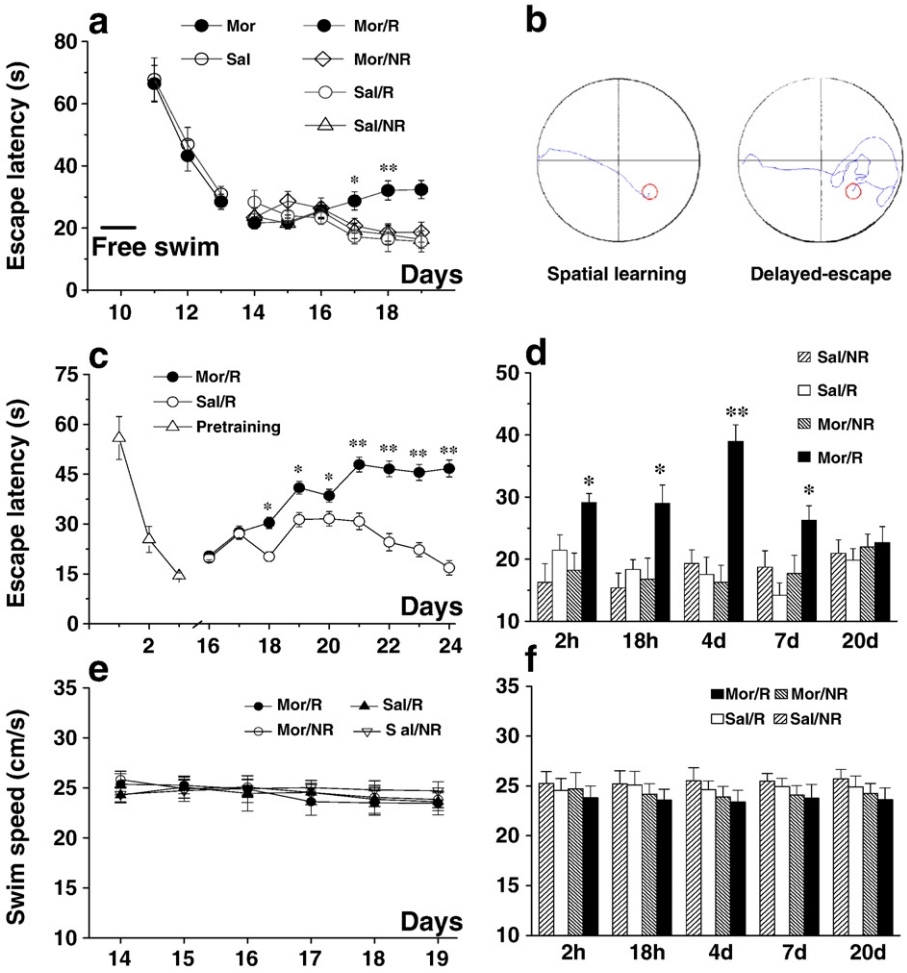

Fig. 1 – Withdrawal from low-dose morphine caused an inverted-*U* curve in delayed-escape behaviour. a, Repeated low-dose morphine (Mor) or saline (Sal) treatment did not influence spatial learning task of Morris water maze. A reinforced procedure (R), rewarded morphine or treated saline if escape latencies showed longer or shorter than 30 s, enabled Mor/R but not Sal/R to learn a new strategy of escape. In contrast, a non-reinforced procedure (NR), randomly treated morphine or saline, failed to affect escape behaviour in Mor/NR and Sal/NR (*$p<0.05$, **$p<0.01$ Mor/R vs. Mor/NR or Sal/R). b, Representative tracking traces for spatial learning (Mor/NR) and delayed-escape behaviour (Mor/R). c, Delayed-escape behaviour was confirmed in the fixed location/visible platform version of the Morris water maze. Likewise, low-dose morphine (1 mg/kg/trial) effectively reinforced delayed-escape behaviour in rats repeatedly treated with morphine (Mor/R) but not with saline (Sal/R) ($p<0.01$, *$p<0.05$ Mor/R vs. Sal/R). d, The learned delayed-escape behaviour showed inverted-*U* curve over a 20-day withdrawal period and peaked on 4-day withdrawal in the Mor/R group, but escape behaviour in the three control groups, Mor/NR, Sal/R and Sal/NR, remained unchanged (*$p<0.05$, **$p<0.01$ Mor/R vs. Mor/NR or Sal/R). e, Swimming speed during morphine-reinforced training did not differ among groups. f, Swimming speed during the withdrawal period did not differ among groups.**

