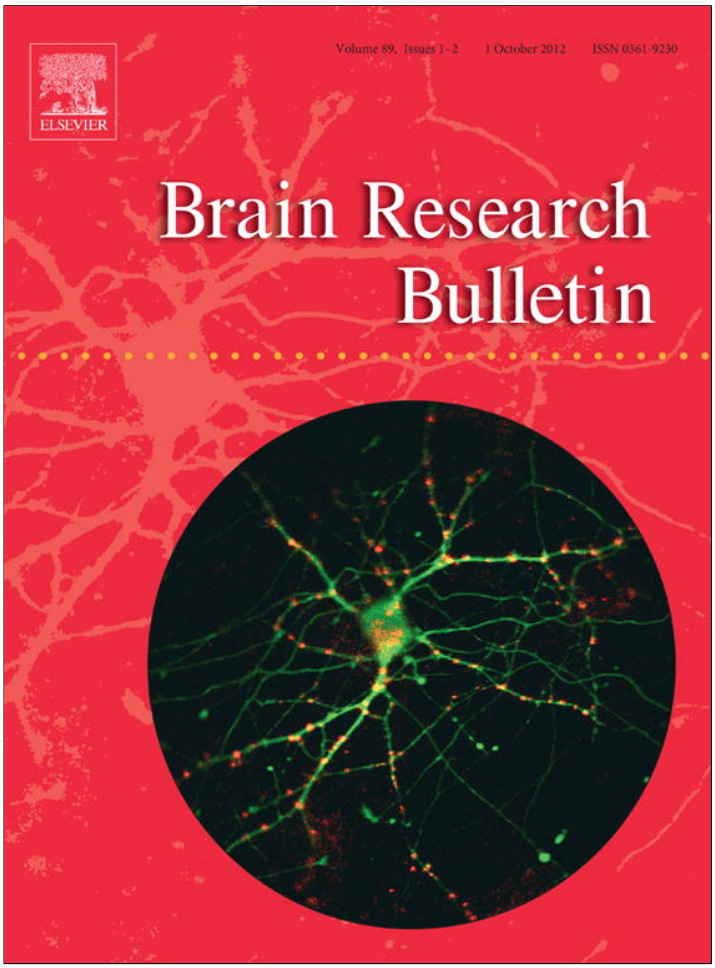

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Research report

Essential role of NR2B-containing NMDA receptor–ERK pathway in nucleus accumbens shell in morphine-associated contextual memory

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ABSTRACT

Learned associations between the rewarding effect of addictive drugs and drug-paired contexts resist extinction and contribute to the high rate of relapse observed in drug addicts. Although it has been shown that extracellular signal-regulated kinase 1/2 (ERK1/2) activity in the nucleus accumbens (NAc) is modulated by the primary rewarding effect of opiates, little is known as to its role in the morphine-associated contextual memory. In the present study, we investigated the ERK1/2 activity indicated by phosphorylated ERK1/2 (pERK1/2) levels in rats using a morphine-induced conditioned place preference (CPP) procedure. Our results showed that, in rats that had undergone morphine conditioning, after testing (expression phase) pERK1/2 in the NAc shell but not the NAc core or the adjacent caudate putamen was specifically increased. pERK1/2 levels in several other parts of the brain involved in drug-seeking, such as the medial prefrontal cortex, dorsal hippocampus, and basolateral amygdala, showed no significant changes. A significant positive correlation was observed between the elevated pERK1/2 level in the NAc shell and the degree of conditioned preference for morphine-associated contexts. Bilateral injection of an inhibitor of ERK activation into the NAc shell attenuated ERK1/2 phosphorylation and prevented the expression of morphine CPP, but injections into the core did not. Selective inhibition of NR2B-containing NMDA receptor in the NAc shell by ifenprodil prevented CPP expression and down-regulated local ERK1/2 phosphorylation. These findings collectively suggest that recall of morphine-associated contextual memory depends specifically upon ERK1/2 activation in the NAc shell and that ERK1/2 phosphorylation is regulated by the upstream NR2B-containing NMDA receptor.

1. Introduction

It has become clear that mechanisms involved in drug addiction and those underlying learning and memory seem to converge over the past decades (Hyman, 2005; Kelley, 2004; Nestler, 2002). Once a relationship has been established between an environmental cue and the rewarding effect of an addictive drug, the cue triggers the same responses as the drug itself (Schultz, 1998; Schultz et al., 1992). Memory for the drug-paired cues is highly resistant to extinction and often provokes relapse to drug use in humans and prompts drug-seeking behavior in other animals (O'Brien et al., 1992; Shaham et al., 2003; Wikler, 1973). Although dopamine (DA) and opiate receptor systems have been demonstrated to be involved in the memory for drug-associated cues, recent work had focused more on the role of glutamate system and its interaction with dopamine (Hallett et al., 2006; Hyman et al., 2006). Conditioned place preference (CPP) used in our study is a model commonly used to study the conditioned incentive value of morphine-associated contextual cues. In this method, behavioral experiments were conducted in three phases: pretesting phase, conditioning (training) phase and expression phase (testing). During the pretesting phase, baseline of the animal's preference was measured. During the training phase, animals learn to associate the rewarding effect of morphine with one context—in this case, a specific chamber—and to associate the control vehicle with another. Responses are then measured during the expression phase (testing) by evaluating the degree to which conditioned animals increase their contact with an environment in which they have previously encountered drug rewards (Tzschentke, 1998).

Previous studies on neural circuitries associated with CPP have implicated the involvement of multiple brain regions, including NAc (Bechara and van der Kooy, 1989). Exposure to environmental cues associated with morphine administration elicits neuronal activation, which is indicated by immediate early gene c-fos product protein Fos in the nucleus accumbens (Schroeder et al.,

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2000; Schroeder and Kelley, 2002). Place conditioning can change the firing activity of NAc neurons and this population of neurons can persistently encode location preference, which manifests as morphine CPP (German and Fields, 2007). Recent studies have found that NAc subregions, the core and the shell play different roles. Miller and Marshall (2005) have shown that cocaine-paired contextual cues activate ERK solely in the NAc core. However, one microdialysis study has demonstrated that conditioned cues paired with morphine administration increase dopamine release in the NAc shell but not in the core (Bassareo et al., 2007). Reinstatement of drug-seeking behavior induced by heroin-associated contextual cue requires activation of the NAc shell but not the D1-like receptors in the core or glutamate release in the shell (Bossert et al., 2006). Taken together, it can be concluded that it is NAc shell but not the core that is involved in the conditioned responses evoked by opiate-paired contextual cues. However, little is known about the molecular mechanism by which opiate-associated cues initiate drug-seeking behavior. ERK signaling has been implicated in long-term synaptic plasticity (English and Sweatt, 1996), associative learning (Atkins et al., 1998; Kelly et al., 2003), and drug addiction (Zhai et al., 2008). Studies have also revealed that stimulation of D1-like receptors and NMDA receptors can activate ERK in the NAc medium spiny neurons (MSNs) (Valjent et al., 2005). For this reason, we have hypothesized that ERK in the NAc shell is the molecular substrate for CPP induced by opiate-associated contexts. The present study was designed to determine the role of ERK signaling pathway in the NAc subregions, namely the shell and the core, in the expression of morphine-induced CPP, a widely accepted animal model of drug-associated reward memory.

