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N-acetylcysteine modulates hallucinogenic 5-HT$_{2A}$ receptor agonist-mediated responses: Behavioral, molecular, and electrophysiological studies

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ABSTRACT

N-acetylcysteine (NAC) has been reported to reverse the psychotomimetic effects in the rodent phencyclidine model of psychosis and shown beneficial effects in treating patients with schizophrenia. The effect of NAC has been associated with facilitating the activity of cystine-glutamate antiporters on glial cells concomitant with the release of non-vesicular glutamate, which mainly stimulates the presynaptic metabotropic glutamate receptor subtype 2 receptors (mGluR2). Recent evidence demonstrated that functional interactions between serotonin 5-HT$_{2A}$ receptor (5-HT$_{2A}$R) and mGluR2 are responsible to unique cellular responses when targeted by hallucinogenic drugs. The present study determined the effects of NAC on hallucinogenic 5-HT$_{2A}$R agonist (±)1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI)-elicited behavioral and molecular responses in mice and DOI-evoked field potentials in the mouse cortical slices. NAC significantly attenuated DOI-induced head twitch response and expression of c-Fos and Egr-2 in the infralimbic and motor cortex and suppressed the increase in the frequency of excitatory field potentials elicited by DOI in the medial prefrontal cortex. In addition, the cystine-glutamate antiporter inhibitor (S)-4-carboxyphenylglycine (CPG) and the mGluR2 antagonist LY341495 reversed the suppressing effects of NAC on DOI-induced head twitch and molecular responses and increased frequency of excitatory field potentials, supporting that NAC attenuates the 5-HT$_{2A}$R-mediated hallucinogenic effects via increased activity of cystine-glutamate antiporter followed by activation of mGluR2 receptors. These findings implicate NAC as a potential therapeutic agent for hallucinations and psychosis associated with hallucinogen use and schizophrenia.

1. Introduction

Serotonin 5-HT$_{2A}$ receptor (5-HT$_{2A}$R) is involved in behavioral abnormalities induced by hallucinogenic substances such as lysergic acid diethylamide (LSD), mescaline, and psilocybin (Aghajanian and Marek, 1999) as well as symptoms in psychiatric disorders including schizophrenia (Barnes and Sharp, 1999). In fact, many of the psychotomimetic properties of the NMDA receptor antagonists, e.g., phencyclidine (PCP) and ketamine, previously believed to be entirely distinct from that of the psychedelic hallucinogens, may also involve 5-HT$_{2A}$Rs (Meltzer et al., 2011). Pharmacological manipulations of glutamate transmission e.g., by inhibitory metabotropic glutamate mGluR2/3 receptor agonists provide parallels between the actions of these two classes of drugs. The agonists or positive allosteric modulators of mGluR2 effectively block the behavioral and electrophysiological responses and signal

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pathway induced by PCP-like (Galici et al., 2005; Lorrain et al., 2003; Woolley et al., 2008; Xi et al., 2011) and LSD-like (Benneyworth et al., 2007; Marek et al., 2000; Molinaro et al., 2009; Zhai et al., 2003) drugs. Because the psychoactive effects of both PCP-like and LSD-like drugs in rodents require increased levels of synaptic glutamate in the prefrontal cortex, one hypothesis to explain how mGluR2/3 activation abolishes the responses induced by LSD-like drugs or PCP-like drugs is that the activation of the presynaptic mGluR2/3 inhibits glutamate release from cortical terminals either induced by activation of 5-HT_{2A}Rs or by blockade of NMDA receptors in the GABAergic interneurons (Gonzalez-Burgos and Lewis, 2008).

On the other hand, recent evidence has shown that 5-HT_{2A}R and mGluR2 are colocalized in the cortical pyramidal neurons and may form 5-HT_{2A}R-mGluR2 complex which is responsible to unique cellular responses when targeted by hallucinogenic drugs (Fribourg et al., 2011; Gonzalez-Maeso et al., 2008; Moreno et al., 2012). The studies with mGluR2 and mGluR3 knockout mice demonstrated that mGluR2, but not mGluR3 is necessary for the pharmacological and behavioral effects induced by LSD-like drugs (Moreno et al., 2011). Thus, the 5-HT_{2A}R-mGluR2 complex in the cortical pyramidal neurons has been speculated as the target site of atypical antipsychotics and mGluR2 agonists for treatment of schizophrenia (Gonzalez-Maeso et al., 2008).

N-acetylcysteine (NAC), a widely practiced mucolytic drug and antidote for acetaminophen overdose, is emerging as a useful agent in the treatment of psychiatric disorders including addiction, compulsive and grooming disorders, schizophrenia, and bipolar disorder (Dean et al., 2011). The mechanisms underlying the beneficial effects of NAC on psychiatric conditions are only beginning to be understood. In addition to being a precursor to the antioxidant, glutathione, NAC, a prodrug of cysteine, has been proved to modulate glutamatergic system by facilitating the activity of cystine-glutamate antiporters in glial cells concomitant with the release of non-vesicular glutamate in extrasynaptic compartment, thereby stimulating proximal receptors, such as mGluR2/3 (Conn and Pin, 1997). Accordingly, the blunting effects of NAC on PCP-induced psychomimetic behaviors (Baker et al., 2008), cocaine-induced drug seeking (Moran et al., 2005) and metaplasticity (Moussawi et al., 2009) have been suggested to be associated with negative modulation of synaptic release of glutamate through activation of presynaptic mGluR2/3. It is of interest to elucidate whether NAC can modify the hallucinogenic 5-HT_{2A}R agonist-induced responses in the same manner.

The present study evaluated the effects of NAC on the hallucinogenic 5-HT_{2A}R agonist (±)1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI)–induced behavioral, molecular, and electrophysiological responses. Head twitch response induced by DOI and its structural analogs in mice was used to predict the hallucinogenic-specific effects in humans (Dursun and Handley, 1996; Kleven et al., 1997; Schreiber et al., 1995) since it is reliably elicited by a variety of hallucinogenic 5-HT_{2A}R agonists, but not induced by non-hallucinogenic 5-HT_{2A}R agonists, such as R-lisuride, S-lisuride and ergotamine (Gonzalez-Maeso et al., 2007, 2003).

