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Betaine prevents and reverses the behavioral deficits and synaptic dysfunction induced by repeated ketamine exposure in mice

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ABSTRACT

As an N-methyl-D-aspartate (NMDA) receptor inhibitor, ketamine has become a popular recreational substance and currently is used to address treatment-resistant depression. Since heavy ketamine use is associated with persisting psychosis, cognitive impairments, and neuronal damage, the safety of ketamine treatment for depression should be concerned. The nutrient supplement betaine has been shown to counteract the acute ketamine-induced psychotomimetic effects and cognitive dysfunction through modulating NMDA receptors. This study aimed to determine whether the adjunctive or subsequent betaine treatment would improve the enduring behavioral disturbances and hippocampal synaptic abnormality induced by repeated ketamine exposure. Mice received ketamine twice daily for 14 days, either combined with betaine co-treatment or subsequent betaine post-treatment for 7 days. Thereafter, three-chamber social approach test, reciprocal social interaction, novel location/object recognition test, forced swimming test, and head-twitch response induced by serotonergic hallucinogen were monitored. Data showed that the enduring behavioral abnormalities after repeated ketamine exposure, including disrupted social behaviors, recognition memory impairments, and increased depression-like and hallucinogen-induced head-twitch responses, were remarkably improved by betaine co-treatment or post-treatment. Consistently, betaine protected and reversed the reduced hippocampal synaptic activity, such as decreases in field excitatory post-synaptic potentiation (fEPSP), long-term potentiation (LTP), and PSD-95 levels, after repeated ketamine treatment. These results demonstrated that both co-treatment and post-treatment with betaine could effectively prevent and reverse the adverse behavioral manifestations and hippocampal synaptic plasticity after repeated ketamine use, suggesting that betaine can be used as a novel adjunct therapy with ketamine for treatment-resistant depression and provide benefits for ketamine use disorders.

1. Introduction

Ketamine, a non-competitive antagonist at glutamate N-methyl-D-aspartate (NMDA) receptors, is primarily used for induction and maintenance of general anesthesia in surgery. The sub-anesthetic doses of ketamine are being used to treat acute and chronic pain. Notably, ketamine and one of its enantiomers S-ketamine have been observed to produce the rapid-onset antidepressant effects in humans [1]. On the other hand, R-ketamine also exhibits the beneficial effects on depression-like symptoms in animal models [2]. Recently, the intranasal spray formulation of S-ketamine has been approved for use in curing treatment-resistant depression. Under these specific interest findings, ketamine can exert a rapid and longer-term clinical antidepressant activities following repeated infusion in treatment-resistant major depression [3]. However, the long-term safety of repeated ketamine treatment is still a matter of concern [4].

In fact, ketamine has become a popular recreational substance. Long-term heavy ketamine use results in cognitive deficits, including spatial

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and pattern recognition memory impairments, greater dissociative symptom, and delusional disorder [5,6]. Moreover, the increased depression scores have been observed not only in frequent ketamine users, but also in those who were abstinent using ketamine over 12 months [6]. Therefore, it is of great importance to develop an add-on therapy to prevent the potential adverse psychiatric effects of repeated ketamine treatment.

Our previous study has revealed that betaine (trimethylglycine), a methyl glycine derivative, could promote the antidepressant-like effects, yet abolish the psychotomimetic action as well as motor- and cognitive-impairing effects of acute ketamine treatment in mice [7], indicating that betaine might potentially enhance the antidepressant efficacy of ketamine and reduce the acute psychotic symptoms in patients when receive ketamine to treat depression. However, it remains unknown if repeated co-treatment with betaine could also minimize the adverse psychiatric effects observed after repeated ketamine use, either medically or recreationally.

Betaine is an important natural component of rich food sources and a commonly used nutrient supplement. In addition to acting as an osmolyte and a methyl donor, betaine regulates lipid, sugar, ethanol, and homocysteine metabolism [8], represents antioxidant and anti-inflammatory [9–11] activities, and mitigates endoplasmic reticulum stress [12] and apoptosis [13]. Actually, there are various neuronal pharmacological activities of betaine, such as anti-seizure [14–16], antidepressant-like [7,17] and memory-improving effects [18–21]. Accordingly, it is hypothesized that betaine is of great benefit when it is administered after repeated ketamine exposure.

Our previous findings demonstrated that repeated ketamine exposure caused animal behavioral disturbances, including cognitive impairments, reduced sociability and social recognition, depression-like behavior, and increased hallucinogen-induced head-twitch responses in mice [22]. The present study aimed to assess the effects of repeated co-treatment with betaine and ketamine on these behavioral impairments and hippocampal synaptic plasticity. Furthermore, we determined whether betaine could improve the behavioral deficits and hippocampal synaptic dysfunction in mice after repeated ketamine exposure. The experimental results showed that betaine co-treatment and post-treatment can exhibit the protective and reversing effects on subchronic ketamine-elicited psychotomimetic behaviors, cognitive impairments, and decreases in synaptic function, LTP, and PSD-95 protein expression. Modulation of glutamatergic NMDA receptor by betaine might be the underlying mechanisms accounting for cognitive dysfunction, behavioral deficits, and synaptic neurotransmission induced by chronic ketamine use. These findings supported that betaine may enhance the therapeutic effectiveness when it is combined or post-treated with ketamine for treatment-resistant depression or other mood disorders, as well as benefit ketamine abuse-associated mental disorders.

2. Materials and methods

2.1. Animals

Male and female ICR mice (3 weeks old) were purchased from the BioLASCO Charles River Technology (Taiwan) and housed 4 per cage in a 12 h light/dark cycle with ad libitum access to water and food. All experiments were carried out between 10:00 and 17:00 h and in accordance with the ROC animal protection law (Chapter III: Scientific Application of Animals) and approved by the Review Committee of the institutional animal care and use committees of Tzu Chi University and National Health Research Institutes, Taiwan.

Ketamine and betaine (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in saline and intraperitoneally (i.p.) injected in volumes of 10 ml/kg. Ketamine (20 mg/kg, i.p.) was administered twice daily, separated by at least 6 h, for 14 days. Experimental timelines were shown in Fig. 1. In experiment 1, male mice received betaine (0, 30, or 100 mg/kg, i.p.) 20 min prior to each saline or ketamine injection to assess the effects of co-treatment with betaine and ketamine. In experiment 2, betaine (0, 30, or 100 mg/kg, i.p.) was administered to female mice once daily for 7 days following 14-day ketamine treatment.

