

# Raphe AMPA receptors and nicotinic acetylcholine receptors mediate ketamine-induced serotonin release in the rat prefrontal cortex



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## Abstract

Several lines of evidence indicate that ketamine has a rapid antidepressant-like effect in rodents and humans, but underlying mechanisms are unclear. In the present study, we investigated the effect of ketamine on serotonin (5-HT) release in the rat prefrontal cortex by *in vivo* microdialysis. A subcutaneous administration of ketamine (5 and 25 mg/kg) significantly increased the prefrontal 5-HT level in a dose-dependent manner, which was attenuated by local injection of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) antagonists into the dorsal raphe nucleus (DRN). Direct stimulation of AMPARs in the DRN significantly increased prefrontal 5-HT level, while intra-DRN injection of ketamine (36.5 nmol) had no effect. Furthermore, intra-DRN injection of an  $\alpha_4\beta_2$ -nicotinic acetylcholine receptor (nAChR) antagonist, dihydro- $\beta$ -erythroidine (10 nmol), significantly attenuated the subcutaneous ketamine-induced increase in prefrontal 5-HT levels. These results suggest that AMPARs and  $\alpha_4\beta_2$ -nAChRs in the DRN play a key role in the ketamine-induced 5-HT release in the prefrontal cortex.

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## Introduction

Major depressive disorder (MDD) is a common mental illness characterized by sustained depressive mood, diminished interest and suicidal ideation. Although around two thirds of MDD patients respond to drug therapy, the presence of treatment-resistant MDD patients is still of concern; therefore, it is considered to be a serious public health problem throughout the world (Belmaker and Agam, 2008). Several clinical studies demonstrate that a single subanesthetic dose of an *N*-methyl-D-aspartate receptor (NMDAR) antagonist, ketamine, produces rapid and sustained antidepressive effects in patients with treatment-resistant MDD (Berman et al., 2000). Also, in rodents, ketamine produces rapid antidepressant-like effects in various behavioral tests, including the learned helplessness, forced swim test and novelty-suppressed feeding test, which can be blocked by systemic administration of  $\alpha$ -amino-3-hydroxy-5-methyl-

4-isoxazolepropionic acid receptor (AMPA) antagonist (Maeng et al., 2008; Autry et al., 2011; Duman and Voleti, 2012). Although the mechanism by which NMDAR antagonism induces AMPAR activation has not been fully delineated, it is suggested that ketamine blocks NMDARs on GABAergic interneuron, resulting in the disinhibition of glutamatergic neurons and an increase of extracellular glutamate (Homayoun and Moghaddam, 2007). On the other hand, most of first-line antidepressants including selective serotonin reuptake inhibitors (SSRIs) increase the extracellular monoamine (i.e. serotonin (5-HT), dopamine and/or norepinephrine) levels in the brain. Hence, it is widely believed that monoaminergic systems, especially serotonergic systems, play an important role in the antidepressive effects (Belmaker and Agam, 2008). Similar to SSRIs, acute administration of ketamine increases 5-HT levels in the medial prefrontal cortex (mPFC), one of the key brain structures in the pathophysiology of MDD (Amargos-Bosch et al., 2006). Furthermore, depletion of 5-HT by *p*-chlorophenylalanine attenuates antidepressant-like effect of ketamine (Gigliucci et al., 2013). Therefore, it is possible that the prefrontal 5-HT increase by ketamine is involved in its antidepressant-like effects, although underlying mechanisms are unclear.

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We previously reported that sustained exposure to SSRIs caused the augmentation of 5-HT release through the activation of AMPARs, but not NMDARs, in the rat raphe organotypic slice cultures, consistent with other reports in which AMPA increases firing frequency of 5-HT neuron in the dorsal raphe nucleus (DRN) (Gartside et al., 2007; Nagayasu et al., 2010). However, the role of glutamate receptors in the DRN in ketamine-induced 5-HT increase is still unknown. Furthermore, recent studies reported that endogenous acetylcholine (ACh) and nicotine increase 5-HT neuronal excitability by increasing glutamate release through presynaptic  $\alpha_4\beta_2$ -nicotinic ACh receptors (nAChRs) in the DRN (Chang et al., 2011; Garduno et al., 2012). In this study, the roles of AMPARs and  $\alpha_4\beta_2$ -nAChRs in the DRN in ketamine-induced 5-HT increase in the mPFC were examined by *in vivo* microdialysis in rats.