2. Materials and methods

2.1. Animals

One hundred and sixty-seven male Sprague-Dawley rats weighing 180–220 g were obtained from the Laboratory Animal Center, Peking University Health Science Center. Rats were kept in a temperature- and humidity-controlled vivarium under a 12 h:12 h reversed light–dark cycle (lights on at 7:00 p.m.) and housed four per cage. Food and water were available ad libitum. Behavioral experiments were performed during the dark phase. All animal procedures were approved by the Institutional Animal Care and Use Committee, Peking University, and consistent with the NIH Guide for the Care and Use of Laboratory Animals. Appropriate efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Morphine CPP conditioning procedure

The three-chamber CPP apparatus contained two large black conditioning chambers, A and C (A and C, 280 mm × 220 mm × 225 mm), which offered the rats different tactile and visual cues; both chambers A and C could be accessed from chamber B (B, 135 mm × 220 mm × 225 mm) in the middle through door-way like openings (Ma et al., 2006, 2007). Fourteen photobeams were placed across the chambers 47.5 mm apart. Through a computer interface, the time spent in each chamber was recorded for each rat. The preference score for each rat was calculated by dividing the time spent in the drug-paired compartment by the time spent in both conditioning compartments. Locomotor activity was estimated by counting the total number of crossings between any two adjacent compartments. The basic behavioral experiment schedule is illustrated in Fig. 1A. On day 0, pretest was performed to determine the baseline of rat's preference: all rats received saline injections and then they were placed in chamber B and allowed to freely explore the apparatus for 15 min. Rats that spent more than 540 s in either chamber A or chamber C were classified as biased and eight of the initial 167 rats were excluded. During the four conditioning days, days 1–4, rats were given twice-daily 45 min sessions in the apparatus. The morphine CPP group was confined to one conditioning chamber after saline injection (1 ml/kg, i.p.) in the morning; 6 h later, morphine CPP rats were given an injection of morphine (4 mg/kg, i.p.) and placed in the other conditioning chamber; these rats were counterbalanced based on pretest score as well as morphine-paired chamber. The saline CPP group received saline injections (1 ml/kg, i.p.) before being placed in either chamber. On day 5, expression phase test was performed: rats were each given a saline injection (1 ml/kg, i.p.) and placed in chamber B with free access to both conditioning chambers for 15 min. To serve as control for detecting effect of the expression test on ERK1/2 activity, we also used two groups receiving morphine or saline conditioning but not expression testing. On day 5 rats that did not receive expression testing were kept in their home cages.

2.3. Tissue preparation and Western blotting

To determine ERK1/2 activity in brain regions of interest, all rats were decapitated rapidly 15 min after the end of the 15 min CPP expression test and their brains were harvested. The 15 min window was chosen based on the previous reports (Miller and Marshall, 2005). Brains were quickly frozen in isopentane (−70 °C) and stored at −80 °C until further use. NAc core and shell, CPU, mPFC, CA1, BLA punches were obtained from 60 μm thick sections taken on a sliding freezing microtome using 16 or 12 gauge needles. Tissues were sonicated in 150–200 μl ice-cold RIPA buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, and 0.1% SDS (Beijing Applygen Technologies Inc., Beijing, China). Homogenates were then centrifuged at 12,000 rcf for 5 min and the supernatant was removed. Thirty micrograms of protein sample was subjected to PAGE and transferred to PVDF membranes for immunodetection using rabbit antisera (Cell Signaling, Beverly, MA, USA) to pERK1/2 (1:2000, #4370s) or total ERK1/2 (1:1000; #4695). All blots were incubated in 1:5000 anti-β-actin mouse polyclonal antibody (Zhongshan Biotechnology, Beijing, China) to correct differences in protein loading. The Western blotting analysis also included the respective nucleus of fifteen naïve rats that served as reference point in the graphical presentation of the data. These naïve rats were used for assessing the basal levels of total and phosphorylated ERK1/2 but did not participant in the behavioral experiments. Band intensities were quantified by densitometry using a TotalLab 2.01 analysis system (Phoretix, UK).

2.4. Cannula implantation and microinfusions

To assess the involvement of ERK and NR2B-containing NMDA receptor in the expression of morphine CPP, cannulas were surgically implanted bilaterally to locally infuse U0126 or ifenprodil, and control vehicle into the NAc shell and core. Rats were anesthetized with chloral hydrate (35 mg/kg, i.p.) and secured on a Kopf stereotaxic apparatus (Kopf Instrument, Tujunga, CA, USA). Bilateral stainless steel guide cannulas (0.67 mm in outer diameter) were implanted 1 mm above the NAc shell: anteroposterior (AP), +2.0 mm relative to bregma; lateral (L), ±1.1 mm; dorsoventral (DV), −7.8 mm; from the skull and the NAc core: anteroposterior (AP), +1.6 mm relative to bregma; lateral (L), ±2.3 mm; dorsoventral (DV), −7.6 mm from the skull. The guide cannulas were glued to the skull with dental cement. Internal cannulas were replaced with dummy cannulas to ensure clearance. Antibiotic ointment was applied to prevent infections. Rats were allowed at least 5 days to recover before the behavioral procedures began. All rats were then morphine conditioned for 4 days and on day 5, the expression test day the dummy cannulas were removed and infusion cannulas (0.3 mm in outer diameter) inserted. Drugs were infused with an infusion pump at 0.25 μl/min. A total volume of 0.5 μl of 2 μg/μl U0126 or ifenprodil and vehicle was infused into each side of the nucleus. After infusion, the cannula was left in place for an additional 1 min to allow the solution to diffuse from the cannula tip. Infusions occurred 30 min before testing. The dummy cannula was then replaced and the rat put into the place preference apparatus to assess preference for morphine-associated contextual cues. The time and dosage of U0126 or ifenprodil infusion were based on the results of a previous report (Ma et al., 2006; Miller and Marshall, 2005; Sotres-Bayon et al., 2007). U0126 (Upstate, Temecula, CA, USA) was dissolved with 5% DMSO and 6% Tween 80 in 0.1 M PBS. Ifenprodil (Sigma, USA) was dissolved with 5% DMSO and 9% Tween 80 in 0.9% saline.