2.2. Three-chamber social approach test

The apparatus for three-chamber social test (TCT) is a box made of acrylic (60 cm L × 40 cm W × 35 cm H) and divided into three chambers with two clear acrylic walls. Dividing walls have retractable doorways allowing access into each chamber. The wire cup used to contain the stranger mice is made of cylindrical chrome bars spaced 1 cm apart (21 cm H; bottom diameter: 12.3 cm). The mice were confined in the center chamber at the beginning of each phase. Then, the doorways to the side chambers were opened and allowed the mice to explore freely. During the 5-min habituation phase, each of the two side chambers contained an inverted empty wire cup. During the sociability phase, an unfamiliar mouse (stranger 1) was enclosed in one of the wire cup in a side chamber. During the social novelty phase, a new unfamiliar mouse (stranger 2) was enclosed in the wire cup that had been empty during the sociability phase. Exploration of an enclosed mouse or a wire cup was defined as when a test mouse oriented toward the cup with the distance between the nose and the cup less than 1 cm, or as climbing on the cup. The time spent in each chamber and time spent exploring enclosed novel

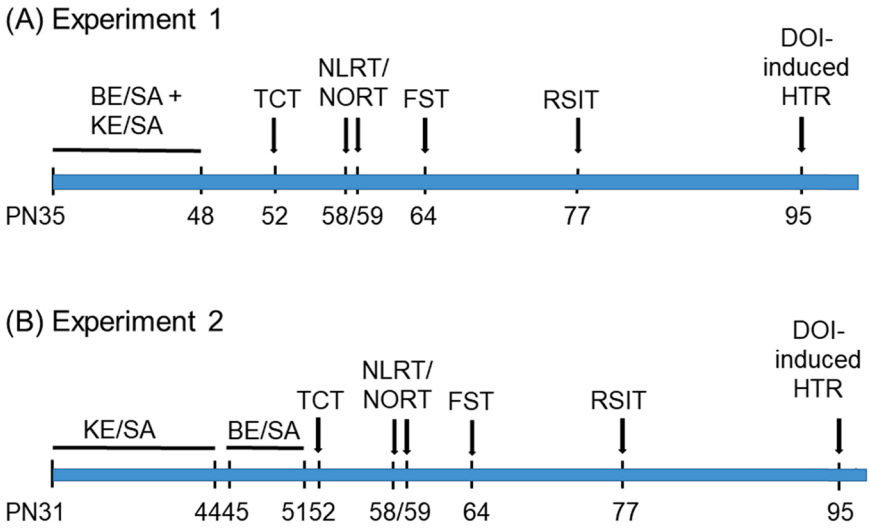

Fig. 1. Timeline of treatment and behavioral testing for betaine and ketamine administration. (A) The mice received betaine (BE, 0, 30 and 100 mg/kg, i.p.) 20 min prior to ketamine (KE, 20 mg/kg, i.p.) or saline (SA) twice daily for 14 days. (B) The mice were administrated with ketamine (KE, 20 mg/kg, i.p.) or vehicle (SA) twice daily for 14 days. Then betaine (BE, 0, 30 and 100 mg/kg, i.p.) was administrated once daily for 7 days following ketamine treatment. A battery of behavioral tests including three-chamber social approach test (TCT), novel location/object recognition test (NLRT/NORT), forced swimming test (FST), reciprocal social interaction test (RSIT), and DOI-induced head twitch response (HTR) were individually assessed in the indicated postnatal day (PN) as shown in the diagrams.

mice or empty cups were recorded and analyzed for the first 5 min of each session. All stranger mice were habituated to being enclosed in inverted wire cups in the three chamber apparatus for 15 min daily on two consecutive days prior to the experiment. The chambers were cleaned with 70% ethanol after each use.

2.3. Reciprocal social interaction test

Reciprocal social interaction test (RSIT) was performed as described [23]. Two unfamiliar mice from the same treatment group were placed in an open-field box (35 × 35 × 30 cm). The duration of social contacts including sniffing and grooming the partner, following, mounting, and crawling under or over the partner was recorded over a period of 10 min. The chambers and wire cups were cleaned with 70% ethanol after each use.

2.4. Novel location and novel object recognition tests

The novel location recognition test (NLRT) and novel object recognition test (NORT) were examined in a Plexiglas open field box (35 × 35 × 30 cm) as described previously [24]. The novel location and novel object recognition procedure consisted of habituation, sample phase, and test sessions. Habituation was conducted in two consecutive daily sessions, during which each mouse was allowed to individually explore the box in the absence of objects for 20 min. The animal was then removed from the arena and placed in its home cage. During the sample phase, each animal was placed in the box, and after 5 min, two identical sample objects (A+A1) were simultaneously introduced in two corners. Each animal was allowed to explore the objects for 5 min. An animal was considered to explore the object when its head was facing the object at a distance of approximately 1 cm or less between the head and object or when it was touching or sniffing the object. The time spent exploring each object was recorded using stopwatches by an experimenter blind to the treatment condition. After the sample phase, the mice were immediately returned to their home cages. The novel location recognition test was conducted 30 min after sample phase. The animals were returned to the same box as during the sample phase, and one of the two identical objects was replaced with a novel local corner (N object) to test the location-based recognition memory. After 24 h, novel object recognition test was performed. The mice were allowed to explore the open field with one identical sample object (A) and a novel object (N1) to assess the novel object recognition memory. The animals were allowed to explore the box freely for 5 min, and the time spent exploring each object (A or N1) was recorded as described above. The objects and chambers were cleaned with 70% ethanol after each use. The time spent exploring the two objects was individually quantified in the sample phase, NLRT, and NORT sessions. A recognition index (RI), a ratio of the amount of time spent exploring the original object (A1) or the novel location/object (N/N1) over the total time spent exploring both objects (A+A1, A+N, or A+N1) was used to evaluate recognition memory. RI=A1/(A+A1), N/(A+N), or N1/(A+N1). Values of RI (%) close to 50% indicate that animals spent equal time exploring both objects, while RI values greater than 50% denote a preference to explore the object (N or N1) that was moved or replaced over the familiar one. The data were excluded if the mouse explored the objects less than 10 s during each session.

2.5. Forced swimming test

Animals were subjected to forced swimming test (FST) for 2 consecutive days. The mice were placed in a Plexiglas cylinder (33.5 cm height, 20 cm diameter) filled with 25 ± 1 °C water to a height of 20 cm for 15 min (pre-test session). Twenty-four hours later, mice were placed in the water again for a 6 min session (test session), the first 2 min has elapsed. Immobility was recorded when no additional activity was observed other than that required to keep the mouse's head above the water. After the test, animals were dried by towels and under a lamp.

2.6. DOI-induced head-twitch response

Mice were injected with serotonergic hallucinogen (±)1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) at 1 mg/kg and immediately placed in an acrylic cylinder container (40 cm height and 20 cm diameter). The number of head-twitch response (HTR, 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).

2.7. Electrophysiological recordings

The procedure for hippocampal slice recordings was modified from the previous description [25]. After behavioral tests, the mice were sacrificed and the brain tissues were dissected and immersed in ice-cold artificial cerebrospinal fluid (ACSF) with the following composition (in mM): 128 NaCl, 1.9 KCl, 2.2 $CaCl_2$, 2.0 $MgSO_4$, 26 $NaHCO_3$, 1.2 KH_2PO_4 and 10 D-glucose, pH 7.38, bubbled with a mixture of 95% O_2/5% CO_2. Dorsal hippocampus (1.2–2.8 mm posterior to bregma) was immediately cut into small pieces (400 μm) in a coronal orientation by using a vibratome (VT1000S, Leica). The pyramidal layer and stratum radiatum of CA1 were positioned to cover the 8 × 8 microelectrode array in the coated MED probe (Alpha MED Scientific Inc.). The hippocampal slice was continuously perfused with ACSF (2 ml/min) through the perfusion cap (MED-KCAP01, Alpha MED Scientific Inc.). Bipolar electrical stimulation (0.05 or 0.1 ms duration) in CA1 at Schaffer collaterals was used to evoke the field excitatory post-synaptic potentials (fEPSPs) which is 300 μm from the stimulation site. Amplifier (SU-MED640, Alpha MED Scientific Inc.) was used to transduce voltage signals and digitize at 20 kHz.

