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ORIGINAL INVESTIGATION

Effects of central activation of serotonin 5-$HT_{2A/2C}$ or dopamine $D_{2/3}$ receptors on the acute and repeated effects of clozapine in the conditioned avoidance response test

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Abstract
Rationale Acute administration of clozapine (a gold standard of atypical antipsychotics) disrupts avoidance response in rodents, while repeated administration often causes a tolerance effect.
Objective The present study investigated the neuroanatomical basis and receptor mechanisms of acute and repeated effects of clozapine treatment in the conditioned avoidance response test in male Sprague-Dawley rats.
Methods 2,5-dimethoxy-4-iodo-amphetamine (DOI, a preferential 5-$HT_{2A/2C}$ agonist) or quinpirole (a preferential dopamine $D_{2/3}$ agonist) was microinjected into the medial prefrontal cortex (mPFC) or nucleus accumbens shell (NAs), and their effects on the acute and long-term avoidance disruptive effect of clozapine were tested.
Results Intra-mPFC microinjection of quinpirole enhanced the acute avoidance disruptive effect of clozapine (10 mg/kg, sc), while DOI microinjections reduced it marginally. Repeated administration of clozapine (10 mg/kg, sc) daily for 5 days caused a progressive decrease in its inhibition of avoidance responding, indicating tolerance development. Intra-mPFC microinjection of DOI at 25.0 (but not 5.0) μg/side during this period completely abolished the expression of clozapine tolerance. This was indicated by the finding that clozapine-treated rats centrally infused with 25.0 μg/side DOI did not show higher levels of avoidance responses than the vehicle-treated rats in the clozapine challenge test. Microinjection of DOI into the mPFC immediately before the challenge test also decreased the expression of clozapine tolerance.
Conclusions Acute behavioral effect of clozapine can be enhanced by activation of the $D_{2/3}$ receptors in the mPFC. Clozapine tolerance expression relies on the neuroplasticity initiated by its antagonist action against 5-$HT_{2A/2C}$ receptors in the mPFC.

Keywords 5-$HT_{2A/2C}$ receptor · $D_{2/3}$ receptor · Medial prefrontal cortex · Clozapine · Conditioned avoidance response · Tolerance

Introduction

As a prototypical atypical antipsychotic drug, clozapine possesses a superior efficacy in the treatment of schizophrenia, especially for refractory patients who respond poorly to other antipsychotic medications and patients with a high suicide risk (Kane et al. 1988; McEvoy et al. 2006). The neurobiological mechanisms responsible for its superiority are not known. At the receptor level, clozapine's mechanism of action is thought to include more potent blockade of serotonin 5-HT_{2A} than of dopamine D_2 receptor (Meltzer 2002). However, this property does not distinguish it from other atypical antipsychotic drugs, nor could it be used to explain its therapeutic effects. This is because the unique clinical therapeutic effects of clozapine manifest only after some period of repeated drug treatment,

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which inevitably induces long-term plastic changes in the brain beyond its acute receptor binding actions. In addition, recent evidence does suggest that the receptor mechanisms underlying the acute effect of clozapine are distinct from that of its chronic effect (Li et al. 2010, 2012). Neuroanatomically, clozapine also does not seem to possess a higher regional specificity than other drugs (Borison and Diamond 1983; Kuroki et al. 1999). Furthermore, the exact neural network upon which clozapine exerts its therapeutic effects has not been elucidated, although the medial prefrontal cortex (mPFC) has been implicated as an important brain region (Ohashi et al. 2000; Pehek and Yamamoto 1994). Overall, it seems difficult to distinguish clozapine from other antipsychotic drugs based on current understandings of its neurobiological mechanisms of action.

Remarkably, at the behavioral level, clozapine can be singled out on the basis of its effect in the conditioned avoidance response (CAR) and phencyclidine (PCP)-induced hyperlocomotion tests, two widely used and validated behavioral measures of antipsychotic activity (Li et al. 2007; Sanger 1985; Wadenberg and Hicks 1999; Zhao et al. 2012). Repeated and intermittent exposures to most antipsychotic drugs (e.g., haloperidol, olanzapine, aripiprazole, risperidone, or asenapine) often lead to a progressive and persistent increase in their ability to suppress avoidance response and PCP-induced hyperlocomotion, known as *antipsychotic sensitization*. However, clozapine is the only drug that causes a decrease in its ability to do so (termed *clozapine tolerance*) (Feng et al. 2013; Li et al. 2007; Li et al. 2010, 2012; Qiao et al. 2013; Qin et al. 2013; Zhang and Li 2012). Clozapine tolerance thus represents an interesting form of neuroplasticity. It might be one characteristic effect that distinguishes this drug from other antipsychotic drugs, although its clinical implications are still not clear. We hypothesized that clozapine tolerance may be a unique feature linked to its superior therapeutic efficacy and may reflect its procognitive effect. Therefore, if we could understand the neuroanatomical basis and receptor mechanisms of clozapine tolerance in these preclinical behavioral tests, we may be able to delineate the neurobiological mechanisms responsible for clozapine's unique therapeutic effect. In the present study, we addressed this issue using a combination of microinjection and pharmacological techniques in the CAR test. We first determined that the mPFC is one critical brain region where clozapine acts to achieve its *acute* disruptive effect of avoidance, likely through its actions on $D_{2/3}$ and 5-$HT_{2A/2C}$ receptors (to a lesser extent). Next, we showed that the *expression* of clozapine tolerance, but not the tolerance *induction* is dependent on 5-$HT_{2A/2C}$ receptors in the mPFC. This study illustrates an interesting dissociated receptor mechanism underlying the acute effect of clozapine and its repeated effect.

Materials and methods

Animals

Male Sprague-Dawley rats (226–250 g upon arrival, Charles River, Portage, MI) were housed in pairs in transparent polycarbonate cages (48.3 cm×26.7 cm×20.3 cm) and maintained on a 12:12 light/dark schedule. Food and water were provided ad libitum. Room temperature was maintained at 22±1 °C with a relative humidity of 45–60 %. All procedures were approved by the IACUC at the University of Nebraska-Lincoln. All behavioral tests were conducted in the light cycle between 9:00 and 17:00.

Conditioned avoidance response training procedure

After 5 days of acclimation to the animal facility, rats were first handled and habituated to the custom-built two-compartment shuttle boxes (Med Associates, VT, USA) for 2 days (20 min/day). Over the next 2 weeks, they were trained to acquire avoidance responding in 10 sessions (1 session/day) (Feng et al. 2013; Li et al. 2010). Each training session consisted of 30 trials and each trial started with a presentation of a conditioned stimulus (CS, 76 dB white noise) for 10 s. If a subject moved from one compartment into the other during the CS presentation, the CS was terminated and an avoidance response was recorded. If the rat did not move across the chambers during the CS, a footshock (unconditioned stimulus, US, 0.8 mA) was immediately delivered to the metal grid floor for a maximum of 5 s. A shuttling response during this period was recorded as an escape. If the rat did not respond during the entire 5 s presentation of the shock, the trial was terminated and the next trial started after an intertrial interval of 30–60 s. Only those rats (119 out of 156) that reached the training criterion (minimum 70 % avoidance response in each of the last two sessions) were used in the subsequent drug tests.