## Materials and methods

All animal care and experimental procedures were in accordance with the ethical guidelines of the Kyoto University Animal Research Committee. Male Wistar/ST rats (250–320 g; Nihon SLC, Japan) were used. The animals were housed in groups of two per cage with free access to food and water, and kept under controlled conditions (12 h light/dark cycle;  $24 \pm 1$  °C). Under sodium pentobarbital anesthesia (60 mg/kg i.p.), each rat was stereotaxically implanted with guide cannula (Eicom, Japan) in the mPFC (AP +3.2 mm, ML +0.6 mm, DV +4.0 mm, from bregma, according to the Brain Atlas (Paxinos and Watson, 2007)) for *in vivo* microdialysis and in the DRN (AP –7.7 mm, ML +2.4 mm, DV +7.0 mm, from bregma) for local drug microinjection. The guide cannulae were secured to the skull with dental cement. After 1–2 d recovery period, microdialysis experiments were performed in unanesthetized and freely moving rats. Microdialysis probes (membrane length 2 mm, o.d. 0.22 mm; Eicom) were inserted through the guide cannula and perfused at a constant flow of 1  $\mu$ l/min with Ringer buffer (147 mM NaCl, 4 mM KCl, 2.3 mM CaCl<sub>2</sub>), containing 1  $\mu$ M citalopram. Dialysates were collected every 10 min and immediately analyzed by high-performance liquid chromatography with electrochemical detector (HPLC-ECD, Eicom). 5-HT was separated on a reverse-phase column (PPII-ODS column, 3.0  $\phi$   $\times$  150 mm; Eicom), and quantified by reference to a linear calibration curve, as previously described (Nagayasu et al., 2010).

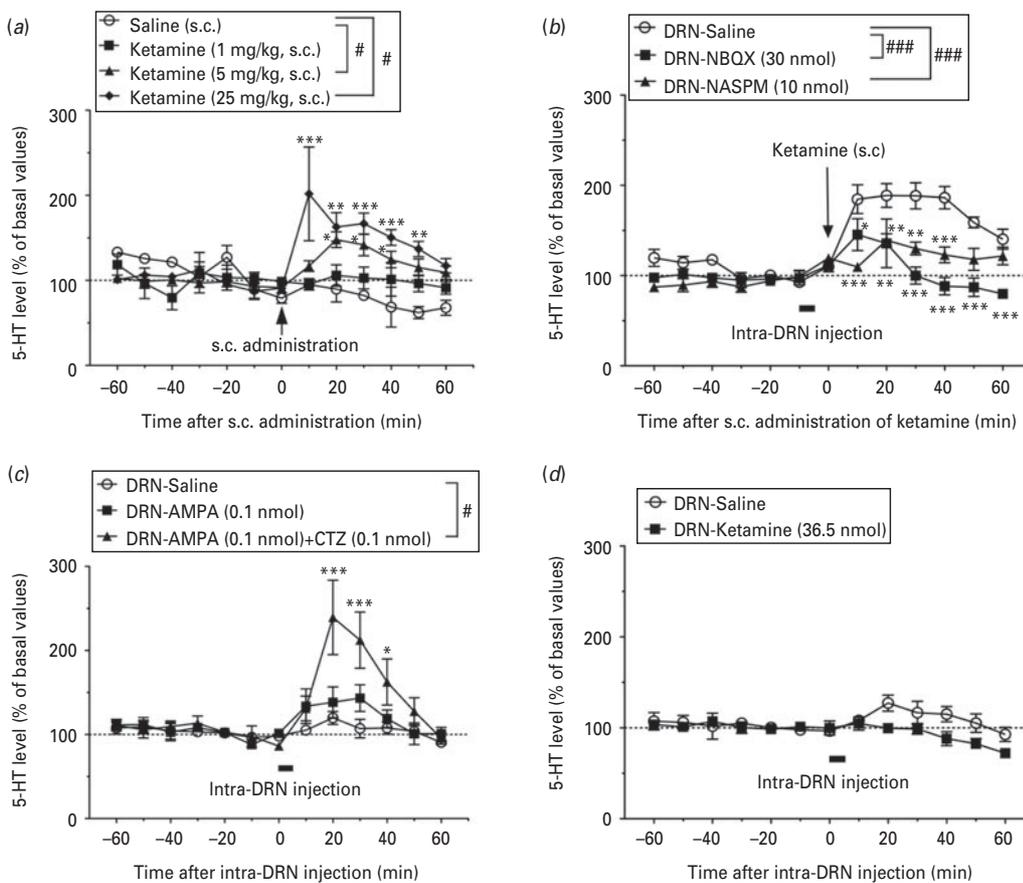
Ketamine (Ketalar; Daiichi Sankyo Propharma Co., Ltd., Japan) was administered subcutaneously (s.c.) or microinjected into the DRN through stainless-steel injection cannula (28 gauge). For microinjection of ketamine into the DRN, we used the concentration of 36.5 nmol/1  $\mu$ l, which is the highest concentration achievable with Ketalar. 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), cyclothiazide, 1-naphthylacetyl spermine (NASPM) (Sigma-Aldrich,