An input-output (I/O) curve was determined using the measurements of fEPSP slope in response to stimulation current from the respective 10–80 μA and 20–140 μA in sequential increments of 10 and 20 μA. Each current intensity was repeated at 5 times at 0.033 Hz. Before long-term potentiation (LTP) induction, test stimulation was first performed by using the currents that could induce one-third of the maximum amplitude at 0.033 Hz for more than 30 min until the fEPSP slope was stable. LTP induction was then carried out by using the theta burst stimulation (TBS) protocol, which is four-pulse, 100 Hz bursts repeated ten times at 200 ms intervals [26]. The LTP response was then recorded by test stimulation at 0.033 Hz for 60 min

Data were obtained and saved using NI-DAQmx14.0 software (Alpha MED Scientific Inc). Digital filtering and initial slope of fEPSPs measurement were then operated using pCLAMP10 software (Molecular Devices). The degree of LTP was evaluated by normalizing to the mean slope of baseline during the last 10 min. The mean fEPSP slope averaged among 2 min was present.

2.8. Immunofluorescence staining

After animal behavioral experiments, brain tissues were fixed with 4% paraformaldehyde and dehydrated by 30% sucrose. Cryosections (10 μm) were cut in a coronal orientation and collected from Bregma −1.40 to −1.70 mm. Slices were retrieved in 10 mM sodium citrate, pH 6.0, at 95 °C for 15 min and then incubated by blocking solution (Bio Future) for 1 h. Incubation of primary rabbit monoclonal antibody against PSD95 (1:1000, Abcam) was operated at 4 °C for 16 h followed by secondary antibody goat-anti-rabbit IgG conjugated with Alexa Fluro 555 (2 μg/ml, Invitrogen) for 1 h. Tissues were counterstained with 1 μg/ml Hoechst 33258 (Invitrogen) for 1 h and then mounted on slides using a mounting medium (Vector). Images were captured using a fluorescence microscope (Leica).

Immunosignal of postsynaptic density protein 95 (PSD-95) in stratum radiatum of CA1 were observed, and 5 rectangles regions

(150 × 150 μm^2) were randomly selected to quantify the optical density by ImageJ software. The density values from 5 regions in each slice were averaged and the mean value for each animal resulted from the analysis of 6 sections and compared in all groups. Three animals in each group were operated and examined independently with similar observations.

2.9. Statistical analyses

Data were obtained and expressed as mean±S.E.M. The data were analyzed using one-way ANOVAs followed by a post hoc Student-Newman-Keuls test. Two-way mixed-design ANOVAs were used in the I//O curve and LTP results. The within-subject factors included current in the I/O curve and time in the LTP. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of repeated ketamine exposure on animal behavioral performance

The experimental timeline for repeated ketamine exposure was shown in Fig. 1A. In experiment 1, male mice were intraperitoneally (i.p.) administrated twice daily with ketamine at 20 mg/kg for 14 days from PN35–48. Then, a battery of animal behavioral tests, including three-chamber social test (TCT) at PN52, novel location recognition test/novel object recognition test (NLRT/NORT) at PN58/59, forced swimming test (FST) at PN64, reciprocal social interaction test (RSIT) at PN77, and DOI-induced head-twitch response (HTR) at PN95, were conducted in different postnatal day (PN).

The three-chamber social test was used to assess cognition in the form of general sociability and interest in social novelty in mice. Both sociability index (Fig. 2A) and social novelty index (Fig. 2B) were lower in ketamine-treated mice under repeated ketamine administration for two weeks than the saline group. The results revealed a significant social behavioral deficit in the ketamine-treated mice compared to the control animals.

Following 14 days repeated administration, ketamine also significantly attenuated the animal recognition index in NLRT and NORT sessions, but did not affect those in the sample phase (Fig. 2C). Notably, there was no significant difference of the total exploration time between saline and ketamine-treated group in each session (Fig. 2D). In the forced swimming test, the mice treated with ketamine exhibited the depression-like behavior with a significant increase in immobility (Fig. 2E). In the reciprocal social interaction test, the reduced interaction time was observed in the mice treated with repeated ketamine (Fig. 2F). DOI which is a hallucinogenic 5-HT2A/2C receptor agonist was used to elicit head-twitch response in mice under repeated ketamine exposure. Importantly, the number of head-twitch responses induced by DOI was remarkably increased in the ketamine-treated mice compared to vehicle controls (Fig. 2G). It was noted that there was no difference in locomotor activity contributed to total distances traveled between the control and ketamine-treated mice (data not shown).

3.2. Effects of betaine co-treated with ketamine on animal behavioral disturbances

The experimental procedure for repeated co-treatment of betaine with ketamine was shown in Fig. 1A. Betaine was administrated (i.p.) at doses of 30 and 100 mg/kg 20 min prior to each ketamine injection (20 mg/kg, i.p.), administered twice daily and separated by about 6 h for 14 days. Then, a series of behavioral tests were performed as described above.

The decreased social novelty index-induced by ketamine exposure was protected by betaine pretreatment at 30 and 100 mg/kg ($F_{(4,34)} = 2.89$, $p < 0.05$) (Fig. 2B). Betaine alone at 100 mg/kg did not alter animal sociability and social novelty. Moreover, pretreatment of betaine at 100 mg/kg, but not 30 mg/kg significantly improved the reduced recognition index in both NLRT ($F_{(4,34)} = 2.89$, $p < 0.05$) and NORT ($F_{(4,34)} = 4.90$, $p < 0.05$) sessions in the ketamine-treated group (Fig. 2C). Betaine alone at 100 mg/kg did not influence the location and object recognition activity. However, there was no any significant difference of total exploration time under these distinct treatments in each session (Fig. 2D).

Depressive-like behavioral performance under betaine and ketamine co-treatment was also detected by FST. The increased immobility time induced by ketamine exposure ($F_{(4,34)} = 7.33$, $p < 0.01$) was significantly attenuated by betaine at 100 mg/kg, but not 30 mg/kg (Fig. 2E). As to the reciprocal social interaction, the decreased contact time in ketamine-treated group ($F_{(4,34)} = 14.91$, $p < 0.01$) was remarkably prevented by betaine at 100 mg/kg, but not 30 mg/kg (Fig. 2F).

The number of head-twitch induced by DOI was significantly increased in ketamine-treated mice compared with control mice ($F_{(4,34)} = 14.18$, $p < 0.001$) (Fig. 2G). Betaine at the doses of 30 and 100 mg/kg remarkably attenuated the enhancing action of ketamine on DOI-elicited head-twitch responses (Fig. 2G). Notably, betaine alone at 100 mg/kg did not affect DOI-induced head twitch response.

3.3. Effects of betaine treatment after ketamine exposure on behavioral disturbances

The experimental procedure for betaine post-treatment after ketamine exposure was shown in Fig. 1B. The female mice were also administered with ketamine (20 mg/kg, i.p.) twice daily for 14 days. Betaine was then given with 30 and 100 mg/kg (i.p.) once daily for 7 days posterior to ketamine treatment. A battery of behavioral assays was performed as previous described.

The one-way ANOVAs revealed a significant effect of repeated ketamine treatment on the TCT ($F_{(4,34)} = 7.36$, $p < 0.001$), NLRT ($F_{(4,34)} = 7.36$, $p < 0.001$), NORT ($F_{(4,34)} = 11.22$, $p < 0.001$), FST ($F_{(4,34)} = 10.5$, $p < 0.001$), RSI test ($F_{(4,34)} = 14.91$, $p < 0.01$), and DOI-induced HTR ($F_{(4,34)} = 10.06$, $p < 0.001$) (Fig. 3).

