

RESEARCH ARTICLE

3'-Deoxyadenosine (Cordycepin) Produces a Rapid and Robust Antidepressant Effect via Enhancing Prefrontal AMPA Receptor Signaling Pathway

Bai Li, MSc^{*}; Yangyang Hou, BSc^{*}; Ming Zhu, PhD^{*}; Hongkun Bao, MSc; Jun Nie, BSc; Grace Y. Zhang, BSc; Liping Shan, MSc; Yao Yao, BSc; Kai Du, MSc; Hongju Yang, PhD; Meizhang Li, PhD; Bingrong Zheng, PhD; Xiufeng Xu, MD, Chunjie Xiao, PhD[#]; and Jing Du, MD, PhD[#]

School of Medicine, Yunnan University, Kunming, Yunnan, China (Mr B Li, Bao, MS Hou, Nie, and Dr Zhu, Yang, Zheng, Xiao, and J Du); Beijing Gragen Biotechnology Co. Ltd., Beijing, China (Ms Zhang, Shan, and Yao); Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China (Mr K Du); Department of Biochemistry & Molecular Biology, School of Life Sciences, Yunnan University, Kunming, Yunnan, China (Dr M Li); Department of Psychiatry, The First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, China (Dr Xu).

^{*}These authors contributed equally to this work.

[#]These two authors are co-corresponding authors.

Correspondence: Jing Du, MD, PhD, Professor, Yunnan University, School of Medicine, 2 Cuihu North Road, Kunming, Yunnan, P. R. China, 650091 (dujing@ynu.edu.cn).

Abstract

Background: The development of rapid and safe antidepressants for the treatment of major depression is in urgent demand. Converging evidence suggests that glutamatergic signaling seems to play important roles in the pathophysiology of depression.

Methods: We studied the antidepressant effects of 3'-deoxyadenosine (3'-dA, Cordycepin) and the critical role of the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor in male CD-1 mice via behavioral and biochemical experiments. After 3'-dA treatment, the phosphorylation and synaptic localization of the AMPA receptors GluR1 and GluR2 were determined in the prefrontal cortex (PFC) and hippocampus (HIP). The traditional antidepressant imipramine was applied as a positive control.

Results: We found that an injection of 3'-dA led to a rapid and robust antidepressant effect, which was significantly faster and stronger than imipramine, after 45 min in tail suspension and forced swim tests. This antidepressant effect remained after 5 days of treatment with 3'-dA. Unlike the psycho-stimulants, 3'-dA did not show a hyperactive effect in the open field test. After 45 min or 5 days of treatment, 3'-dA enhanced GluR1 S845 phosphorylation in both the PFC and HIP. In addition, after 45 min of treatment, 3'-dA significantly up-regulated GluR1 S845 phosphorylation and GluR1, but not GluR2 levels, at the synapses in the PFC. After 5 days of treatment, 3'-dA significantly enhanced GluR1 S845 phosphorylation and GluR1, but not GluR2, at the synapses in the PFC and HIP. Moreover, the AMPA-specific antagonist GYKI 52466 was able to block the rapid antidepressant effects of 3'-dA.

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Conclusion: This study identified 3'-dA as a novel rapid antidepressant with clinical potential and multiple beneficial mechanisms, particularly in regulating the prefrontal AMPA receptor signaling pathway.

Keywords: 3'-deoxyadenosine, synapse, animal behavior, GluR1, rapid antidepressant

Introduction

Due to the millions of individuals who suffer from major depression today, and the delayed efficacy of the existing therapeutic agents, there exists an interest in finding a safer compound with robust and rapid antidepressant effects (Krystal et al., 2013; Caddy et al., 2014). The most famous rapid antidepressant, Ketamine, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, was found to rely on increasing α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) signaling to exert its antidepressant efficacy (Maeng et al., 2008). A previous study has shown that a traditional Chinese medicine, Cordyceps Militaris powder, demonstrated an antidepressant effect in the tail suspension test (TST; Nishizawa et al., 2007). Moreover, 3'-deoxyadenosine (3'-dA, Cordycepin), one of the major bioactive metabolites in the Cordyceps Militaris (Tuli et al., 2013), exerted a beneficial effect in a 6-week chronic unpredictable mild stress (CUMS) mouse model via the forced swim test (FST; Tianzhu et al., 2014). 3'-dA possesses a wide range of beneficial effects of therapeutic potential, including anti-insomnia, anti-inflammatory, anti-cancer, anti-viral, and anti-fungal activities (Hu et al., 2013; Tuli et al., 2013). However, whether or not 3'-dA has a fast-acting antidepressant effect remains unknown.

Glutamate mediates the vast majority of excitatory transmissions and about 80% of neurons form 85% of the synapses, which are spiny and excitatory in the central nervous system (CNS; Douglas and Martin, 2007; Sanacora et al., 2012). A growing body of evidence suggests that the glutamatergic system might be very important in the pathophysiology and treatment of depression (Krystal et al., 2002; Sanacora et al., 2012; Musazzi et al., 2013; Pilc et al., 2013). The AMPA receptor channel is a tetramer assembled from the four subunits, including GluR1, GluR2, GluR3, and GluR4. Clinical evidence has shown that the expression of AMPA receptors, particularly GluR1, is decreased in the brains of patients with depression (Beneyto et al., 2007; Duric et al., 2013). Additionally, the trafficking of AMPA receptor subunit GluR1 and synaptic plasticity are regulated by the phosphorylation of specific subunits (Malinow and Malenka, 2002; Esteban et al., 2003). Phosphorylation of GluR1 at serine 845 (S845) was increased following antidepressant treatment with fluoxetine or tianeptine in mice (Svenningsson et al., 2002, 2007), suggesting that phosphorylation of GluR1 might be associated with the potentiation of AMPA receptor currents (Wang et al., 2005). Previous studies have shown that phosphorylation of GluR1 at S845 and GluR1 synaptic localization might be common mechanisms for the agents with antidepressant efficacy, such as imipramine, lamotrigine, and riluzol (Du et al., 2007; Gould et al., 2008). However, whether 3'-dA regulates AMPA synaptic plasticity is still unknown.