USA), AMPA, dihydro- $\beta$ -erythroidine (DH $\beta$ E) and RJR-2403 (Tocris Bioscience, UK) were dissolved in sterile saline (Otsuka Pharmaceutical Co., Ltd, Japan) and microinjected into the DRN through the injection cannula. In microdialysis experiments, all drugs were administered after 3–4 h stabilization period. Intra-DRN drug microinjection (1  $\mu$ l) was performed at 0.2  $\mu$ l/min for 5 min by microinfusion pump 10 min before s.c. administration of ketamine. After all tests, histological analyses were performed to verify the placement of each tip of microdialysis probe (Nissl stain) or injection cannula (Evans Blue injection). The animals in which the tips incorrectly located were excluded from the final analysis.

Data are presented as means  $\pm$  S.E.M of the percentage of the basal values calculated as an average of four consecutive dialysate 0–30 min before drug administration. Data were analyzed by two-way analysis of variance (ANOVA) for repeated measures, followed by Bonferroni *post-hoc* test. The area under the curve (AUC) for dose-response of ketamine was analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. Differences of  $p < 0.05$  were considered statistically significant.

## Results

We investigated the effect of ketamine (1, 5 or 25 mg/kg, s.c.) on the 5-HT levels in the mPFC (Fig. 1a). Consistent with previous studies (Amargos-Bosch et al., 2006; Lopez-Gil et al., 2012), s.c. administration of ketamine (5 and 25 mg/kg) rapidly increased 5-HT levels in the mPFC in a dose-dependent manner (5 mg/kg;  $F_{1,4} = 9.49$ ,  $p < 0.05$ , 25 mg/kg;  $F_{1,4} = 15.1$ ,  $p < 0.05$ ), although 1 mg/kg ketamine did not affect 5-HT levels in the mPFC ( $F_{1,4} = 0.17$ ,  $p = 0.70$ ). AUC for 5-HT concentration 0–60 min after the administration was increased by ketamine in a dose-dependent manner ( $F_{3,8} = 10.84$ ,  $p < 0.01$ ; Supplementary Fig. 1). Since systemic administration of AMPAR antagonists attenuates antidepressant-like efficacy of ketamine (Maeng et al., 2008; Autry et al., 2011), we investigated whether AMPARs in the DRN mediates ketamine-induced 5-HT release in the mPFC (Fig. 1b). A microinjection of an AMPAR antagonist, NBQX (30 nmol), into the DRN 10 min before s.c. ketamine administration significantly attenuated the ketamine-induced 5-HT release ( $F_{1,6} = 44.7$ ,  $p < 0.001$ ). Likewise, a microinjection of a Ca<sup>2+</sup>-permeable AMPAR antagonist, NASPM (10 nmol), also attenuated ketamine-induced 5-HT release ( $F_{1,5} = 58.5$ ,  $p < 0.001$ ). Consistently, co-application of AMPA (0.1 nmol) and an inhibitor of AMPAR desensitization, cyclothiazide (0.1 nmol, cyclothiazide (CTZ)), into the DRN significantly enhanced 5-HT levels in the mPFC to about 200% of the baseline. Significant increases were observed at 20 and 30 min after the microinjection of AMPA and CTZ (Fig. 1c), whereas AMPA (0.1 nmol) alone did not. However, a microinjection of ketamine (36.5 nmol/1  $\mu$ l) into the