The reduced sociability index (Fig. 3A) and social novelty index (Fig. 3B) in TCT, and recognition index in NLRT/NORT (Fig. 3C) under repeated ketamine exposure were reversed by subsequent chronic treatment of betaine at both 30 and 100 mg/kg. The post-treatment of betaine at 100 mg/kg, but not 30 mg/kg recovered the decreased interaction time in RSIT (Fig. 3F) and abolished the increased immobility time in FST (Fig. 3E) after repeated ketamine exposure. Moreover, the potentiation effect of ketamine on DOI-induced HTR was attenuated by chronic post-treatment of betaine at both 30 and 100 mg/kg (Fig. 3G).

3.4. Effects of betaine co-treated with ketamine on synaptic functions

The effects of betaine co-treatment with ketamine on hippocampal fEPSPs in response to electrical stimulation were shown in Fig. 4A. Two-way mixed-design ANOVA revealed main effects of treatment ($F_{4,\ 15} = 16.45$, $p < 0.01$), current intensity ($F_{7,\ 105} = 112.50$, $p < 0.001$), and significant interaction ($F_{28,\ 105} = 8.73$, $p < 0.001$). *Post hoc* tests showed that betaine at 100 mg/kg, but not 30 mg/kg prevented repeated ketamine-induced reduction in the slope of I/O curves (Fig. 4B). However, betaine alone at 100 mg/kg slightly but not significantly impaired fEPSP in control.

The effects of betaine co-treatment on ketamine-induced long-term potentiation (LTP) inhibition were shown in Fig. 4C. Two-way mixed-design ANOVA showed significant effects of treatment ($F_{4,15} = 5.01$, $p < 0.05$), time ($F_{29,\ 435} = 15.02$, $p < 0.05$), and interaction ($F_{116,\ 435} = 3.88$, $p < 0.05$). The fEPSP slope after TBS was significantly reduced by repeated ketamine exposure and the inhibition was significantly protected by betaine at 100 mg/kg, but not 30 mg/kg (Fig. 4D).

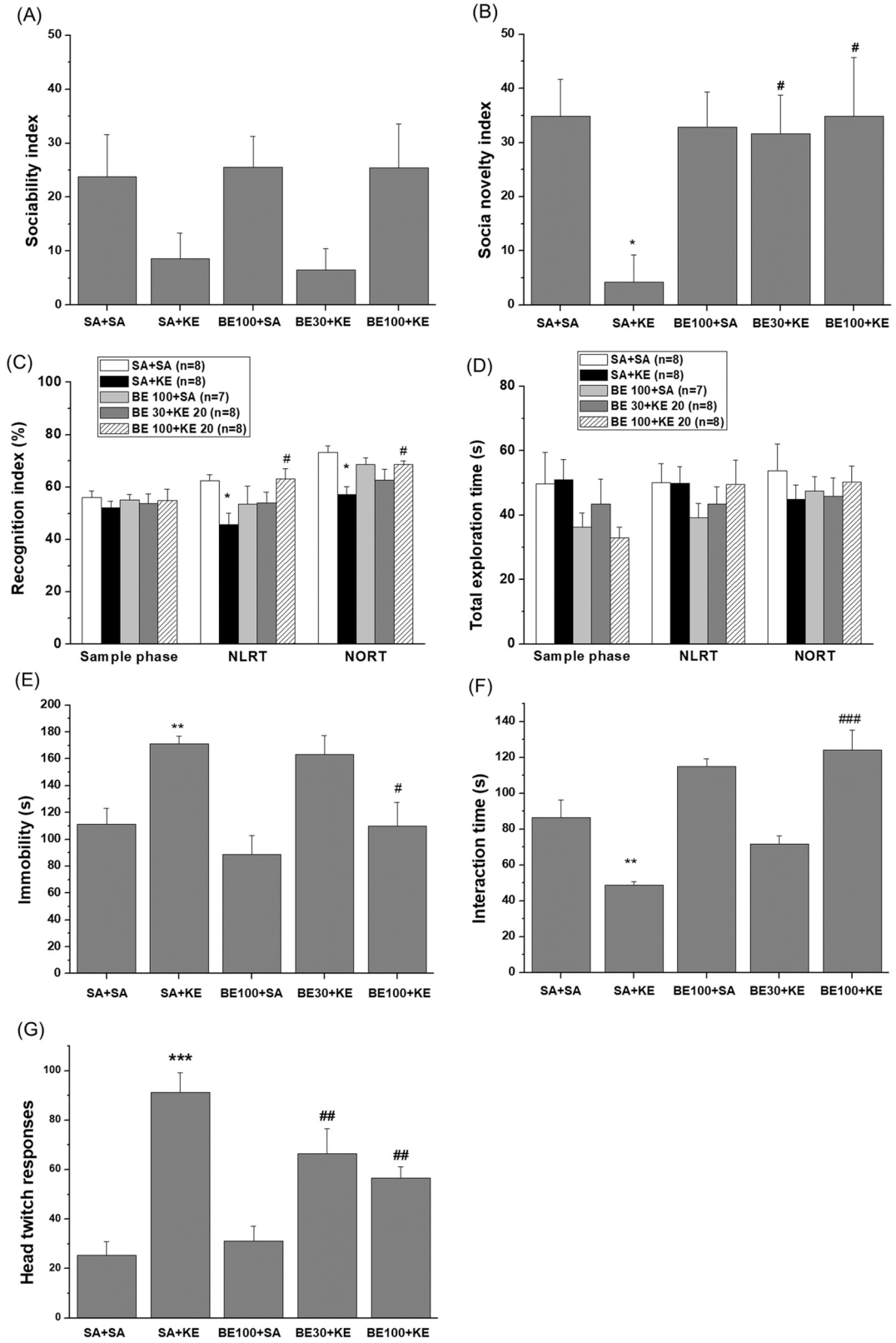

Fig. 2. The combined effects of betaine and ketamine on the behavioral disturbances induced by repeated administrated ketamine. After co-treatment with betaine (30 and 100 mg/kg) and ketamine (20 mg/kg), the psychotic-like and depression-like behavioral tests were determined. The (A) sociability index, (B) social novelty index, (C) recognition index, (D) total exploration time, (E) immobility time, (F) interaction time, and (G) number of DOI-induced head-twitch responses were assessed compared among different treatment groups. The data are expressed as mean±SEM (n = 7–8). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with saline+saline (SA+SA) group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with saline+ketamine (SA+KE) group.