Based on these premises, we designed a series of behavioral and biochemical experiments to investigate the antidepressant effects of 3'-dA in animal models of depression. We studied the rapid and chronic effects of various concentrations of 3'-dA via behavioral tests, including the TST, FST, and open field test (OFT). The phosphorylation of GluR1 S845 and synaptic localization of GluR1 and GluR2 were determined in the prefrontal cortex (PFC) and hippocampus (HIP) after 45-min and 5-day treatments. In addition, the role of the enhanced AMPA function in the rapid antidepressant effect was addressed by pretreatment with AMPA receptor-specific antagonist GYKI 52466 followed by TST.

Materials and Methods

Animal Behavioral Studies

All animal treatments, procedures, and care were approved by the medical ethics committee of the School of Medicine, Yunnan University, and followed the *Guide for the Care and Use of Laboratory Animals* (ISBN 0-309-05377-3). Male CD-1 mice (6 weeks; starting weight, 23–28 g; Vital River) were group housed ($n = 4/\text{cage}$) in an animal room with a constant temperature ($21 \pm 3^\circ\text{C}$) and maintained on a 12-hour light/dark cycle (lights on/off at 09:00 and 21:00 hours), with constant humidity ($55 \pm 10\%$) and free access to water and food. After a 1-week acclimatization period, the mice were treated with drugs or vehicle in a volume of $10 \mu\text{l/g}$ by intraperitoneal (i.p.) injection and tested between 10:00 and 14:00 hours.

To examine the rapid (45-min treatment) and chronic (5-day treatment) antidepressant effects of 3'-dA (Sigma), mice were randomly assigned to four treatment groups: saline (0.9% sterile sodium chloride solution), low dose of 3'-dA (5 mg/kg, dissolved in saline), high dose of 3'-dA (12.5 mg/kg, in saline), and imipramine (15 mg/kg, in saline; Sigma). Animal behavioral tests were performed 45 minutes after drug or vehicle administration, TST was performed on the first day, OFT on the third day, and FST on the fifth day. To confirm the rapid and chronic antidepressant effects of 3'-dA, another two batches of mice were subjected to the FST and TST, respectively, under similar settings.

To examine whether the rapid antidepressant effect of 3'-dA could be blocked by GYKI 52466 (a selective non-competitive AMPA receptor antagonist; TOCRIS Bioscience, R&D), mice were treated with GYKI 52466 (30 mg/kg in 26% DMSO/74% saline), followed by a high dose of 3'-dA treatment. Since a previous study showed that plasma levels of GYKI 52466 peaked at 15 minutes after i.p. injection and then fell to 21% of the peak value at 60 minutes (De Sarro et al., 1998), we administered GYKI 15 minutes before 3'-dA treatment and performed the TST 45 minutes later. For each drug treatment, the control mice received the respective vehicle alone.

Tail Suspension Test

A 7 cm long and 2 cm wide tape was positioned with approximately 2 mm of tail protruding. In a series of pilot experiments using this procedure, we observed that no mice climbed their tails. Each mouse was individually suspended by the tail from a bar (30 cm high) and videotaped during a 6-min test session. Immobility time was quantified by a naive observer for the last 4 minutes.

Open Field Test

An activity chamber (60 × 60 × 30 cm) with a black floor divided into 16 squares of an equal area (15 × 15 cm) by white lines was used to study 3'-dA-induced hyperactivity. After 3 days of i.p. injection of drugs, mice were placed in the center of the chamber and their behavior was recorded for 60 minutes. Total distance traveled and the amount of distance traveled in the center area (the 4-square area in the middle of the chamber) were analyzed by Anymaze system (Stoelting).

Forced Swim Test

Mice were placed in a cylinder ($\Phi = 20 \text{ cm}$) with water 20 cm in depth (temperature between $23 \pm 1^\circ\text{C}$). Mice were videotaped

during a 6-min test session, which was later analyzed by a naive observer for activity during the final 4 minutes. Mobility was defined as any movement beyond what was necessary to maintain the head above water.

After the behavioral experiments, all mice were decapitated immediately. The PFC and HIP were dissected, frozen rapidly in liquid nitrogen, and stored at -80°C until further analysis.

Western Blot Analysis

PFC or HIP tissue was lysed with an ice-cold radioimmuno-precipitation assay buffer (20mM Tris [pH 7.5], 150mM NaCl, 1% Triton X-100, sodium pyrophosphate, β -glycerophosphate, EDTA, Na_3VO_4 , leupeptin plus a Protease Inhibitor Cocktail Tablet [Roche], and Phosphatase Inhibitor Cocktail Tablet [Roche]) in a tissue grinder (Wheaton). Protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology). Equal amounts of proteins were subjected to 7–10% SDS-PAGE gels electrophoresis, then transferred to polyvinylidene difluoride (PVDF) membranes (Pall) and blocked with 1% BSA in TBST (0.1% Tween). Antibodies against Phos-GluR1 S845 (Rabbit mAb, 1:1000, Cell Signaling Technology) and GluR1 (Goat pAb, 1:1000, Santa Cruz) in 5% BSA-TBST were applied to the membranes. The secondary antibodies were horseradish peroxidase conjugated goat anti-rabbit (1:5000, Affinity Bioscience) or donkey anti-goat antibodies (1:5000, Santa Cruz). Antibody for β -actin (Mouse mAb, 0.2 $\mu\text{g}/\text{ml}$, Affinity Bioscience) was applied for loading calibration. Immunoreactive bands were visualized using the ECL detection system (Millipore). Images were acquired by the FlourChem E image system (FE0511, ProteinSimple) and quantified by the Image-Pro Plus Version 6.0 software (Media Cybernetics).

Synaptosomes Preparation

Synaptosomal fractions were prepared from PFC or HIP tissue using the differential and discontinuous Ficoll gradient centrifugation method (Pozzo-Miller et al., 1999; Du et al., 2004a, 2008). Tissue was homogenized by tissue grinder and then electronic polytron homogenizer (AutoScience) in cold Syn buffer (300mM mannitol and 1mM EDTA, pH 7.4). The crude homogenates were centrifuged at $5000 \times g$ for 10 minutes and the supernatants were again centrifuged at $15000 \times g$ for 30 minutes. The resuspended pellets were loaded on Ficoll

gradient tubes and centrifuged at $22000 \times g$ for 90 minutes. The 8/12% and 12/16% interfaces were carefully removed, diluted in Syn buffer in a ratio of 1:4, and centrifuged for 20 minutes at $15000 \times g$. Pellets were resuspended and lysed with Lysis buffer. All samples were constantly maintained at 4°C during all steps. Western blot was applied to analyze the synaptic expressions of Phos-GluR1 S845, GluR1, and GluR2 (Rabbit mAb, 1:2000, Abcam).

