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The Role of Dopamine D1 and D3 Receptors in N-Methyl-D-Aspartate (NMDA)/Glycine$_B$ Site-Regulated Complex Cognitive Behaviors following Repeated Morphine Administration

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Abstract

Background: Opiate addiction is associated with complex cognitive impairment, which contributes to the development of compulsive drug use and relapses. Dopamine and N-methyl-D-aspartate receptors play critical roles in opiate-induced cognitive deficits. However, the roles of D1 and D3 receptors in the N-methyl-D-aspartate/glycine$_B$ receptor-regulated cognitive behaviors induced by morphine remain unknown.

Methods: The 5-choice serial reaction time task was used to investigate the cognitive profiles associated with repeated morphine administration in D1 (D1$^{-/-}$)- and D3 (D3$^{-/-}$)-receptor knockout mice. The expression of phosphorylated NR1, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and cAMP response element-binding protein (CREB) in the brain was examined by western blotting. D1$^{-/-}$ and D3$^{-/-}$ mice were treated with the N-methyl-D-aspartate/glycine$_B$ site agonist l-aminocyclopropanecarboxylic acid and the antagonist L-701,324 to chronically disrupt N-methyl-D-aspartate receptor function and investigate their effects on morphine-induced cognitive changes.

Results: Repeated morphine administration impaired attentional function and caused impulsive and compulsive behaviors. D1$^{-/-}$ mice exhibited hardly any premature nosepokes. D3$^{-/-}$ mice showed robustly increased morphine-induced impulsive behavior. The numbers of premature responses were decreased by L-701,324 administration and increased by ACPC administration; these effects were completely abolished in D1$^{-/-}$ mice due to their inability to perform reward-based tasks. In contrast, the inhibitory effects of L-701,324 on impulsive behavior were significantly augmented in D3$^{-/-}$ mice.

Conclusions: N-methyl-D-aspartate/glycine$_B$ site functions may contribute to morphine-induced cognitive deficits, especially those related to impulsive behavior. D1 and D3 receptors may have contrasting effects with respect to modulating impulsive behavior. D3 receptors have inhibitory effects on impulsive behaviors, and these effects are clearly mediated by N-methyl-D-aspartate/glycine$_B$ receptor and μ-opioid receptor interactions.

Keywords: dopamine receptor, NMDA receptor, morphine, impulsive behavior

Significance Statement

Dopamine and N-methyl-D-aspartate (NMDA) receptors play critical roles in opiate-induced cognitive deficits, but the roles of D1 and D3 receptors in the NMDA/glycine receptor-regulated complex cognitive behaviors induced by morphine remain unknown. The present study suggests that NMDA/glycine$_B$ receptor functions contribute to impaired cognition, particularly with respect to impulsive behaviors that are associated with opiate addiction. Dopamine D1 and D3 receptors may have contrasting effects on impulsive behavior. The D1 receptor is essential for the expression of impulsive and motivational endophenotypes. D3 receptors may attenuate morphine-induced impulsive behaviors, and these regulatory effects mediate NMDA/glycine$_B$ receptor and μ-opioid receptor interactions. Our data may have important therapeutic implications in opiate-induced cognitive impairment.

Introduction

Clinical observations suggest that a number of cognitive processes are compromised in individuals with opiate addiction (van Holst and Schilt, 2011; Segala et al., 2015; P. W. Wang et al., 2015a). These maladaptive processes are seen clinically as reduced inhibition of inappropriate responses regardless of negative consequences (impulsive behavior) and a loss of control over drug intake (compulsive drug use) (Robbins, 2002; Kalivas and Volkow, 2005). Moreover, excessive attentional bias to drug-related cues and the motivation to alleviate drug withdrawal-induced negative affect (i.e., stress, anxiety, and dysphoria) may contribute to relapse (Franken et al., 2000; Koob and Le Moal, 2001). Therefore, development and validation of cognitive deficits characterizing opiate addiction, especially in animal models, are crucial for clarifying the underlying neuropathology.

The 5-choice serial reaction time task (5-CSRTT) is a well-established cognitive task used in rodents for measuring effects of systemic drug treatments on multiple aspects of cognition, including attention, behavioral inhibition, motivation, and processing speed (see Amitai and Markou, 2010 for the review). These neurocognitive functions are dependent on several brain regions, including frontal cortex (FC), nucleus accumbens (NAc), and hippocampus (HIP) (Robbins, 2002). In 5-CSRTT, animals attend to an array of nosepoke apertures; correct detection of a brief visual stimulus across 5 spatial locations provides a measure of attentional performance. Premature responses (i.e., responses before the onset of the light cue stimulus) gauge impulsivity or response disinhibition. Perseverative responses (continued nose pokes after a correct response) and timeout responses (i.e., persistent responding after the onset of a penalizing timeout period) reflect inability to shift out of an initiated behavioral pattern and therefore are considered indicators of compulsivity. Finally, the animal's latency to respond correctly presents a measure of its speed of processing, whereas, the animal's latency to retrieve a food reward permits control for nonspecific effects on locomotion or motivation.

The dopamine (DA) receptors in the cortical-mesolimbic region play an indispensable role in modulating cognitive functions (Nakajima et al., 2013; Homberg et al., 2016). For example, DA depletion from NAc modulates 5-CSRTT performance (Cole and Robbins, 1987, 1989). The attentional accuracy on 5-CSRTT in rats can be disrupted by intra-FC infusion of the D1 agonist and antagonist (Granon et al., 2000). Furthermore, activation of D2/3 receptors in NAc increases premature responding in highly impulsive rats on the 5-CSRTT (Moreno et al., 2013). In addition to the DA system, the glutamate system also plays a critical role in opiate addiction. Activation of the N-methyl-D-aspartate (NMDA) receptors requires both glutamate and its co-agonist, glycine, which binds to a specific NMDA receptor-coupled, strychnine-insensitive, glycine modulatory site (glycine$_B$ site) on the obligatory NR1 subunit (Danysz and Parsons, 1998). It has been shown that acute NMDA receptor dysfunction in the rat medial FC impairs attention and behavioral inhibition in the 5-CSRTT (Mirjana et al., 2004; Pozzi et al., 2011). However, effects of NMDA/glycine$_B$ sites, particularly on opiate-induced cognitive impairment, have not yet been examined.

Previous studies indicated that DA and glutamate system interact in the mesocorticolimbic circuitry (Lorrain et al., 2003; Del Arco and Mora, 2008; Philibin et al., 2011); thus, a dysfunctional relationship in their transmission in this circuitry may underlie the complex cognitive deficits observed in drug abuse. However, there have been several debates regarding the possible functional interactions between the NMDA receptor and the DA-ergic neural pathways underlying opiate addiction (Bishop et al., 2011; Tan et al., 2014). The present study focused on the regulatory effects of NMDA/glycine$_B$ sites on D1 and D3 receptors and the inherent cognitive consequences of these effects in a mouse model of repeated morphine administration. Three sequential experiments were carried out. Firstly, 5-CSRTT was used to investigate the cognitive profiles associated with repeated morphine administration in D1 (D1$^{-/-}$)- and D3 (D3$^{-/-}$)- receptor knockout mice. The expression of phosphorylated NR1, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and cAMP response element-binding protein (CREB) in the brain was examined by western blotting. Secondly, a partial strychnine-insensitive NMDA/glycine$_B$ site agonist, l-aminocyclopropane-carboxylic acid (ACPC), and a highly selective NMDA/glycine$_B$ site full antagonist, 7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1H)-quinolone (L-701,324), were tested to determine their effects on cognition. Thirdly, the effects of ACPC and L-701,324 on morphine-induced cognitive and behavioral changes were investigated in D1$^{-/-}$ and D3$^{-/-}$ mice via the 5-CSRTT.