Surgery

One day after the CAR training, rats were anesthetized using a mixture of ketamine HCl (90 mg/kg) and xylazine (4 mg/kg) (ip), and implanted with bilateral stainless-steel guide cannulas (22 gauge; Plastics One) into the NAs or the mPFC. To avoid the lateral ventricles and to allow a slanted cannula angle aimed at the NAs, the incisor bar was set at 5.0 mm above interaural zero and the coordinates were: anteroposterior (AP) +3.4 mm, mediolateral (ML) ±1.0 mm, dorsoventral (DV) −5.7 mm (Reynolds and Berridge 2001; Richard and Berridge 2011). For mPFC cannulation, the incisor bar was set at −3.4 mm and the coordinates were: AP + 3.0 mm, ML ±0.75 mm, DV −2.2 mm (Paxinos and Watson 2004). All rats were allowed 6–8 days of recovery time before being used in the subsequent drug tests.

Drugs and microinjections

Clozapine (CLZ, a gift from the NIMH drug supply program) was dissolved in 1.0 % glacial acetic acid in sterile distilled water and administrated subcutaneously at 10.0 mg/kg in all experiments. This dose of CLZ produces a reliable disruption on avoidance responding and is commonly used in the comparative study of antipsychotic drugs (Feng et al. 2013; Li et al. 2010, 2011, 2012; Mead and Li 2009; Qiao et al. 2013; Sun et al. 2009; Zhang and Li 2012; Zhao et al. 2012). It also gives rise to clinically relevant striatal dopamine D_2 occupancies in rats (40–60 %) (Kapur et al. 2003; Wadenberg et al. 2001b). Quinpirole (QUI) and 2,5-dimethoxy-4-iodo-amphetamine (DOI) (RBI-Sigma, Natick, MA) were dissolved in 0.9 % saline (SAL) and were microinjected through 10-μl Hamilton syringes mounted on infusion pumps (Fisher Scientific) via polyethylene tubing (PE 10) attached to a 28-gauge injector (Plastics One), which extended 2.0 or 1.5 mm below the tips of the guide cannulas in the NAs or mPFC, respectively. We tested QUI at 0.0, 1.0, 5.0, and 10.0 μg/0.5 μl/side, and DOI at 0.0, 1.0, 5.0, and 25.0 μg/0.5 μl/side, based on previous work showing that these doses are effective at producing behavioral alterations in rats, including prepulse inhibition, impulsive behavior, psychomotor response to cocaine (Beyer and Steketee 2000; Sipes and Geyer 1997; Sotoyama et al. 2011; Wan and Swerdlow 1993; Wischhof et al. 2011). The bilateral microinjection (0.5 μl at 0.5 μl/min) started 1 min after injector insertions, and the injectors remained in place for an additional 1 min post infusion to allow for drug diffusion.

Experiment 1: Effects of intra-NAs or intra-mPFC infusions of QUI or DOI on acute CLZ-induced avoidance disruption

In this experiment, we intended to determine the possible brain sites where CLZ acts to disrupt avoidance responding by centrally infusing a preferential dopamine $D_{2/3}$ receptor agonist (QUI) or serotonin 5-$HT_{2A/2C}$ receptor agonist (DOI) into the NAs or mPFC, two possible sites implicated in the action of CLZ (Atkins et al. 1999; Robertson et al. 1994; Young et al. 1999). After the CAR training, 46 rats were implanted with sterile guide cannulas bilaterally into the NAs or mPFC. After recovery, they were first given a predrug retraining session to ensure a high level of avoidance responding (>70 % avoidance response) before drug testing. Four groups of rats were tested: NAs-QUI (n=12), NAs-DOI (n=12), mPFC-QUI (n=11), and mPFC-DOI (n=11). On the first drug test day, rats were injected with CLZ 10 mg/kg. Thirty minutes later, they were centrally infused with one of four doses of QUI (0.0, 1.0, 5.0, and 10.0 μg/0.5 μl/side) or DOI (0.0, 1.0, 5.0, and 25.0 μg/0.5 μl/side). Rats were then placed in the CAR boxes and tested for avoidance (20 trials) at 45 and 95 min after the CLZ injection in an attempt to capture the time course of acute action of CLZ. Rats were returned to their home cages during the test interval. Rats remained in their home cages for the following day. On the third day, rats were given a retraining session (30 trials) to return their avoidance response to the predrug levels. One day later, the second round of drug testing under a different dose of QUI or DOI was initiated. This 3-day cycle (day 1: drug test, day 2: rest, day 3: retraining) was repeated four times until all four doses of QUI or DOI had been tested based on a Latin-square design, which provided a random sequence of administered doses in rats.

Experiment 2: Effects of intra-mPFC infusions of DOI during the induction phase on CLZ tolerance

Results from Experiment 1 indicated that the mPFC is one likely brain site where CLZ acts to disrupt avoidance responding. Given the preferential antagonist action of CLZ on 5-$HT_{2A/2C}$ over $D_{2/3}$, and the finding that $D_{2/3}$ activation by QUI potentiates rather than diminishes the acute and repeated effects of CLZ (Li et al. 2010), we hypothesized that 5-$HT_{2A/2C}$ receptor activation in the mPFC would decrease CLZ tolerance. In Experiment 2, we tested this hypothesis using a between-subjects design. After the CAR training, 48 rats were implanted with sterile guide cannulas bilaterally into the mPFC. After recovery, they were first given a predrug retraining session to ensure a high level of avoidance responding (>70 % avoidance response) before drug testing. Then, they were allocated to the following six groups (n=8/group) based on a complete factorial design [2 systemic injection (vehicle (VEH) and CLZ)×3 central injection (VEH, DOI 5, and DOI 25)]: VEH-SAL, VEH-DOI 5, VEH-DOI 25 and CLZ-SAL, CLZ-DOI 5, CLZ-DOI 25. All rats were first tested under CLZ or vehicle every other day for five sessions (30 CS-US trials/session). At the beginning of each test session, rats were first injected with CLZ (10 mg/kg, sc) or sterile water with 1.0 % glacial acetic acid (VEH), then centrally infused with SAL, DOI 5, or DOI 25 μg/0.5 μl/side into the mPFC 30 min later. Ten minutes later, they were placed in the avoidance boxes and tested for 30 trials. One day after the 5th CLZ test, all rats were retrained drug-free under the CS-only condition for 1 session and under the CS-US condition for another session the following day to bring their avoidance back to the predrug level before the final challenge test to assess the expression of CLZ tolerance (Feng et al. 2013; Li et al. 2010, 2012). On the challenge test, all rats were injected with CLZ 10 mg/kg (sc) and tested for avoidance performance in the CS-only condition (30 trials) 1 h later. No central injection was conducted.