To assess ERK1/2 activity regulated by U0126 or ifenprodil microinfusions, the rats were decapitated without anesthesia 15 min after the end of the 15 min CPP expression testing. After decapitation, the NAc cores and shells of the experimental rats were dissected for Western blotting assays. During tissue dissection, we also verified the location of the injection site, only those NAc samples with correct cannula placements were used for data collection. Seventeen rats with misplaced canulae were excluded. Fig. 5A shows the locations of cannula tips of the rats.

2.5. Statistical analysis

Data were processed using Graph Pad Prism 5.0 software. Results were analyzed using two-way ANOVA with the between- and within-subjects factors followed by the Bonferroni post hoc correction. In all analyses, statistical significance was all set at $P < 0.05$. Results are presented as mean ± SEM.

3. Results

3.1. Effects of morphine CPP expression testing on ERK1/2 activity in NAc, CPU, mPFC, CA1 and BLA

Fig. 1B shows that, in our experiment, rats displayed reliable place preference for drug-paired contexts after morphine conditioning but not after control saline treatment. Statistical analysis indicated that treatment had a significant effect on CPP score (pretest vs. test) ($F(1, 13) = 10.07$, $P < 0.01$). Bonferroni post hoc analysis demonstrated that rats subjected to morphine conditioning expressed significant preferences for morphine-associated

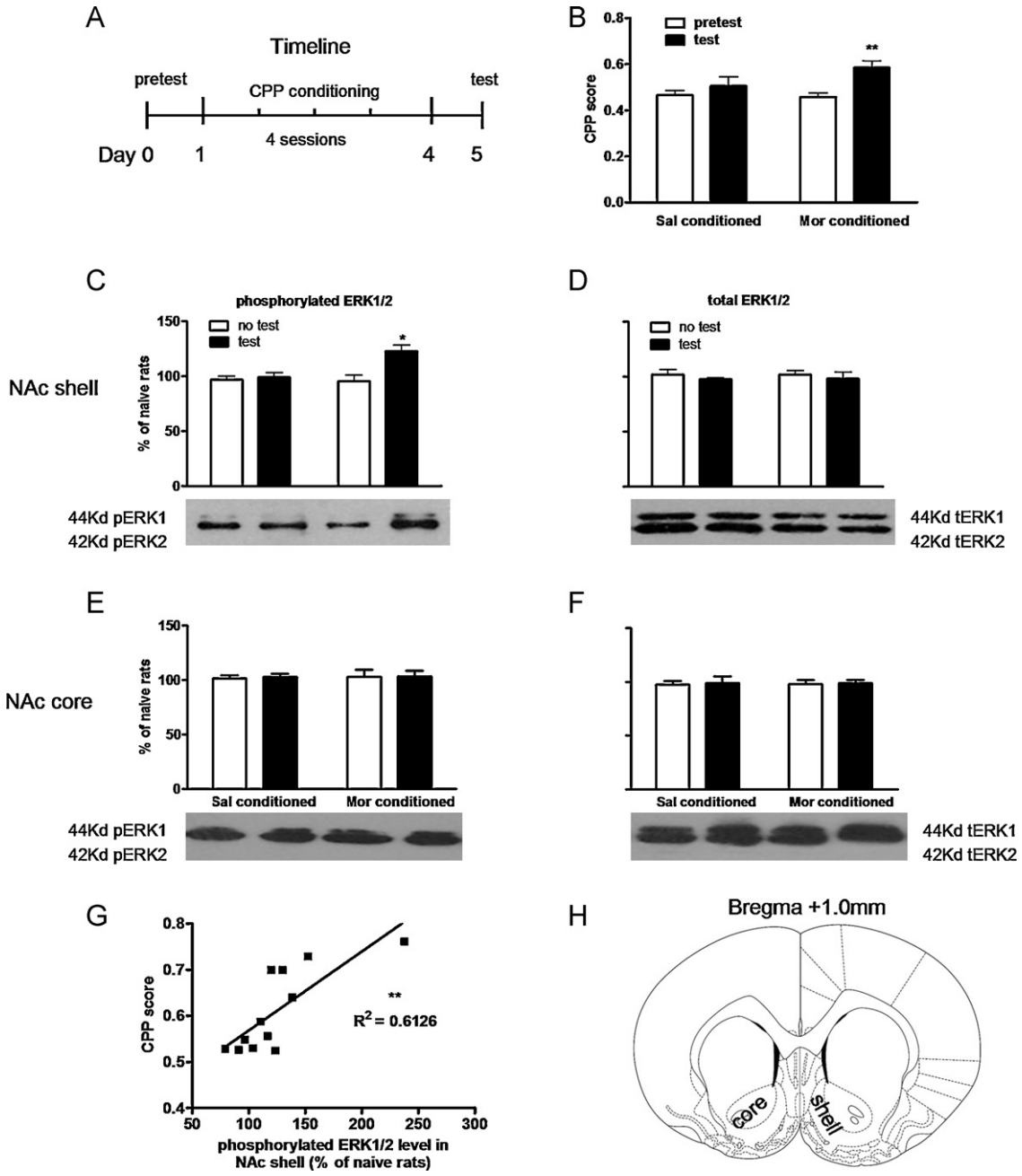

Fig. 1. Specific ERK1/2 activation in NAc shell following morphine CPP expression test. (A) Timeline for CPP conditioning procedures. (B) Morphine (4 mg/kg, i.p.) conditioning induced reliable conditioned place preference. Preference for morphine-paired contextual cues indicated by CPP score in morphine-conditioned ($n = 8$) and saline-conditioned animals ($n = 7$) during the pretest and expression test phase. $^{**}P < 0.01$, different from pretest. (C–F) Levels of NAc core and shell pERK1/2 and tERK1/2 in morphine and saline-conditioned rats with test and no test. Data are presented as a percentage of pERK1/2 and tERK1/2 of naïve rats. Representative pERK1/2 and tERK1/2 immunoblots are shown for each treatment group. $^{*}P < 0.05$, different from morphine conditioned rats without test, $n = 5$ per group. (G) pERK1/2 induction in the NAc shell positively correlated with morphine CPP score. NAc shell ERK1/2 activation induced by morphine-paired contexts is indicated by phosho-ERK1/2 content normalized by naive rats. **Statistical significance is indicated by two asterisks with $P < 0.01$, $n = 12$. (H) Location of sample taken from NAc core and shell. pERK1/2 and tERK1/2 refer to phosphorylated and total ERK1/2.

contexts ($t = 3.543$, $P < 0.01$), while saline-conditioned rats did not ($t = 1.031$, $P > 0.05$).