Materials and Methods

Animals

Age-matched (8 weeks old on arrival, weight 18–21 g) male C57BL/6J wild-type (WT) mice and D1- or D3-receptor knockout mice (generated by Xu et al.) were housed in cages in groups of 3 in a humidity- (50±5%) and temperature-controlled (22±3°C) room (Xu et al., 1994, 1997). The mice were allowed to acclimatize for 5 days before the experiments. All training and testing sessions were conducted during the light phase (lights on from 7:00 AM to 7:00 PM). The experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University. All mice were used only once, and each experiment was carried out on a separate cohort of animals.

Drugs and Antibodies

Morphine hydrochloride (First Pharmaceutical Factory of Shenyang, Shenyang, China) was dissolved in 0.9% saline to a final concentration of 1.0 mg/mL. ACPC and L-701,324 (Sigma-Aldrich, St. Louis, MO) were suspended in a 1% aqueous solution

of Tween-80 to obtain concentrations of 20 mg/mL and 5 mg/mL, respectively. All drugs were injected i.p. at a volume of 10 mL/kg. Drug doses were chosen based on previous works (T. Li et al., 2010; Y. P. Wang et al., 2015c), pilot experiments, and other behavioral studies (Poleszak et al., 2007; Labrie et al., 2008; Skolnick et al., 2015). Rabbit polyclonal antibodies against phospho-NR1 (Ser890), phospho-CaMKII (Thr286), phospho-CREB (Ser133), and their total proteins were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibodies against GAPDH and the horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Technology (Santa Cruz, CA).

5-CSRTT Apparatus and Training Procedures

Mice were trained on the 5-CSRTT as previously described, with minor modifications (Finlay et al., 2015). The 10% condensed milk was used as the reinforcer of the task. To habituate the mice to the milk and rule out any preexisting differences in motivation, a 10-day milk preference test was conducted. The detailed information about the initial shaping and 5-CSRTT training and testing can be found in the supplementary Materials. The body weight and milk preference of mice are shown in supplementary Figure 1.

Western Blotting

To investigate the expression levels of phosphorylated NR1, CaMKII, and CREB in the brain following repeated morphine administration, the mice in experiment 1 were sacrificed immediately after completion of the final 5-CSRTT test. The brains were rapidly removed. According to Paxino and Franklin's Stereotaxic Atlas, 2nd edition (George Paxinos, 2001), brain samples of the FC [including area 1 of cingulate cortex, prelimbic cortex, and infralimbic cortex], NAc (including core and shell) and dorsal hippocampus (dHIP) were carefully dissected out on dry ice and were then frozen in liquid nitrogen and put into a -80°C freezer. The brain samples of FC, NAc, and dHIP were then processed for protein extraction as described before (Y. Wang et al., 2015b).
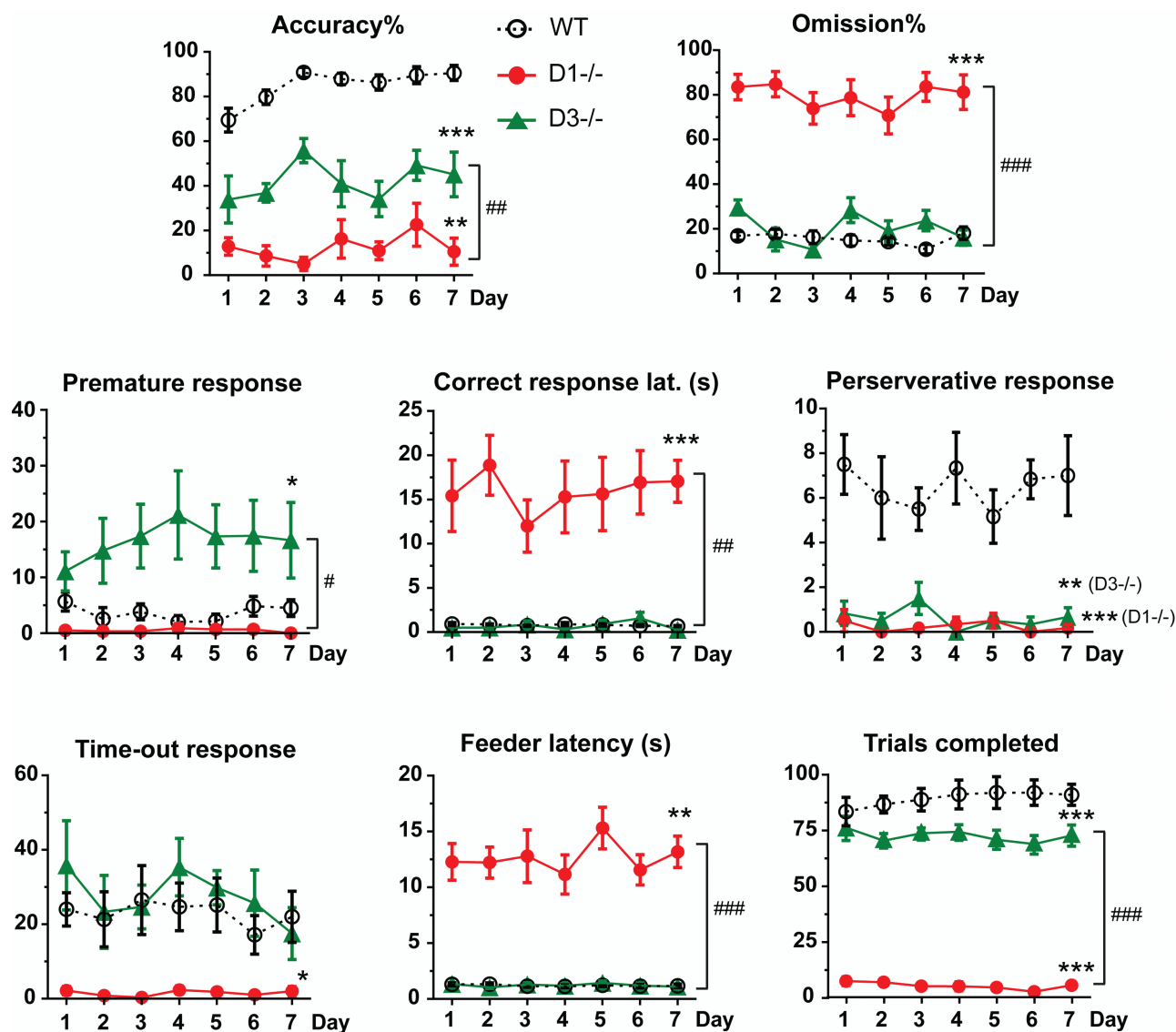

Figure 1. 5-Choice serial reaction time task (5-CSRTT) baseline performance in the D1^{-/-} and D3^{-/-} mice. The training sessions were performed from days 1 to 7. The values represent the mean ± SEM. The final performance on day 7 was analyzed by 1-way ANOVA. Sidak's posthoc test was used to analyze difference between genotypes. *$P < .05$, **$P < .01$, ***$P < .0001$, compared with WT mice. #$P < .05$, ##$P < .01$, ###$P < .0001$, compared between D1^{-/-} and D3^{-/-} mice.

Protein samples were separated by 12% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% BSA and then incubated with different primary antibodies (all at 1:1000 dilution) overnight at 4°C. Then the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. An enhanced chemiluminescence kit (Millipore, Billerica, MA) was used to detect immunoreactive protein bands. Band intensities were analyzed using QuantityOne software (BioRad, Hercules, CA) to calculate the target protein-to-internal control (GAPDH) ratio for each protein.

Experiment 1: Cognitive Effects of Repeated Morphine Administration in D1-/- and D3-/- Mice

The WT, D1$^{-/-}$, and D3$^{-/-}$ mice were trained on the 5-CSRTT until they achieved a stable baseline performance level (n = 16/group for each genotype). They were then randomly assigned to 6 groups (n = 8/group) and injected with morphine (10 mg/kg, i.p.) or saline 15 minutes before 5-CSRTT testing. Drug administration and 5-CSRTT testing were carried out for 7 consecutive days. After completion of the tests, the mice were immediately killed by decapitation, and their brains were quickly removed. Protein expression in the FC, NAc, and dHIP was determined by western blotting.