DOI induces several immediately early gene expression including c-Fos and early growth response-2 (Egr-2) in mouse cortex (Garcia et al., 2007; Gonzalez-Maeso et al., 2008). It has been reported that Egr-2 induction is a downstream event of a 5-HT_{2A}R-mediated Gi/Go-dependent pathway, which is selectively activated by hallucinogens. In contrast, c-Fos induction follows the activation of a Gq-dependent pathway, which is activated by both hallucinogenic and nonhallucinogenic 5-HT_{2A}R agonists (Gonzalez-Maeso et al., 2008, 2007, 2003). The induction of Egr-2, correlated with the generation of a head-twitch response and dependent on mGluR2 (Moreno et al., 2011), has been thought as a specific molecular marker for hallucinogenic response. The effects of NAC on DOI-induced c-Fos and Egr-2 expression were examined by immunohistochemistry. Moreover, DOI induces a robust increase in the frequency of spontaneous excitatory postsynaptic potentials (EPSPs) in the medial prefrontal cortex (Aghajanian and Marek, 1997), which is counteracted by mGluR2 agonists (Klodzinska et al., 2002). Accordingly, the effects of NAC on DOI-evoked excitatory field potentials were assessed in mouse prefrontal cortical slices.

After demonstrating that NAC blocked the DOI-induced responses, a cystine-glutamate antiporter inhibitor (S)-4-carboxyphenylglycine (CPG) and an mGluR2 antagonist LY341495 were used to determine whether stimulation of mGluR2 by glutamate derived from cystine-glutamate antiporters is contributed to the capacity of the NAC to inhibit DOI-elicited head twitch and electrophysiological responses.

2. Materials & methods

2.1. Animals

Male NMRI mice (8–10 weeks) were supplied from the Laboratory Animal Center of Tzu Chi University (Hualien, Taiwan) and housed 4 to 5 per cage in a 12 h light/dark cycle (lights on 0700 h) with ad libitum access to water and food during the time the animals were in their home cages. All experiments were performed in accordance with the Republic of China animal protection law (Chapter III: Scientific Application of Animals) and approved by the Review Committee of the Tzu Chi University.

2.2. Drug treatment

DOI and NAC (Sigma, St. Louis, MO) were dissolved in 0.9% saline. CPG and LY341495 (Tocris Cookson Ltd., Bristol, UK) were dissolved in double distilled water and titrated to pH 7.4 by using 1 N NaOH. CPG was administered intracerebroventricularly (i.c.v.) in an injection volume of 5 μl. Other compounds were administered intraperitoneally (i.p.) in an injection volume of 10 ml/kg.

2.3. Head twitch response

Mice were transferred from the colony room to the observation room and allowed to habituate for 30 min. After injection of DOI or saline, mice were immediately placed in an acrylic cylinder container. The number of head twitch response (rapid movements of the head with little or no involvement of the trunk) was counted for 30 min following DOI or saline administration by two observers (blind to the treatment).

2.4. Immunohistochemical analysis

The mice completed the 30-min observation of head-twitch response were deeply anesthetized with thiamylal sodium (150 mg/kg, i.p.) and then perfused transcardially with 0.1 M phosphate-buffered saline (PBS), subsequently with 0.1 M PBS containing 4% paraformaldehyde 60 min after administration of DOI. The brains were removed and post-fixed for 24 h and then stored in 30% sucrose solution until it sank. The brains were frozen and cut into 25 μm thick coronal section in a microtome. Free–floating sections were processed for c-Fos and Egr-2 immunohistochemistry. The sections were incubated for 30 min in 1% H_2O_2 to inactivate endogenous peroxidase and decreased non-specific staining. Sections were washed three times (10 min per wash) in PBS containing 0.4% Triton X-100 and 2% Bovine serum albumin (BSA). Blocking was performed with 10% BSA for 1 h and then incubated on rocking table with c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA, 1:1000) or Egr-2 (Santa Cruz Biotechnology, Santa Cruz, CA, 1:100) polyclonal antibody for overnight at 4 °C. Sections were washed again three times in PBS prior to being incubated with the biotinylated anti-rabbit IgG (Sigma, 1:200) for 2 h at room temperature. The sections were then washed three times in PBS buffer, and then incubated with 0.2% avidin-biotinylated horseradish peroxidase complex (ABC solution, Vector Laboratories, Burlingame, CA) for 1 h. After three 10 min washes in PBS, the sections were placed in the chromogen 3, 3′-diaminobezidine tetrahydrochloride (DAB, Sigma) for 15 min. The sections were again washed three times in PBS, mounted on gelatinized slides, air-dried overnight and coverslipped.

c-Fos and Egr-2 immunoreactivity was observed in the cell nuclei and brain sections were shown brownish round dots under a light microscope. According to the atlas (Paxinos and Franklin, 2004), the coronal sections (+1.70 mm relative to bregma) including cingulate cortex area 1 (Cg1), prelimbic cortex (PrL), infralimbic cortex (IL), somatosensory cortex (S1), and motor cortex area 2 (MC2) were defined and analyzed. Bright-field images were captured with a digital camera (Canon EOD50) mounted on a Nikon Eclipse 800 microscope at 100× magnification. An image of 0.12 mm^2 area was analyzed for the number of c-Fos and Egr-2 immunoreactive cells. Cell counts were made with the help of Image J and manually counted

by an observer who was blind to group arrangement. For each brain area, data were obtained from three slices per mice ($n = 4$–6).

2.5. Cannulation and intracerebroventricular injections

Ketamine/xylazine (80/10 mg/kg i.p.)-anesthetized mice were mounted on a stereotaxic alignment system (David Kopf Instruments, Tujunga, CA), guide cannulas (26 GA, 2.4 mm from pedestal) were placed in the lateral ventricle at 1.0 mm lateral, 0.4 mm posterior from bregma, and 3.0 mm below the skull (Paxinos and Franklin, 2004). Cannulas were anchored with screws using dental cement. After surgery, mice recovered in their home cage for 6–7 days. Mice ($n = 7$) were injected with CPG (100 nmol, icv) 5 min prior to either NAC (100 mg/kg, i.p.) or saline by placing a 33 guage internal cannula into the guide cannula and attached to a flared PE20 tube connected to a 10 μl Hamilton syringe. Injections of CPG (5 μl) of or vehicle were performed during a 2 min period. Internal cannulas were removed 1 min after injection.

2.6. Preparation of prefrontal cortex (PFC) slices for electrophysiological recordings

The brain of NMRI mice were removed and immersed in an ice-cold artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl (127), KCl (1.9), $CaCl_2$ (2.4), $MgCl_2$ (1.3), $NaHCO_3$ (26), KH_2PO_4 (1.2), and D-glucose, bubbled with a mixture of 95% O_2/5% CO_2, pH 7.4. Coronal slices (300 μm) were cut from the frontal cortex (3–5.16 mm anterior to bregma) using a vibrating tissue slicer. After recovery for at least 1 h at room temperature, a single slice was transferred to the center area of the coated MED probe and positioned to cover the 8 × 8 microelectrode array by a paint brush. The positioned slice was superfused at 2.0 ml/min with ACSF saturated with O_2.