Experiment 3: Effects of intra-mPFC infusions of DOI during the expression phase on CLZ tolerance

Experiment 2 showed that intra-mPFC infusion of DOI (25 μg/0.5 μl/side) during the tolerance induction phase completely abolished the expression of CLZ tolerance. Experiment 3 examined how activation of prefrontal 5-$HT_{2A/2C}$ receptor by DOI immediately prior to the challenge test would affect the expression of CLZ tolerance. After the CAR training, 25 rats that reached the training criterion were implanted with sterile guide cannulas bilaterally into the mPFC. After recovery, they were first given a predrug retraining session to ensure a high level of avoidance responding (>70 % avoidance response) before drug testing. Then, they were matched and assigned into three groups (n= 8-9/group): VEH-SAL, CLZ-SAL, and CLZ-DOI 25. They were first repeatedly tested for avoidance response under CLZ (10 mg/kg, sc, −45 min) or vehicle every other day for five sessions (30 CS-US trials/session). After 2 days of retraining following the last test session, all rats were challenged with CLZ (10 mg/kg, sc) and tested for avoidance response in the CS-only condition (30 trials) 1 h later. Fifteen minutes before the challenge test, rats in the CLZ-DOI 25 group were centrally infused with DOI 25 μg/0.5 μl/side into the mPFC, whereas rats in the other groups (the VEH-SAL and CLZ-SAL) were infused with saline.

Histology

At the end of behavioral tests, rats were sacrificed and perfused (Gao et al. 2013). Their brains were extracted and the injection sites were verified as previously reported (Gao et al. 2013). The location of the injection site was mapped onto a stereotaxic atlas (Paxinos and Watson 2004) (Fig. 1).

Statistical analysis

The avoidance data were expressed as the mean percent (i.e., number of avoidances/total number of trials)+SEM. Data from Experiment 1 were analyzed using two-way repeated-measures analysis of variance (ANOVA) followed by post hoc LSD pairwise tests and/or one-way repeated-measures ANOVA when needed. The two within-subjects factors were "test time" (three levels: predrug day, 45 min, and 95 min on the drug day) and "treatment" (QUI, or DOI). In Experiments 2 and 3, avoidance data from the five drug tests were analyzed with repeated-measures ANOVA followed by post hoc Tukey's test. The between-subjects factor were "CLZ" and "DOI" treatment (Experiment 2), or "DOI" treatment (Experiment 3), while within-subjects factor was "test day". Data from the challenge tests were analyzed with two-way ANOVA with the between-subjects factors being "CLZ" and "DOI" treatment (Experiment 2), or one-way ANOVA (Experiment 3), followed by post hoc Tukey's tests. Avoidance data on the predrug day in Experiments 2 and 3 were analyzed using one-way ANOVA. For all comparisons, significant difference was assumed at $p<0.05$, and all data were analyzed using SPSS version 21. Thirteen rats from all three experiments showed either sickness, or cannula failure, or misplacement, and they were excluded from data analysis. The final number of rats in different groups is indicated in the figure legends.

Results

Experiment 1: Effects of intra-NAs or intra-mPFC infusions of QUI or DOI on acute CLZ-induced avoidance disruption

Figure 2a, b shows the mean percentage of avoidance response on the predrug and CLZ test days from rats that received central infusions of QUI or DOI into the NAs. For QUI (Fig. 2a), two-way repeated-measures ANOVA showed a main effect of *test time* ($F(2, 16)=76.549$, $p<0.001$), but no main effect of QUI ($F(3, 24)=0.992$, $p=0.445$), nor their interaction ($F(6, 48)=0.600$, $p=0.729$). Post hoc pairwise tests for *test time* confirmed that acute CLZ treatment significantly suppressed avoidance response at 45 and 95 min on the drug test days (both $ps<0.001$), compared to the predrug day. However, intra-NAs infusion of QUI at all three doses (1, 5, 10 μg/0.5 μl/side) had no effect on the avoidance suppressive effect of CLZ. For DOI (Fig. 2b), the same analysis showed a main effect of *test time* ($F(2, 14)=185.846$, $p<0.001$), but no main effect of DOI ($F(3, 21)=0.992$, $p=0.445$), nor their interaction ($F(6, 42)=1.224$, $p=0.313$). Post hoc pairwise tests for *test time* confirmed that acute CLZ treatment significantly suppressed avoidance response at 45 and 95 min on the drug test days (both $ps<0.001$), compared to the predrug day. Again, intra-NAs infusion of DOI at all three doses (1, 5, 25 μg/0.5 μl/side) did not alter the avoidance suppressive effect of CLZ.

Figure 2c, d shows the mean percentage of avoidance response on the predrug and drug test days from rats that received central infusions of QUI or DOI into the mPFC. For QUI (Fig. 2c), two-way repeated-measures ANOVA showed a main effect of *test time* ($F(2, 20)=256.925$, $p<0.001$) and a main treatment effect of QUI ($F(3, 30)=3.058$, $p=0.043$), but no interaction between the two ($F(6, 60)=2.216$, $p=0.054$). Post hoc pairwise tests for *test time* confirmed that acute CLZ treatment significantly suppressed avoidance response at 45 and 95 min on the drug test days (both $ps<0.001$), compared to the predrug day. Intra-mPFC infusion of QUI dose-dependently potentiated the avoidance suppressive effect of CLZ, as post hoc pairwise tests for *treatment* revealed that CLZ-treated rats centrally infused with QUI at 10 μg/0.5 μl/side ($p=0.030$), but not