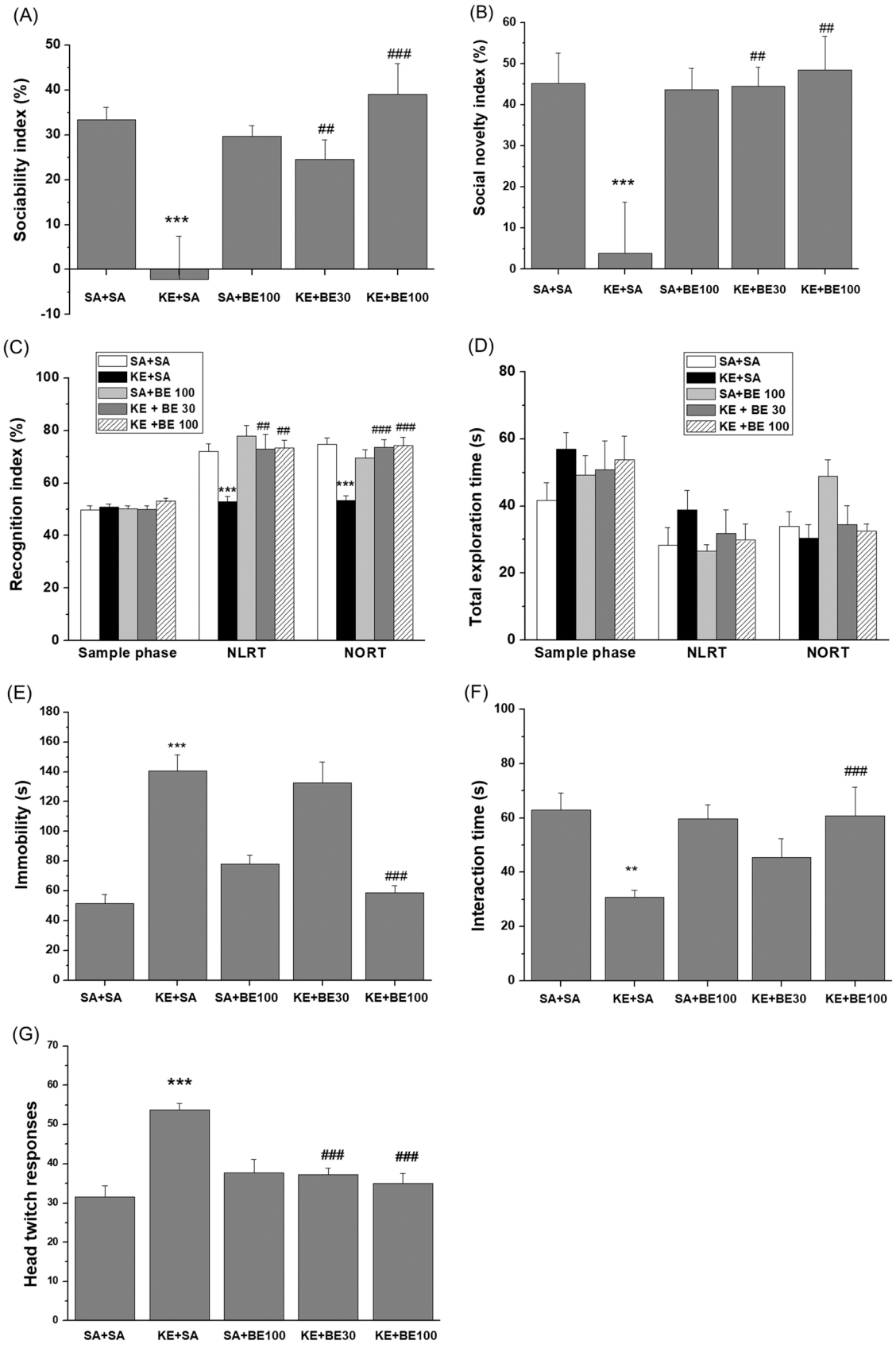

Fig. 3. Post-treatment effects of betaine on the chronic administration of ketamine-induced behavioral disturbances. After subsequent treatment of betaine (30 and 100 mg/kg) following ketamine (20 mg/kg) exposure, the psychotic-like and depression-like behavioral tests were examined. The (A) sociability index, (B) social novelty index, (C) recognition index, (D) total exploration time, (E) immobility time, (F) interaction time, and (G) number of DOI-induced head twitch responses were assessed compared among different treatment groups. The data are expressed as mean±SEM (n = 7–8). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with saline+saline (SA+SA) group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with saline+ketamine (KE+SA) group.

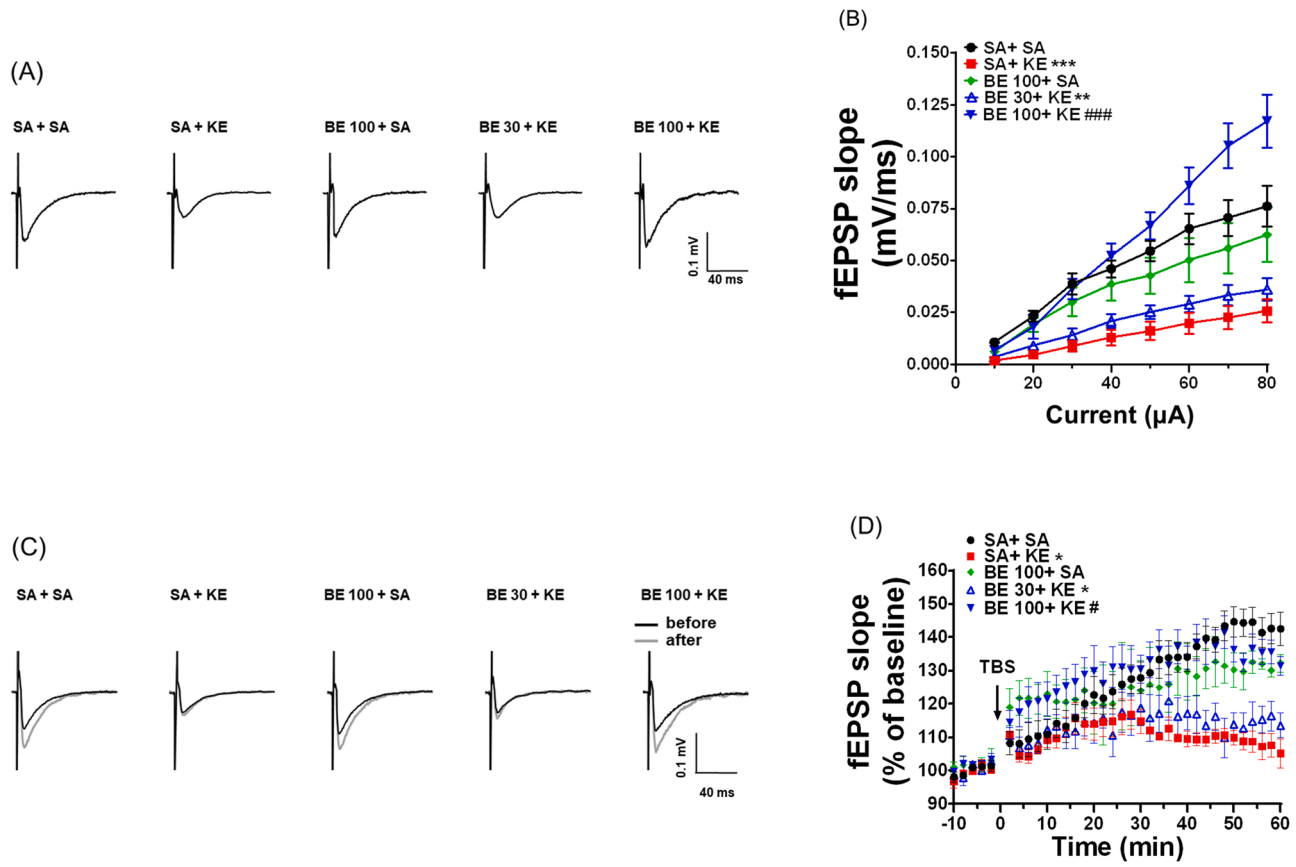

Fig. 4. Co-treatment effects of betaine on subchronic ketamine-induced impairments in basal synaptic transmission and synaptic plasticity. Combined treatment of betaine at 30 and 100 mg/kg with ketamine modulated the fEPSPs in Schaffer collateral-CA1 synapses. (A) Representative traces show the fEPSPs under electrical stimulation at 50 μA. (B) Input/output (I/O) curves obtained by plotting the fEPSP slope in the hippocampal CA1 area under various stimulation intensity from 20 to 80 μA. (C) Typical recordings of LTP induction of fEPSPs evoked by TBS in the stratum radiatum layer of the CA1 area. (D) The plot presents the fEPSP slope in TBS-evoked LTP at different time from 0 to 60 min. Arrow indicates application of TBS to induce LTP. The data are expressed as mean±SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with saline+saline (SA+SA) group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$comapred with saline+ketamine (SA+KE) group. n = 4–5 in each group.