To evaluate ERK1/2 activity in the brain following morphine CPP expression, we divided twenty rats into four groups of rats in a 2×2 factorial design (between-subject treatment factors: saline vs. morphine conditioned $\times$ within-subject expression test factors: test vs. no test). As shown in Fig. 1C–F, in contrast to that of NAc core, ERK1/2 in NAc shell was activated after morphine CPP expression test. There were significant main effects in test factor ($F(1, 8) = 5.831$, $P < 0.05$) and treatment ($F(1, 8) = 18.15$, $P < 0.01$) when the amount of pERK1/2 in the NAc shell was analyzed. Bonferroni post hoc analysis confirmed that levels of pERK1/2 was significantly higher in morphine-conditioned rats that received expression test than in those not subjected to test ($t = 3.198$, $P < 0.05$, Fig. 1C). Among saline-treated rats, no significant difference was observed between rats subjected to expression test and those not subjected to test ($t = 0.217$, $P > 0.05$, Fig. 1C). In contrast, detection of total ERK1/2 immunoreactivity showed no inter-group difference in the NAc shell between treatments ($F(1, 8) = 0.01$, $P > 0.05$) or test factor ($F(1, 8) = 1.62$, $P > 0.05$, Fig. 1D). In the NAc core, however,

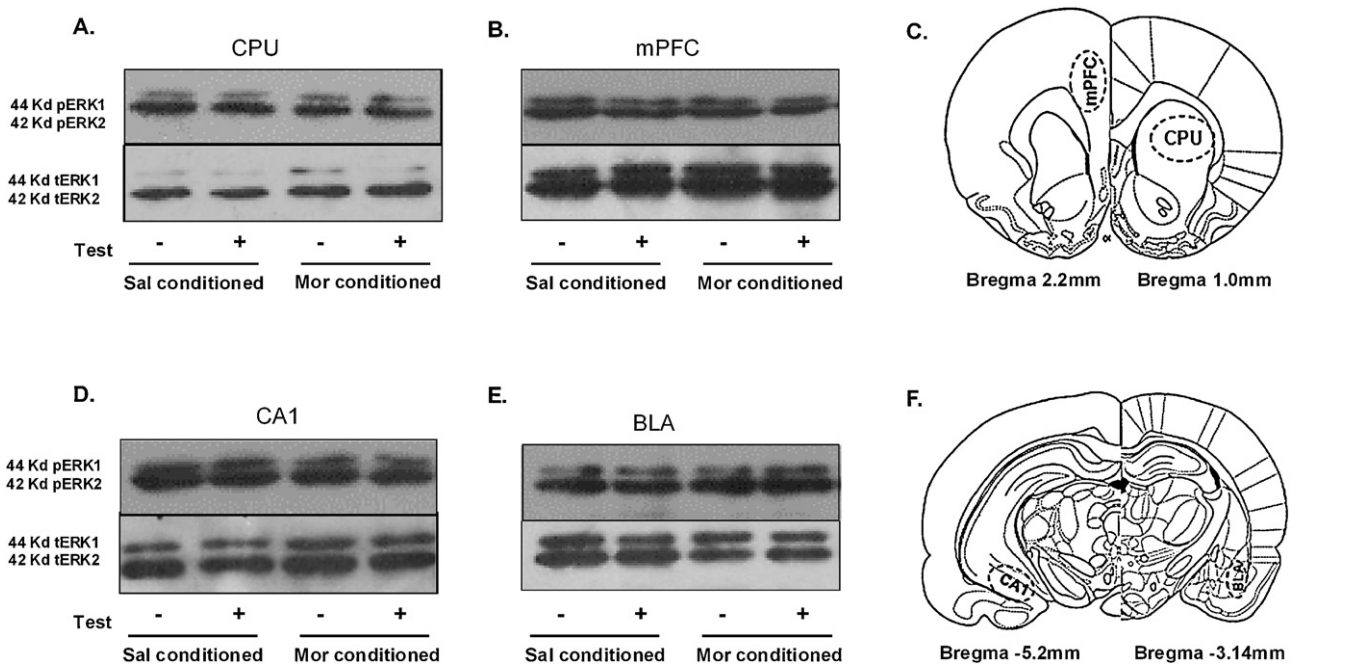

Fig. 2. Morphine CPP expression testing did not change the levels of pERK1/2 and tERK1/2 in mPFC, BLA, or the hippocampus CA1. There is no significant difference between saline or morphine conditioned rats with test or no test. Representative blots of pERK1/2 and corresponding tERK1/2, β-actin bands were obtained from the samples of the (A) CPU, (B) mPFC, (D) BLA, and (E) hippocampus CA1. (C and F) Circles in the coronal section insets indicate the locations of samples from the CPU, mPFC, BLA, and hippocampus CA1. CPU refers to caudate putamen. BLA refers to basolateral amygdala. "−" refers to no test, "+" refers to test.

statistical analysis showed no inter-group differences in the pERK1/2 levels between treatments ($F(1, 8) = 0.05$, $P > 0.05$) or test factor ($F(1, 8) = 0.04$, $P > 0.05$, Fig. 1E); examination of total ERK1/2 levels also revealed no significant difference between treatments ($F(1, 8) = 0.01$, $P > 0.05$) or test factor ($F(1, 8) = 0.06$, $P > 0.05$, Fig. 1F).