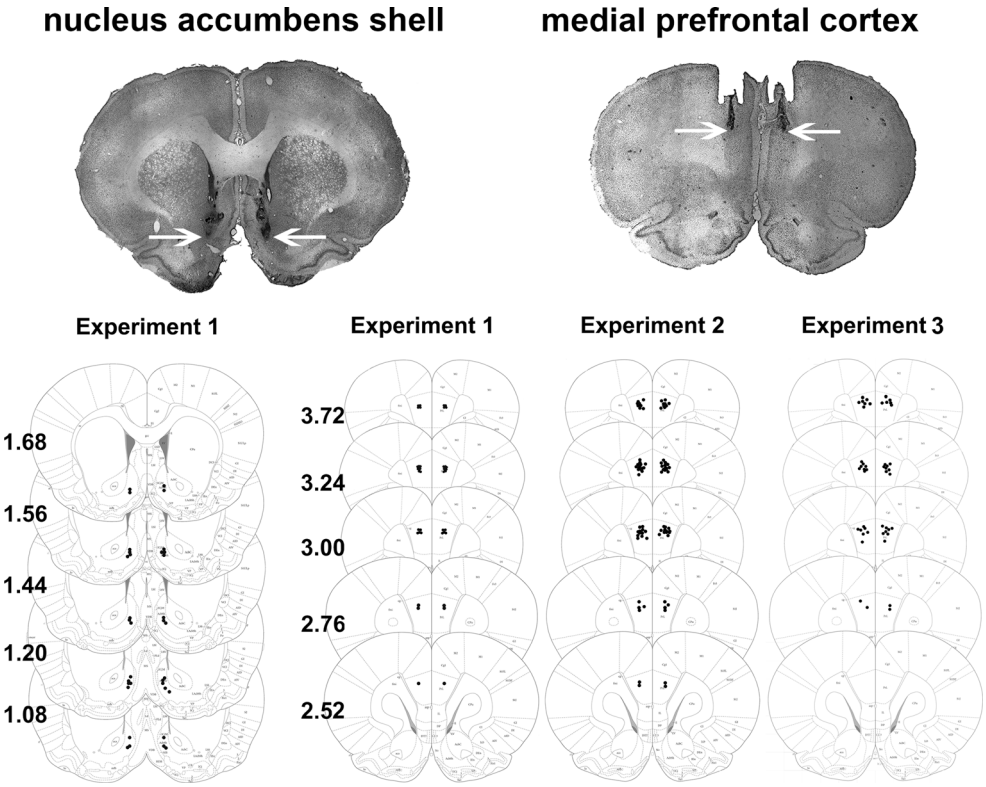

Fig. 1 Histological representations of infusion sites and schematic diagrams showing the location of the injector tips in the nucleus accumbens shell (*Experiment 1*) and medial prefrontal cortex (Experiments 1, 2, and 3). Data are reconstructed from Paxinos and Watson (2004). *Numbers to the left of the sections* indicate anteroposterior distance from bregma in millimeters. The *arrow* denotes the infusion placement

at 1 and 5 μg/0.5 μl/side (both *p*s>0.137) had significantly fewer avoidance responses than the vehicle controls. For DOI (Fig. 2d), two-way repeated-measures ANOVA showed a main effect of *test time* ($F(2, 16)=81.247$, $p<0.001$) and the main

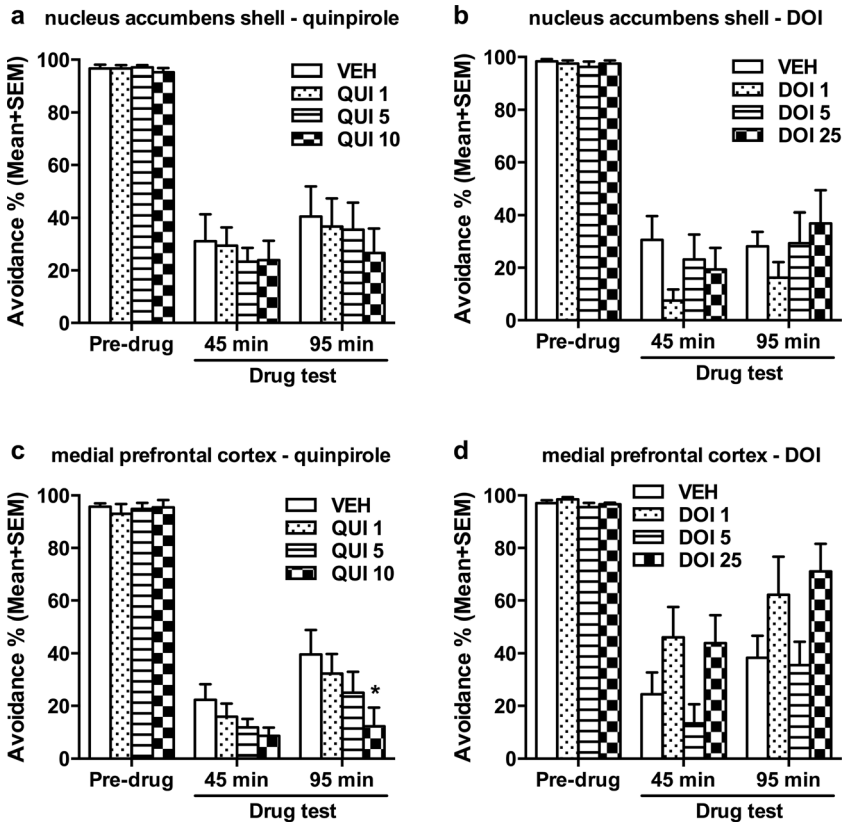

Fig. 2 Effects of microinjection of quinpirole (*QUI*) at 0, 1, 5, or 10 μg/side or DOI at 0, 1, 5, or 25 μg/side into the nucleus accumbens shell (NAs; **a**, **b**) or medial prefrontal cortex (mPFC; **c**, **d**) on the acute CLZ (10.0 mg/kg, sc)-induced avoidance disruption. $n=9$, 8 for NAs—QUI or DOI, and $n=11$, 9 for mPFC—QUI or DOI, respectively. Each data point represents the percentage avoidance response (Mean+SEM, number of avoidance responses divided by the total number of trials) made by rats during the predrug and the drug test days . Rats received a central infusion 30 min after systematic CLZ injection (10.0 mg/kg, sc), and were tested at 45 and 95 min after CLZ injection (10 and 60 min after the central infusion). *$p<0.05$ in comparison to the VEH group during each test

effect of DOI ($F(3, 24)=3.156$, $p=0.043$), and an interaction between the two ($F(6, 48)=2.340$, $p=0.046$). Post hoc pairwise tests for *test time* confirmed that acute CLZ treatment significantly suppressed avoidance response at 45 and 95 min on the drug test days (both $ps<0.001$), compared to the predrug day. The main DOI effect was due to the differences between DOI 5 and DOI 1 ($p=0.033$) and between DOI 5 and DOI 25 ($p=0.010$). Because of the significant interaction between DOI and test time, one-way ANOVA was used to analyze the group differences at each test time point. There was no significant group difference on the predrug day ($F(3, 24)=1.926$, $p=0.152$). However, there was a marginal significant effect of DOI dose at 45 min ($F(3, 24)=2.760$, $p=0.064$) and 95 min ($F(3, 24)=2.904$, $p=0.056$). These findings suggest that acute effect of CLZ can be potentiated by activation of $D_{2/3}$ receptors in the mPFC.