Statistical Analysis

All data were analyzed by one-way ANOVA and post hoc Bonferroni (unequal sample) or Tukey (equal sample) tests and presented as the mean \pm standard error via SPSS 17. A p -value less than 0.05 was considered a significant difference. Figures were generated by Graphpad Prism Version 5 software.

Results

3'-dA Demonstrated a Rapid and Significant Antidepressant Effect

To investigate whether 3'-dA plays a role in regulating depression-like behavior, we i.p. injected the 7-week-old CD-1 mice with a low (5 mg/kg) or high (12.5 mg/kg) dose of 3'-dA for 45 minutes or 5 days. 45 minutes after the treatment, mice were subjected to either the TST or FST. The data showed that the immobility times in the 3'-dA-treated groups were significantly lower than in the controls (101.74 ± 6.49 sec) in a dose dependent manner: 54.60 ± 7.73 sec (for 12.5 mg/kg of 3'-dA) and 76.57 ± 6.49 sec (for 5 mg/kg of 3'-dA) in the TST (Figure 1A). It is noteworthy that the high dose of 3'-dA (12.5 mg/kg) reduced the immobility time of TST to a much larger extent than that of the comparable traditional antidepressant imipramine (15 mg/kg; Figure 1A). To confirm the data in the TST, the FST was performed under similar settings. After 45 minutes of treatment with 3'-dA, the high dose group also demonstrated a stronger antidepressant effect, as low as 48.23 ± 10.11 sec, compared with the control (92.69 ± 9.24 sec) and the imipramine group (79.92 ± 9.92 sec; Figure 1B).

To further study this antidepressant effect for long-term treatment, we treated the mice with a low (5 mg/kg) or high (12.5 mg/kg) dose of 3'-dA for 5 days, then performed the TST and FST. The data showed that both low and high doses of 3'-dA significantly decreased immobility time, to 50.35 % and 55.36 %

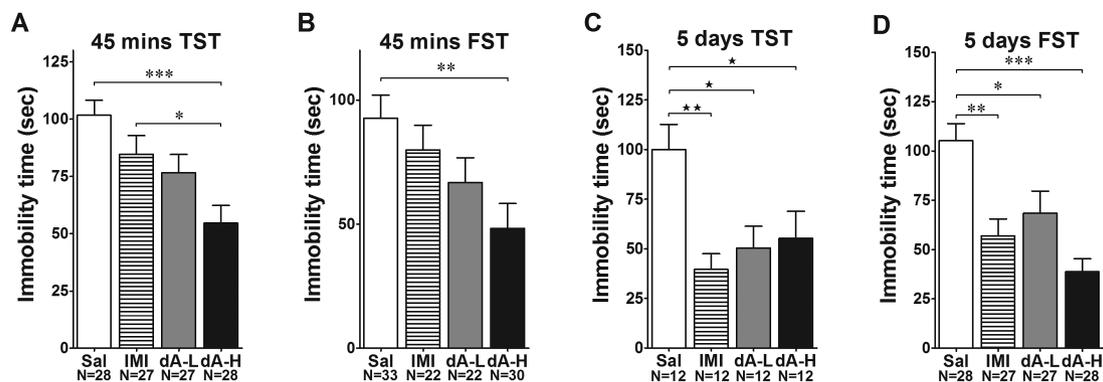


Figure 1. 3'-deoxyadenosine (3'-dA) demonstrated rapid and significant antidepressant effects in the tail suspension test (TST) and forced swim test (FST). CD-1 mice were i.p. injected with a low dose of 3'-dA (5 mg/kg/day, dA-L), a high dose of 3'-dA (12.5 mg/kg/day, dA-H), imipramine (15 mg/kg/day, IMI), or saline (Sal). 45 minutes after the drug injection, mice were subjected to the TST or FST. The number of mice per group is indicated in each individual graph. Data were analyzed by one-way ANOVA and presented as mean \pm standard error (post hoc Bonferroni test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; post hoc Tukey test, $\star p < 0.05$, $\star\star p < 0.01$). (A, B) 45 minutes after the injection, 3'-dA significantly reduced the immobility time in the TST and FST. (C, D) After 5 consecutive days of injection, 3'-dA significantly reduced the immobility time in the TST and FST.

of control in the TST and 64.95 % and 36.78 % of control in the FST, respectively, in a dose-dependent manner. Imipramine also showed a stable and strong antidepressant effect in both the TST and FST (Figure 1C and D).

Unlike the Psycho-Stimulants, 3'-dA Did Not Show Locomotor Hyperactivity in the OFT

To further determine whether 3'-dA causes locomotor hyperactivity, one of the core features of mania-like symptoms, we performed the OFT after 3 days of treatment with a low or high dose of 3'-dA. The total distance traveled showed no significant difference in 3'-dA-treated groups compared with controls, suggesting that 3'-dA does not cause hyperactivity in mice (Figure 2A). This is different from the psycho-stimulant amphetamine, which significantly increased the total distance travelled in rats during the OFT (Labonte et al., 2012). 3'-dA showed a trend to increase the distance travelled in the center area, but it was not statistically significant (Figure 2B). The weights of mice after 5 days of treatment did not show a significant difference (Figure 2C).

After 45 Min of Treatment, High Concentration of 3'-dA Enhanced AMPA GluR1 S845 Phosphorylation in the PFC and HIP

Previous studies from our group showed that phosphorylation of AMPA GluR1 S845 presented a common mechanism for the treatment of depression (Du et al., 2007). In this context, we further addressed whether Phos-GluR1 S845 levels were changed after treatment with 3'-dA. We found that after 45 minutes of i.p. injection, the phosphorylation of GluR1 S845 was rapidly and significantly increased in both the PFC and HIP from the high-dose 3'-dA-treated group (Figure 3A). In contrast, the phosphorylation of GluR1 S845 in the imipramine-treated group remained unchanged after 45 minutes of treatment, which is consistent with the fact that the antidepressant effects of imipramine were not significant after 45 minutes of treatment (Figures 1A and B and 3A). The levels of total GluR1 proteins in these brain regions remained unchanged (Figure 3B).