widely believed to be dependent on experience- or activity-dependent synaptic plasticity (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Martin et al., 2000), e.g. long-term potentiation (LTP) and long-term depression (LTD). Recent studies suggest that NR2A/2B-containing NMDA receptors (NMDAR) may govern hippocampal LTP and LTD, respectively (Liu et al., 2004). On the other hand, ample evidence has demonstrated that the hippocampus plays critical roles in regulating the stress effect on learning and memory (Kim and Diamond, 2002; McEwen, 1999). The underlying mechanisms are associated with impaired LTP (Foy et al., 1987; Shors et al., 1989) but facilitated LTD (Kim et al., 1996; Xu et al., 1997, 1998) through NR2B-containing NMDAR (Wang et al., 2006; Yang et al., 2005) or a glucocorticoid receptor (Diamond et al., 1992; McEwen et al., 1986; Xu et al., 1998). These scientific advances provide unique aspects/models to test memories associated with addictive behaviours. For example, Vorel et al. report that electric stimulation in the hippocampus may read out addictive memories and thus induce cocaine-seeking behaviour (Vorel et al., 2001). This memory mechanism helps to understand why drug craving and relapse can be often triggered by cues or stress. However, recent studies on hippocampal plasticity show that repeated morphine treatment impairs LTP, but morphine re-exposure restores LTP (Pu et al., 2002); either repeated morphine treatment or morphine re-exposure impairs LTD (Yang et al., 2004). These findings raise questions whether hippocampal plasticity is impaired by repeated drug uses, and how addictive memories are formed. In marked contrast, morphine withdrawal enhances hippocampal LTP, and further study reveals an inverted-*U* curve over a 20-day withdrawal period, in which the largest LTP is on 4-day withdrawal (Dong et al., 2007, 2006b). Thus, it seems that the hippocampus may play more important roles in addictive memories associated with the withdrawal state.

To avoid significant withdrawal symptoms, and the influences on motor activity and cognition following high-dose morphine treatment, we have developed a delayed-escape paradigm of the Morris water maze using repeated low-dose morphine (Mor) (Yang et al., 2004), which enables subsequent low-dose morphine to reinforce the Mor group but not the saline (Sal) group to learn delayed-escape behaviour that lasts for over a 20-day withdrawal period. Remarkably, here we found that delayed-escape behaviour and NR2A/2B expression ratio showed an inverted-*U* curve during the withdrawal period and peaked on 4-day withdrawal, which were prevented by either antagonist NMDAR or glucocorticoid receptor. These findings suggest that delayed-escape behaviour enabled by repeated low-dose morphine treatment may be a useful and simple rat model for studying addictive memories to be retrieved by stress exposure.

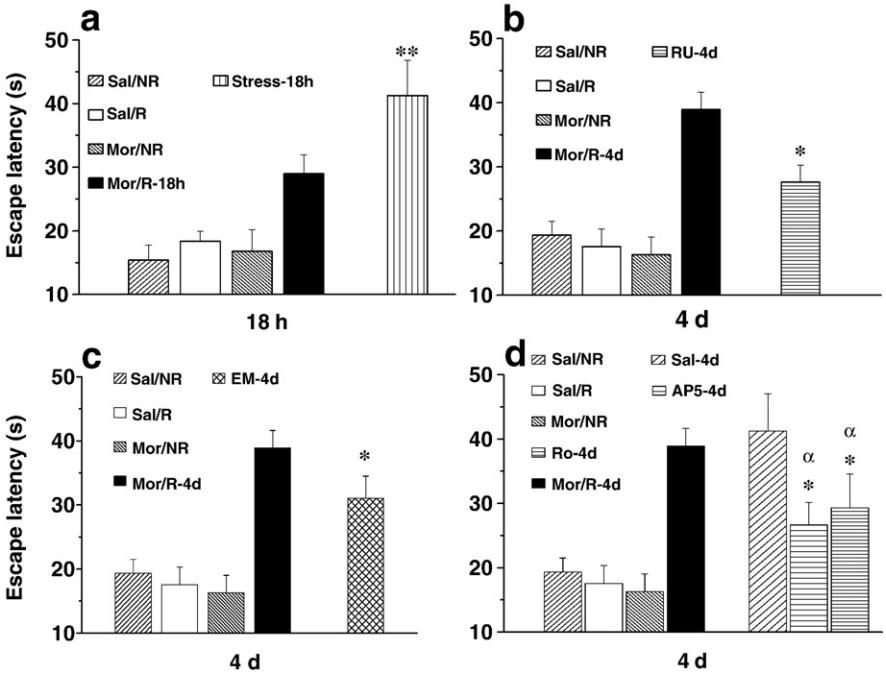

Fig. 2 – NMDAR and glucocorticoid receptor mechanisms in delayed-escape behaviour during withdrawal. a, Stress applied at 16-h withdrawal enhanced delayed-escape behaviour at 18-h withdrawal (Str-18 h) ($p<0.01$ Str-18 h vs. Mor/R-18 h). b, Conversely, the glucocorticoid receptor antagonist RU38486 (RU-4d) given twice per day for the first 3-day suppressed delayed-escape behaviour on 4-day withdrawal (*$p<0.05$ RU-4d vs. Mor/R-4d). c, Similarly, morphine re-exposure (EM-4d) 12-h before test suppressed delayed-escape behaviour on 4-day withdrawal (*$p<0.05$ EM-4d vs. Mor/R-4d). d, Moreover, bilateral intrahippocampal infusion of the NMDAR antagonist AP-5 (AP5-4d) or the NR2B-containing NMDAR antagonist Ro25-6981 (Ro-4d) for the first 3-day withdrawal suppressed delayed-escape behaviour on 4-day withdrawal (*$p<0.05$ AP5-4d or Ro-4d vs. Mor/R-4d; $^{\alpha}p<0.05$ AP5-4d or Ro-4d vs. Sal-4d). Infusions of saline (Sal-4d) with the same procedure had no effect ($p>0.05$ Sal-4d vs. Mor/R-4d).**