QUI and DOI microinjected into the NAs or mPFC did not affect the CLZ's suppression of intertrial crossing (Fig. 3), nor escape responding (see Fig. SI for details). Two-way repeated-measures ANOVA followed by post hoc tests revealed that the number of intertrial crossing decreased significantly at 45 and 95 min (all $p<0.001$) on the drug days than the predrug day, but infusion of QUI or DOI into NAs or mPFC had no effect. The number of escape responses increased significantly at 45 and 95 min on the drug day than the predrug day (all $p<0.001$). These data suggest that the $D_{2/3}$ receptors and 5-$HT_{2A/2C}$ in the mPFC had no effect on CLZ's effect on the intertrial crossing and escape responses in this experimental setup.

Experiment 2: Effects of intra-mPFC infusions of DOI during the induction phase on CLZ tolerance

Figure 4a shows the mean percentage of avoidance response on the predrug day and throughout the five drug test days. All groups had a high level of avoidance responding on the predrug day (one-way ANOVA: $F(5, 38)=0.142$, $p=0.981$). Throughout the five drug test days, systemic CLZ treatment significantly decreased avoidance response, but this avoidance suppressive effect was gradually attenuated over time, a clear sign of CLZ tolerance development. Intra-mPFC DOI infusion did not affect the acute effect of CLZ nor the tolerance development. Repeated-measures ANOVA (i.e., "CLZ and DOI treatments" as two between-subject factors and "test day" as a repeated within-subjects factor) revealed a main effect of CLZ treatment ($F(1, 38)=184.674$, $p<0.001$), test day ($F(4, 152)=18.538$, $p<0.001$), and a significant interaction between the two ($F(4, 152)=10.868$, $p<0.001$), but there was no main effect of DOI ($F(2, 38)=1.058$, $p=0.357$), no DOI×CLZ interaction ($F(2, 38)=0.735$, $p=0.486$), nor test day×DOI×CLZ interaction ($F(2, 38)=2.931$, $p=0.065$). CLZ treatment also decreased the intertrial crossing through the five drug test days. Repeated-measures ANOVA revealed a main effect of CLZ treatment ($F(1, 38)=79.63$, $p<0.001$),

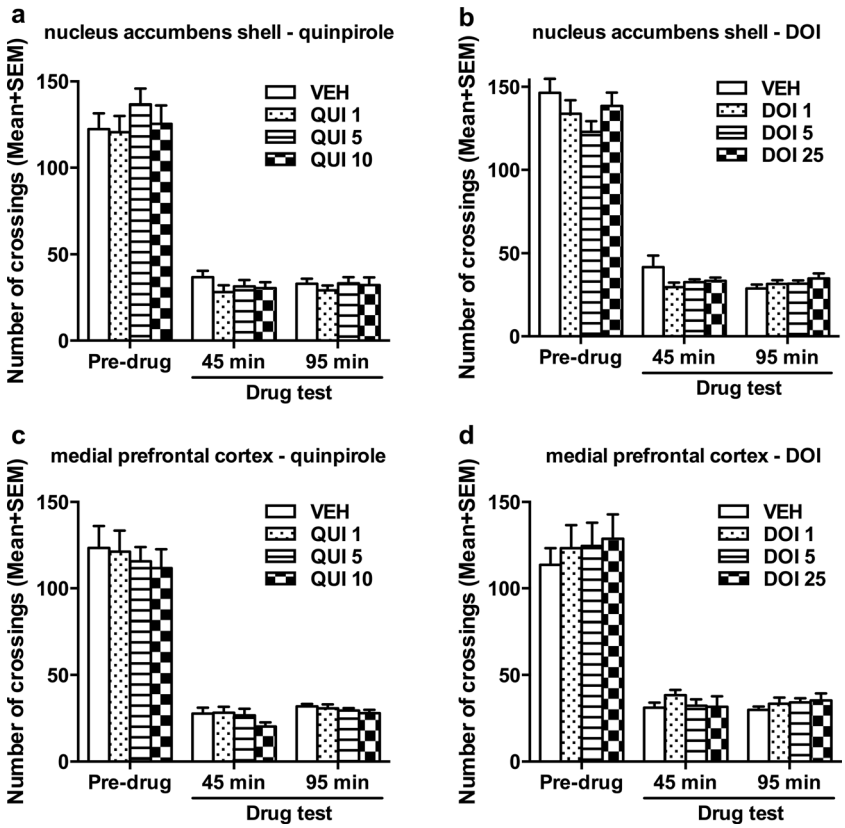

Fig. 3 Number of intertrial crossings made by rats on the predrug and five drug test days. On each drug test day, rats received a central infusion of quinpirole (QUI) at 0, 1, 5, or 10 μg/side or DOI at 0, 1, 5, or 25 μg/side into the nucleus accumbens shell (NAs; **a**, **b**) or medial prefrontal cortex (mPFC; **c**, **d**) 30 min after systematic CLZ injection (10.0 mg/kg, sc), and were tested at 45 and 95 min after CLZ injection (10 and 60 min after the central infusion)