3.5. *Effects of betaine post-treatment after ketamine exposure on synaptic functions*

The effects of betaine post-treatment after repeated ketamine exposure on hippocampal fEPSPs in response to electrical stimulation were shown in Fig. 5A. The I/O curves results revealed main effects of treatment ($F_{4, 16} = 4.34$, $p < 0.05$), current intensity ($F_{6, 96} = 227.70$, $p < 0.05$), and significant interaction ($F_{24, 96} = 6.61$, $p < 0.05$). The ketamine-induced reduction in the slope of I/O curves was reversed following post-treatment of betaine at 30 and 100 mg/kg (Fig. 5B).

The effects of betaine post-treatment on ketamine-induced LTP inhibition were shown in Fig. 5C. Two-way mixed-design ANOVA showed significant effects of treatment ($F_{4,16} = 4.8$, $p < 0.05$) and time ($F_{29, 465} = 6.71$, $p < 0.05$). The fEPSP slope under TBS was significantly attenuated by repeated ketamine exposure and the inhibition was remarkably reversed by betaine at 30 and 100 mg/kg (Fig. 5D).

3.6. *Effects of betaine post-treatment after ketamine exposure on PSD-95 expression*

The postsynaptic glutamatergic marker protein PSD-95 in stratum radiatum of CA1 were further determined and compared by immunohistochemistry staining (Fig. 6). The reduced optical density was significantly observed in ketamine treated group which was reversed by post-treatment of betaine at 30 and 100 mg/kg. In addition, betaine alone at 100 mg/kg increased the PSD-95 level (Fig. 6B).

4. Discussion

The present study was undertaken to reveal the protective and reversing efficacy of betaine against psychotic-like defects induced by repeated ketamine administration in mice. Our findings demonstrated that repeated ketamine exposure bi-daily at sub-anesthetic dose (20 mg/kg) for 2 weeks contributed to behavioral disturbances, including the decreased sociability and social novelty in TCT, recognition memory impairments in NLRT/NORT, social withdrawal in RSIT, depression-like response in FST, and psychedelic action in hallucinogen-induced HTR in both male and female ICR mice. There is considerable correlation between ketamine-elicited psychotic behaviors and reduction in hippocampal synaptic function, such as fEPSP, LTP, and PSD-95 protein levels. Importantly, these animal behavioral disturbances and synaptic defects induced by repeated ketamine exposure can be protected by betaine co-treatment and reversed by betaine post-treatment at 30 and/or 100 mg/kg. These results suggest that betaine co-treatment could be helpful to avoid the adverse-effects of ketamine for chronic treatment of major depression. Furthermore, betaine post-treatment might reverse the psychotic-like disorders induced by repeated ketamine exposure, indicating that betaine could be a valuable antipsychotic candidate.

The behavioral abnormalities induced by repeated ketamine administration represent the predictive validity for positive, negative, and cognitive symptoms of schizophrenia [27,28]. Currently, ketamine is commonly used to provoke schizophrenia-like disorders in rodent models [29]. Consistent to our present results, supporting evidence demonstrates that subchronic administration of ketamine with

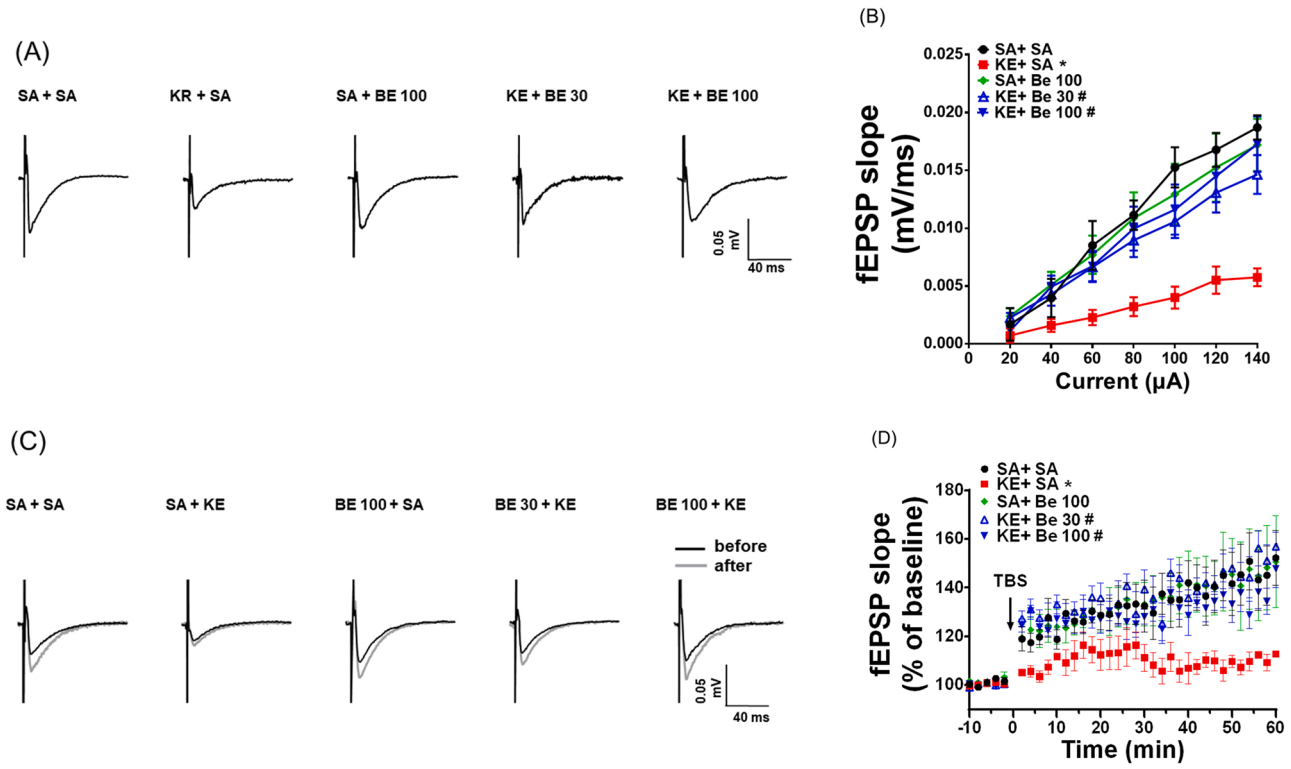

Fig. 5. Post-treatment effects of betaine on the subchronic ketamine-induced impairments of basal synaptic transmission and synaptic plasticity. Post-treatment of betaine at 30 and 100 mg/kg (BE30 & BE100) and ketamine were examined to record fEPSPs in the Schaffer collateral-CA1 synapses. (A) Representative traces show the fEPSPs under electrical stimulation at 50 μA. (B) I/O curves obtained by plotting the fEPSP slope in the hippocampal CA1 area under various stimulation intensity from 20–140 μA. (C) Typical recordings of LTP induction of fEPSPs evoked by TBS in the stratum radiatum layer of the CA1 area. (D) The plot presents the fEPSP slope in TBS-evoked LTP at different time from 0 to 60 min. Arrow indicates application of TBS to induce LTP. The data are expressed as mean±SEM. *$p < 0.05$ compared with saline+saline (SA+SA) group; $^{\#}p < 0.05$ compared with ketamine+saline (KE+SA) group, $n = 4–5$ in each group.

subanesthetic doses could cause psychosis-related alterations in rodents, particularly sociability and social novelty defects in TCT [30], social withdrawal in RSIT [31], cognitive dysfunction in NLRT/NORT [32], enhanced immobility in FST [33], and psychotic-like behavior in DOI-induced head-twitch response [34]. It was further noted that these psychotic disorder behaviors induced by repeated ketamine applications may be not due to motor dysfunction, since the locomotor activity and motor coordination are not altered in present studies and previous reports.