Experiment 2: Cognitive Effects of ACPC/L-701,324 Administration in WT Mice

WT mice were trained on the 5-CSRTT and then were randomly assigned into 4 groups and treated with ACPC (200 mg/kg, n = 8), L-701,324 (3 mg/kg, n = 8), L-701,324+ACPC (n = 8), or vehicle (1% Tween-80, n = 16) 30 minutes before the 5-CSRTT testing. To confirm the pharmacological specificity of the agonist and antagonist on the NMDA/glycine$_B$ site, mice in the L-701,324+ACPC group were sequentially injected with 3 mg/kg of L-701,324 and 200 mg/kg of ACPC, with a 15-minute interval between the 2 injections. Drug administration and 5-CSRTT testing were carried out for 7 days.

A new cohort of WT mice was separated into 4 groups as follows (n = 8/group): a vehicle+saline control group, a vehicle+morphine group, an ACPC+morphine group, and a L-701,324+morphine group. After 5-CSRTT baseline training, the mice were treated with ACPC (200 mg/kg), L-701,324 (5 mg/kg), or vehicle (1% Tween-80) 15 minutes before 10 mg/kg morphine treatment. The interval between morphine/saline injection and the 5-CSRTT testing was 15 minutes. All drugs were administered i.p., and 5-CSRTT testing was carried out for 7 days.

Experiment 3: Effects of ACPC/L-701,324 Administration on Morphine-Induced Cognitive Changes in D1-/- and D3-/- Mice

D1$^{-/-}$ or D3$^{-/-}$ mice were randomly assigned to 6 groups (n = 8/group) and were trained on the 5-CSRTT. Then they were injected i.p. with ACPC, L-701,324, or vehicle, followed by morphine or saline. Drug administration and 5-CSRTT testing were carried out for 7 days.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). The results are presented as the mean±SEM. Behavioral data were analyzed by using 2-way ANOVA, followed by Sidak's multiple comparisons test. In some cases, to demonstrate the effect of repeated morphine or ACPC/L-701,324 administration on cognitive behavior throughout the entire treatment period, repeated-measures (RM) 2-way ANOVA was used, with time serving as a within-subjects factor and treatment (or genotype) serving as a between-subjects factor. For western blotting, unpaired t tests were used to determine the effect of repeated morphine administration on protein expression in different brain regions. In experiment 3, to better illustrate the difference between D3$^{-/-}$ and WT mice with respect to ACPC- and L-701,324-regulated impulsive and compulsive behaviors, the behavioral data of the saline group from each genotype were set as 1, and the data from the other treatment groups were expressed as the relative fold change vs the saline group. Statistical significance was set at $P < .05$.

Results

Effects of Repeated Morphine Administration on 5-CSRTT Performance in D1$^{-/-}$ and D3$^{-/-}$ Mice

After baseline training (Figure 1), the WT mice showed high levels of accuracy (≥80%) while omitting few trials (≤20%). There were very few premature nosepokes, and the mice exhibited consistent latencies when reacting to and collecting rewards, demonstrating adequate motivation and good stimulus control. RM 2-way ANOVA revealed significant main effects of genotype on all 8 parameters observed (see supplementary Table 1 for statistical results). The final baseline performance was further analyzed by 1-way ANOVA, followed by Tukey's multiple comparisons test. As shown in Table 1, D1$^{-/-}$ mice presented significant attentional impairments, demonstrated by lower response accuracy (posthoc, $P = .0001$) and higher numbers of omissions (posthoc, $P = .0007$). Premature, perseverative, and timeout responses were hardly observed in D1$^{-/-}$ mice, suggesting less impulsive behavior and cognitive flexibility. Moreover, feeder latency in D1$^{-/-}$ mice was significantly increased (posthoc, $P = .0002$), accompanied by decreased numbers of completed trials (posthoc, $P < .0001$), indicating reduced motivation to obtain rewards. In D3$^{-/-}$ mice, response accuracy (posthoc, $P = .0009$), but not the number of omissions (posthoc, $P = .944$) was affected. The number of premature responses was significantly increased (posthoc, $P = .039$). The D3$^{-/-}$ mice showed a robust decrease in the perseverative responses (posthoc, $P = .0015$) but not the

Table 1. 5-CSRTT Baseline Performance in the WT, D1$^{-/-}$, and D3$^{-/-}$ Mice

	WT	D1-/-	D3-/-	ANOVA
Accuracy (%)	90.58±3.39	10.51±2.43***	45.11±16.43**,##	$F_{2,45} = 32.56, P < .0001$
Omission (%)	18.08±2.79	81.24±7.76***	16.03±3.43###	$F_{2,45} = 51.7, P < .0001$
Premature response	7.0±1.89	0.21±0.08	14.57±4.22*,#	$F_{2,45} = 5.77, P = .0138$
Correct response lat. (s)	0.68±0.06	17.05±4.38***	0.28±0.16##	$F_{2,45} = 34.45, P < .0001$
Perseverative response	7.1±1.79	0.16±0.1***	0.77±0.24**	$F_{2,45} = 12.78, P = .0006$
Timeout response	22±6.89	2.1±1.26*	17.5±6.95	$F_{2,45} = 3.884, P = .0412$
Feeder lat. (s)	1.18±0.16	13.18±1.4**	1.11±0.21###	$F_{2,45} = 70.52, P < .0001$
Trials completed	91.67±4.02	5.72±1.28***	67.19±3.96***,###	$F_{2,45} = 271.5, P < .0001$

Data are mean±SEM. One-way ANOVA followed by Sidak's posthoc test was used to analyze difference between genotypes. *$P < .05$, **$P < .01$, ***$P < .0001$, compared with WT mice. #$P < .05$, ##$P < .01$, ###$P < .0001$, compared between D1$^{-/-}$ and D3$^{-/-}$ mice.
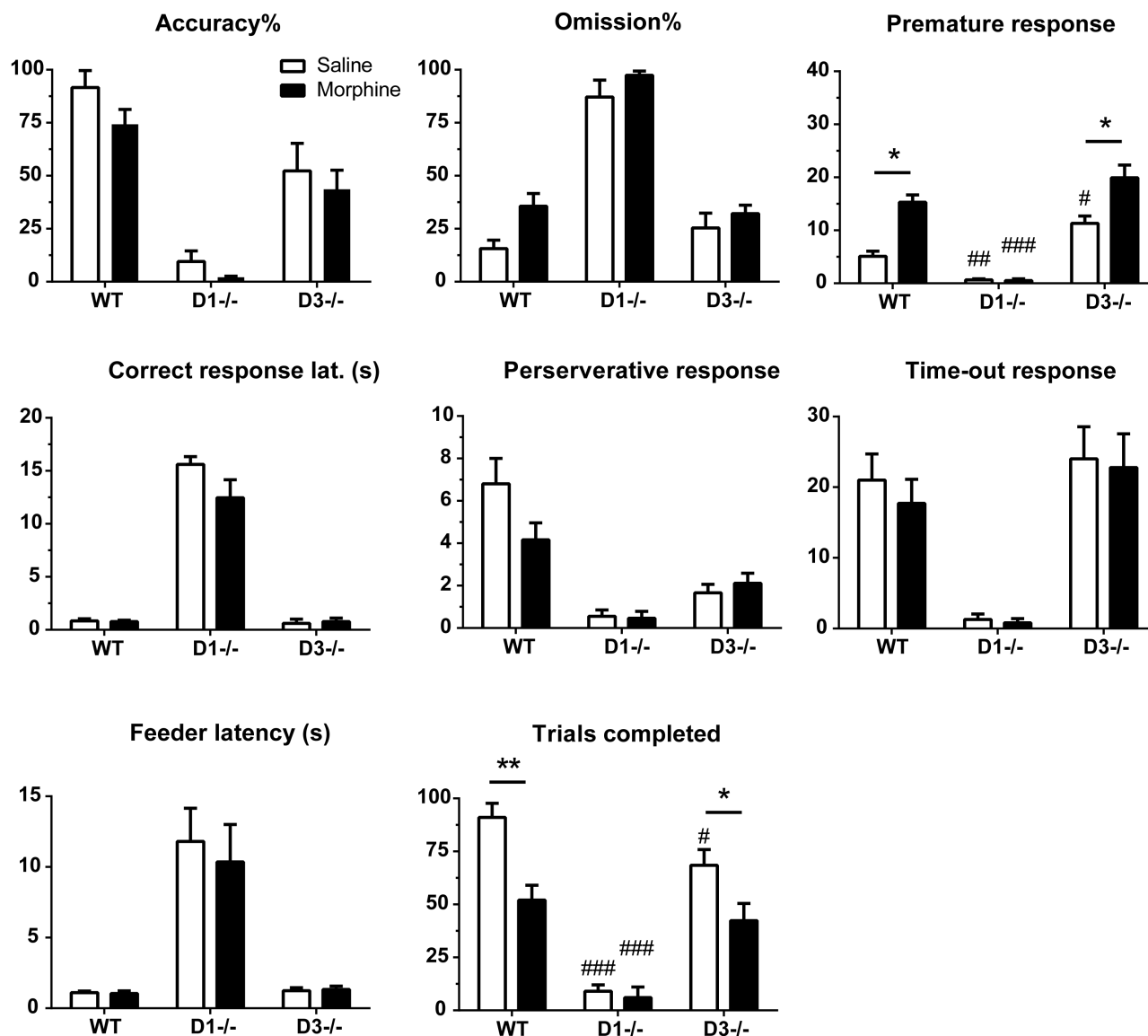
Figure 2. Effects of repeated morphine administration in D1$^{-/-}$ or D3$^{-/-}$ mice. Mice were administered 10 mg/kg morphine i.p. for 7 days. The values represent the mean ± SEM. Two-way ANOVA followed by Sidak's multiple comparisons test was used to reveal difference between groups. *P < .05, **P < .01, morphine-treated mice were compared with their saline-treated counterparts. #P < .05, ##P < .01, ###P < .0001, D1$^{-/-}$ and D3$^{-/-}$ mice were compared with WT mice with the morphine and saline treatments.