2. Results

2.1. *Withdrawal caused inverted-U curve of delayed-escape behaviour*

Rats were treated with low-dose morphine (Mor, 3 mg/kg/day, i.p.) or saline (Sal, 0.6 ml/kg/day, i.p.) for 13 days, while adapted to a water maze on day 10 (free swimming, Fig. 1a) and trained in the spatial learning task at 3 trials per day on days 11–13. Both groups equally learned the task as indicated by shorter latencies on day 13 compared with those on day 11 (Sal and Mor, days 11–13; Fig. 1a). Then, each group was divided into 2 subgroups and subjected to reinforced and non-reinforced training, 3 trials per day on days 14–19. In reinforced training, animals (Mor/R, $n=89$; Sal/R, $n=10$) were rewarded with morphine (1 mg/kg/trial) if escape latencies were longer than 30 s; otherwise, they were treated with saline (0.2 ml/kg/trial). In non-reinforced training, animals (Mor/NR, $n=8$; Sal/NR, $n=10$) were randomly treated with low-dose morphine or saline regardless of their escape latencies. We found that low-dose morphine reward had no effect on the Sal/R group ($n=10$, days 14–19; Fig. 1a) but enabled the Mor group to learn a new strategy, by which animals swam around the hidden platform for morphine reward until a rapid escape (Mor/R, $n=89$; Fig. 1a and b). We termed this strategy as delayed-escape behaviour. Furthermore, in the non-reinforced training groups, random low-dose morphine reward failed to enable delayed-escape behaviour in both Sal and Mor groups (Mor/NR, $n=8$; Sal/NR, $n=10$; days 14–19; Fig. 1a). In a fixed location/visible platform task, a similar reinforced procedure enabled the Mor (Mor/R, $n=10$) but not the Sal group (Sal/R, $n=10$) to form delayed-escape behaviour (Fig. 1c), in which the criterion was set 30 s on days 19–21 and 40 s on days 22–24. These results further confirmed our previous report (Yang et al., 2004). Since delayed-escape behaviour depended on repeated low-dose morphine treatment and later low-dose morphine-reinforced training (Mor/R), it may be a unique animal model indicating drug-seeking behaviour.