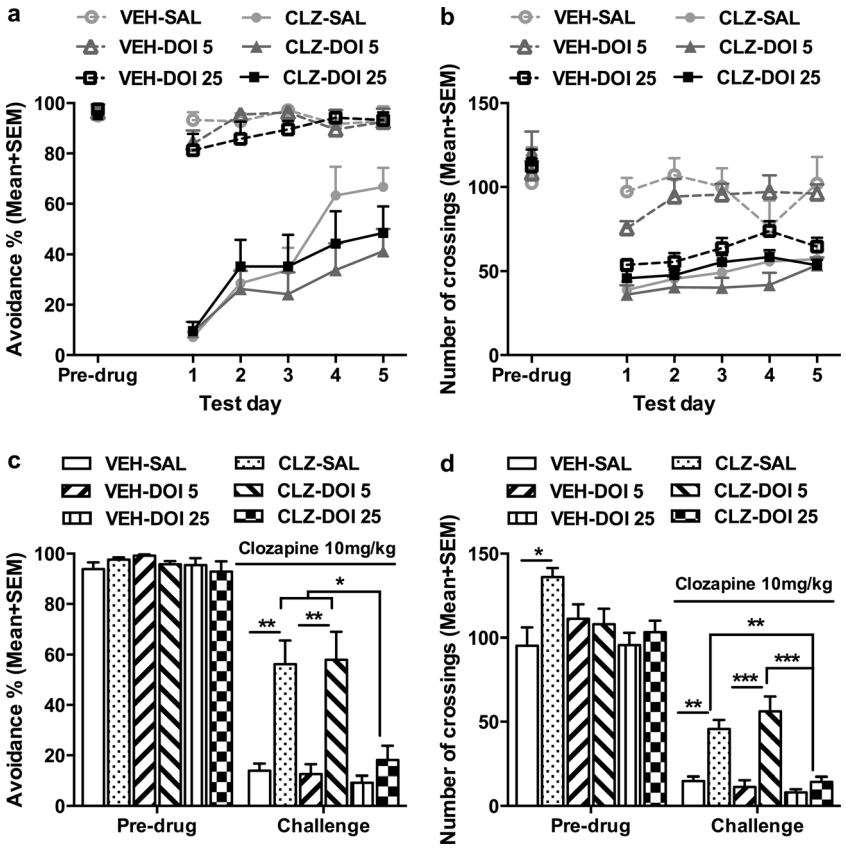

Fig. 4 Effects of microinjection of DOI into the medial prefrontal cortex during the induction phase of CLZ tolerance. The data represent the percentage avoidance response and number of intertrial crossings (Mean+SEM) made by rats during the predrug and five drug test days (**a**, **b**), and on the retraining day and the challenge test day (**c**, **d**). Rats received either systemic vehicle or CLZ (10.0 mg/kg, sc) treatment in combination with central DOI treatment (0, 5 or 25 μg/side; n=6, 8, 8 for VEH; n=7, 8, 7 for CLZ) for five test days and were challenged with CLZ (10.0 mg/kg) after two drug-free retraining days. ***p<0.001, **p<0.01 in comparison to the corresponding group (**c**, **d**)

DOI (F(2, 38)=5.102, p=0.011), test day (F(4, 152)=4.209, p=0.003), and a significant interaction of CLZ×DOI (F(2, 38)=10.473, p<0.001) (Fig. 4b). Inspection of Fig. 4b reveals that DOI at 25 μg suppressed the intertrial crossing in a similar manner as did CLZ, suggesting that either activation or suppression of cortical 5-$HT_{2A/2C}$ receptors could decrease motor or motivational suppressing effects of CLZ.

Figure 4c shows the mean percentage of avoidance response on the predrug day (F(5, 38)=1.006, p=0.428) and on the CLZ challenge day. Two-way ANOVA of the avoidance performance on the challenge day revealed a significant effect of CLZ treatment (F(1, 38)=31.796, p<0.001), DOI treatment (F(2, 38)=6.412, p=0.004), and a significant interaction between the two (F(2, 38)=4.295, p=0.021). Subsequent one-way ANOVA followed by post hoc Tukey's tests revealed that the CLZ-SAL group and CLZ-DOI 5 group made significantly more avoidance responses than the corresponding VEH groups (both ps<0.001), confirming the CLZ tolerance effect. More importantly, this tolerance effect was absent in the CLZ-DOI 25 group, as it did not differ significantly from the VEH-DOI 25 (p=0.366), suggesting that intra-mPFC infusion of DOI at 25.0 μg/0.5 μl/side during the induction period of CLZ tolerance completely abolished CLZ tolerance, despite the fact that DOI at this dose had no effect on the day-to-day avoidance disruptive effect of CLZ.

On the intertrial crossing, two-way ANOVA revealed a main effect of CLZ treatment (F(1, 38)=42.013, p<0.001), DOI treatment (F(2, 38)=11.451, p<0.001), and a significant interaction between the two (F(2, 38)=7.552, p=0.002) on the CLZ challenge day. Subsequent one-way ANOVA followed by post hoc tests revealed that the CLZ-SAL group (p=0.004) and CLZ-DOI 5 group (p=0.001), but not the CLZ-DOI 25 group (p=0.951) made significantly more crossings than the corresponding VEH groups, indicating a tolerance effect of CLZ on intertrial crossing which was abolished by DOI (at 25 μg/0.5 μl/side) (Fig. 4d). Throughout the 5 days of drug treatment, CLZ-treated groups had higher levels of escapes. However, with repeated treatment and the increase of avoidance responses, it was gradually reduced. Intra-mPFC infusion of DOI at all three doses had no effect on this behavior (see Fig. SII for details).

Experiment 3: Effects of intra-mPFC infusions of DOI during the expression phase on CLZ tolerance

Figure 5a shows the mean percentage of avoidance response on the predrug day and during the five drug test days. We replicated the acute avoidance disruptive effect of CLZ and the developmental process of CLZ tolerance, as confirmed by the repeated-measures ANOVA showing a main effect of CLZ treatment (F(2, 21)=9.091, p=0.001), test day (F(4, 84)=

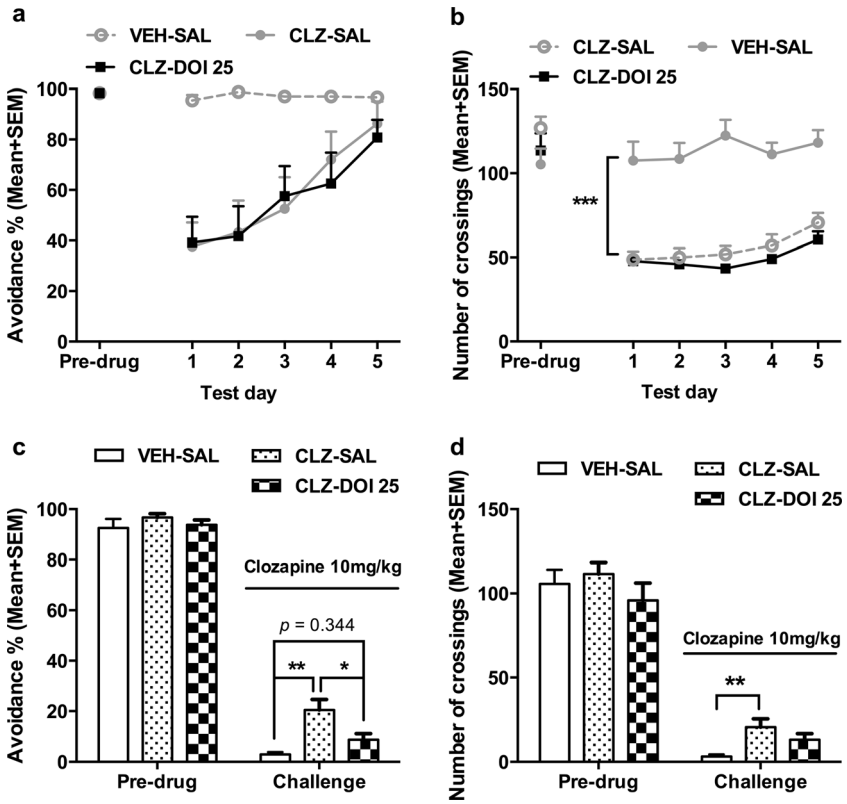

Fig. 5 Effects of microinjection of DOI into the medial prefrontal cortex on the challenge day on the expression of clozapine tolerance. The data represent the percentage avoidance response and number of intertrial crossings (Mean+SEM) made by rats during the predrug and five drug test days (**a**, **b**), and on the retraining day and the challenge test day (**c**, **d**), respectively. Rats received systemic vehicle or CLZ (10.0 mg/kg, sc) injection for five test days and were challenged with CLZ (10.0 mg/kg) after two drug-free retraining days. On the challenge test day, rats received a central infusion of saline (VEH-SAL and CLZ-SAL, n=8/group) or DOI 25 μg/side (CLZ-DOI 25, n=8) 15 min before the test. **p<0.01, *p<0.05 in comparison to the corresponding group (**c**, **d**)

15.438, p<0.001), and a significant interaction between the two (F(8, 84)=4.293, p<0.001). Correspondingly, the escape responses were higher in the CLZ-treated groups and gradually decreased with the increase of avoidance responses (see Fig. SIII for details).