2.7. Electrophysiological recordings

For electrophysiological recordings, the MED probe containing the brain slice was placed in a small incubator which was superfused with Mg^{2+} free-ACSF in 5% CO_2/95% O_2 at 25 °C and connected to the stimulation/recording component of MED8 multi-electrode array system (Panasonic). Mg^{2+}-free ACSF was used to minimize Mg^{2+} block of NMDA receptors. The preparation of the multi-electrode dish has been described previously (Chen et al., 2011). Briefly, the MED probe is an array of 64 planar microelectrodes, where each microelectrode has a size of 50 × 50 μm and is arranged in an 8 × 8 pattern. The interpolar distance in this type of probe (MED-P515A) is 150 μm. For sufficient adhesion of the brain slice to the MED probe, the surface of probe was treated with 0.1% polyethylenimine in 25 mM borate buffer for 8 h at room temperature. Then the probe surface was rinsed three times with distilled water for future experiments.

The spontaneous field potentials or DOI-evoked field potentials at 8 sites in the 64 multi-electrode probe were recorded simultaneously with the multi-channel recording system at a 20 kHz sampling rate. The electrodes in the prefrontal cortex were selected as the recording electrodes. In order to prevent the sodium channel-mediated action potential activity, all the experiments were performed with 0.3 μM TTX. The recording of spontaneous field potentials was first carried out to establish a baseline. The frequency changes induced by DOI and NAC were expressed as percentages of baseline, which were set at 100%.

2.8. Statistical analysis

Data were obtained and expressed as mean ± S.E.M. The data for head twitch and immunohistochemical test were analyzed by two-way or three-way ANOVA followed by Newman–Keuls post hoc test. The data for DOI-induced frequency changes in field potentials were analyzed by repeated measured one-way ANOVA, The level of statistical significance was set as $p < 0.05$.

3. Results

3.1. Dose-dependent effect of NAC on DOI-induced head twitch response

Mice ($n = 8$) were treated with NAC (30 or 100 mg/kg) or saline 90 min prior to DOI (1 mg/kg, i.p.) or saline administration. Two-way ANOVA indicated that there are significant main effects (DOI: $F_{1,42} = 111.96$, $p < 0.001$:NAC $F_{2,42} = 4.64$, $p < 0.05$) and interaction between DOI and NAC ($F_{2,42} = 4.993$, $p < 0.05$). As illustrated in Fig. 1, DOI (1 mg/kg) significantly increased head twitch response in mice. Pretreatment with NAC at high dose (100 mg/kg), but not low dose (30 mg/kg), significantly decreased the DOI-induced head twitch response.

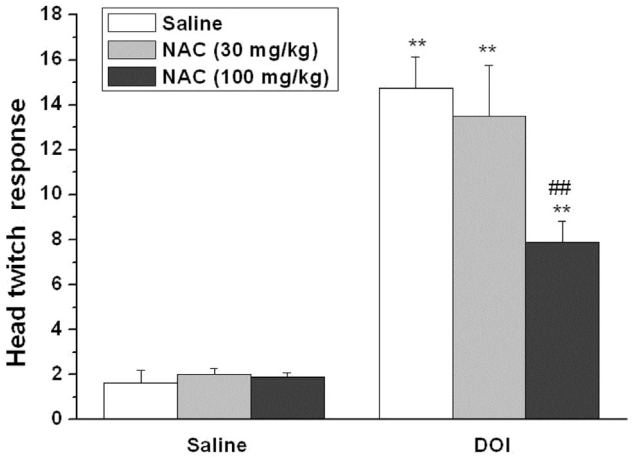

Fig. 1. Dose-dependent effects of NAC on DOI-induced head twitch response. Mice were treated with NAC (0, 30 and 100 mg/kg) 90 min prior to DOI (1 mg/kg) or saline. Head twitch response was observed for 30 min. Data are expressed as mean ± SEM ($n = 8$). *$p < 0.05$ compared with saline + DOI group.

3.2. Dose-dependent effect of NAC on DOI-induced c-Fos and Egr-2 expression

c-Fos and Egr-2 induction by DOI and saline was examined in the infralimbic cortex (IL), motor cortex area 2 (MC2), cingulate cortex area 1 (Cg1), prelimbic cortex (PrL), and somatosensory cortex (S1) by immunohistochemistry (Fig. 2A). First, three way-ANOVA was used to analyze the effects of DOI, NAC, and brain region on the expression of cFos and Egr-2. There is significant interactions between three factors (cFos: DOI × NAC × brain region: $F_{8,130} = 9.68$, $p < 0.001$; DOI × NAC: $F_{2,130} = 10.23$, $p < 0.001$; DOI × brain region $F_{8,130} = 27.57$, $p < 0.001$; NAC × brain region: $F_{4,130} = 4.59$, $p < 0.001$; Egr-2: DOI × NAC × brain region: $F_{8,130} = 5.472$, $p < 0.001$; DOI × NAC: $F_{2,130} = 4.279$, $p < 0.01$; DOI × brain region $F_{8,130} = 5.215$, $p < 0.001$; NAC × brain region: $F_{5.405} = 3.22$, $p < 0.001$). Post-hoc test in the individual brain regions demonstrated that DOI significantly increased c-Fos expression in the IL, MC2, and Cg1 and Egr-2 in the IL, MC2, and S1. NAC (100 mg/kg) reversed the enhancement effects of DOI on c-Fos and Egr-2 expression in the IL and MC2, whereas it did not affect the saline-treated groups (Fig. 2B–E).

3.3. Effects of NAC on DOI-evoked field potentials

The baseline of field potentials recorded in the frontal cortex included excitatory and inhibitory components, which were blocked by ketamine (10 μM) and bicuculline (10 μM), respectively (Fig. 3A). After stabilization of baseline activity, slices were superfused for 5–7 min with modified ACSF containing 1 μM DOI, which was subsequently washed out for 5 min. To evaluate the effect of NAC, 10 μM NAC was applied for 15 min before the second administration of DOI (Fig. 3B). DOI (1 μM) increased the frequency of excitatory, but not inhibitory field potentials. NAC (10 μM) significantly suppressed the DOI-elicited enhancement of frequency in excitatory field potentials (Fig. 3C–E).