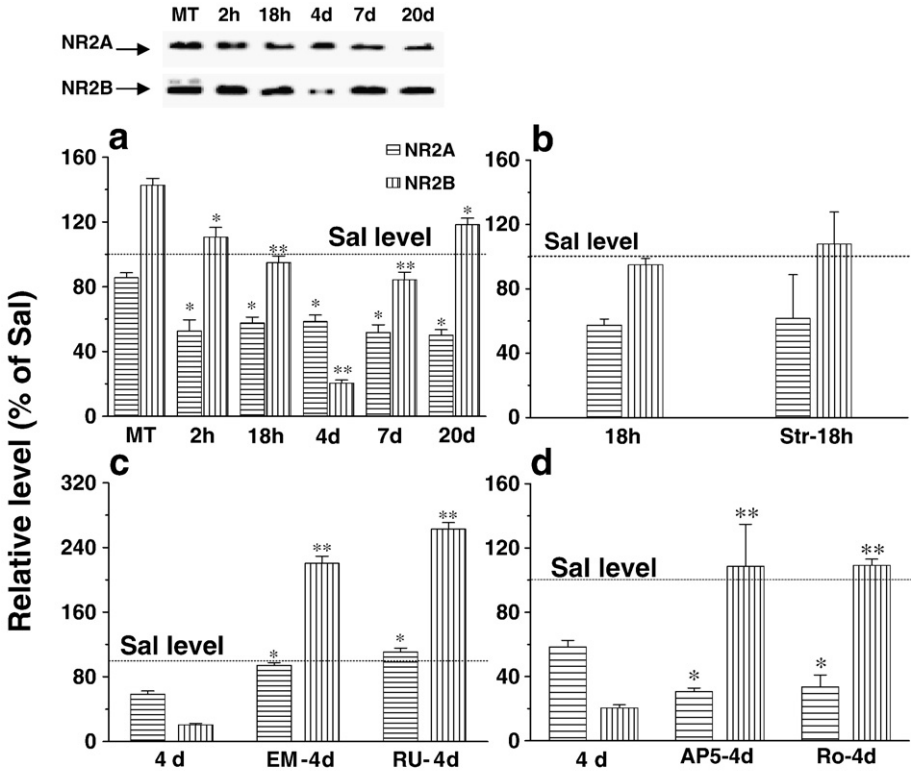

Fig. 3 – NR2A/2B expression in hippocampal synapses of rats after delayed-escape behaviour tests. a, Repeated low-dose morphine treatment for 13 days (MT) decreased NR2A but largely increased NR2B at hippocampal synapses compared with those found in the saline (Sal) group with the same procedure. Remarkably, over the 20-day withdrawal period, NR2A remained at low levels of expression, but NR2B showed a *U* curve and the minimal level occurred on 4-day withdrawal. Upper panel: Western blots of NR2A and NR2B levels in groups. Lower panel: Summary of the NR2A and NR2B levels in synaptosomes in groups of morphine treatment for 13 days (MT), morphine withdrawal for 2 h, 18 h, 4 days, 7 days and 20 days ($n=6$ per group) ($^{**}p<0.01$, $^{*}p<0.05$ vs. MT). b, NR2A and NR2B subunit of NMDARs at hippocampal synapses remained unchanged at 18-h withdrawal of rats exposed to behavioural stress for 30 min (Str-18 h), 2 h before behavioural test. c, However, NR2A was increased and NR2B was largely increased on 4-day withdrawal of rats treated with the glucocorticoid receptor antagonist RU38486 (RU-4d) twice per day at 12 h intervals for the first 3 days of withdrawal or with a single dose of morphine re-exposure (EM-4d) at 12 h before behavioural test ($^{**}p<0.01$, $^{*}p<0.05$ RU-4d or EM-4d vs. 4d). d, NR2A was decreased but NR2B was increased on 4-day withdrawal of rats infused with the antagonist to NMDARs (AP5-4d) or to NR2B-containing NMDARs (Ro-4d), twice per day at 12 h intervals for the first 3 days of withdrawal ($^{**}p<0.01$, $^{*}p<0.05$ AP5-4d or Ro-4d vs. 4d).

Then, the Mor/R group ($n=89$) was further divided into 5 subgroups and subjected to morphine withdrawal for 2 and 18 h, and 4, 7 and 20 days, respectively. Delayed-escape behaviour was accordingly examined for 3 trials without morphine reward. Remarkably, although these animals did not show significant withdrawal symptoms, delayed-escape behaviour exhibited an inverted-*U* curve over the withdrawal period and peaked on 4-day withdrawal (on 2 h, 18 h, 4 day, 7 day and 20 day: $n=10$, 29.1±1.5 s; $n=9$, 30.0±3.0 s; $n=7$, 39.0±2.7 s; $n=7$, 26.3±2.4 s; $n=8$, 22.7±2.6 s; Fig. 1d), while escape behaviour remained unchanged in the other three groups (Mor/NR, Sal/R and Sal/NR; Fig. 1d). Swimming speed was not different among groups during delayed-escape behaviour training (Fig. 1e) and withdrawal period (Fig. 1f). Thus, withdrawal from repeated low-dose morphine (3 mg/kg/day, i.p.) for 4 days enabled a strongest delayed-escape behaviour, which is highly consistent with previous reports that withdrawal from repeated high-dose morphine (20 mg/kg/day, s.c.) for 4 days enabled a largest LTP in the hippocampus (Dong et al., 2006b; Pu et al., 2002), thereby indicating drug-seeking behaviour driven by hippocampal memory mechanism.

2.2. *Stress/glucocorticoid receptor mechanism in delayed-escape behaviour*

Since stress can often trigger addictive behaviours, we further examined whether stress influenced delayed-escape behaviour during withdrawal. Rats were exposed to elevated-platform stress (16-h) and then delayed-escape behaviour was examined at 18-h withdrawal. We found that elevated-platform stress enhanced delayed-escape behaviour (Str-18 h, $n=11$; Fig. 2a) to a level similar as that found on 4-day withdrawal ($p>0.05$ vs. Mor/R-4d). Conversely, when the glucocorticoid receptor antagonist RU38486 (20 mg/kg, s.c.) was given twice per day for the first 3 days of withdrawal, delayed-escape behaviour on 4-day withdrawal was reduced (RU-4d, $n=8$; Fig. 2b) to a level similar as that found on 18-h withdrawal ($p>0.05$ vs. Mor/R-18 h). These results suggest that withdrawal may activate the glucocorticoid receptor to enhance delayed-escape behaviour, suggesting that chronic mild stress occurs without significant withdrawal symptoms. Furthermore, when morphine re-exposure (3 mg/kg, i.p.) was given at 12 h before the 4-day withdrawal, delayed-escape behaviour on 4-day withdrawal was reduced (EM-4d, $n=6$; Fig. 2c) to a level similar as that found on 18-h withdrawal ($p>0.05$ vs. Mor/R-18 h), indicating that delayed-escape behaviour was reversibly dependent on the time period of morphine withdrawal.

2.3. *NMDAR mechanism in delayed-escape behaviour*

Since addictive behaviours are also triggered by cues and our previous report demonstrates that the magnitude of hippocampal LTP shows an inverted-*U* curve during withdrawal and peaked on 4-day withdrawal, we further examined whether