To further evaluate the relationship between ERK1/2 activity in the NAc shell and morphine CPP behavior, an independent group of rats was morphine-conditioned and their NAc shell samples were probed for ERK1/2 activity. It was found that CPP score was positively correlated with levels of pERK1/2 expression in the NAc shell ($R^2 = 0.6126$, $P < 0.01$, $n = 12$, Fig. 1G).

To validate whether the specific change of ERK activity in NAc shell would spread to the whole stratum, samples from the CPU, which is adjacent to the NAc, was detected and showed no differences in pERK1/2 levels between groups of different treatment or test factor (Fig. 2A). Considering the important role of mPFC, hippocampus CA1, or BLA in the contextual-cue induced conditioned response, we also detected samples from these brain structures. Similarly, no significant changes in pERK1/2 levels were found in the mPFC, hippocampus CA1, or BLA (all: $P > 0.05$, Fig. 2B, D and E). Taken together, the above results indicate that morphine CPP expression could increase pERK1/2 levels in the NAc shell but not the NAc core or other brain structures.

3.2. Effects of selective MEK1/2 inhibitor U0126 on morphine CPP expression and ERK1/2 activity

In order to determine whether ERK1/2 activity is necessary for morphine CPP expression, thirty-six rats with correct cannula implantation were trained on morphine CPP and given bilateral infusions of the MEK1/2 inhibitor U0126 or its vehicle to the NAc shell or the NAc core. Infusions were administered 30 min before CPP expression testing. Statistical analysis included the between-subject factors of the drug (U0126 vs. vehicle) and within-subject factors of the test session (pretest vs. test). When drugs were administered into the NAc shell, ANOVA revealed a significant main effect of test session factor on the CPP score ($F(1, 16) = 7.471$, $P < 0.05$). As shown in Fig. 3A, Bonferroni post hoc analysis confirmed that CPP score of animals infused with control vehicle significantly increased during test compared to pretest ($t = 3.216$, $P < 0.05$), but this was not the case for animals infused with U0126 in the NAc shell ($t = 0.6491$, $P > 0.05$). There was also significant main effect of test session factor on the CPP score ($F(1, 16) = 21.45$; $P < 0.001$) when drugs were infused into the NAc core. However, rats infused with U0126 into the NAc core still expressed strong preferences for morphine-associated contexts ($t = 3.411$, $P < 0.01$) as those that received control vehicle infusions ($t = 3.139$, $P < 0.05$, Fig. 3B).

To rule out the possibility that U0126 may affect morphine CPP expression by influencing locomotion, we analyzed the shuttle times in all groups of rats using one-way ANOVA during test. Morphine-conditioned rats pretreated with U0126 did not show any significant difference in the number of crossings between adjacent chambers during the testing period compared to control rats treated with vehicle (data not shown).

To verify the effect of U0126 on ERK1/2 activity, rats were killed 15 min after testing and Western blotting analyses were performed on the isolated NAc cores and shells. Statistical analysis revealed that when drugs were infused into the NAc shell there were significant main effects of treatment factor (U0126 vs. vehicle, $F(1, 16) = 11.40$, $P < 0.01$) and of nucleus factor (NAc shell vs. NAc core, $F(1, 16) = 37.31$, $P < 0.0001$) as well as significant interaction between the treatment and nucleus ($F(1, 16) = 5.569$, $P < 0.05$); pERK1/2 levels were significantly more down-regulated in the NAc shells of rats that received U0126 infusions than in rats that had received vehicle infusions ($t = 4.056$, $P < 0.01$), but pERK1/2 levels in the NAc core were not significantly different ($t = 0.7191$, $P > 0.05$, Fig. 3C). When drugs were infused into the NAc core, the pERK1/2 level in the NAc shell was significantly different from that in the core ($F(1, 16) = 113.6$, $P < 0.0001$). As Fig. 3D shows, U0126 infusion