3.4. NAC-mediated block of DOI-induced head twitch response and expression of c-Fos and Egr-2 is reversed by a cystine-glutamate antiporter inhibitor and an mGluR2 antagonist

NAC is a prodrug for the amino acid cysteine. Cysteine was then converted to dimeric cystine by oxidative enzymes (Meister, 1985;

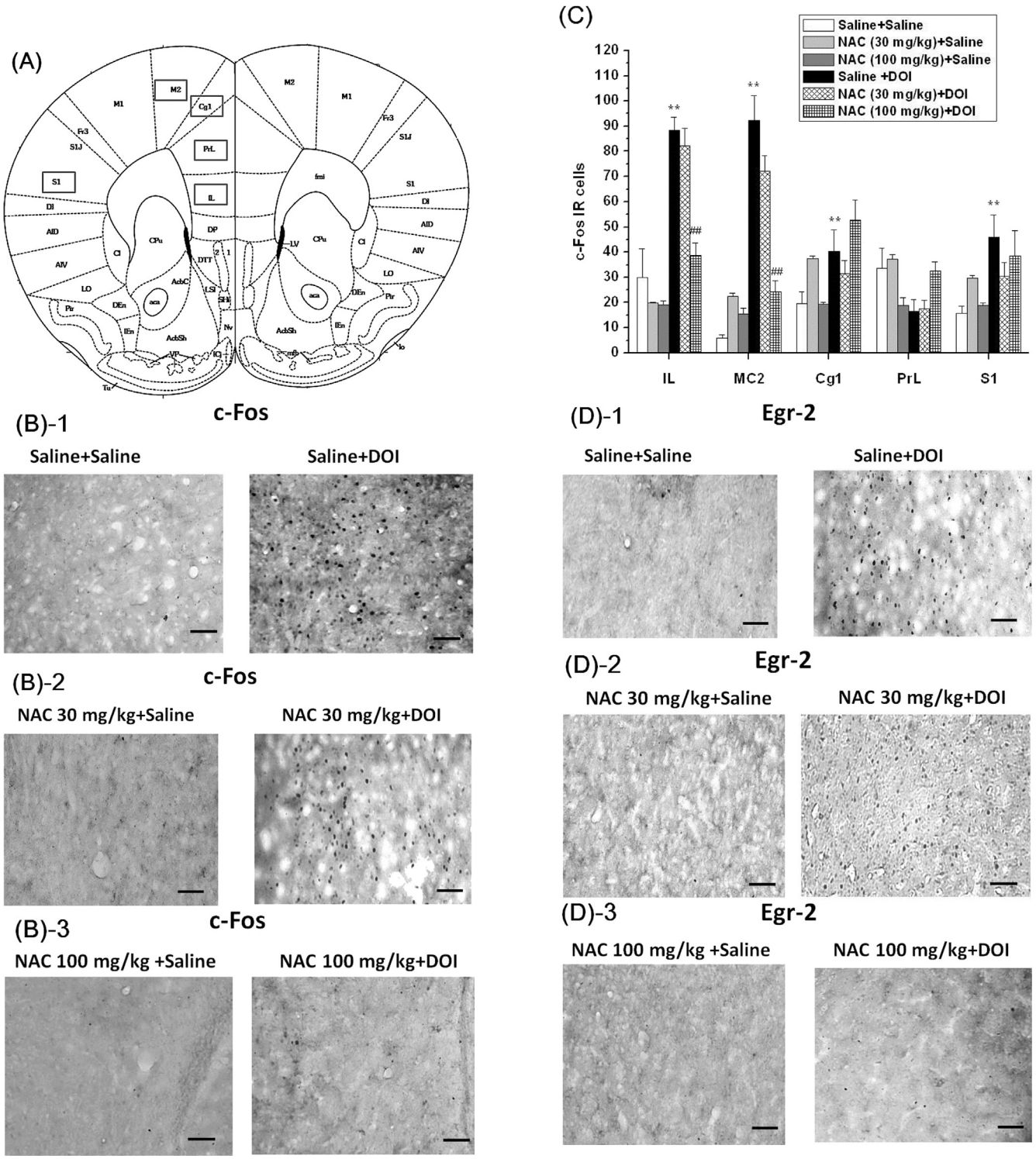

Fig. 2. Schematic illustration of the areas in which expression of c-Fos and Egr-2 was analyzed (A), the representative photos in the infralimbic cortex (B, D) and quantitative analyses of the number of c-Fos (C) and Egr-2 (E) immunoreactive cells in the cortices following DOI (1 mg/kg) or saline with pretreatment with NAC (0, 30, and 100 mg/kg). The c-Fos and Egr-2 immunoreactive cells were counted per 0.12 mm^2 area in the highlighted regions of the coronal sections (+1.70 mm relative to bregma, based on Paxinos and Franklin, 2004) including cingulate cortex area 1 (Cg1), prelimbic cortex (PrL), infralimbic cortex (IL), somatosensory cortex (S1), and motor cortex area 2 (MC2). Data are expressed as mean ± SEM ($n = 6$). $^{**}p < 0.01$ compared with Saline + Saline group.$^{\#\#}p < 0.01$ compared with Saline + DOI group. Scale bar = 10 μm.

Fig. 2. (*continued*).

Pileblad and Magnusson, 1992) to activate cystine-glutamate antiporters. However, cysteine can also be transported by the sodium-dependent neuronal glutamate transporter, excitatory amino acid carrier 1 (EAAC1) (Aoyama et al., 2006). To reveal the critical role of cystine-glutamate antiporter in the effect of NAC, we evaluated whether the cystine-glutamate antiporter inhibitor CPG can effectively suppress the effects of NAC. Mice ($n = 7$) were injected with CPG (0 and 100 nmol, icv) 5 min prior to NAC (0 and 100 mg/kg, i.p.).

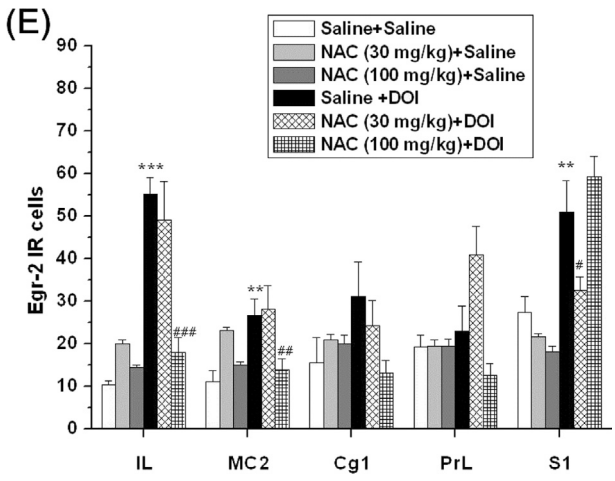

Fig. 2. (*continued*).

DOI (1 mg/kg, i.p.) was given 90 min after NAC injection. Two-way ANOVA yielded a main effect of NAC ($F_{1,24} = 4.17$, $p < 0.05$), and a significant interaction between CPG and NAC treatment ($F_{1,24} = 4.76$, $p < 0.05$). CPG had no effect alone, but significantly reversed the reducing effects of NAC on DOI-induced head twitch response (Fig. 4A).