timeout responses (posthoc, P = .802). Motivation to obtain food rewards was intact in D3$^{-/-}$ mice (posthoc, P = .987). There were no differences among the WT, D1$^{-/-}$, and D3$^{-/-}$ groups regarding body weight during the shaping and training phase (genotypes: $F_{2, 45}$ = 1.198, P = .391; time: $F_{10, 450}$ = 1.77, P = .136, data not shown).

After the 5-CSRTT training sessions, the D1$^{-/-}$, D3$^{-/-}$, and WT mice were subjected to 7 days of repeated morphine treatment (Figure 2). Two-way ANOVA reported significant effects exerted by both morphine treatment and genotype on omission% (morphine: $F_{1, 42}$ = 7.400, P = .0094; genotype: $F_{2, 42}$ = 46.12, P < .0001) but not accuracy% (morphine: $F_{1, 42}$=2.873, P=.0975; genotype: $F_{2, 42}$= 91.72, P<.0001). Premature nosepokes were significantly affected by both morphine treatment ($F_{1, 42}$ = 10.94, P = .0019) and genotype ($F_{2, 42}$ = 37.35, P<.0001) as well as an interaction between them ($F_{2, 42}$ = 3.249, P = .041). In morphine-treated WT mice, premature responses were increased compared with the saline group (15.16±0.61 vs 5.15 ± 0.5; posthoc, P = .024). Morphine-treated D3$^{-/-}$ mice also showed more premature responses than their saline counterparts (20.13±4.82 vs 12.1±5.0; posthoc, P = .014). No change in the

number of premature nosepokes was found in D1$^{-/-}$ mice (0.5±0.07 vs 0.66±0.12; posthoc, P=.78). Perseverative responses were significantly affected by genotypes ($F_{2, 42}$ = 29.66, P<.0001) but not morphine ($F_{1, 42}$ = 1.947, P=.172). Significant effects were exerted by both morphine ($F_{1, 42}$ = 16.62, P=.0071) and genotype ($F_{2,42}$=38.17, P<.0001) as well as morphine×genotype interaction ($F_{2, 42}$ = 3.27, P = .0459), on the number of trials completed. Posthoc analysis showed a significant decrease in the numbers of trials completed by both WT (posthoc, P = .0031) and D3$^{-/-}$ (posthoc, P = .0066) mice. Regarding other cognitive parameters, such as correct response latency, time-out responses, and feeder latency, no significant treatment effects were observed after repeated morphine administration.

Phosphorylated NR1, CaMKII, and CREB Expression in the Brain following Repeated Morphine Administration

Figure 3a shows that repeated morphine administration induced significant NR1 phosphorylation in the NAc (t_{14} = 4.97,
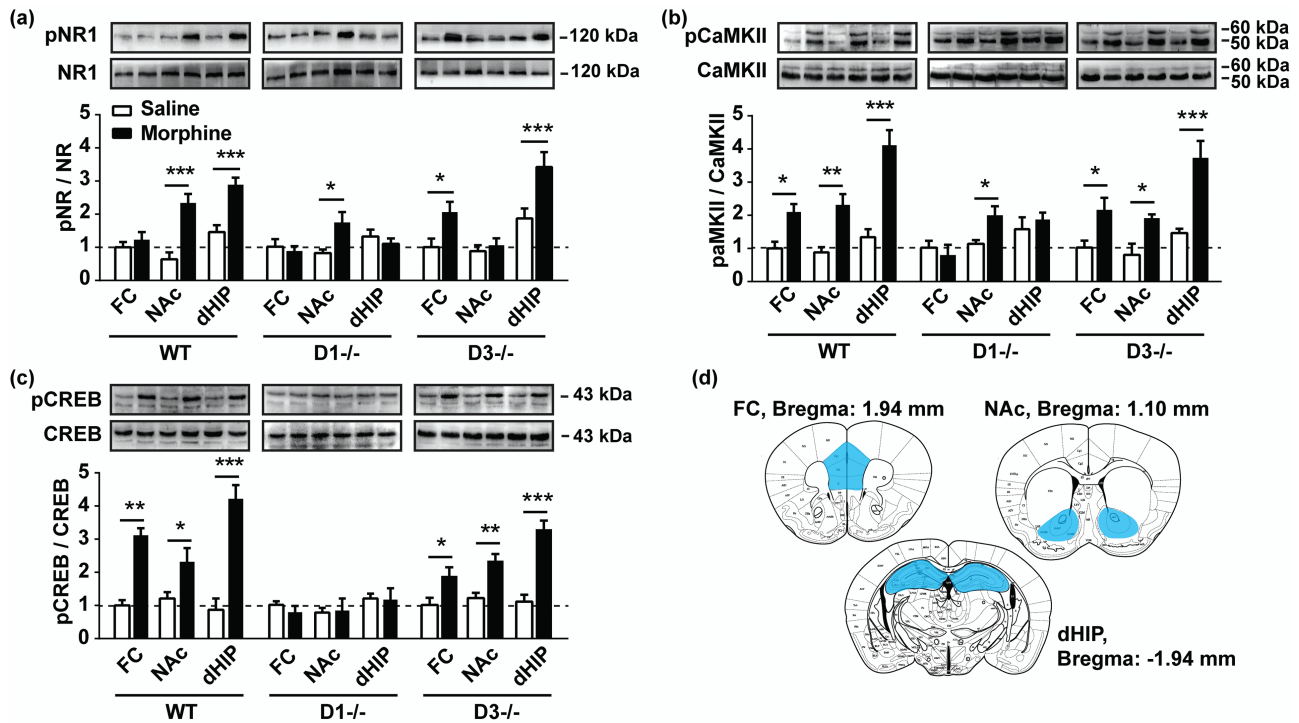
Figure 3. Effects of repeated morphine administration on NR1-Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-cAMP response element-binding protein (CREB) phosphorylation. Mice were administered 10 mg/kg morphine i.p. for 7 days. The relative fold changes in the levels of phosphorylated (a) NR1, (b) CaMKII, and (c) CREB protein were analyzed. The protein levels in the frontal cortex (FC) of the saline group were set as 1. The values represent the mean±SEM. Morphine-treated mice were compared with their saline-treated counterparts, *P<.05, **P<.01, ***P<.0001. (d) Schematic representation showing the locations of the excised brain regions (blue area). Figures were adapted from atlas of Paxinos and Watson (George Paxinos, 2001). NAc, nucleus accumbens; dHIP, dorsal hippocampus.