Acute administration of ketamine at sub-anesthetic doses has been developed for depressant treatment [1]. However, healthy human subjects treated with ketamine at sub-anesthetic doses exhibit cognitive and memory impairments in many tasks and produce behavioral disturbances similar to the positive or negative symptoms of schizophrenia [35–37]. Ketamine also provokes exacerbation of preexisting psychotic symptoms and cognitive impairments resembling those seen in schizophrenics [38]. Unfortunately, these side-effects associated with acute use of ketamine in treatment-refractory depression are common and similar to those observed in healthy subjects [39]. Furthermore, chronic ketamine users usually experience psychiatric disorders with body image aberrations [40] and cognitive impairments [41], more akin to schizophrenia. The brain lesions in ketamine addicts have been revealed by magnetic resonance imaging (MRI) after ketamine abuse for 2–4 years [42]. Cortical atrophy that presents the diffuse effects of ketamine in many brain regions could explain the pervasive dysfunction similar to schizophrenic patients. The frontal gray matter volume is reduced in patients after chronic ketamine use [43]. In agreement with clinical studies, the white matter microstructural abnormality is induced by repeated exposures to ketamine in a non-human primate model [44]. Therefore, the safety of repeated ketamine treatment for patients with treatment-resistant depression (TRD) is warranted to consider, since TRD patients might exhibit additive, hallucinations, and cognitive deficits during ketamine infusion [45].

Ketamine has been reported to increase 5-HT-provoked EPSC in mPFC neurons in rat [46]. It postulates that ketamine exerts its antidepressant efficacy in TRD associated with activation of 5-HT neurotransmission in mPFC [47,48]. Importantly, activation of serotonin 2 A receptor (5-HT_{2A}R) could provoke hallucinations and schizophrenia-like symptoms in humans [49]. The hallucinogen-like and psychotomimetic behavior associated with 5-HT_{2A}R activation can be blocked by 5-HT_{2A} receptor antagonists [50]. The role for 5-HT_{2A}R in the actions of ketamine is further supported by evidence that the discriminative stimulus effects of ketamine are partially blocked by a selective 5-HT2A antagonist [51]. Moreover, direct injection of DOI, a hallucinogenic 5-HT_{2A}R agonist, into the medial prefrontal cortex in rats has been shown to induce head-twitch response (HTR) [52]. Notably, ketamine could increase 5-HT2 receptor mediated HTR, which is blocked by a 5-HT_{2A}R antagonist [53]. Consistently, our findings demonstrate that repeated ketamine exposure significantly enhanced DOI-induced HTR, mimicking the development of psychotic hallucination in chronic ketamine users. It is noted that betaine both prevented and reversed hypersensitivity to DOI-induced head-twitch behavior. However, it still remains further study whether the exact inhibitory mechanism of betaine against repeated ketamine evoked greater responsiveness to DOI-induced HTR is associated with modulation of 5-HT2 receptor activity.

Ketamine at subanesthetic doses has been shown to disrupt dopamine neurotransmission in the PFC and cognitive functions associated with activation of glutamatergic neurotransmission in rat [54]. Current evidence further indicates that ketamine increases prefrontal glutamate neurotransmission in healthy and depressed subjects [55]. These results demonstrate that ketamine-induced psychiatric behaviors and cognitive deficits may be associated with relative overabundance of glutamate and

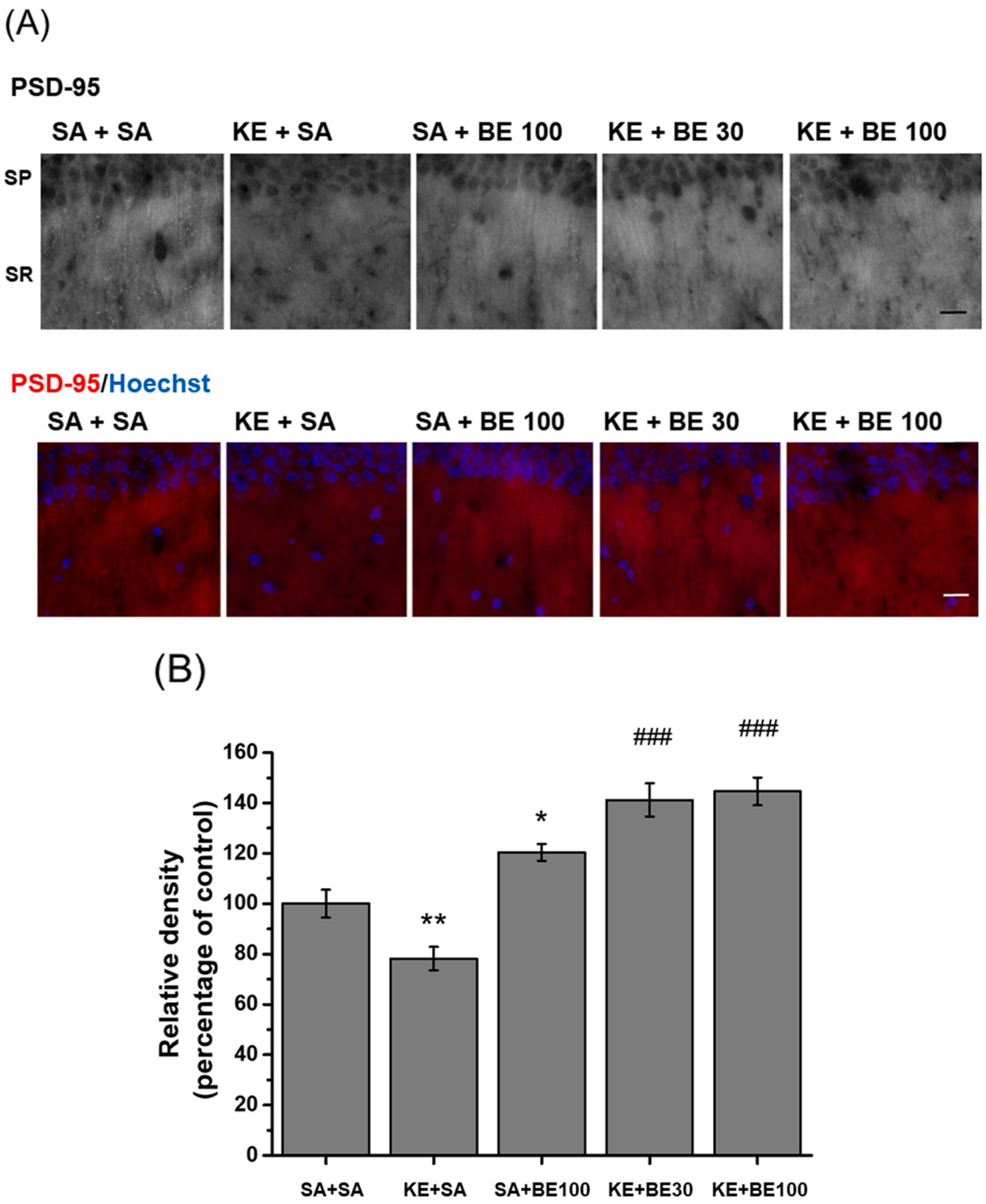

Fig. 6. Post-treatment effects of betaine on the reduced PSD95 expression induced by subchronic ketamine exposure in hippocampal CA1. Subsequent treatment of betaine at 30 and 100 mg/kg (BE 30 & BE 100) following ketamine (KE) exposure was utilized to determine the PSD95 expression in hippocampal CA1. (A) The coronal sections of hippocampal CA1 were immunofluorescent staining with an antibody against PSD95 (red: PSD95; blue: DAPI) in the stratum radiatum (SR). (B) The mean relative fluorescent density of PSD95 immunoreactivity was quantified. The data are expressed as mean ± SEM. *$p < 0.05$ and **$p < 0.01$ compared with saline+saline (SA+SA) group; $^{###}p < 0.001$ compared with ketamine+saline (KE+SA) group, n = 3 animals in each group. SP, stratum pyramidale.