Figure 5c shows the mean percentage of avoidance response on the predrug day (F(2, 21)=0.703, p=0.507) and the CLZ challenge day (F(2, 21)=9.556, p=0.001). The CLZ-SAL group had significantly higher avoidance than the VEH-SAL group (p=0.001), confirming the CLZ tolerance effect. More interestingly, the CLZ-DOI 25 group did not differ from the VEH-SAL group (p=0.344), but had significantly lower avoidance than the CLZ-SAL group (p=0.024), suggesting the intra-mPFC infusion of DOI at 25.0 μg/0.5 μl/side prior to the challenge test blocked the expression of CLZ tolerance.

CLZ treatment decreased the number of intertrial crossings through the five drug test days (Fig. 5b). Repeated-measures ANOVA revealed a main effect of CLZ treatment (F(2, 21)=48.273; p<0.001), test day (F(4, 84)=6.077; p<0.001), but no significant interaction between the two (F(8, 84)=1.402; p=0.208). Post hoc Tukey's test confirmed that the VEH-SAL group made significant more crossings than the CLZ-DOI 25 and CLZ-SAL (both ps<0.001). On the CLZ challenge day, the CLZ effect was still significant (F(2, 21)=5.871, p=0.009), with the CLZ-SAL group making more crossings than the VEH-SAL group (p=0.007), confirming the CLZ tolerance effect on intertrial crossing. Interestingly, DOI did not significantly block this tolerance (p=0.328) (Fig. 5d).

Discussion

Using a pharmacological and microinjection approach, we first demonstrated that the acute avoidance disruptive effect of CLZ could be enhanced by activating the $D_{2/3}$ receptors in the mPFC and reduced to some extent by activating the prefrontal 5-$HT_{2A/2C}$ receptors. A more important finding is that the expression of CLZ tolerance relies on the neuroplasticity initiated by its antagonist action against 5-$HT_{2A/2C}$ receptors in the mPFC. Specifically, in Experiment 1, we showed that intra-mPFC infusion of QUI dose-dependently potentiated the avoidance suppressive effect of CLZ, as CLZ-treated rats centrally infused with QUI at 10 μg/0.5 μl/side but not at 1 and 5 μg/0.5 μl/side into the mPFC made significantly fewer avoidance responses than the CLZ-treated ones but centrally infused with vehicle. Intra-mPFC infusion of DOI appeared to have an opposite effect as did QUI. It marginally reduced the avoidance suppressive effect of CLZ in this within-subjects design. Both drugs infused into the NAs did not affect this effect of CLZ, thus, whether the NAs is involved in the behavioral effects of CLZ is equivocal and need to be further

investigated. Regarding the effects of repeated CLZ administrations, we showed that microinjection of DOI (a preferential 5-$HT_{2A/2C}$ agonist) into the mPFC completely abolished the tolerance expression when it was infused during the induction phase (Experiment 2), and reduced it when it was infused right before the challenge test (Experiment 3). The same central treatment did not affect the acute avoidance suppressive effect of CLZ and the tolerance development. These findings imply that the receptor mechanisms (e.g., primarily $D_{2/3}$ and 5-$HT_{2A/2C}$ receptors in the mPFC) that regulate the acute effect of CLZ are likely different from those (primarily 5-$HT_{2A/2C}$ receptors in the mPFC) that modulate CLZ tolerance.

Our previous studies suggest that 5-HT_{2A} receptors appear to be critical for CLZ's acute disruptive effect on avoidance responding (Li et al. 2010, 2012). In both studies, we found that systemic pretreatment of DOI attenuated acute CLZ-induced disruption of avoidance responding, possibly by activating 5-HT_{2A} receptor in the prefrontal cortex (McOmish et al. 2012). Others also reported that systemic DOI at 10 mg/kg reversed the avoidance disruptive effect of 10 mg/kg CLZ (Browning et al. 2005), and its disruption of maternal behavior (Zhao and Li 2009, 2010), suggesting the reversal effect of DOI on CLZ is quite robust and is a generalized effect. It is thus surprising that central infusion of DOI into the mPFC had only a marginal reversal effect on the acute effect of CLZ. It could mean that our selected doses of DOI or the drug test conditions were not optimal. Since DOI does not differentiate between 5-HT_{2A} and 5-HT_{2C} receptor subtypes, and 5-HT_{2A} and 5- HT_{2C} receptors play *opposing roles* in various brain functions and psychological processes (Di Giovanni et al. 2000; Di Matteo et al. 2002; Winstanley et al. 2004), including in the CAR (Grauer et al. 2009; Wadenberg and Hicks 1999), it is conceivable that the chosen doses of DOI might have activated both receptors, resulting in a lowered reversal effect of DOI. Alternatively, it could mean that 5-$HT_{2A/2C}$ receptor in other brain regions (e.g., lateral septum, hippocampus, or ventral tegmental area) may play a more important role in the regulation of CLZ's acute effect (Ichikawa et al. 2001b). Finally, because CLZ also has high affinity for adrenergic α_1 receptor, muscarinic M_1 receptor and histamine H_1 receptor and moderate affinity for the D_4 and 5-HT_6 receptors, its actions on these receptors may also contribute to its acute avoidance disruptive effect.

Another surprising finding is that microinjection of QUI in the mPFC actually enhanced the acute effect of CLZ, an effect opposite to that of DOI. Our previous systemic studies failed to find such an effect in the CAR (Li et al. 2010) and in maternal behavior (Zhao and Li 2009; 2010). The exact reason for this discrepancy is not clear. One possibility is that QUI might have different effects when it reaches different brain areas. Because systemic QUI administration presumably impacts a broader range of brain areas than central infusion, some of its effects might have canceled each other out, yielding no obvious net effect on CLZ when it is administrated systemically. As to why QUI and DOI produced opposite effects on the acute effect of CLZ, we speculate that activation of $D_{2/3}$ receptors in the mPFC by QUI may cause a decrease in 5-$HT_{2A/2C}$ receptor-mediated neurotransmission, which could lead to a potentiation of the acute disruption of avoidance of CLZ, as demonstrated in several studies involving other antipsychotic drugs (Wadenberg et al. 1998, 2001a). Furthermore, both receptors are colocalized in the mPFC and they form hetero-5-HT_{2A}/D_2 dimers and homo-5-HT_{2A}/5-HT_{2A} dimers (Lukasiewicz et al. 2010), providing a physical basis for their interactions. Therefore, the acute behavioral effect of CLZ is likely mediated by its action on 5-$HT_{2A/2C}$ receptors superimposed by its action on $D_{2/3}$ receptors.