P < .0001) and dHIP (t$_{14}$ = 4.82, P < .0001) of WT mice. D3$^{-/-}$ mice exhibited increased phospho-NR1 levels in the FC (t$_{14}$ = 2.62, P = .0202) and dHIP (t$_{14}$ = 4.96, P < .0001). Significantly increased phospho-NR1 levels were observed in the NAc of D1$^{-/-}$ mice following repeated morphine administration (t$_{14}$ = 3.15, P = .013). We next analyzed the levels of downstream CaMKII phosphorylation (Figure 3b). In WT and D3$^{-/-}$ mice, phospho-CaMKII was augmented by morphine administration in all 3 regions. In D1$^{-/-}$ mice, increased phosphorylation was observed only in the NAc (t$_{14}$ = 2.59, P = .021). Phospho-CREB levels were also increased in WT and D3$^{-/-}$ mice in all the observed regions (in the WT mice, FC: t$_{14}$ = 2.66, P=.017, NAc: t$_{14}$ = 3.37, P = .011, dHIP: t$_{14}$ = 6.28, P < .0001; in the D3$^{-/-}$ mice, FC: t$_{14}$ = 2.58, P = .021, NAc: t$_{14}$ = 3.74, P = .008, dHIP: t$_{14}$ = 6.55, P<.0001) (Figure 3c), suggesting elevated transcriptional activity induced by morphine. No significant changes in phospho-CREB levels were observed in D1$^{-/-}$ mice following morphine treatment.

Effects of ACPC and L-701,324 Administration on Morphine-Induced Cognitive and Behavioral Changes

Because the phosphorylated active form of NR1 was increased by repeated morphine administration, we investigated whether activation of the glycine site on NR1 contributed to morphine-induced cognitive changes. The WT mice were trained on the 5-CSRTT and then randomly assigned to 4 groups. They were treated with ACPC (200 mg/kg), L-701,324 (5 mg/kg), L-701,324+ACPC, or vehicle (1% Tween-80) 30 minutes before 5-CSRTT testing. RM 2-way ANOVA reported that premature responses were significantly affected by treatment (F$_{3, 36}$ = 37.7, P<.0001) and time (F$_{9, 324}$ = 5.42, P < .0001) without significant interaction between them (F$_{27, 324}$ = 1.382, P = .139) (Figure 4a).

Following 7 days of drug administration, ACPC induced an upward trend in premature responses (5.93±0.48, compared with 4.11±0.49 of saline mice), while L-701,324 exerted the opposite effect (1.82±0.41) The perseverative responses were also significantly affected by drug treatment (F$_{3, 36}$ = 81.79, P <.0001) but not time (F$_{9, 324}$ = 1.62, P = .057). In the final test session, the mean values of perseverative responses in the saline, ACPC, and L-701,324 groups were 5.24±0.41, 8.68±0.22, and 2.88±0.45. It is noteworthy that the premature and perseverative responses in the L-701,324+ACPC group dod not significantly differ from L-701,324 alone, thus suggesting a full blocking effect of L-701,324 at the NMDA/glycine$_B$ site. During the experimental period, no significant effects were exerted by ACPC or L-701,324 treatment on other parameters, such as accuracy%, omission%, or correct response latency (supplementary Figure S2).

Then we examined the effects of ACPC and L-701,324 administration on morphine-induced 5-CSRTT performance (Figure 4b). The drug treatment and testing were carried out in a separate cohort of WT mice. One-way ANOVA followed by Sidak's multiple comparisons tests were performed to determine the difference between groups. Neither ACPC nor L-701,324 affected morphine-induced response accuracy. However, the main treatment effect on omission was significant (F$_{3, 28}$=10.34, P<.0001). Morphine-induced premature nosepokes were significantly altered by ACPC and L-701,324 administration (F$_{3, 28}$ = 27.1, P < .0001). The number of premature nosepokes increased following ACPC+morphine treatment (posthoc, P=.001) and decreased following L-701,324+morphine treatment (posthoc, P=.0181) compared with the vehicle+morphine group. The main treatment effect on perseverative responses was also significant (F$_{3, 28}$ = 16.2, P < .0001). ACPC not only reversed the effects of morphine on perseverative responses (posthoc, ACPC+morphine
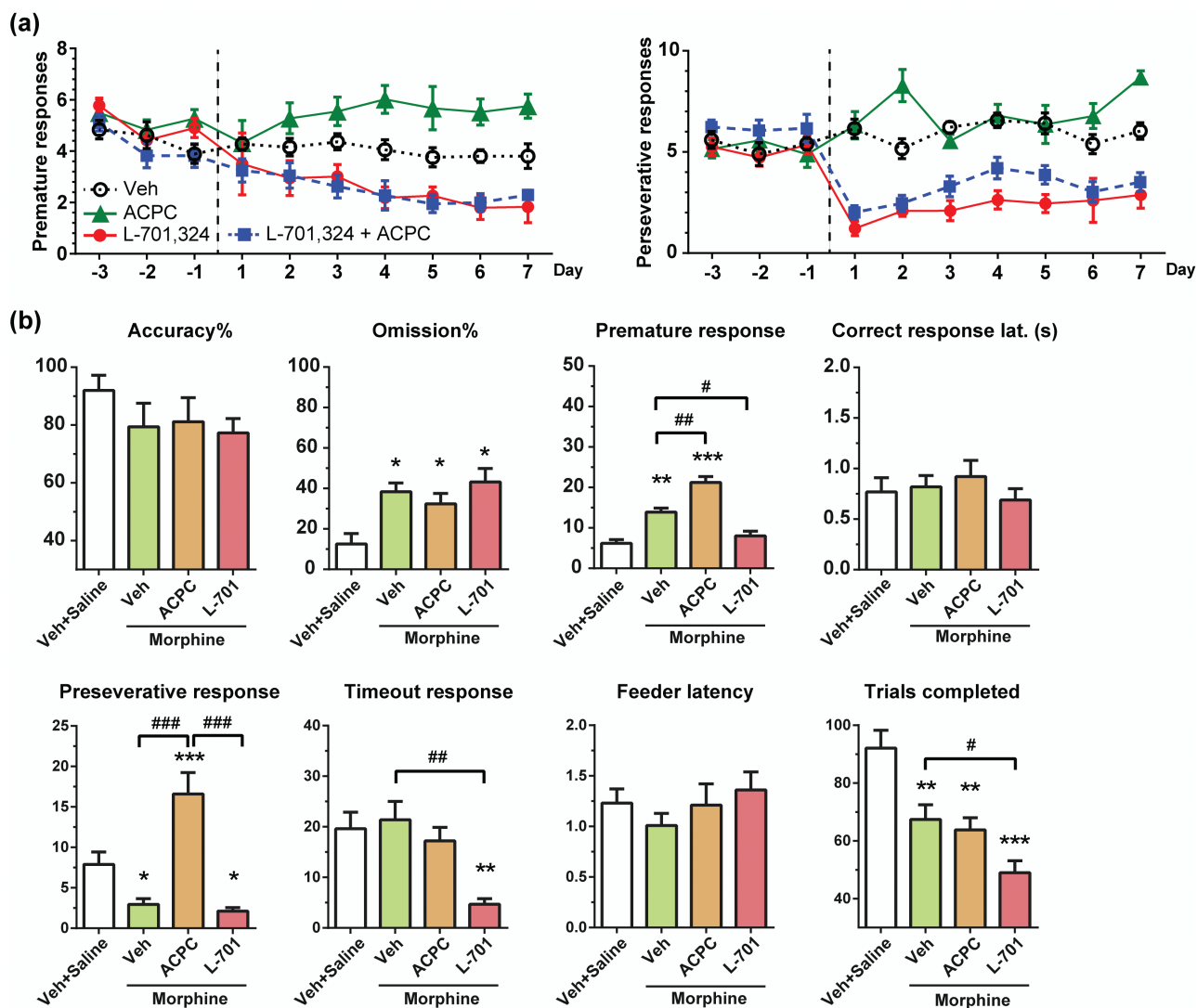
Figure 4. Effects of l-aminocyclopropanecarboxylic acid (ACPC) and 7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1H)-quinolone (L-701,324) administration on morphine-induced cognitive and behavioral changes. (a) Mice received ACPC (200 mg/kg), L-701,324 (3 mg/kg), L-701,324+ACPC, or Veh (1% Tween-80 vehicle) i.p. once per day for 7 consecutive days. Premature responses and perseverative responses are showed. (b) Mice received ACPC, L-701,324, or vehicle 15 minutes before 10 mg/kg morphine or saline administration. 5-Choice serial reaction time task (5-CSRTT) performance was analyzed after 7 days of treatment. *$P < .05$, **$P < .01$, ***$P < .0001$, compared with Veh+Saline controls. #$P < .05$, ##$P < .01$, ###$P < .0001$, comparison between the 2 indicated groups.