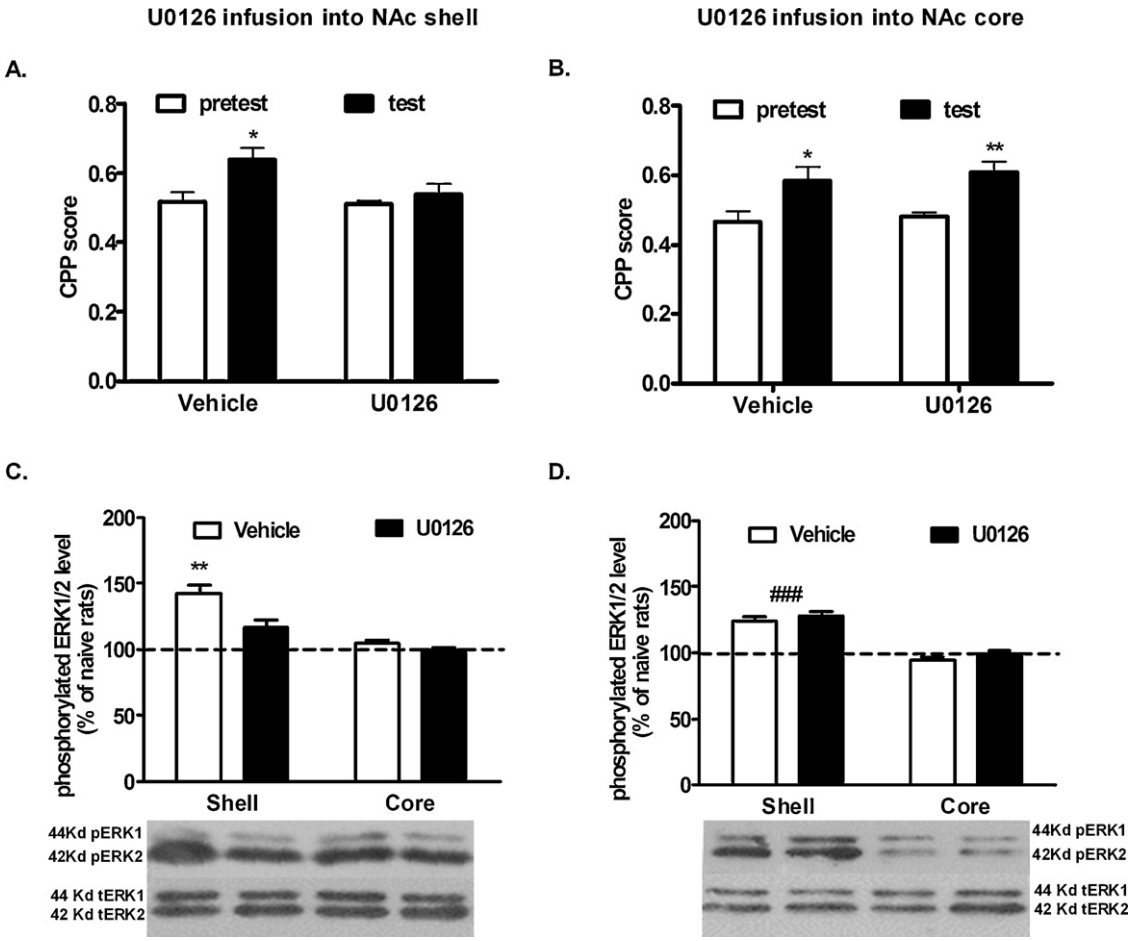

Fig. 3. Effects of intra-NAc shell or core infusion of U0126 (1 μg/side) on morphine CPP expression and pERK1/2. (A and B) U0126 microinjections into the NAc shell abolished the expression of morphine CPP but injection into the core did not. $^{*}P<0.05$; $^{**}P<0.01$; different from pretest, $n=9$ per group. (C and D) Bilateral intra-NAc shell infusions of U0126 significantly decreased the amount of NAc shell but not core pERK1/2 relative to vehicle controls. $^{**}P<0.01$, different from pERK1/2 level in NAc shell from U0126 treated rats; $^{\#\#\#}$significantly different from pERK1/2 level in NAc core, $n=5$ per group.

into the NAc core did not significantly change pERK1/2 level in the NAc shell ($t=1.057$, $P>0.05$) or in the core ($t=1.03$, $P>0.05$). Furthermore, neither U0126 infusions into the NAc core nor those into the shell had significant effect on total ERK level ($P>0.05$, data not shown).

3.3. Effects of NR2B-containing NMDA receptor selective antagonist ifenprodil on morphine CPP expression and ERK1/2 activity

Although NMDA receptors containing the NR2B subunit were found to reside in brain regions related to reward and reinforcement, the role of NR2B in the conditioned response induced by morphine-associated contexts is not known. In this experiment, we assessed whether blockage of NR2B-containing NMDA receptors would affect morphine CPP expression and ERK1/2 activity.

Five days after surgical implantation of guide cannulas, thirty-six rats with correct cannula implantation undergoing morphine conditioning were divided into four groups: either ifenprodil or control vehicle was infused into the NAc core or NAc shell. These rats were matched for unconditioned CPP scores during pretest. For CPP scores after infusions into the NAc shell, there were significant main effects of treatment factor (ifenprodil vs. vehicle, $F(1, 16)=13.81$, $P<0.01$), test session factor (pretest vs. test, $F(1, 16)=7.522$, $P<0.05$), as well as significant interaction between the two factors ($F(1, 16)=7.131$, $P<0.05$). For infusions into NAc core, significant effect was only observed in test session factor ($F(1, 16)=89.29$, $P<0.0001$). Importantly, after receiving ifenprodil into the NAc shell, there was no longer significant difference in CPP score between test and pretest ($t=0.051$, $P>0.05$, Fig. 4A), yet for rats receiving intra-NAc core infusion of ifenprodil, the difference in CPP score between test and pretest was still significant ($t=6.585$, $P<0.001$, Fig. 4B).

Fifteen minutes after the behavioral tests, rats were killed for analysis of ERK1/2 activity in the NAc shell and core. For ERK1/2 activity after infusions into the NAc shell, there were significant main effects of treatment factor (ifenprodil vs. vehicle, $F(1, 16)=30.69$, $P<0.0001$), the nucleus factor (NAc shell vs. NAc core, $F(1, 16)=26.53$, $P<0.0001$), as well as significant treatment × nucleus interaction ($F(1, 16)=16.88$, $P<0.001$). For infusions into NAc core, there was significant main effect of treatment factor ($F(1, 16)=32.34$, $P<0.0001$). Importantly, as Fig. 4D shows, intra-NAc-core infusions of ifenprodil did not change the level to which pERK1/2 in the NAc shell was upregulated by morphine-paired contexts relative to vehicle-infused controls; however, ifenprodil infusion into the NAc shell significantly reduced the amount of pERK1/2 present there relative to amounts in the core ($t=6.823$, $P<0.001$, Fig. 4C).