It has been reported that cystine-glutamate antiporters regulate synaptic glutamate through activation of mGluR2 (Baker et al., 2002; Moran et al., 2005) and the mGluR2 agonist LY379268 significantly reduces the DOI-induced head twitch response (Gonzalez-Maeso et al., 2008). Accordingly, we examined whether activation of mGluR2 signaling is required for NAC to reverse the DOI-induced head twitch response by administration of mGluR2 antagonist LY341495 prior to NAC. Mice ($n = 9$) were pretreated with NAC (0 and 100 mg/kg, 90 min) and the mGluR2/3 antagonist LY341495 (0 and 3 mg/kg, 45 min) prior to DOI (1 mg/kg) administration. Two-way ANOVA yielded a main effect of NAC ($F_{1,32} = 7.226$, $p < 0.05$), and a significant interaction between LY341495 and NAC treatment ($F_{1,32} = 7.562$, $p < 0.01$). LY341495 had no effect alone; whereas it significantly reversed the reducing effects of NAC on DOI-induced head twitch response (Fig. 4B). After observation of head-twitch response, the mice were sacrificed for evaluation of the c-Fos and Egr-2 expression.

The IL and MC2 were examined because DOI and NAC affected head twitch response in parallel with the c-Fos and Egr-2 expression pattern in these two regions. Two-way ANOVA revealed that there are significant main effects and interaction between CPG and NAC on DOI-induced c-Fos expression in the IL (CPG: $F_{1,12} = 11.68$, $p < 0.01$, NAC $F_{1,12} = 11.05$, $p < 0.01$; interaction: $F_{1,12} = 7.84$, $p < 0.05$) and MC (CPG: $F_{1,12} = 12.09$, $p < 0.01$, NAC $F_{1,12} = 9.62$, $p < 0.01$; interaction: $F_{1,12} = 3.42$, $p < 0.05$) (Fig. 5A). For DOI-induced Egr-2 expression, significant main effects and interaction between CPG and NAC showed in the IL (CPG: $F_{1,12} = 14.03$, $p < 0.01$, NAC $F_{1,12} = 20.99$, $p < 0.01$; interaction: $F_{1,12} = 5.63$, $p < 0.05$), whereas there is no interaction between CPG and NAC in MC2 (CPG: $F_{1,12} = 63.9$, $p < 0.01$, NAC $F_{1,12} = 23.72$, $p < 0.01$) (Fig. 5B). Post-hoc tests demonstrated that CPG reversed the reducing effects of NAC on c-Fos and Egr-2 expression induced by DOI in both brain regions.

Similarly, LY341495 reversed the effects of NAC on c-Fos and Egr-2 expression. Two-way ANOVA revealed that there is significant main effect and interaction between LY341495 and NAC on DOI-induced c-Fos expression in the IL (LY341495: $F_{1,12} = 7.38$, $p < 0.05$, NAC $F_{1,12} = 6.74$, $p < 0.05$; interaction: $F_{1,12} = 6.57$, $p < 0.05$) and MC (LY341495: $F_{1,12} = 19.74$, $p < 0.01$, NAC $F_{1,12} = 5.03$, $p < 0.05$; interaction: $F_{1,12} = 8.581$, $p < 0.05$) (Fig. 5C). For DOI-induced Egr-2 expression, significant main effect and interaction between LY341495 and NAC showed in the IL (LY341495: $F_{1,12} = 10.78$, $p < 0.01$, NAC $F_{1,12} = 25.96$, $p < 0.01$; interaction: $F_{1,12} = 5.11$, $p < 0.05$), whereas there is no interaction between LY341495 and NAC in MC2 (LY341495: $F_{1,12} = 4.546$, $p = 0.054$, NAC $F_{1,12} = 6.89$, $p < 0.05$) (Fig. 5D). Post-hoc tests demonstrated that LY341495 reversed the reducing effects of NAC on c-Fos and Egr-2 expression induced by DOI in both brain regions.

3.5. Inhibitory effects of NAC on DOI-evoked excitatory field potentials is reversed by a cystine-glutamate antiporter inhibitor and an mGluR2 antagonist

In order to determine whether cystine-glutamate antiporters and mGluR2 are involved in the inhibitory effect of NAC on DOI-evoked field potentials, the cystine-glutamate antiporter inhibitor CPG and the mGluR2 antagonist LY341495 were applied 10 min prior to NAC perfusion, respectively (Fig. 6A). There was no significant difference between the frequency of the excitatory field potentials evoked by DOI alone and DOI with pre-application of CPG/LY341495 with NAC (Fig. 6B–D), supporting that the inhibitory effect of NAC on DOI-induced enhancement of the frequency of excitatory field potentials can be reversed by CPG or LY341495.

4. Discussion

The present study demonstrated that NAC attenuates hallucinogen DOI-induced head twitch response and c-Fos and Egr-2 protein expression in the infralimbic and motor cortices in mice. Furthermore, NAC suppressed the increased frequency of excitatory field potentials induced by bath-applied DOI recorded in the mouse medial prefrontal cortical slices. These results revealed that NAC negatively modulates the responses to hallucinogen DOI. NAC, a precursor of the antioxidant glutathione and also a prodrug of cysteine, is known to increase the activity of cystine-glutamate antiporter in glial cells, thereby promoting glutathione synthesis (e.g. in treating acetaminophen overdose), as well as increasing glutamatergic tone on mGluR2 autoreceptors or postsynaptic mGluR2 in close proximity with the 5-HT_{2A}Rs to antagonize behavioral dysfunction, such as cocaine-induced drug seeking (Moran et al., 2005) or the psychotomimetic effects of PCP (Baker et al., 2008, 2002). In fact, our data demonstrated that cystine-glutamate antiporter inhibitor CPG and mGluR2 receptor antagonist LY341495 suppressed the inhibitory effects of NAC on head twitch and field potentials in response to DOI. Therefore, the suppressing effect of NAC might be mainly mediated by activation of the cystine-glutamate antiporters in the glial cells to increase extrasynaptic glutamate levels, thereby stimulating the mGluR2 activity to reduce the behavioral, molecular, and electrophysiological responses associated with activation of 5-HT_{2A}Rs in response to DOI.