After 5 Days of Treatment, 3'-dA and Imipramine Enhanced AMPA GluR1 S845 Phosphorylation in Both the PFC and HIP

To determine the long-term effects of 3'-dA, we treated the mice with 3'-dA for 5 days, and found that the phosphorylation of GluR1

S845 was significantly increased in both the low ($175.44 \pm 14.32\%$; $169.86 \pm 11.83\%$) and high ($174.75 \pm 13.00\%$; $158.06 \pm 11.62\%$) dose groups in the PFC and HIP, respectively (Figure 3C). The imipramine-treated group also demonstrated a significant increase in Phos-GluR1 S845 levels in both the PFC ($163.02 \pm 12.08\%$) and HIP ($148.52 \pm 12.16\%$) after 5 days of treatment (Figure 3C), which is consistent with a previous publication (Du et al., 2007). The total GluR1 levels in the PFC and HIP remained unchanged (Figure 3D).

After 45 Min of Treatment, 3'-dA Significantly Up-Regulated AMPA GluR1 S845 Phosphorylation and GluR1 Levels at the Synapses in the PFC

To further determine whether GluR1 localization at the synapses in the PFC or HIP was increased, we isolated the synaptosomal fractions from the PFC and HIP tissue. Western blot analysis of Phos-GluR1 S845, GluR1, and GluR2 were performed with the synaptic fractions. Here, we found that levels of GluR1 and GluR2 were significantly enriched at the synapses, compared with the total protein (Figure 4A). After 45 minutes of treatment with the high dose of 3'-dA (12.5 mg/kg), Phos-GluR1 S845 and GluR1 levels, but not GluR2 levels, were significantly increased in the synaptosomal fractions in the PFC (Figures 4B and C and 5A). It is noteworthy that the acute treatment of the traditional antidepressant imipramine did not lead to a significant increase in GluR1 levels at the synapses (Figure 4C), which is consistent with the results in the TST and FST after 45 minutes of treatment (Figure 1A and B).

After 5 Days of Treatment, 3'-dA Significantly Enhanced AMPA Receptor GluR1 S845 Phosphorylation and GluR1, But Not GluR2 Levels at the Synapses

After 5 days of treatment with drugs, 3'-dA and imipramine enhanced the phosphorylation levels of GluR1 S845 in the PFC and HIP (Figure 4D). We found that both the low and high doses of 3'-dA induced an increase in GluR1 S845 phosphorylation (low dose: $304.01 \pm 24.46\%$ in PFC, $244.82 \pm 28.91\%$ in HIP; high dose: $285.14 \pm 24.54\%$ in PFC, $245.27 \pm 26.39\%$ in HIP). Both the low and high doses of 3'-dA induced an increase in GluR1 levels (low dose: $144.12 \pm 10.21\%$ in PFC, $129.02 \pm 5.04\%$ in HIP; high dose: $152.37 \pm 12.47\%$ in PFC, $147.48 \pm 10.98\%$ in HIP), but not GluR2 levels, in the PFC and HIP, suggesting that the regulation of 3'-dA to AMPA was subtype-specific. For the imipramine-treated group, chronic treatment resulted in both GluR1 ($157.46 \pm 7.69\%$ in PFC) and GluR2 ($138.01 \pm 9.20\%$ in PFC) increases at the synapses, suggesting the

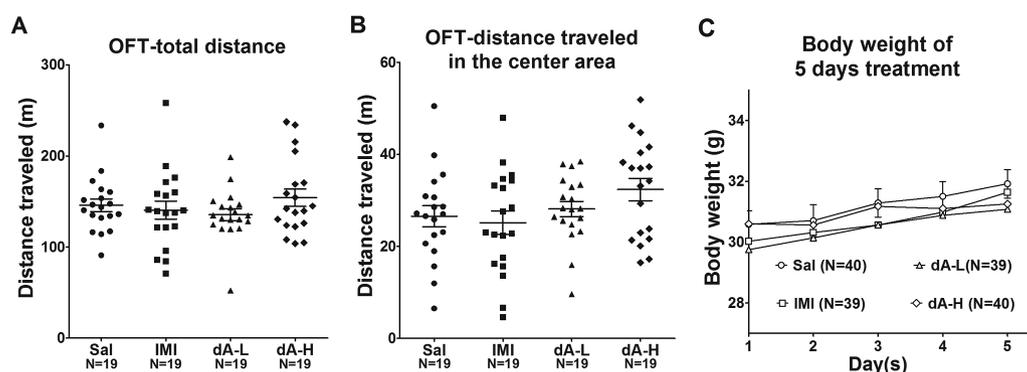


Figure 2. An open field test (OFT) of animals after 3 days of treatment with 3'-deoxyadenosine (3'-dA). After 3 days of treatment, mice were subjected to the OFT. Total distance traveled and the distance traveled in the center area was determined by automated tracking system. The number of mice per group is indicated in each individual graph. Data were analyzed by one-way ANOVA and presented as mean \pm standard error. (A) The total distance traveled in the field after 3'-dA treatment. (B) The distance traveled in the center area of field after 3'-dA treatment. (C) Body weight of the 5 consecutive days after 3'-dA treatment.