vs vehicle+morphine, $P < .0001$) but also increased perseverative responses above that of controls. Furthermore, a significant difference was found between the ACPC+morphine group and the L-701+morphine group (posthoc, $P < .0001$). L-701,324 significantly decreased the timeout responses (main effect: $F_{3,28} = 7.1$, $P = .0011$) compared with its vehicle controls (posthoc, $P = .0008$). Nevertheless, L-701,324 exacerbated the morphine-induced decreases in the number of trials completed (main effect: $F_{3,28} = 12.87$, $P < .0001$; posthoc, L-701+morphine vs vehicle+morphine, $P = .03$). The remaining parameters showed no obvious changes between the groups.

Effects of ACPC/L-701,324 Administration on Morphine-Induced Cognitive and Behavioral Changes in D1⁻/⁻ and D3⁻/⁻ Mice

After 7 days of drug administration, the D1⁻/⁻ and D3⁻/⁻ mice underwent 5-CSRTT testing. Unfortunately, D1⁻/⁻ mice did not show any behavioral responses to ACPC or L-701,324 administration

with respect to any parameters, whether combined with saline or morphine treatment (supplementary Figure S3).

Compared with the general lack of responsiveness exhibited by D1⁻/⁻ mice, D3⁻/⁻ mice responded differently to ACPC and L-701,324 administration (Figure 5a). Premature responses were significantly affected by both morphine and NR1 glycine site agonist/antagonist administration (morphine: $F_{1,42} = 8.923$, $P = .0047$; agonist/antagonist effect: $F_{2,42} = 51.61$, $P < .0001$; interaction: $F_{2,42} = 6.811$, $P = .0027$). Sidak's multiple comparisons revealed that ACPC robustly increased the premature responses in saline-treated D3⁻/⁻ mice (posthoc, $P = .032$). However, L-701,324 administration decreased the premature responses in those mice (posthoc, $P = .026$). This cutback effect was significantly enhanced by morphine treatment (posthoc, $P < .0001$). In addition, the perseverative responses were significantly affected by ACPC ($F_{2,42} = 52.21$, $P < .0001$) but not morphine ($F_{1,42} = 0.5435$, $P = .4651$). The ACPC-treated mice (21.6 ± 3.34 and 17.66 ± 3.62, in the ACPC+saline and ACPC+morphine group, respectively) showed an upward trend in the perseverative responses than the
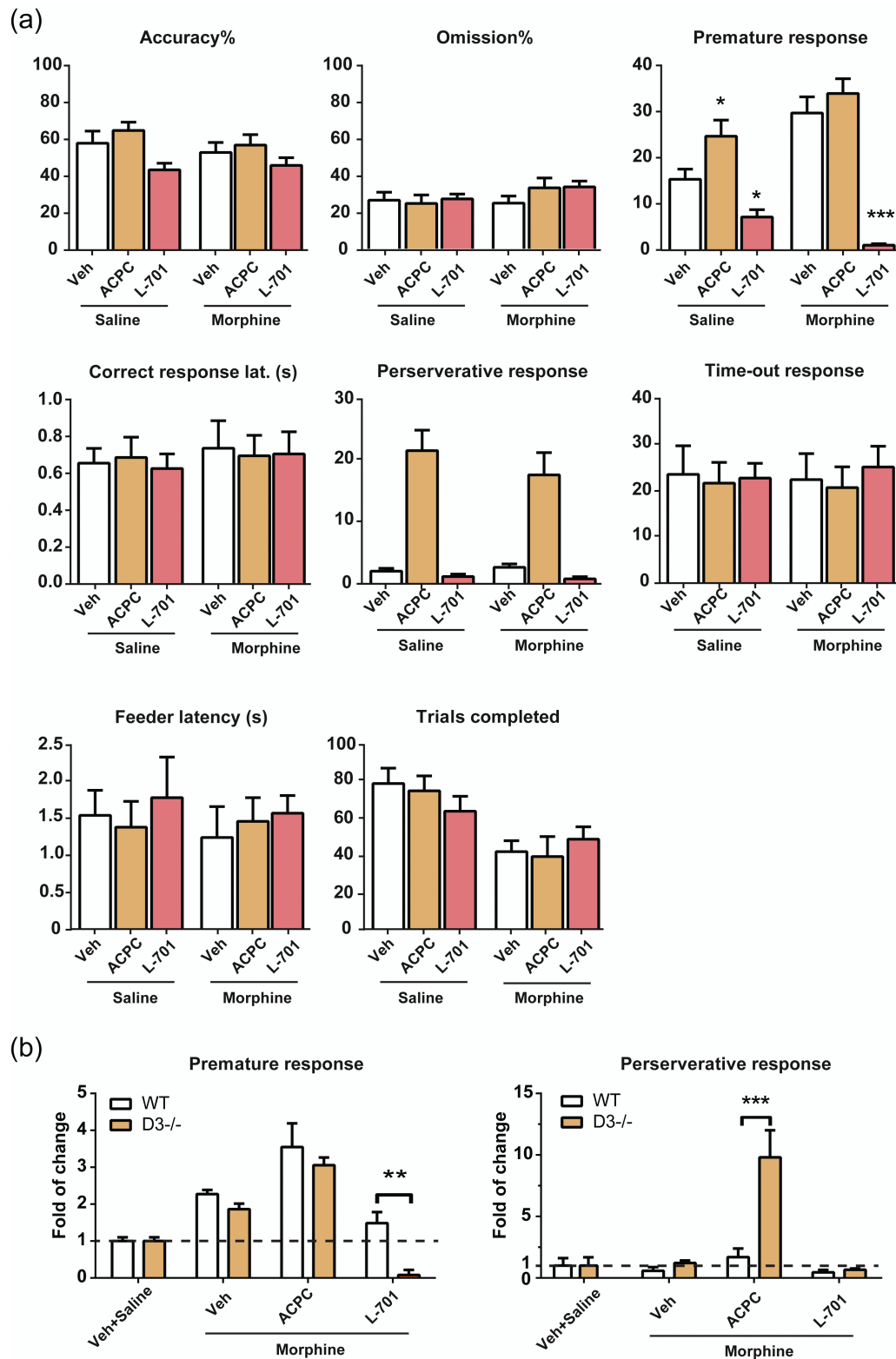
Figure 5. Effects of l-aminocyclopropanecarboxylic acid (ACPC)/7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1H)-quinolone (L-701,324) on morphine-induced cognitive and behavioral changes in D3^{−/−} mice. (a) D3^{−/−} mice were administered ACPC (200 mg/kg), L-701,324 (3 mg/kg), or Veh (1% Tween-80) i.p. in combination with 10 mg/kg morphine or saline for 7 days. *P < .05, ***P < .0001, vs Veh controls within the saline- or morphine-treated groups. (b) The fold changes in the numbers of premature and perseverative responses in wild-type (WT) and D3^{−/−} mice. In each genotype, the data from the Veh + Saline group were set as 1, and the data from the other treatment groups are expressed as the relative fold change vs the Veh + Saline group. *P < .05, ***P < .0001, comparison between the 2 indicated groups.

vehicle-treated mice (2.01±0.25 and 2.64±0.55), whereas no obvious effects of L-701,324 treatment were observed (1.13±0.42 and 0.76±0.34). Neither ACPC nor L-701,324 administration exerted any obvious effects on the remaining parameters in either group.