NMDAR activity. Moreover, chronic ketamine exposure could excessively increase synaptic glutamate release in cortex and striatum and deregulate neuronal calcium homeostasis [56]. In our present experiments, the glutamate outflow might be more likely occurred after repeated ketamine exposure. The glutamate overflow could bind with extra-synaptic NMDARs of the post-synaptic neurons which preferentially initiate neurotoxicity and cell death pathways, pivotally resulting in psychosis evoked by ketamine exposure [57].

According to the pharmacological, clinical, post-mortem, and animal studies, the glutamatergic NMDA receptors are suggest to play a significant role in cognitive and psychotic behavioral changes [58–60]. Thus, NMDAR antagonists, such as MK801, PCP, and ketamine, elicit feelings of dissociation and psychotomimetic reactions linked to antagonism of NMDAR [61]. Chronic ketamine exposure also effectively induces schizophrenia associated cognitive impairments and related to NMDAR hypofunction [62]. Considering ketamine-induced psychiatric effects, the increasing evidence indicates that the molecular mechanism of ketamine is more complicated than just inhibition of NMDAR. In addition, ketamine appears to modulate glutamatergic regulation via actions at AMPA receptors and downstream signaling pathways for

synaptic plasticity. Thus, development of antipsychotic drugs by modulating the NMDA receptor function is believed to be appropriately effective for treatment of behavioral impairments in ketamine-induced relevant phychotomimetic disorders.

With regard to increasing glycine level and stimulating glycine site of NMDAR, the glycine transporter inhibitor has been reported to attenuate the psychotomimetic effects of ketamine in healthy male subjects [63]. Acting as an NMDAR modulator with glycine binding site partial agonist properties, betaine might have potential to be an adjunct to ketamine treatment for depression. Our previous findings show that betaine could enhance anti-depressant-like, but alleviate psychotomimetic adverse-effects elicited by a single ketamine administration [7]. The present study further indicates that betaine co-treatment and post-treatment could effectively prevent and reverse the adverse behavioral impairments induced by repeated ketamine use, including the decreased sociability and social novelty, recognition memory impairments, social withdrawal, depression-like response, and psychedelic action elicited by hallucinogen in both male and female ICR mice. These results are in agreement with the expected impact of betaine on modulating NMDA receptors. It further suggests that long-term reduction in NMDAR function, leading to synaptic remodeling and dysfunction, may play a critical role in persistent behavioral disturbances caused by repeated ketamine exposure. Therefore, betaine might protect and recover the behavioral disturbances and deficits in synaptic transmission associated with repeated ketamine exposure through modulation of NMDAR activity. The exact mechanisms underlying the protective and improving effects of betaine against ketamine-induced psychiatric disorders remain incompletely resolved. Analyzing the cellular and/or molecular signaling in detail brain regions and circuitry involved in its pharmacological action is crucial.

There is considerable correlation between ketamine-elicited psychotic behaviors and reduction in hippocampal synaptic function. Acute ketamine administration has been shown to inhibit the induction of LTP in rodent hippocampal-prefrontal synapse [64], hippocampal synapse [65,66], and lateral entorhinal cortex-dentate gyrus synapses [67], probably associated with blockade of NMDAR activity. However, it is wondered if the inhibitory effect of acute ketamine on hippocampal LTP could persist after recovery from anesthesia. Moreover, we are not yet fully understood whether chronic exposure of ketamine at subanesthetic doses sufficient to cause persistent alterations of hippocampal LTP and synaptic plasticity. Current report showed that neonatal exposure of ketamine could inhibit the induction of hippocampal LTP in adult rats [68]. Regarding the effects of chronic ketamine treatment on basal excitatory synaptic transmission and plasticity, our present results indicated that fEPSP and LTP were attenuated after repeated ketamine exposure. Furthermore, the inhibition of hippocampal synaptic plasticity was correlated with reduction in PSD-95 expression elicited by repeated ketamine exposure. Since PSD-95 is a key scaffolding protein implicated in excitatory synaptic signaling, cognition, and memory, the reduced PSD-95 protein levels may be associated with cognitive dysfunction. Consistent to our findings, the repeated long-term ketamine administration has been shown to produce cognitive impairments associated with decreased expression of glutamate receptor subunits and synaptic protein PSD-95 and reduction of hippocampal LTP and synaptic transmission [69]. Our results further highlight the interactions of betaine and ketamine in behavioral disturbances and synaptic dysfunction. Importantly, betaine with co-treatment and post-treatment can mediate the preventive and reversing effects on the ketamine-induced behavioral disturbances and reduction of hippocampal synaptic plasticity and PSD-95 protein expression. The glycine partial agonists, D-cycloserine and GLYX-13, on NMDAR have been shown to enhance LTP in hippocampus [70,71]. In contrast, betaine alone slightly but not significantly impaired fEPSP, suggesting that betaine possibly acts as a partial agonist at NMDAR glycine binding site. Thus, repeated treatment of betaine slightly affect the neurotransmission, reflecting in the I/O curves might be due to its antagonistic action without ketamine treatment. However, betaine might modulate the NMDAR function to reduce the blocking effect of ketamine on NMDA receptor. Moreover, GLYX-13 could prevent acute and subchronic phencyclidine- and ketamine-induced memory impairments determined by NORT in mice [72]. Therefore, these results further support that the pharmacological action of betaine with glycine site partial agonist properties may promote the synaptic plasticity in hippocampus. These effects of betaine on plasticity-related processes could be linked to its cognitive enhancing and antidepressant actions, as well as to recover the inhibitory effects of ketamine in a cognition and memory task.

In conclusion, betaine with co-treatment and post-treatment can mediate the preventive and reversing effects on subchronic ketamine-induced psychotic behavioral deficits, cognitive impairments, and reduction of synaptic function, LTP, and PSD-95 protein expression. These findings of this study lay the groundwork for conducting clinical studies to verify the effectiveness of betaine as an adjunct therapy to resolve the safety concern of clinical ketamine use for the management of treatment-resistant depression and other psychiatric disorders.

CRediT authorship contribution statement

Shao-Tsu Chen, Ming-Huan Chan, Hwei-Hsien Chen: Participated in the study concept. **Mei-Yi Lee, Chien-Min Huang**: Contributed to the acquisition and analysis of the behavioral data. **Chung-Pin Hsieh**: Conducted the electrophysiological assay, Analyzed the data. **Liao-Chen Chen**: Conducted the immunofluorescence staining. **Shao-Tsu Chen, Chung-Pin Hsieh**: Partly drafted the manuscript. **Ming-Huan Chan, Hwei-Hsien Chen**: Provided critical revision of the manuscript. All Authors have approved the manuscript being submitted.

Conflict of interest statement

The authors report no conflicts of interest.

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