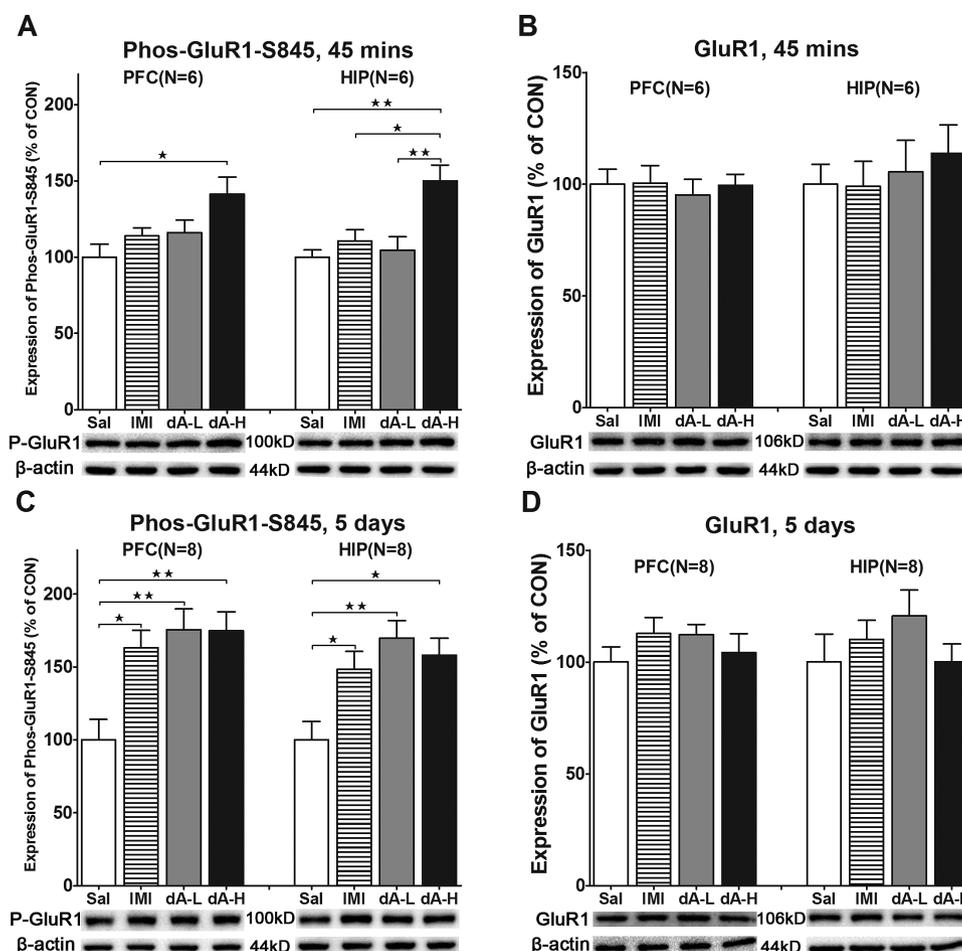


Figure 3. Enhanced phosphorylation of GluR1 S845 after 45-minute and 5-day treatments of 3'-deoxyadenosine (3'-dA) in the prefrontal cortex (PFC) and hippocampus (HIP). CD-1 mice were i.p. injected with a low dose of 3'-dA (5 mg/kg/day, dA-L), a high dose of 3'-dA (12.5 mg/kg/day, dA-H), imipramine (15 mg/kg/day, IMI), or saline (Sal) for 45 minutes or 5 days. Western blot analyses of the proteins from the PFC or HIP were performed with anti-Phos-GluR1 S845 or anti-GluR1 antibodies. The number of mice per group is indicated in each individual graph. Data were analyzed by one-way ANOVA and presented as mean \pm standard error (post hoc Tukey test, $\star p < 0.05$, $\star\star p < 0.01$). (A, B) The expression levels of Phos-GluR1 S845 or GluR1 in both the PFC and HIP after 45 minutes of treatment with 3'-dA. (C, D) The expression levels of Phos-GluR1 S845 and GluR1 in both the PFC and HIP after 5 days of treatment with 3'-dA.

regulation is in a GluR1/2 subtype-specific manner, which is different from the 3'-dA regulation (Figure 4E and 5B).

The Rapid Antidepressant Effect of 3'-dA was Blocked by the AMPA Receptor Specific Antagonist GYKI 52466

We hypothesized that the AMPA receptor increase is critical for the rapid antidepressant effects of 3'-dA. To determine whether the rapid antidepressant effects of 3'-dA could be blocked by the AMPA receptor-specific antagonist GYKI 52466, we pretreated the mice with 30 mg/kg of GYKI, followed by the i.p. injection of 3'-dA (12.5 mg/kg). The mice were subjected to TST 45 minutes after the 3'-dA injection. The pretreatment of GYKI 52466 almost completely blocked the decrease of immobility time caused by 3'-dA (control: 130.06 ± 11.67 sec; 3'-dA: 65.69 ± 12.02 sec; GYKI + 3'-dA: 122.91 ± 8.65 sec; Figure 6), suggesting that enhanced AMPA receptor excitability at the synapses might play an important role in the rapid antidepressant effects of 3'-dA.

Discussion

This study sought to determine the rapid and robust antidepressant effects of 3'-dA and its possible underlying mechanisms

in regulating AMPA receptor signaling. We found that: (1) 3'-dA had rapid and robust antidepressant effects in the TST and FST after 45 min of treatment, and this effect remained after 5 days of treatment; (2) unlike the psycho-stimulants, 3'-dA had no hyperactive effect in the OFT; (3) after 45 min of treatment, only high doses of 3'-dA enhanced Phos-GluR1 S845 in the PFC and HIP, and after 5 days of treatment, 3'-dA, similar to imipramine, enhanced Phos-GluR1 S845 in both the PFC and HIP; (4) after 45 min of treatment, only high doses of 3'-dA enhanced Phos-GluR1 S845 and GluR1, but not GluR2 levels, at the synapses in the PFC, and after 5 days of treatment, 3'-dA enhanced Phos-GluR1 S845 and GluR1, but not GluR2 levels, at the synapse in both the PFC and HIP; and (5) AMPA receptor-specific antagonist GYKI 52466 was able to block the rapid and robust antidepressant effects of 3'-dA, suggesting enhanced AMPA synaptic transmission is essential for the rapid antidepressant effects.