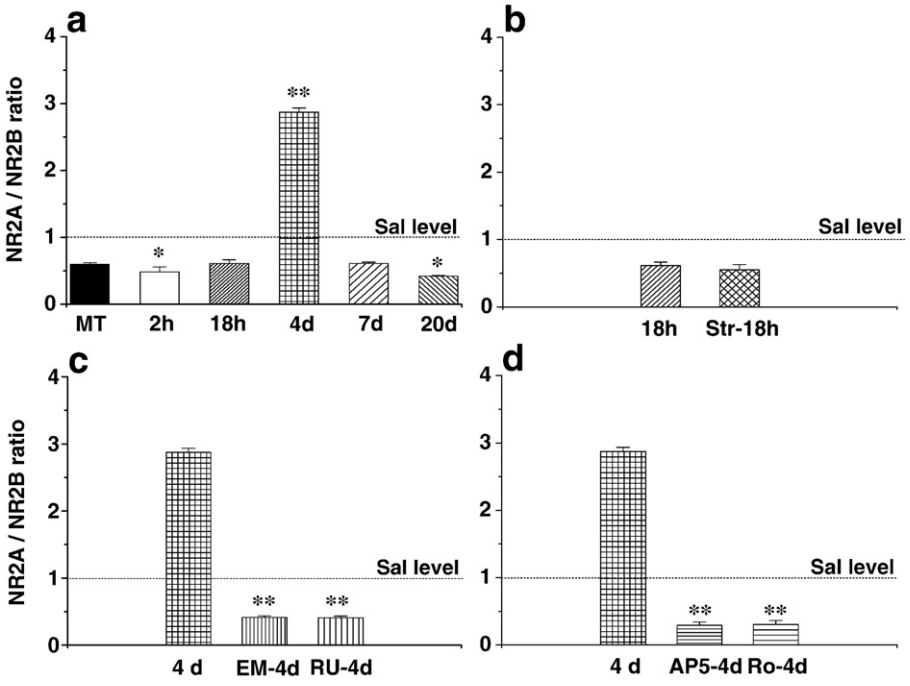

Fig. 4 – Withdrawal from low-dose morphine caused inverted-*U* curve in NR2A/2B expression ratio. a, A maximal level of NR2A/NR2B expression ratio at hippocampal synapses was on 4-day withdrawal ($p<0.01$, *$p<0.05$ vs. MT). b, Behavioural stress at 18 withdrawal did not affect NR2A/2B expression ratio of NMDARs at hippocampal synapses. c, Either the glucocorticoid receptor antagonist RU38486 treatment twice per day for the first 3 days of withdrawal or morphine re-exposure at 12 h before opiate seeking test resulted in a minimal level of NR2A/2B expression ratio at hippocampal synapses (**$p<0.01$ RU-4d or EM-4d vs. 4d). d, Intrahippocampal infusions of the NMDAR antagonist AP-5 or the NR2B-containing NMDAR antagonist Ro25-6981, twice per day for the first 3 days of withdrawal, resulted in a minimal level of NR2A/2B expression ratio at hippocampal synapses (**$p<0.01$ AP5-4d or Ro-4d vs. 4d).**

hippocampal NMDARs were involved in delayed-escape behaviour. We found that intrahippocampal infusions of the NMDAR antagonist AP-5 or the NR2B-containing NMDAR antagonist Ro25-6981, twice per day for the first 3 days of withdrawal, reduced delayed-escape behaviour on 4-day withdrawal (AP5-4d, $n=7$, Ro-4d, $n=8$; Fig. 2d) to a level similar as that found at 18-h withdrawal, whereas the infusions of saline with the same procedure had no effect on delayed-escape behaviour (Sal-4d, $n=8$; Fig. 2d).

2.4. *Withdrawal caused inverted-U curve of NR2A/2B ratio*

Since ratio of NR2A/2B may govern LTP and LTD (Liu et al., 2004), we examined the expression of NR2A and NR2B subunits of NMDARs at hippocampal synaptosomes of rats ($n=6$ per group) after the behavioural studies described above. We found that NR2B was increased but NR2A was decreased after repeated low-dose morphine treatment compared with that found in the repeated saline group (Sal) (Fig. 3a). Following morphine withdrawal, however, NR2B exhibited a marked reduction on 4-day (4d; Fig. 3a), while NR2A remained low levels of expression (2h–20d; Fig. 3a), resulting in an inverted-*U* curve of NR2A/NR2B expression ratio peaked on 4-day withdrawal (Fig. 4a). Furthermore, elevated-platform stress at 18-h withdrawal slightly increased NR2B and had no effect on NR2A compared to those found at 18-h withdrawal without elevated-platform stress (Figs. 3b, 4b). However, opiate withdrawal for 4 days with the treatment of the glucocorticoid receptor antagonist RU38486 for the first 3 days increased NR2A, similar as that found in the Sal group ($p>0.05$ RU-4d vs. Sal), and largely increased NR2B, to a maximal level ever found at hippocampal synapses (Fig. 3c). It resulted in a large reduction in NR2A/2B ratio on 4-day withdrawal (Fig. 4c). Morphine re-exposure given at 12 h before 4-day withdrawal increased NR2A and increased NR2B largely on 4-day withdrawal (Fig. 3c). Similarly, this resulted in a large reduction in NR2A/2B ratio on 4-day withdrawal (Fig. 4c). In addition, intrahippocampal infusions of AP-5 or Ro25-6981 reduced NR2A, but increased NR2B, to a level similar as that found in the Sal group (Fig. 3d). However, this still resulted in a large reduction in NR2A/NR2B ratio on 4-day withdrawal (Fig. 4d). Thus, withdrawal caused an inverted-*U* curve of both delayed-escape behaviour and NR2A/2B ratio, which depended on the activation of hippocampal NMDAR and glucocorticoid receptor.

3. Discussion

The major findings were that withdrawal from repeated low-dose morphine treatment caused an inverted-*U* curve in both delayed-escape behaviour and hippocampal NR2A/2B ratio over a 20-day withdrawal period, and peaked on 4-day withdrawal. The effects were dependent on glucocorticoid receptor and NMADR mechanisms.