DOI-induced head twitch response has been commonly used as a hallucinogenic 5-HT_{2A} receptor agonist-specific behavioral model, which may correspond to some human-like conditions such as schizophrenia, hallucinogen addiction (Dursun and Handley, 1996; Garcia et al., 2007), and tics of Tourette syndrome (Hayslett and Tizabi, 2003; Tizabi et al., 2001). NAC (100 mg/kg) significantly attenuated this response. The immunohistochemical data demonstrated that NAC also suppressed DOI-induced c-Fos and Egr-2 expression in the infralimbic (a subdivision of medial prefrontal cortex) and motor cortices in the same manner. In agreement with our results, earlier work demonstrated that activation of mGluR2 prevents the induction of c-Fos by hallucinogens in the medial prefrontal cortex, but not in the somatosensory cortex

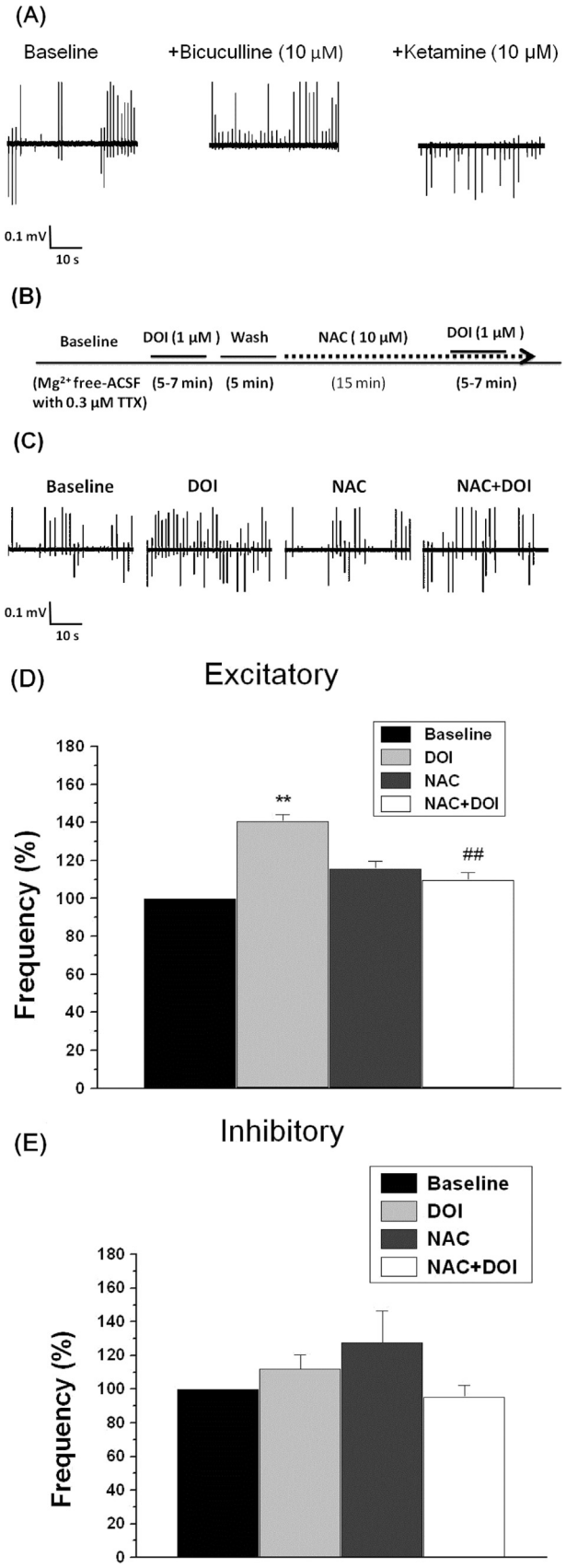

Fig. 3. Effects of NAC on DOI-evoked field potentials. Spontaneous field potentials recorded in the medial frontal cortex including excitatory and inhibitory components, which were blocked by ketamine (10 μM) and bicuculline (10 μM), respectively (A). The experimental protocol (B), the representative field potential recordings (C), and the frequency of excitatory (D) and inhibitory field potentials (E) recorded after perfusion with NAC (10 μM) and/or DOI (1 μM). Data are expressed as percentage of baseline, mean ± SEM ($n = 4$). $^{**}p < 0.01$ compared with baseline. $^{\#\#}p < 0.01$ compared with DOI.

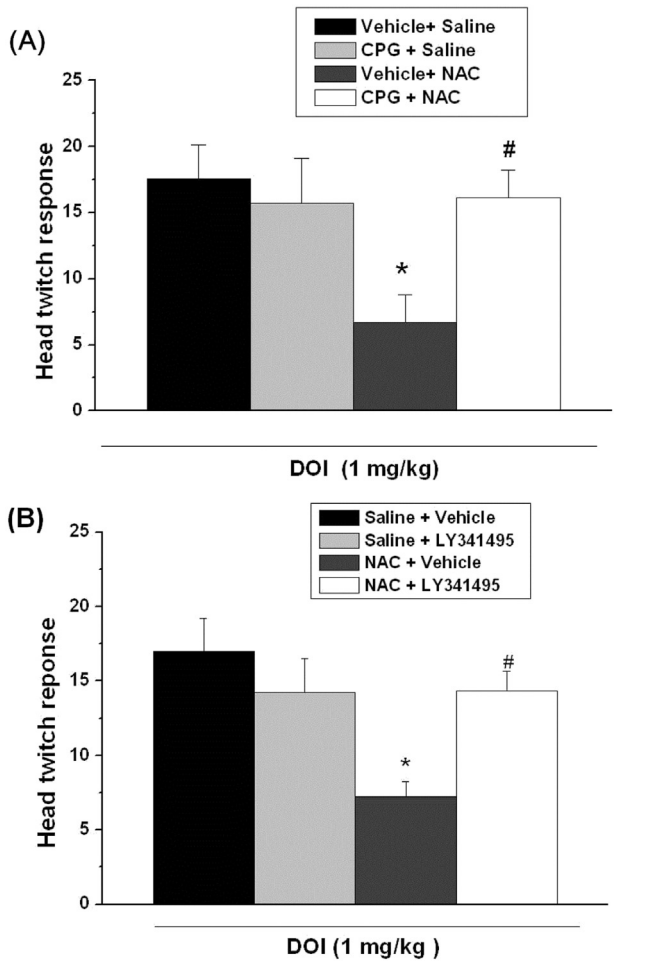

Fig. 4. Suppressing effects of the cystine-glutamate antiporter inhibitor CPG (100 nmol) (A) and the mGluR2 antagonist LY341495 (3 mg/kg) (B) on NAC (100 mg/kg)-mediated reduction of DOI (1 mg/kg)-induced head twitch response. Data are expressed as mean ± SEM $p < 0.05$ compared with vehicle control group, $\#p < 0.05$ compared with NAC group.