The Rapid and Robust Antidepressant Effect of 3'-dA was Unique to the Psycho-Stimulant Antidepressant Effect

The most famous rapid antidepressant is ketamine, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist that elicits a rapid antidepressant response in patients with

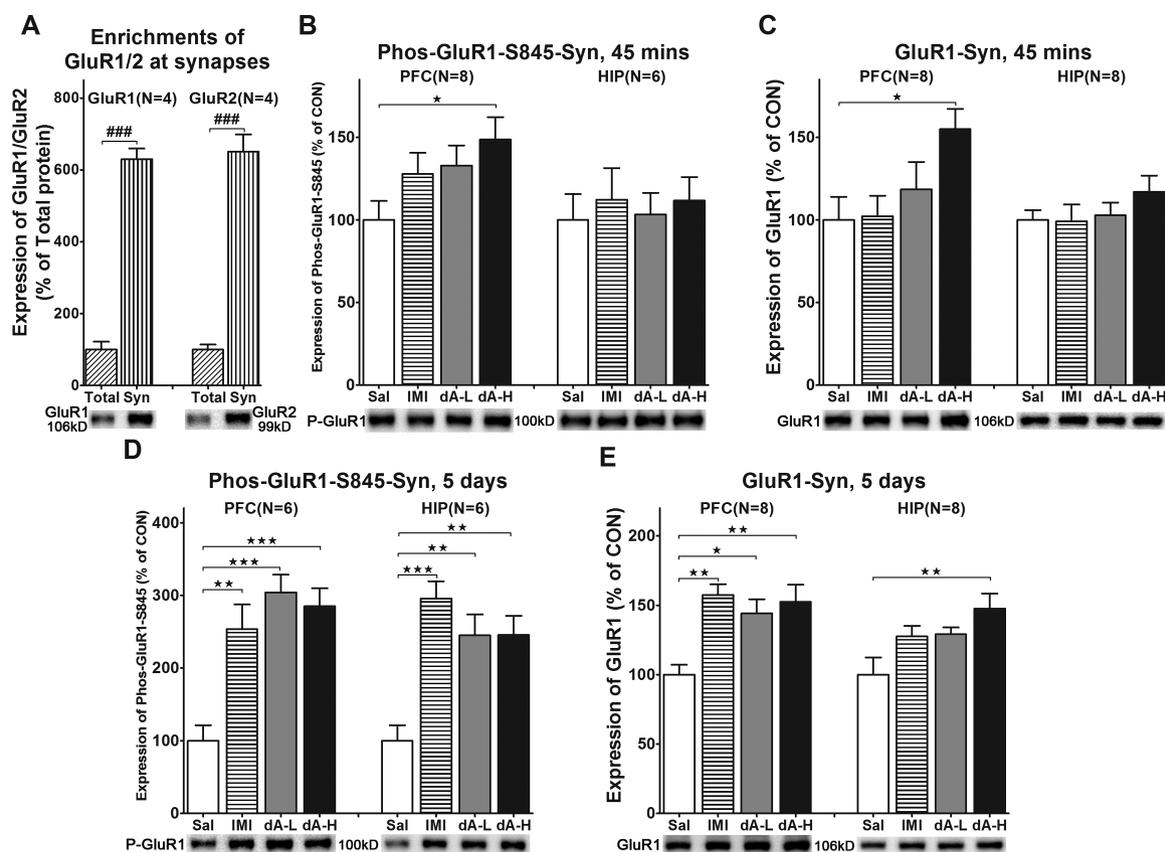


Figure 4. Rapid and chronic effects of 3'-deoxyadenosine (3'-dA) on synaptic phosphorylation of GluR1 S845 and GluR1 in the prefrontal cortex (PFC) and hippocampus (HIP). CD-1 mice were i.p. injected with a low dose of 3'-dA (5 mg/kg/day, dA-L), a high dose of 3'-dA (12.5 mg/kg/day, dA-H), imipramine (15 mg/kg/day, IMI), or saline (Sal) for 45 minutes or 5 days. Synaptosomal fractions from the PFC or the HIP were prepared and subjected to Western blot analyses with anti-Phos-GluR1 S845 or anti-GluR1 antibodies. The number of mice per group is indicated in each individual graph. Data were analyzed by one-way ANOVA and presented as mean \pm standard error (post hoc Tukey test, $\star p < 0.05$, $\star\star p < 0.01$, $\star\star\star p < 0.001$; two-tail t-test, $\#p < 0.05$, $\#\#\#p < 0.001$). (A) Enrichments of GluR1 and GluR2 at synapses compared with the total protein. (B, C) The expression levels of synaptic Phos-GluR1 S845 and GluR1 were increased in the PFC, but not in the HIP, after 45 minutes of treatment with 3'-dA. (D, E) After 5 days of treatment, 3'-dA enhanced synaptic Phos-GluR1 S845 and GluR1 expressions in both the PFC and HIP.

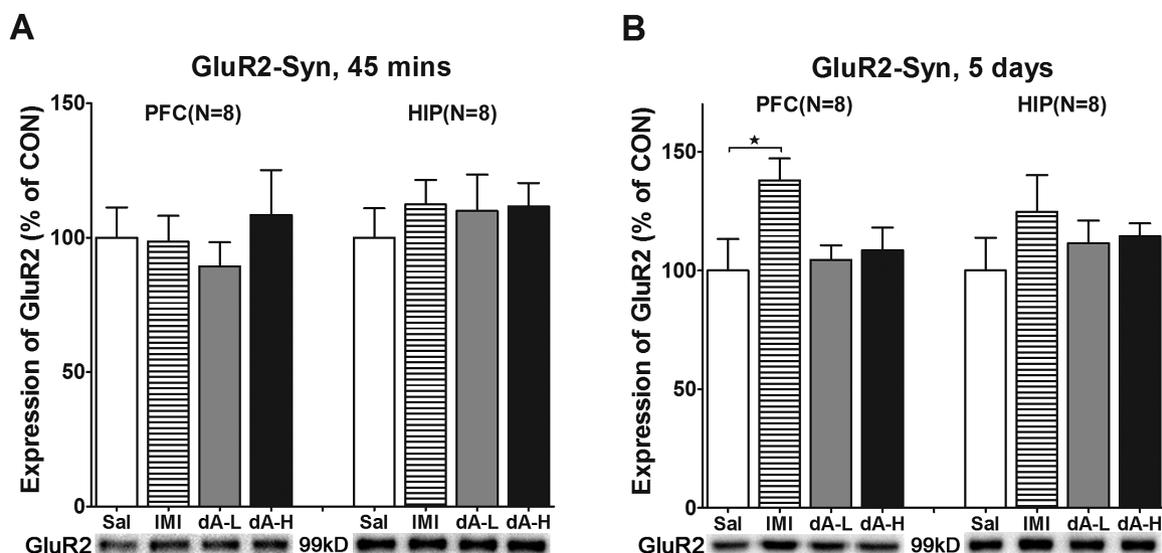


Figure 5. Rapid and chronic effects of 3'-deoxyadenosine (3'-dA) on synaptic GluR2 in the prefrontal cortex (PFC) and hippocampus (HIP). CD-1 mice were i.p. injected with a low dose of 3'-dA (5 mg/kg/day, dA-L), a high dose of 3'-dA (12.5 mg/kg/day, dA-H), imipramine (15 mg/kg/day, IMI), or saline (Sal) for 45 minutes or 5 days. Synaptosomal fractions from the PFC or HIP were prepared and subjected to Western blot analyses with anti-GluR2 antibodies. The number of mice per group is indicated in each individual graph. Data were analyzed by one-way ANOVA and presented as mean \pm standard error (post hoc Tukey test, $\star p < 0.05$). (A, B) The expression levels of synaptic GluR2 were not increased in the PFC and HIP after 45-minute or 5-day treatments with 3'-dA.