3.1. *Delayed-escape behaviour*

It is known that the reinforcing/rewarding effect of addictive drugs can be tested by animal models of drug addiction, e.g. morphine conditioned place preference, as indicated by the rats which preferred to stay in morphine-paired compartment. In the Morris water maze, survival motivation drives rats to readily learn escape onto a hidden platform, which type of memory is known to be dependent on the hippocampus (Morris et al., 1982). However, we used delayed-escape paradigm to enable rats to learn a delayed-escape strategy for morphine reward, while a rapid escape resulted in a saline treatment. Remarkably, only the rats repeatedly treated with morphine formed delayed-escape behaviour, for which rats swam around the hidden platform until a rapid escape. Delayed-escape behaviour may have resulted from the competition between rewarding and survival motivation. Delayed-escape behaviour is persistent for at least 20 days after withdrawal. Since delayed-escape behaviour is dependent on both repeated morphine treatment and morphine-reinforced training, it thus may indicate drug-seeking behaviour when there is no morphine rewarding in withdrawal state.

3.2. *Low-dose morphine treatment for delayed-escape behaviour*

Although repeated low-dose morphine treatment may not produce significant tolerance and dependence as well as significant withdrawal symptoms, the fundamental problem of drug addiction is persistent drug craving and compulsive relapse. However, repeated low-dose morphine treatment may provide unique insight into drug addiction when there are no significant tolerance and dependence, and importantly no significant withdrawal symptoms after withdrawal. Here, we demonstrated that repeated low-dose morphine enabled low-dose morphine to reinforce delayed-escape behaviour, which lasted at least for 20-day withdrawal. Furthermore, with re-exposure to the water maze, rats still showed delayed-escape behaviour without morphine rewarding. This could resemble addictive behaviours often triggered by drug-taking associated cues. Furthermore, although no significant physical symptoms were evoked by withdrawal because of the regimen of repeated low-dose morphine treatments, delayed-escape behaviour showed an inverted-*U* curve over a 20-day withdrawal and peaked on 4-day withdrawal. However, chronic mild stress still occurred since it was dependent on glucocorticoid receptor and additional acute stress produced similar effect as that found on 4-day withdrawal. This suggests that the motivational or stress-like effects of withdrawal itself, but not the physical symptoms, may further modify delayed-escape behaviour. It is highly consistent with our previous report that the magnitude of hippocampal LTP shows inverted-*U* curve over a 20-day withdrawal and peaks on 4-day withdrawal in rats repeatedly treated with high-dose morphine (10 mg/kg, s. c.) twice per day for 12 days, a procedure known to produce significant tolerance and dependence to the drug. Therefore, withdrawal from repeated low-dose morphine treatment may cause adaptation of hippocampal functions similar to withdrawal from drugs of abuses.

3.3. *Withdrawal caused inverted-U curve of NR2A/2B ratio*

Recent evidence suggests that NR2A/2B-containing NMDAR may govern hippocampal LTP and LTD (Liu et al., 2004). Notably, stress-impaired LTP and facilitated LTD in hippocampal

CA1 area can be prevented by the NR2B-containing NMDAR antagonist Ro25-6981 (Wang et al., 2006; Yang et al., 2005). Here, we found that NR2A/2B ratio in hippocampal synaptosomes showed an inverted-*U* curve and peaked on 4-day withdrawal. It is highly consistent with our previous report that 4-day withdrawal largely increases the ratio of NR2A/2B-containing NMDAR mediated whole-cell currents, due to a large reduction of the NR2B component current (Dong et al., 2006b). Therefore, either the altered expression or current ratio of NR2A/2B subunits in hippocampal synapses may be responsible for the largest LTP on 4-day withdrawal. Since LTP is widely regarded to be the mechanism underlying certain types of learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Martin et al., 2000), we provided strong evidence supporting that withdrawal may directly modify delayed-escape behaviour, a type of memory that may be associated with drug-seeking behaviour to be retrieved by cues or stress.

3.4. *NMADR and glucocorticoid receptor mechanisms for delayed-escape behaviour*

Ample evidence suggests that hippocampus-dependent memory and hippocampal plasticity are sensitive to repeated drug uses and behavioural stress (Kim and Diamond, 2002; McEwen, 1999), and the underlying mechanisms are associated with NR2B-containing NMDAR and glucocorticoid receptor (Kim and Diamond, 2002; Wang et al., 2006; Xu et al., 1998; Yang et al., 2005). Here we demonstrated that both delayed-escape behaviour and NR2A/2B ratio showed inverted-*U* curve over a 20-day withdrawal and peaked on 4-day withdrawal. Furthermore, injection of the glucocorticoid receptor antagonist RU 38486 for the first 3 days of withdrawal, the largest delayed-escape behaviour and NR2A/2B ratio on 4-day withdrawal were prevented. This is consistent with our previous report that morphine conditioned place preference is prevented by intrahippocampal infusion of RU 38486 in a dose-dependent manner (Dong et al., 2006a). Moreover, intrahippocampal infusion of the NMDAR antagonist AP-5 or NR2B-containing NMDAR antagonist Ro25-6981 suppressed both delayed-escape behaviour and NR2A/2B ratio found on 4-day withdrawal. These findings suggest that hippocampal memory and stress mechanisms are involved in withdrawal state-associated memories, which provide a possible explanation why addictive behaviours can be often triggered by cues or stress. Although we mainly focused on hippocampal NMDAR and glucocorticoid receptor mechanisms after low-dose morphine withdrawal, other mechanisms such as mechanisms involving GABAergic system cannot be ruled out. Furthermore, other brain regions can also contribute to withdrawal state-associated memories, such as the ventral tegmental area that is known to be critical in drug addiction and relevant to the effect of stress or glucocorticoids on cocaine addiction (Saal et al., 2003).