Consistent with our previous studies, repeated administration of CLZ caused a behavioral tolerance in the CAR test, an effect opposite to behavioral sensitization typically associated with many other antipsychotic drugs. As previously mentioned, we and others have repeatedly demonstrated CLZ tolerance in the CAR test (Feng et al. 2013; Li et al. 2010, 2012; Qiao et al. 2013; Qin et al. 2013; Sanger 1985) and in the PCP-induced hyperlocomotion test (Shu et al. 2014). CLZ tolerance has also been reported in other behavioral domains. For example, in a motor function and attention test, Stanford and Fowler (1997) reported that CLZ-treated rats exhibited tolerance to the drug's suppressive effect on the amount of time that rats were in contact with a force-sensing target disk. In a fixed ratio 5 lever-pressing test, Trevitt et al. (1998) found that acute CLZ treatment significantly suppressed lever pressing but this effect was attenuated with repeated drug administration. Similarly, Varvel et al. (2002) and Villanueva and Porter (1993) also found that repeated dosing with CLZ produced tolerance to the rate-suppressing effects of CLZ in a lever pressing task for food reward. CLZ-induced tolerance has also been observed in a drug discrimination task (Goudie et al. 2007a, b). Future studies could examine how the interoceptive drug state contributes to CLZ tolerance and what other behavioral mechanisms (e.g., associative learning and contextual control) are involved.

The major contribution of the present study is its identification of the neuroanatomical basis and receptor mechanisms of CLZ tolerance. Previously, we showed that CLZ tolerance development may be mediated by its $D_{2/3}$ blockade-initiated neural processes, as pretreatment of QUI during the 3-day repeated avoidance test period enhanced the expression of CLZ tolerance in the challenge test (Li et al. 2010). We are currently examining the possible brain regions where QUI may act to modulate CLZ tolerance. In light of the present findings, we will focus on the mPFC as it is one likely target.

In the present study, we revealed that although intra-mPFC infusion of DOI did not affect the acute avoidance disruptive effect of CLZ as well as the tolerance development, it did

dose-dependently suppress the expression of CLZ tolerance. This finding highlights the importance of prefrontal 5-$HT_{2A/2C}$ receptors in CLZ tolerance and is also consistent with a recent finding showing that 5-HT_{2A} receptors located on forebrain glutamatergic neurons are critical for the motor-suppressive effect of CLZ (McOmish et al. 2012). In that study, McOmish et al. (2012) found that 5-HT_{2A} KO mice lack the locomotor-suppressing response to acute CLZ, suggesting that the drug's motor suppression effect (in wild-type animals) is normally mediated by a blockade of 5-HT_{2A} receptors in the prefrontal cortex. This conclusion is strengthened by the observation that restoring 5-HT_{2A} expression in forebrain glutamatergic neurons was sufficient to restore the locomotor-suppressing effect of CLZ. In light of these observations, we can conclude that CLZ's acute suppression of avoidance responding and intertrial crossing is mediated by its antagonistic action on 5-HT_{2A} receptor on forebrain glutamatergic neurons. Thus, by countering the antagonistic action of CLZ on $5HT_{2A}$ receptors, intra-mPFC infusions of DOI might block the tolerance to CLZ. Because of the known functional interaction between 5-HT_{2A} receptor and D_2 receptor in the mPFC (Ichikawa et al. 2001b), these two receptor mechanisms may diametrically regulate the development/expression of CLZ tolerance. Ichikawa et al. (2001a, b) showed that DOI treatment attenuates acute CLZ-induced cortical dopamine release and combined blockade of 5-HT_{2A} and D_2 receptors produces a greater increase in dopamine release than that by each alone. It is thus conceivable that DOI treatment in the mPFC would reduce D_2-mediated neurotransmission, possibly via the mPFC to ventral tegmental area pathway (Vazquez-Borsetti et al. 2009) and/or NA pathway, leading to a reduction in CLZ tolerance. This would easily explain why QUI pretreatment potentiates CLZ tolerance (Li et al. 2010), as QUI stimulates $D_{2/3}$ receptor and increases associated dopamine neurotransmission. Based on the available evidence, we would propose the following hypothesis regarding the neuroreceptor mechanisms of CLZ tolerance: CLZ tolerance is a form of neuroplasticity resulting from CLZ's dual action on 5-$HT_{2A/2c}$ and $D_{2/3}$ receptors with a *yin–yang-like* relation: the magnitude of CLZ tolerance can be increased via stimulating $D_{2/3}$ receptor (e.g., by QUI) and decreased via stimulating 5-$HT_{2A/2c}$ receptor in the mPFC (e.g., by DOI). This hypothesis is supported by the evidence that repeated CLZ treatment causes an upregulation of D_2 receptor level (Moran-Gates et al. 2006; Tarazi et al. 1998) and a downregulation of 5-HT_{2A} receptor in the mPFC (Steward et al. 2004).

Finally, we want to comment on the finding that the absolute magnitude of CLZ tolerance appears to be weaker in Experiment 3 than in Experiment 2 (Fig. 4c, d versus Fig. 5c, d). There are several procedural differences that may contribute to this difference. In Experiment 2, rats were centrally infused with SAL, DOI 5, or DOI 25 μg/0.5 μl/side into the mPFC before each CLZ test, whereas in Experiment 3, no such central infusion was done. In Experiment 2, rats were not centrally injected with SAL or DOI before the challenge test, whereas in Experiment 3, they were. In Experiment 2, DOI was administered five times, whereas in Experiment 3, it was only administered once. Also, Experiment 2 was run by a female experimenter, and Experiment 3 was run by a male, which could affect rats' behavior differently, as shown by a recent study (Sorge et al. 2014). All these differences could potentially contribute to the magnitude difference between these two experiments.

Taken together, we demonstrated that acute behavioral effect of CLZ is likely mediated by its actions on the $D_{2/3}$ and 5-$HT_{2A/2C}$ receptors (to a lesser extent) in the mPFC, whereas its long-term tolerance effect might rely on the neuroplasticity initiated by its antagonist action against 5-$HT_{2A/2C}$ receptors in the mPFC. Because CLZ also has multiple actions on other receptors (e.g., 5-HT_{1A}, D_1, D_3, H_1, M_1, α_1-noradrenergic, etc.), of which some have been implicated in CLZ tolerance, future studies should expand this line of research by systematically investigating the roles of these receptors in the mediation of CLZ tolerance.

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Conflicts of interest The authors declare no conflict financial interests.

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