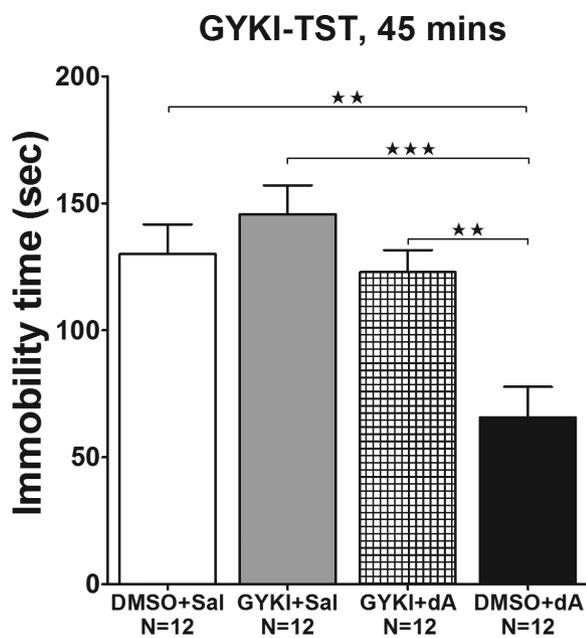


Figure 6. The α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor-specific antagonist GYKI 52466 significantly blocked the 3'-deoxyadenosine (3'-dA)-induced rapid antidepressant effect in the tail suspension test (TST). CD-1 mice were i.p. injected with AMPA-specific antagonist GYKI 52466 (30 mg/kg) or vehicle. After 15 minutes, the high dose of 3'-dA or vehicle was also injected. 45 minutes later, the CD-1 mice were subjected to the TST. Immobility time was determined. The number of mice per group is indicated in each individual graph. Data were analyzed by one-way ANOVA and presented as mean \pm standard error (post hoc Tukey test, $\star p < 0.05$, $\star\star p < 0.01$, $\star\star\star p < 0.001$).

depression (Berman et al., 2000; Zarate et al., 2006; Price et al., 2009) or bipolar depression (DiazGranados et al., 2010; Zarate et al., 2012; Krystal et al., 2013). However, ketamine does not present ideal features as an antidepressant because of its potential psychotomimetic effects and abuse. The underlying antidepressant mechanism of ketamine has been well studied, and emerging evidence shows that ketamine's antidepressant properties rely on blocking NMDA receptors, increasing AMPA signaling, and rapidly inducing synaptogenesis (Maeng et al., 2008; Caddy et al., 2014). The antidepressant effect of ketamine appears to be fast-acting and lasts for a few days to weeks (Aan Het Rot et al., 2012). Some clinical evidence indicates that depressed patients might have side effects of a fast heart rate, high blood pressure, respiratory depression, and hallucinations after treatment with ketamine (Bowdle et al., 1998; Roback et al., 2006; Paul et al., 2009; Aan Het Rot et al., 2010). Other psycho-stimulants, such as cocaine and amphetamine, may also have rapid antidepressant effects: however, these psycho-stimulants usually induce excitatory neural toxicity and drug addiction (Pierce and Kalivas, 1997; McCance-Katz et al., 1998). Moreover, most psycho-stimulants also lead to the development of depressive symptoms during withdrawal (Che et al., 2013; Krzascik et al., 2015).

3'-dA is an analogue of adenosine, which is a neurotransmitter in the nervous system (Haas and Selbach, 2000). It has been shown that adenosine-derived drugs can be transported from blood to the brain via an adenosine transporter across the blood-brain barrier (Li et al., 2001; Pardridge, 2012). Very recently, it has been shown that 3'-dA has a beneficial effect in CUMS-induced behavioral deficits after treatment with doses of 20 mg/kg and 40 mg/kg for 3–6 weeks (Tianzhu et al., 2014). Chronic 3'-dA (20, 40 mg/kg) treatments reversed the behavioral deficiency induced by CUMS exposure in the TST and FST (Tianzhu

et al., 2014). Here, we report a rapid and robust antidepressant effect of 3'-dA alone within a lower dose range (5–12.5 mg/kg). In our study, the 3'-dA resulted in approximately a 45% reduction in immobility time within 45 minutes in both the TST and FST, which is significantly stronger and faster than the traditional antidepressant imipramine (Figure 1A and B). The imipramine dose range commonly used for mouse models of depression is 10–20 mg/kg/day (Solomon et al., 2014; Ichikawa et al., 2015). We chose the middle of this range, 15 mg/kg, to compare with the similar dose of 3'-dA (12.5 mg/kg) and found that after 45 min of treatment, the imipramine (15 mg/kg) did not reach significant antidepressant effects, but 3'-dA demonstrated faster antidepressant effects (Figure 1A and B). It is noteworthy that recently evidence has shown that i.p. injection with a low dose of imipramine (<20 mg/kg) did not lead to fast antidepressant effects in 30 minutes, but a dose of 30 or 40 mg/kg decreased the immobility time in mice in the TST and FST (Villard et al., 2011; Cai et al., 2013; Nguyen and Matsumoto, 2015). Therefore, we only concluded that compared to the imipramine dose of 15 mg/kg, 3'-dA (12.5 mg/kg) demonstrated faster and stronger antidepressant effects (Figure 1A and B). Although 3'-dA demonstrated rapid and strong antidepressant effects, it did not elicit hyperactivity like some of the psycho-stimulants (Figure 2A and B).