3.5. *Hippocampal memory mechanisms in withdrawal state*

The mechanism how stress triggers drug craving and relapse remains unclear. Evidence shows that stress produces enhancement, impairment or no effect on learning and memory (Conrad et al., 1999; de Kloet et al., 1999; de Quervain et al., 1998; Lupien and Lepage, 2001; Payne et al., 2002; Sapolsky, 2000; Shors et al., 1992). Recent evidence shows that withdrawal-associated stress or additional stress can facilitate hippocampal LTP in withdrawal state (Dong et al., 2006b), which is opposite to the impairing effect of stress on LTP normally found in naive/untreated rats (Diamond et al., 1992; Foy et al., 1987; Shors et al., 1989; Xu et al., 1997). Interestingly, a recent report demonstrates that dopamine-deficient mice shows normal morphine conditioned place preference (Hnasko et al., 2005), which suggests the importance of other neurotransmitter systems or brain areas in opiate addiction. Based on our present and previous findings, hippocampal functions in memory and stress regulation could be critical for opiate addiction. A common inverted-*U* curve over a 20-day withdrawal period that peaked on the same 4-day withdrawal was found in NR2A/2B ratio, LTP magnitude and delayed-escape behaviour. Similar interventions by using NMDAR and glucocorticoid receptor antagonists restored these parameters on 4-day withdrawal to a level found at 18-withdrawal. Furthermore, both hippocampal LTP and LTD are impaired after repeated morphine treatment (Pu et al., 2002; Yang et al., 2004). Therefore, our present findings suggest a possibility that withdrawal from low-dose morphine, which may not be physically but psychologically stressful, for 3 days, alters hippocampal functions dramatically, leading to addictive memories often triggered by cues or stress.

Taken together, we examined the mechanisms for delayed-escape behaviour after withdrawal from repeated low-dose morphine treatment, and found that delayed-escape behaviour is sensitive to the time period of withdrawal, which is dependent on both glucocorticoid receptor and NMDAR. It is highly consistent with previous hippocampal plasticity findings after withdrawal from repeated high-dose morphine. Thus, the delayed-escape behaviour may be a useful and simple rat model for drug addiction studies providing new insight into addictive memories to be retrieved by cues or stress exposure.

4. Experimental procedures

4.1. *Subjects*

Male Sprague–Dawley rats, weighing 200–250 g, were used. Animal care and experimental protocol were approved by the Chinese Academy of Sciences, PR China.

4.2. *Stress*

Behavioural stress was given at 16-h withdrawal. After 1.5 h in home cage, delayed-escape behaviour was examined on 18-h withdrawal. Behavioural stress was evoked by placing rats on an elevated platform for 30 min similar to those described previously (Xu et al., 1997, 1998; Yang et al., 2004).

4.3. *Behaviour studies*

4.3.1. *Apparatus*

The Morris water maze consisted of a circular pool (250 cm in diameter, 60 cm deep at the side) filled to a depth of 20-cm water at 25.0±1.0 °C, and the water surface was covered with floating black resin beads. Yellow curtains were drawn around the pool (50 cm from the pool periphery), and displayed distinctive marks that served as visual cues. An automatic tracking system was

used to record latencies and swimming distances (Yang et al., 2003a,b).

4.3.2. *Delayed-escape behaviour*

4.3.2.1. Pre-treatment. Animals were treated with low-dose morphine (3 mg/kg, i.p.) or saline (0.6 ml/kg, i.p.) in the evening (8:00 pm) once per day for 13 days, while they were adapted to the water maze on day 10, and trained in the spatial learning task at 3 trials per day at 2 h inter-trial intervals on days 11–13. Low-dose morphine is used to avoid the disruption of motor activity and cognitive functions, which is also crucial in determining the sensitivity of delayed-escape paradigm and avoiding significant withdrawal symptoms.

4.3.2.2. Delayed-escape paradigm. The animals trained by spatial learning task and showed escape latencies about 30 s on day 13 were divided into 4 subgroups: two for *reinforced training* (R), Mor/R ($n=89$) and Sal/R ($n=10$); two for *non-reinforced training* (NR), Mor/NR ($n=8$) and Sal/NR ($n=10$), which was 3 trials per day at 2 h inter-trial intervals on days 14–19. We chose 30 s as a criterion to determine morphine or saline treatment. In the *reinforced* training, an animal (Mor/R or Sal/R) was given a morphine reward (1 mg/kg, i.p.) or treated with saline (0.2 ml/kg, i.p.) if escape latencies were longer or shorter than 30 s. For comparison, an animal (Mor/NR or Sal/NR) was randomly given morphine (1 mg/kg, i.p.) or saline (0.2 ml/kg, i.p.) in *non-reinforced* training. Except for the duration of repeated morphine treatment and morphine dose which were slightly increased, these procedures were similar as those described previously (Yang et al., 2004). Total morphine exposure (3 mg/kg/day, i.p.) was normalized by giving supplemental injections to animals in the evening (8:00 pm) each day. The *non-reinforced* group received the same treatment as the *reinforced* group, providing comparable information of motor activity and cognitive functions for its counterpart, the *reinforced* group.