To further elucidate the role of the D3 receptor in ACPC- and L-701,324-regulated impulsive and compulsive behaviors, we compared the premature and perseverative responses of D3^{-/-} with those of WT mice (Figure 5b). The data of the vehicle+saline group for each genotype were set as 1, and the data for the other treatment groups are expressed as the relative fold change vs the vehicle+saline group. Data were analyzed by 2-way ANOVA followed Sidak's posthoc tests. There were significant main effects of both drug treatment ($F_{3, 56}$ = 34.57, $P < .0001$) and genotype ($F_{1, 56}$ = 8.609, $P = .0048$) as well as an interaction ($F_{3, 56} = 2.269$, $P = .0404$), on the premature responses. L-701+morphine D3^{-/-} mice exhibited significantly fewer premature responses than their WT counterparts (posthoc, $P = .0029$), demonstrating that the absence of the D3 receptor in the brain strengthened the inhibitory effect of L-701,324 on impulsive behavior. Moreover, perseverative responses were also significantly affected by both drug treatment ($F_{3, 56} = 16.81$, $P < .0001$) and genotype ($F_{1, 56} = 13.85$, $P = .0005$), with an interaction between them ($F_{3, 56} = 10.61$, $P < .0001$). Significantly more perseverative responses were noted in morphine+L-701-treated D3^{-/-} mice than in their WT counterparts (posthoc, $P < .0001$), indicating that the effects of ACPC on morphine-induced compulsive behavior were augmented in D3^{-/-} mice.

Discussion

Repeated Morphine Administration Induced Attentional Deficits and Increased Impulsive Behavior

Impaired attention is a risk factor for abuse of drugs such as psychostimulants, cannabis, and hallucinogen (Vogel et al., 2016). In the 5-CSRTT, an increase in the omission and a decrease in accuracy typically reflect a deficit in attention. We demonstrated that omission, but not accuracy, was significantly affected by repeated morphine administration, suggesting partial impairment of attentional function. Of course, such a pattern can also result from noncognitive disruptions, such as locomotor impairment or reduced motivation, which tend to increase reward collection latencies. Subsequent evaluations showed that the feeder latency was not affected by repeated morphine administration, suggesting intact motivation and locomotor function. Therefore, the disruptions in omission likely reflected a true attentional deficit. Psychostimulants, such as amphetamine (Wong et al., 2016), cocaine (Dandy and Gatch, 2009), and nicotine (Kirshenbaum et al., 2011), had been reported to affect impulsive behavior. Acute morphine was found to cause dose-dependent increases in premature responding and decreases in perseverative responding on 5-CSRTT (Pattij et al., 2009). These findings supported our observation that repeated morphine administration robustly increased the premature responses and decreased the perseverative responses on the 5-CSRTT, suggesting that opiates have multiple effects on impulsive and compulsive behaviors.

D1 and D3 Receptors Exerted Contrasting Effects on Impulsive Behavior

We found that D1^{-/-} mice exhibited hardly any premature nosepokes, even after repeated morphine administration, as well as a severely attenuated motivation (reflected by longer feeder latency and decreased number of trials completed). Recent evidence indicates that D1 receptor activity in the medial FC is positively correlated with impulsive behavior in rats (Loos et al., 2010). Blockade of the D1 receptor in the core of the NAc reduces impulsive behavior on the 5-CSRTT (Pattij et al., 2007), whereas D1 agonist has the opposite effect (Pezze et al., 2007). Moreover, accumulating evidences suggest that D1 receptor in the cortical-mesolimbic system is crucial for reward-based behavioral responses (Andrzejewski et al., 2006; Abraham et al., 2016a, 2016b; Simon et al., 2016). Thus, it can be speculated that the D1 receptor is essential for the expression of impulsive and motivational endophenotypes and serves as a "gateway" regulating the levels of these behaviors.

D3 knockout resulted in robustly increased impulsive behaviors in mice, which can be augmented by repeated morphine administration. Striatal D2/3 receptor availability is negatively correlated with impulsive behaviors both in rodents (Laughlin et al., 2011) and in humans (Ghahremani et al., 2012). These findings are consistent with our observations. The apparent contrasting contributions of D1 and D3 receptors to impulsive behavior may reflect their proposed roles in behavior, as D1 activation is thought to heighten reward cues, while D3 activation triggers the "brakes" on impulsive behavior. Given the inhibitory effects of D2-class receptors, D3 receptors may represent an important way of negatively modulating impulsive behaviors. In addition, morphine-induced increases in the omissions and decreases in the perseverative nosepokes were reversed in D3^{-/-} mice, suggesting that D3 receptors have distinct functions in modulating μ-opioid receptor-mediated attentional deficits and compulsive behavior.

Phospho-NR1 and Its Downstream CaMKII-CREB Pathway Were Altered by Repeated Morphine Administration

Substantial evidence indicates that NMDA receptors influence the effects of chronic opiate treatment. In our study, activation of NR1 and its downstream CaMKII-CREB pathway, a classical molecular signaling pathway associated with drug-induced neuroplasticity, was analyzed in the WT, D1^{-/-}, and D3^{-/-} mice after repeated morphine administration. Phosphorylation of NR1, specifically at amino acid Ser897, results in NMDA receptor activation and Ca^{2+} influx (Dudman et al., 2003). This Ca^{2+} influx in turn activates CaMKII and initiates a series of kinase cascades and CREB activation, thus contributing to drug-induced behavioral effects (Lisman et al., 2002).

We demonstrated that in WT mice, the level of NR1 phosphorylation was elevated in the NAc and dHIP due to repeated morphine administration. Robust increases in the levels of phosphorylated CaMKII and CREB were also observed in those brain regions. These data are consistent with those of several studies reporting increased NR1 expression levels in NAc in morphine-dependent rats (Inoue et al., 2003; Murray et al., 2007). Phospho-NR1 levels in the NAc were found to be increased during both acute (3 day) and extended (2 month) withdrawal from morphine (Anderson et al., 2015). This raises the possibility that morphine may potentiate the phosphorylation of NR1 via certain NMDA and μ-opiate receptor interactions in the mesolimbic DA-ergic circuitry. This process may in turn affect intracellular signaling and ultimately alter the processing of opiate-related cognitive information.

In D1^{-/-} mice, phosphorylation levels of the NR1 and CaMKII were increased in the NAc after morphine; however, no significant changes in NR1, CaMKII, or CREB phosphorylation were noted in the FC and dHIP after repeated morphine administration. These findings may explain the general lack of behavioral responses to morphine by D1^{-/-} mice during the 5-CSRTT. To our

knowledge, there is no direct evidence linking the D1 receptor to NR1 phosphorylation in opiate addiction; however, several studies support the idea that D1-like receptors modulate NR1 phosphorylation (Ser889 and 897) and are associated with some psychiatric disorders (Mouri et al., 2007; Aira et al., 2016). By contrast, there was a general increase in NR1-CaMKII-CREB phosphorylation in D3$^{-/-}$ mice in the 3 brain regions after repeated morphine administration (except for unchanged phospho-NR1 in NAc), which may indicate that D3 receptors are not necessary for morphine-induced NR1-CaMKII-CREB phosphorylation.