Consistent with Traditional Antidepressants, 3'-dA Regulated GluR1 S845 Phosphorylation

A growing body of data suggests that AMPA receptor signaling plays a critical role in regulating synaptic strength, as well as various forms of neural and behavioral plasticity (Carlezon and Nestler, 2002; Malenka, 2003; Kendell et al., 2005; Rumpel et al., 2005; Sun et al., 2005). 3'-dA is an agonist for the adenosine A3 receptor (Kitamura et al., 2011; Nakamura et al., 2015), which regulates synaptic plasticity and long-term potentiation *in vivo* and *in vitro* (Costenla et al., 2001; Maggi et al., 2009). Therefore, we hypothesized that 3'-dA may mediate through the adenosine A3 receptor to regulate the AMPA receptor function, which we will explore in our future research. GluR1 phosphorylation at the S845 site, which is a PKA site, is often viewed as an indicator for GluR1 membrane insertion in neurons and wide channel openings (Roche et al., 1996; Banke et al., 2000; Lee et al., 2000; Esteban et al., 2003). A previous study has shown that the levels of GluR1 in the PFCs of depressed patients were decreased (Beneyto et al., 2007), which was consistent with the animal experiments (Toth et al., 2008; Yuen et al., 2012; Kallarackal et al., 2013). The AMPA receptor may serve as a common mechanism for the treatment of mood disorders, since the two anticonvulsants riluzole (with a predominantly antidepressant profile) and lamotrigine enhanced GluR1 phosphorylation at the S845 site. In contrast, the anticonvulsant valproate (with a predominantly anti-manic profile) exerted the opposite effect on GluR1 phosphorylation at the S845 site (Du et al., 2004a, 2007, 2008, 2010; Gould et al., 2008). Consistent with previous findings, we found that 3'-dA was able to enhance GluR1 S845 phosphorylation and GluR1 synaptic localization in the PFC within 45 minutes (Figures 3A and 4B and C), which is faster than the traditional antidepressant imipramine. After 5 days of treatment, both 3'-dA and imipramine were able to enhance the phosphorylation of GluR1 S845 and synaptic GluR1 significantly in the PFC and HIP (Figures 3C and 4D and E).

3'-dA Modulated GluR1 Synaptic Localization in a Subtype-Specific and Brain Region-Specific Manner

Previous studies showed that there are three major types of AMPA tetramers in most brain regions: GluR1/2, GluR2/3, and GluR1/3

(Du et al., 2004a, 2004b, 2007, 2008; Ampuero et al., 2010; Blanco et al., 2012, 2014). It has been reported that a traditional antidepressant regulated the GluR1/2 tetramers in the PFC (Ampuero et al., 2010). We also found that the chronic treatment of imipramine enhanced GluR1/2 levels at the synapses (Figure 4E), suggesting that imipramine regulates GluR1/2 tetramers of AMPA receptors, which is consistent with previous studies (Du et al., 2004a, 2004b, 2007, 2008). However, it has been shown that drugs mediating the fast-acting antidepressant mechanisms, like cocaine, regulate the GluR1/3, instead of GluR1/2, in the PFC (Blanco et al., 2012, 2014). 3'-dA did not increase GluR2 levels at the synapses, suggesting that this regulation of increased AMPA synaptic transmission may be mediated through a subtype-specific manner and that other subtypes of AMPA tetramers, such as GluR1/3, might be involved. This is consistent with its fast-acting antidepressant effect.

Previous studies found that PFC was one of the most important brain regions involved in depression. Clinical evidence has demonstrated that neuronal activity in the PFC is reduced in depressed patients, and could be restored by selective serotonin reuptake inhibitor antidepressants (Kennedy et al., 2001; Fales et al., 2009). In rodent models, chronic stress decreased the expression levels of AMPA receptors, NMDA receptors, and various synaptic proteins, induced the atrophy of distal dendrites of pyramidal cells, and damaged the dendritic spines in the PFC (Liston et al., 2006; Radley et al., 2008; Li et al., 2011). In addition, a recent study showed that the expression levels of GluR1 were decreased in rat PFCs, but not in HIPs after 7 days of chronic restraint stress, suggesting that the PFC is more susceptible to stress (Yuen et al., 2012). In this paper, we found that within 45 min, 3'-dA enhanced GluR1 synaptic localization in the PFC (Figure 4B).

AMPA Receptor Signaling Regulated by 3'-dA Might be Essential for its Rapid and Robust Antidepressant Effect

A previous study showed that lithium exerts fast (1.5 days) and chronic antidepressant effects in mice TST and FST via up-regulating the AMPA receptor subunits GluR1 and GluR2 in the HIP, and an AMPA inhibitor was able to block the antidepressant effect of lithium (Gould et al., 2008). In this paper, we found that the effect of 3'-dA on up-regulating AMPA receptor signaling at the synapses might be essential for the rapid antidepressant effects (Figures 1A and B, 3A, and 4B and C), and AMPA antagonist GYKI 52466 was able to block 3'-dA-induced rapid antidepressant effects (Figure 6). This is consistent with a recent clinical trial showing that Org 26576 (ionotropic AMPA-type glutamate receptor enhancer) significantly improved symptoms in patients diagnosed as depressed by the Montgomery-Asberg Depression Rating Scale (Nations et al., 2012).

3'-dA is the main effective component of *Cordyceps Militaris*, which has been used as a Chinese herb and food for hundreds of years. Other beneficial effects of 3'-dA, such as the anti-viral and anti-fungal effects, may also provide additional benefits to depressed patients, because viral or fungal infections contribute to or even cause depressive symptoms (Tuli et al., 2013). 3'-dA also improves the quality of sleep by increasing non-rapid eye movement sleep (Hu et al., 2013). Therefore, 3'-dA is considered relatively safe and beneficial for use as a potential novel antidepressant drug. In summary, in this paper we have identified a rapid and relatively safe antidepressant for the treatment of depression, mediated through enhancing prefrontal AMPA receptor synaptic plasticity. This discovery sheds light on the

development of a novel, effective, safe, and rapid drug for the devastating disorder of major depression.

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Statement of Interest

The authors have no conflicts of interest to disclose, financial or otherwise.

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