4.3.2.3. Testing procedures. A new escape strategy was formed as indicated by swimming around the hidden platform until rapid escape and was termed as delayed-escape behaviour. Delayed-escape behaviour was formed only in the Mor/R group, but not in other groups (Mor/NR, Sal/R and Sal/NR). This learned delayed-escape behaviour was long-lasting after withdrawal. Then, we examined delayed-escape behaviour in the Mor/R group after withdrawal for 2 h ($n=10$), 18 h ($n=9$), 4 days ($n=7$), 7 days ($n=7$) and 20 days ($n=8$) at 30-min after a priming dose of morphine (0.1 mg/kg, i.p.) injection. The other three groups did not show delayed-escape behaviour and were also examined (Sal/R ($n=10$), Mor/NR ($n=8$) and Sal/NR ($n=10$)) over the withdrawal period after the morphine priming.

4.3.3. *Delayed-escape behaviour in a fixed location/visible platform task*

We used experimental procedures of the fixed location/visible platform task similar to those described previously (Yang et al., 2003b). A hidden platform was made apparent by attaching a red flag that protruded 15 cm above water surface.

4.3.3.1. Pre-training. Additional 20 rats were treated with low-dose morphine (Mor, $n=10$, 3 mg/kg/day, i.p.) or saline (Sal, $n=10$, 0.6 ml/kg/day, i.p.) in the evening (8:00 pm) for 13 days after trained in the fixed location/visible platform task, 3 trials per day at 2 h inter-trial intervals on day 1–3, which enabled the rats to escape onto a visible platform within about 15 s.

4.3.3.2. Delayed-escape paradigm. In the first 3 days of the reinforced training (days 16–18), we set 20 s as the criterion to determine morphine reward or saline treatment. If animals showed latencies longer than 20 s they were rewarded with morphine (1 mg/kg/trial, i.p.), but they were treated with saline (0.2 ml/kg/trial, i.p.) if they showed latencies shorter than 20 s. Then, the criterion was set at 30 s on days 19–21 and at 40 s on days 22–24. A similar procedure of supplemental injection was performed in the evening to normalize the total amount of morphine exposure for each rat. Likewise, low-dose morphine (1 mg/kg/trial, i.p.) effectively reinforced delayed-escape behaviour in rats repeatedly treated with morphine (Mor/R) but not with saline (Sal/R).

4.4. *Guide cannulae implantation*

Stainless steel guide cannulae were bilaterally implanted into the surface of the dorsal hippocampus by using standard stereotaxic techniques under pentobarbitone anaesthesia (50–60 mg/kg), similar as those describe previously (Day et al., 2003; Dong et al., 2006a,b). Stainless steel guide cannulae (22 gauge, 11 mm) were implanted into the left and right dorsal hippocampus (AP −3.5, L±2.5, V −2 relative to bregma). The animals were then housed individually for 2 weeks for recovery. After these animals had learned delayed-escape behaviour, the NMDA receptor antagonist AP-5 (10 mM per cannulae, 1 μl, 6 min), the NR2B-containing NMDA receptor antagonist Ro25-6981 (1 mM per cannulae, 1 μl, 6 min) or saline (1 μl per cannulae, 6 min) was infused into the dorsal hippocampus by a Hamilton syringe (1 μl, projecting 1 mm beyond the guide cannulae by polyethylene tubing) driven by a mini pump, twice per day at 12 h intervals for the first 3 days of opiate withdrawal and delayed-escape behaviour was examined on 4-day withdrawal. Once animals finished the delayed-escape behaviour test, the guide cannulae placement was histologically verified by infusion of methylene blue dye.

4.5. *Western blot analysis*

After behavioural studies, hippocampal tissues (6 per group) were quickly obtained and frozen by dry ice immediately and stored in −70 °C until use. Synaptosomes were extracted by using techniques similar to those previously described (Luo et al., 1997). Samples containing equivalent amounts of proteins (5 μg) were loaded onto 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels and resolved by standard electrophoresis (Bio-Rad, Hercules, CA, USA). Proteins were transferred to nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine (pH 8.3), 20% methanol and 0.05% SDS). The membranes were incubated with a blocking buffer of 5% non-fat dry milk in TBST (20 mM Tris–HCl (pH 7.4), 140 mM NaCl, 0.1% Tween-20) for 1 h at room temperature. The membranes were then incubated with anti-NR2A, anti-NR2B antibodies (1:500; Chemicon, Temecula, CA) in blocking buffer overnight at 4 °C. After several washes with TBST, the membranes were

incubated with horseradish peroxidase-conjugated secondary antibody in blocking buffer for 1 h at room temperature. Following washes for 20 min with four intermediate changes of TBST, proteins were visualized with enhanced chemiluminescence. Autoradiographs were scanned using a Bio-Rad imaging densitometry and quantified by using Quantity One-4.4.0 Analysis software. In order to make samples on different gel comparable, every gel was run with three lanes of the same cortex proteins as a standard (Luo et al., 1997). All results were normalized to the Sal samples to obtain the percentage and the ratio of NR2A/NR2B level, and expressed as the mean ± s.e.m.%.

4.6. *Data analysis*

Statistical comparisons were made by using *t*-test or least significant difference test of one-way ANOVA (SPSS 10.0). Significance level was set at $p<0.05$.

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