NMDA/Glycine$_B$ Site Was Involved in Regulating Morphine-Induced Impulsive Behavior

Because the phospho-NR1 was altered by repeated morphine administration, we further examined the role of NMDA-NR1 glycine$_B$ binding site in morphine-induced cognitive deficits. ACPC is one of the first glycine$_B$ site partial agonists described and has an intrinsic activity of 80~92% and a potency of approximately 0.09~0.4 mM (Watson and Lanthorn, 1990; Karcz-Kubicha et al., 1997). Unlike other typical glycine$_B$ site agonists, such as glycine and D-serine, ACPC shows sufficient penetration to the blood-brain barrier of the central nervous system. Moreover, this agent has a half-life of 6 hours longer in the human brain and is currently being tested for clinical treatment of stroke and depression (Cherkofsky, 1995). On the other hand, L-701,324 is a novel antagonist that binds with high affinity and selectivity to the NMDA/glycine$_B$ site (Bristow et al., 1996a). L-701,324 belongs to the 4-Hydroxy-2-quinolones class and penetrates well in the central nervous system. It is also a potent, orally active anti-convulsant (Bristow et al., 1996b). In rodents, L-701,324 has been shown to block the psychostimulant-induced activation of mesolimbic dopaminergic systems (Bristow et al., 1995).

Although it is well documented that memory (Viu et al., 2000), spatial learning (Popik and Rygielska, 1999), and anxiety (Przegalinski et al., 2000) can be influenced by NMDA/glycine$_B$ site agonists or antagonists, to the best of our knowledge, the understanding of the cognitive effects of these agents, particularly with respect to impulsive behavior, remains limited. We showed that both premature and perseverative responses were decreased by L-701,324 administration and increased by ACPC administration, indicating that L-701,324 attenuated the impulsivity and compulsivity, while ACPC has the opposite effect. Clinical observation proposes a transition from impulsive to compulsive behavior during progression from recreational drug use to addiction (Belin et al., 2009). Indeed, impulsivity (rapid or without adequate planning or forethought, carried out without considering the negative consequences of these actions) and compulsivity (repeated performance of a behavior continues despite of adverse consequences) are often defined as a lack of "behavioral inhibition" (Eagle and Baunez, 2010). Thus, our result may suggest an important role of NMDA/glycine$_B$ site in modulation of behavioral inhibition. In addition, our data also demonstrated that neither L-701,324 nor ACPC affect attentional and motivational performance. Interestingly, a recent study showed that ACPC (400 mg/kg, i.p.) enhanced cognitive flexibility, but failed to affect impulsivity and attentional performance in the 5-CSRTT (Popik et al., 2015). This inconsistent observation may be related to species and methodological differences between the previous study (performed with rats that received acute drug administration prior to only one test session) and the present study (performed with mice that received chronic drug administration in conjunction with 7 consecutive test sessions). Although according to the definition that, the glycineB

site agonist, ACPC and the antagonist, L-701,324, are all recognized at the same site of the NMDA receptor complex (Danysz and Parsons, 1998), it should be considered that the agonist and antagonist glycine$_B$ sites of the NMDA receptor are overlapping but probably not exactly the same, as evidenced by mutation analysis of the receptor protein and by comparison of the structures of these two groups of agents (Danysz and Parsons, 1998). In our hand, the premature and perseverative responses in the L-701,324 + ACPC group did not differ from L-701,324 alone, suggesting that L-701,324, as a selective NMDA/glycine$_B$ receptor antagonist, can reverse the effects of the selective agonist ACPC. Thus, our data may suggest that L-701,324 reversed the effect of the ACPC at the glycine$_B$ site of the NMDA receptor complex.

Impaired behavioral inhibition has been identified as one of the most robust behavioral effects induced by cocaine (Ma et al., 2015), methamphetamine (Furlong et al., 2016), and heroin (Cheng et al., 2012). In our study, co-administration of L-701,324 and morphine effectively suppressed morphine-induced premature, perseverative, and timeout responses, suggesting a preventive effect of L-701,324 on morphine-induced deficient behavioral inhibition. Because increased impulsive and compulsive behaviors may contribute to the development of uncontrolled drug use (C. S. Li and Sinha, 2008; Chambers et al., 2009), our data may have important therapeutic implications with respect to L-701,324 administration in opiate-induced cognitive impairment.

D3 Receptors Augmented NMDA/Glycine$_B$ Receptor-Mediated Regulation of Impulsive Behaviors Induced by Morphine

It has been shown that NMDA/glycine$_B$ site antagonists are able to block psychostimulant-induced activation of mesolimbic DA-ergic systems in rodents (Bristow et al., 1996a, 1996b). We examined the role of D1 and D3 receptors with respect to the regulatory effects of NMDA/glycine$_B$ receptors on morphine-induced cognitive changes. Systemic disruption of NMDA/glycine$_B$ receptor function by ACPC or L-701,324 administration did not affect attention, processing speed, or motivation in D1$^{-/-}$ mice, irrespective of repeated morphine administration. Moreover, behavioral inhibition was not affected by ACPC or L-701,324 administration. Based on the observations from experiment 1, it appears that the D1 receptor is essential for the expression of complex cognitive behaviors. Thus, the cognitive effects of ACPC/L-701,324 on 5-CSRTT performance may be completely abolished due to the inability of D1$^{-/-}$ mice to appropriately perform reward-based behavioral tasks. Further specific investigations are required to address whether the D1 receptor plays a role in regulating NMDA/glycine$_B$ receptor-mediated cognitive behaviors.

We are not aware of any published research examining the roles of the D1 and D3 receptors in NMDA/glycine$_B$ receptor-regulated cognitive behaviors. However, a recent study from our laboratory showed that intermittent morphine-induced hyperlocomotion and locomotor sensitization were significantly suppressed by co-administration of ifenprodil (a selective NR2B-containing NMDA receptor antagonist) or nafadotride (a selective D3 receptor antagonist) with morphine (Liu et al., 2014). Besides, systemic blockade of the D3 receptor by nafadotride not only attenuated morphine-induced locomotor sensitization but also reversed the phosphorylation of NR2B in the NAc. Others have reported that RU24213 (a D2/3 receptor agonist)-induced locomotion was dose dependently depressed by HA-966 (an NMDA/glycine$_B$ receptor antagonist) (Starr and Starr, 1994).

As for the present study, L-701,324 effectively prevented morphine-induced premature responses on 5-CSRTT. This inhibitory effect was significantly stronger in D3$^{-/-}$ mice than in WT mice, indicating that (1) D3 receptor attenuates morphine-induced impulsive behaviors and that (2) this regulatory effect mediates NMDA/glycine$_B$ receptor and μ-opioid receptor interactions. In addition, our results demonstrated that the facilitation of perseverative responses by ACPC was fortified in D3$^{-/-}$ mice, suggesting that the D3 receptor strengthens morphine-induced compulsive behaviors. However, the mechanisms underlying this phenomenon require further investigation.

In conclusion, this study suggests that NMDA/glycine$_B$ receptor functions may contribute to impaired cognition, particularly with respect to impulsive behaviors that are associated with opiate addiction. DA, D1, and D3 receptors may have contrasting effects on impulsive behavior. The D1 receptor is essential for the expression of impulsive and motivational endophenotypes. The D3 receptor has inhibitory effects on morphine-induced impulsive behaviors, and these effects modulate NMDA/glycine$_B$ receptor and μ-opioid receptor interactions.

Supplementary Material

Supplementary data are available at *International Journal of Neuropsychopharmacology* online.

Acknowledgments

Statement of Interest

None.

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