(Benneyworth et al., 2007; Zhai et al., 2003). Similarly, González-Maeso et al. (2008) revealed that mGluR2 agonist LY379268 did not inhibit the induction of c-Fos mediated by the activation of 5-HT_{2A}R both in neurons of the mouse somatosensory cortex in vivo and in cultured cortical neurons. The differential effect of mGluR2 activation on the induction of c-Fos in prefrontal and somatosensory cortical regions may result from differential localization of mGluR2 to intrinsic pyramidal and/or nonpyramidal neurons within the prefrontal and somatosensory cortical regions.

It has been reported that Egr-2 induction is a downstream event of a 5-HT_{2A}-receptor mediated Gi/Go-dependent pathway, which is selectively activated by hallucinogens. In contrast, c-Fos induction follows Gq-dependent activation of polyphosphoinositide hydrolysis, which leads to an increased formation of inositol-1,4,5-trisphosphate and diacylglycerol, and is activated by both hallucinogenic and nonhallucinogenic 5-HT_{2A} receptor agonists (Gonzalez-Maeso et al., 2008, 2007, 2003). Activation of mGluR2 has been shown to inhibit hallucinogens-induced Egr-2 expression

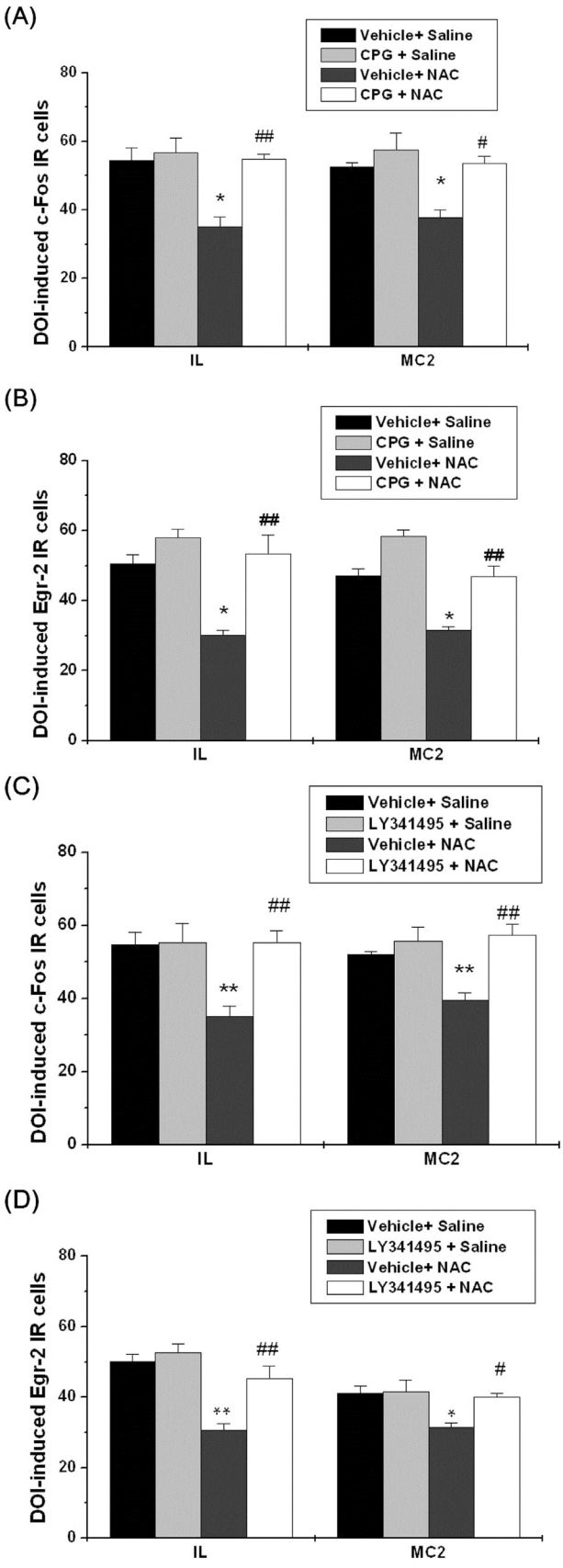

Fig. 5. Suppressing effects of the cystine-glutamate antiporter inhibitor CPG (100 nmol) and the mGluR2 antagonist LY341495 (3 mg/kg) on NAC (100 mg/kg)-mediated reduction of DOI (1 mg/kg)-induced c-Fos (A, C) and Egr-2 (B, D) expression in the IL and MC2. Data are expressed as mean ± SEM *. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with NAC group.

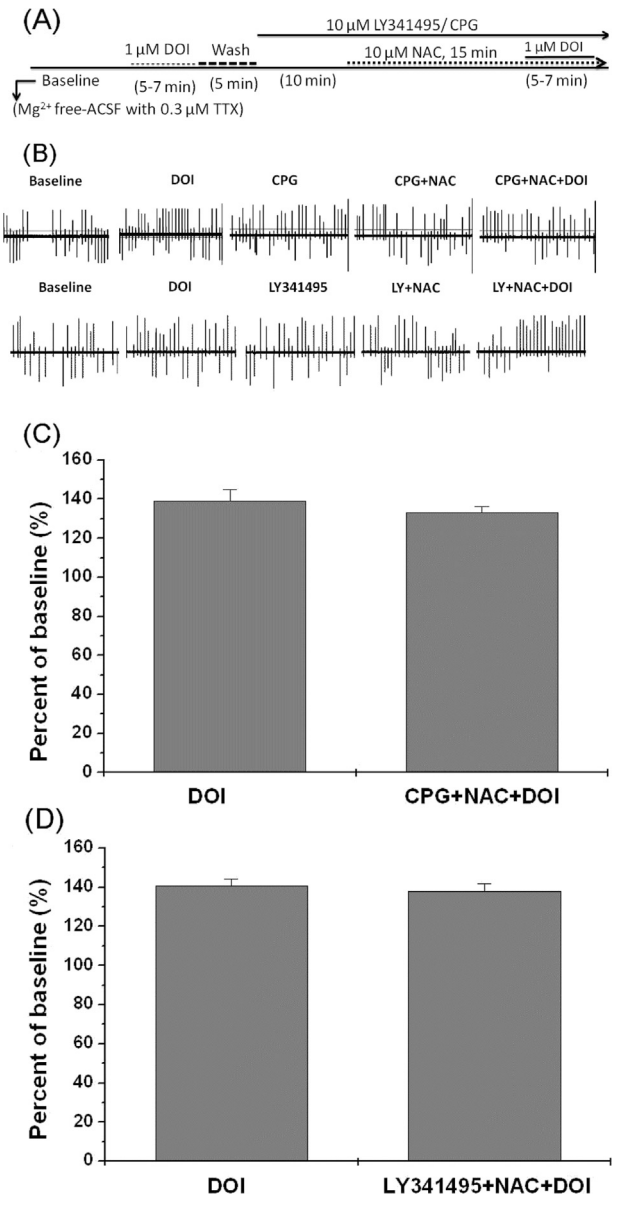

Fig. 6. Suppressing effects of the cystine-glutamate antiporter inhibitor CPG and the mGluR2 antagonist LY341495 on NAC-attenuated the increase in the frequency of excitatory field potentials induced by bath-applied DOI in the medial prefrontal cortex. The experimental protocol (A), the representative field potential recordings (B), and the changes in the frequency of excitatory field potentials recorded after superfusion with CPG (10 μM) (C) or LY341495 (10 μM) (D) combined with NAC (10 μM) and DOI (1 μM). Data are expressed as percentage of baseline, mean ± SEM ($n = 4$).