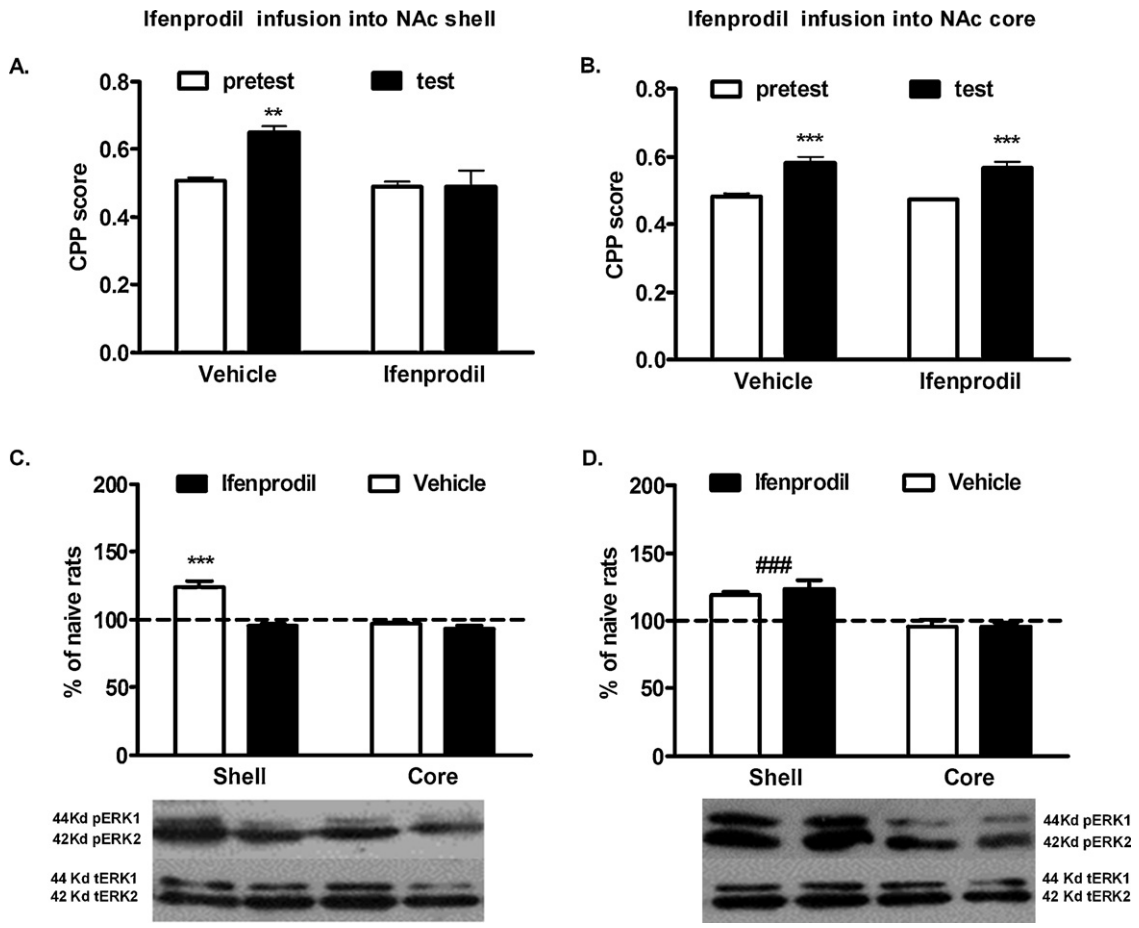

Fig. 4. Effects of intra-NAc shell or core ifenprodil infusion (1 μg/side) on morphine CPP expression and pERK1/2. (A and B) Ifenprodil microinjections into the NAc shell abolished the expression of morphine CPP but injections into the core did not. $^{**}P < 0.01$; $^{***}P < 0.001$; different from pretest, $n = 9$ per group. (C and D) Bilateral intra-NAc shell infusions of ifenprodil decreased the amount of NAc shell but not core pERK1/2 relative to the vehicle. $^{***}P < 0.001$, different from pERK1/2 level in NAc shell from ifenprodil treated rats; $^{###}$significantly different from pERK1/2 level in NAc core, $n = 5$ per group.

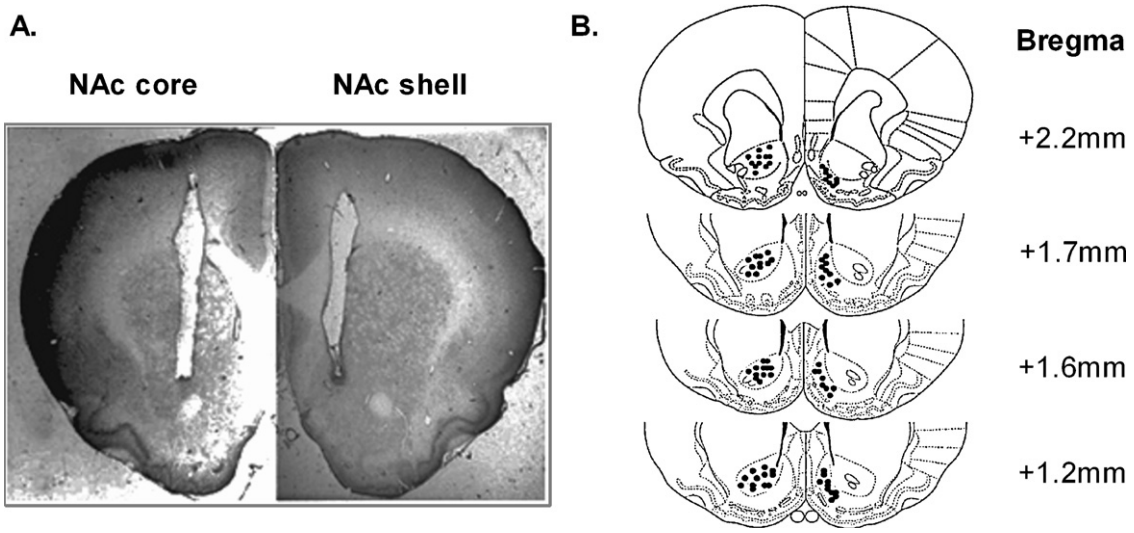

Fig. 5. Representative cannula placements and microinjection sites in the NAc. (A) The NAc core and shell cannula placements of rats. (B) Locations of needle tips for all intra-NAc core and shell infusions. Because of extensive overlap between the infusion needle tips, not all tip locations are illustrated in this diagram.

4. Discussion

4.1. Specific ERK1/2 activation in the NAc shell following morphine-induced CPP expression

This study showed that pERK1/2 levels in the NAc shell specifically increased in rats subjected to morphine conditioning and re-exposed to motivationally relevant contextual cues. However, changes in pERK1/2 level did not spread throughout the mesolimbic system to nuclei such as CPU, mPFC, CA1, or BLA. This provides new evidence for the critical role of NAc in morphine-related contextual responses. Some of previous studies have shown that context-induced cocaine- and alcohol-seeking behavior could activate neurons in the lateral hippocampus and medial prefrontal cortex (mPFC) (Hamlin et al., 2007; Neisewander et al., 2000), which are also elementary constructs of drug seeking-associated neurocircuitry. Fuchs et al. (2002) have shown that BLA also plays an important role in the motivation for cocaine elicited by cocaine-associated cues. We reason that ERK activation occurs only in the NAc in this study due to the followings. Specific elevation of ERK1/2 phosphorylation in the NAc can be attributed to specific substantial bases. Ortiz et al. (1995) have demonstrated that the aggregate total ERK1/2 concentration is significantly higher in the NAc than in the frontal cortex, caudate putamen, hippocampus, or hypothalamus using quantitative blot immunolabeling. The catalytic activity of ERK kinase (MEK) also parallels the ERK protein level. Specifically, it has the same regional distribution (Ortiz et al., 1995). The relationship between contexts and reward relies on the neural circuitry, involving several structures such as the prefrontal cortex, basolateral amygdala, and NAc. Reward-related information is always integrated in the limbic and cortical area and delivered to the motor output structure NAc (Carelli and Ijames, 2001; Lee et al., 2005; Miller and Marshall, 2005; Weiss et al., 2000; Zavala et al., 2003). In fact, the expression of morphine CPP requires activation of the NAc ERK1/2 suggests that NAc is the main site to response to incentive contextual cues. In parallel with this point, the NAc neurons have been shown to increase their activity (indexed by fos-like protein) after exposure to the morphine-paired environment (Schroeder et al., 2000). Even after two weeks of abstinence these neurons could still be activated by morphine-conditioned cues (Harris and Aston-Jones, 2003).

Unlike the drug of abuse, which activates ERK in both the NAc core and shell (Valjent et al., 2004), we found that morphine-paired cues activated ERK solely in the NAc shell. Our finding that ERK1/2 activation was specific to the shell of NAc expanded upon earlier research, which did not discriminate the subregion of NAc. It was the different structure with distinct afferent and efferent connections that led to the different roles of the NAc core and shell in motivated behavior (Heimer et al., 1991; Voorn et al., 1989). In parallel with our results, dopamine or glutamate transmitters in the NAc shell have been shown to mediate the context-induced reinstatement of heroin seeking (Bossert et al., 2006, 2007). NAc shell is also the brain region where CREB activity is necessary for nicotine CPP and the motivation for cocaine (Brunzell et al., 2009; Larson et al., 2011). CREB activity in NAc shell can also control the behavioral responses to rewarding stimuli associated with morphine and sucrose (Barrot et al., 2002).

4.2. Deactivation of the NAc shell ERK1/2 blocked the expression of morphine CPP

Our results showed that NAc shell ERK1/2 activity positively correlated to the degree of preference for morphine-associated contexts. When we used MEK1/2 inhibitor U0126 to inhibit ERK1/2 activation, morphine CPP expression was also abolished. These results show that NAc shell ERK1/2 activation is necessary for morphine CPP expression.

Blockage of morphine CPP expression by intra-NAc shell U0126 infusion may contribute to the effects of the drug on several psychological processes. First, we considered the possibility that U0126 might have disrupted the acquisition of context's incentive motivation. However this was ruled out because U0126 was given 30 min before expression testing and 24 h after 4 days of morphine conditioning, after the acquisition process was complete. ERK1/2 became activated only in the NAc shells of rats that had undergone morphine conditioning and shown Pavlovian approach behavior. Second, we considered whether the lack of preference for morphine-paired contexts observed in some animals that had been given intra-NAc shell U0126 infusions may have been due to decreased performance of the conditioned approach behavior. This possibility can be excluded because the U0126 treated animals did not show significantly less shuffle activity than the vehicle-treated animals. Third, we considered whether the effects of U0126 might reflect decreased conditioned value of morphine-paired contexts. Context was found to induce drug seeking because it had previously acquired motivational properties via direct association with drug rewarding during training. Many investigators have applied self-administration procedures with second-order schedules of reinforcement or acquisition of a new response to study each cue's control over reward seeking. Shiflett et al. (2008) recently reported that presentation with an auditory stimulus previously paired with food reward could activate ERK in the NAc and U0126 administered into NAc could impair acquisition of new instrumental responses induced by auditory cues. This suggests that disruption of ERK activation could interfere with the incentive-motivational effects of conditioned stimuli. Similar studies have shown that MEK inhibition can impair methamphetamine-induced CPP recall (Mizoguchi et al., 2004). In our study, saline-conditioned animals that did not show preference for the same tactile and visual cues also did not show ERK activation in the NAc shell. Sensory stimuli that by themselves were unable to activate ERK after only one exposure were found to do so only after they had been repeatedly associated with morphine. Combined with those of previous studies, our results show that the relationship between the NAc shell ERK1/2 activity and morphine-induced CPP support the hypothesis that ERK1/2 is required to retrieve the value of incentive motivation properties of morphine-associated contexts.

4.3. Activation of NR2B-containing NMDA receptor triggered ERK1/2 phosphorylation

In the striatum neurons, MEK-sensitive ERK1/2 and its downstream transcription factor CREB have been shown to be activated by ionotropic glutamate receptors (Mao et al., 2004). The current study is the first to discuss the subtype of glutamate receptor that mediates ERK1/2 activation in the NAc shell. Our results show that intra-NAc shell infusions of the NR2B-containing NMDA receptor selective antagonist ifenprodil blocked morphine-induced CPP expression by inhibiting ERK1/2 activation. Consistent with our findings, a series of studies have demonstrated that blockage of NMDA receptor can impair drug-induced CPP expression (Mead and Stephens, 1999; Tzschentke and Schmidt, 1995). In most brain regions, studies have shown a decline in the initially high NR2B levels that occur early in development (Monyer et al., 1992, 1994). However, in the striatum, NR2B protein expression remained high in the medium spinal neurons throughout adulthood (Landwehrmeyer et al., 1995; Standaert et al., 1999). The specific distribution of NMDA receptor subunit contributed to the membrane insertion of NR2B-containing NMDA receptor in the NAc shell MSNs in response to cocaine (Huang et al., 2009). In the NAc, morphine-induced CPP also specifically increased the expression

of the NR2B subunit but not that of NR2A (Ma et al., 2007). This molecular event may be the cellular adaptation that occurs prior to the development of addictive behavior. Previous studies have also shown that deactivation of NR2B-containing NMDA receptor by systemic administration of ifenprodil can reduce morphine-induced CPP expression in a dose-dependent manner (Ma et al., 2006; Narita et al., 2000). Altogether, study results show that NR2B-containing NMDA receptors can act as neural substrates underlying long-term drug-associated memory. Activation of the ERK1/2 pathway is linked to the glutamate-dependent long-term memory associated with the addictive properties of habit-forming drugs. In vitro evidence also suggests that NR2B-containing NMDA receptors are the primary activators of the NMDAR-dependent ERK pathway (Krapivinsky et al., 2003). Ras-guanine nucleotide-releasing factor may be the intermediate between the NMDA receptor and downstream ERK signaling in the NAc (Fasano et al., 2009; Krapivinsky et al., 2003). NR2B-containing NMDA receptor and its downstream ERK1/2 activation were found to be necessary for morphine CPP expression and for the retrieval of consolidated memory induced by drug-conditioned reward. Drugs like ifenprodil, which have higher affinity for NR2Bs, do not have confounding effects on the acquisition or retrieval of spatial memory (Cuzick et al., 2011; Ma et al., 2011). For this reason, it may be useful for the treatment of drug addiction.

5. Conclusions

The present study shows that ERK activation in the NAc shell to be involved in the expression of morphine CPP and that this expression is triggered by NR2B-containing NMDA receptor activation. This furthers our understanding of the molecular mechanisms of morphine-associated contextual memory and provides a new view of pharmacotherapy to opiate abuse.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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