(possibly through Gi/Go-dependent pathway) (Gonzalez-Maeso et al., 2008) in the somatosensory cortex and also negatively regulates the canonical Gq/phospholipase Cβ (PLCβ) pathway activated by 5-HT_{2A}Rs in the frontal cortex (Molinaro et al., 2009). Our data demonstrated that the inhibitory effect of NAC on DOI-induced head twitch response is mediated by mGluR2. However, the signal transduction pathways, the immediate early genes, and the brain regions involved in the regulation of hallucinogens-induced head twitch response by those compounds enhancing the activity of mGluR2 including NAC still remain controversial.

Actually, several lines of evidence demonstrated that hallucinogens-induced increases in c-Fos immunoreactivity do not occur in cells expressing 5-HT_{2A}R (Gresch et al., 2002; Scruggs et al., 2000), suggesting that the increased c-Fos expression is not a direct

intracellular response to stimulation of 5-HT_{2A}R, but rather involves glutamatergic transmission indirectly (Reissig et al., 2008). In the prefrontal cortex, 5-HT_{2A}R are found on glutamatergic neurons (Jakab and Goldman-Rakic, 1998; Santana et al., 2004), and hallucinogens increase extracellular glutamate levels in this brain area through stimulation of 5-HT_{2A}R (Muschamp et al., 2004). Furthermore, glutamate release inhibitor riluzole and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor antagonist NBQX significantly inhibit DOI-induced head twitch response (Egashira et al., 2011). Together with our results, these findings support the critical role of glutamatergic transmission in the mechanism of action of DOI to induce head twitch response and c-Fos expression. Although NAC exerted its inhibitory effect on the induction of c-Fos and Egr-2 by DOI similarly in the motor and infralimbic cortices, these two immediately early genes may not be colocalized. It is possible that Egr-2 is induced in the 5-HT_{2A}R-expressing neurons and c-Fos is expressed in the glutamate targeting cells.

In line with the behavioral response, the electrophysiological data demonstrated that NAC attenuated the enhancement of the frequency of excitatory field potentials evoked by DOI. In our experimental condition, the spontaneous filed potentials included excitatory and inhibitory components, which were blocked by ketamine and bicuculline, respectively, demonstrating that the NMDA and $GABA_A$ receptors make major contributions to the excitatory and inhibitory field potentials, respectively. DOI specifically increased the frequency of excitatory field potentials and this effect was suppressed by NAC. It has been reported that the mGlu2/3 agonist LY354740 suppressed glutamate release induced by 5-HT_{2A} receptor activation in the medial prefrontal cortex (Marek et al., 2000) and mGluR2 receptor-selective positive allosteric modulator biphenyl-indanone A (BINA) reduced serotonin-induced increases in spontaneous excitatory postsynaptic currents in the medial prefrontal cortex (Benneyworth et al., 2007). Since the suppressing effects of NAC on DOI-evoked field potentials were blocked by the cystine-glutamate antiporter inhibitor CPG and the mGluR2 antagonist LY341495, NAC might increase extrasynaptic glutamate through activation of cystine-glutamate antiporter and subsequently stimulate mGluR2 to reduce the presynaptic glutamate release elicited by stimulation of 5-HT_{2A}R in response to DOI. Furthermore, mGluR2 has been shown to interact through specific transmembrane helix domains with serotonin 5-HT_{2A} receptors to form functional complexes in brain cortex (Gonzalez-Maeso et al., 2008) although the physiological relevance of 5-HT_{2A}R-mGluR2 heteromeric complex has recently been challenged (Delille et al., 2012). Alternatively, NAC might suppress the activity of 5-HT_{2A}R by indirectly activating the mGluR2 in the 5-HT_{2A}R-mGluR2 complex in the medial prefrontal cortex.

Although DOI also presents high affinity for 5-HT_{2C} receptors (Appel et al., 1990), DOI-elicited head twitch behavior has been proved to be a 5-HT_{2A} agonist-mediated effect, with subsequent inhibition of head twitch behavior being driven by competing 5-HT_{2C} agonist activity (Fantegrossi et al., 2010). There is no evidence demonstrating that NAC could directly interact with 5-HT_{2A} or 5-HT_{2C} receptors. In fact, cystine-glutamate antiporter and mGluR2 play key roles in the suppressing effects of NAC on behavioral, molecular, and electrophysiological responses induced by DOI, reflecting the emerging modulatory role of NAC in the glutamatergic transmission is more critical than glutathione precursor in the regulation of hallucinogen-dependent responses.

In general, the adverse effect of NAC rarely occurs although extremely high dose of NAC has been reported to produce anaphylaxis, including rash, pruritus, angioedema, bronchospasm, and rarely hypotension (Sandilands and Bateman, 2009). NAC effectively suppressed the DOI-induced head twitch behavior, molecular response, and electrophysiological activity in the medial prefrontal cortex. Because increased excitation of the medial prefrontal cortex has been implicated in pathophysiology of schizophrenia, the ability of NAC to reduce the action of hallucinogen DOI in this region is believed to be directly related to its antipsychotic efficacy. Together with clinical report showing that NAC improves the positive and negative symptoms in schizophrenia patients (Berk et al., 2008; Lavoie et al., 2008), our results support that NAC can be used for treatment of hallucinations and psychosis associated hallucinogen use or schizophrenia. Furthermore, DOI-induced head twitch response in mice can also serve as an animal model of tics in Tourette syndrome (Hayslett and Tizabi, 2003; Tizabi et al., 2001). Acute administration of haloperidol and pimozide, the neuroleptics currently used to treat tics of Tourette syndrome, is also effective in reducing DOI-induced head twitch response (Dursun and Handley, 1996; Wettstein et al., 1999). Accordingly, our findings also suggest that NAC may be of therapeutic potential in the treatment of tics of Tourette syndrome.

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