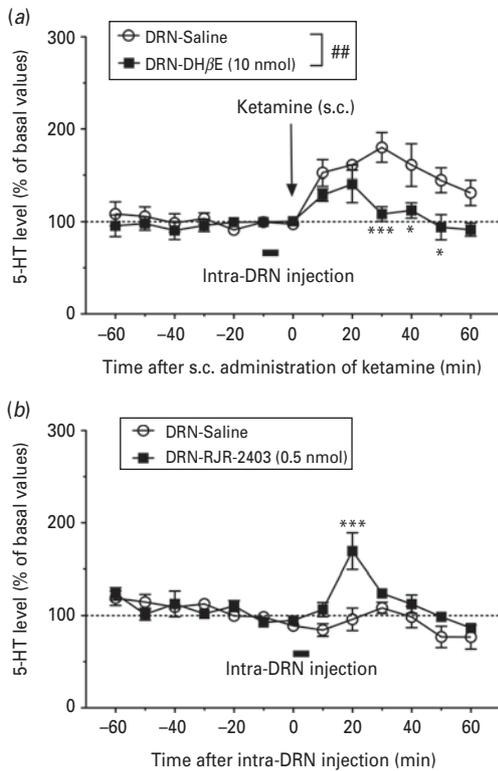
**Fig. 1.** Systemic administration of ketamine increased 5-HT levels in the medial prefrontal cortex (mPFC) through activation of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptors in the dorsal raphe nucleus (DRN). Extracellular 5-HT levels in the mPFC were continuously measured for 120 min before and after drug administration by *in vivo* microdialysis. (a) Saline or ketamine (1, 5 or 25 mg/kg) was administered subcutaneously (s.c.) at 0 min (arrow). # $p < 0.05$  main effect in two-way analysis of variance (ANOVA), \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  vs. saline-administered group (*post-hoc* test).  $n = 3$ . Basal values for 5-HT concentrations were  $0.85 \pm 0.05$  nM (saline),  $1.03 \pm 0.20$  nM (ketamine 1 mg/kg),  $1.26 \pm 0.19$  nM (ketamine 5 mg/kg) and  $0.93 \pm 0.06$  nM (ketamine 25 mg/kg). (b) Saline, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) (30 nmol) or 1-naphthylacetyl spermine (NASPM) (10 nmol) was microinjected into the DRN from -10 to -5 min (bar). Then, ketamine (25 mg/kg) was administered s.c. at 0 min (arrow). ### $p < 0.001$  main effect in two-way ANOVA vs. DRN-saline group, \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  vs. DRN-saline group (*post-hoc* test).  $n = 3-4$ . Basal values for 5-HT concentrations were  $0.56 \pm 0.12$  nM (DRN-saline),  $0.73 \pm 0.03$  nM (DRN-NBQX) and  $0.96 \pm 0.12$  nM (DRN-NASPM). (c) Saline, AMPA (0.1 nmol) or AMPA (0.1 nmol) + cyclothiazide (CTZ; 0.1 nmol) was microinjected into the DRN from 0 to 5 min (bar). Then, ketamine (25 mg/kg) was administered s.c. at 0 min (arrow). \*\*\* $p < 0.001$ , \* $p < 0.05$  vs. DRN-saline group (*post-hoc* test).  $n = 3-6$ . Basal values for 5-HT concentrations were  $0.87 \pm 0.17$  nM (DRN-saline),  $0.85 \pm 0.09$  nM (DRN-AMPA) and  $0.57 \pm 0.09$  nM (DRN-AMPA + CTZ). (d) Saline or ketamine (36.5 nmol) was microinjected into the DRN from 0 to 5 min (bar).  $n = 4$ . Basal values for 5-HT concentrations were  $0.70 \pm 0.13$  nM (DRN-saline) and  $0.54 \pm 0.08$  nM (DRN-ketamine).

DRN did not increase, but rather tended to decrease 5-HT levels in the mPFC ( $F_{1,6} = 4.13$ ,  $p = 0.0884$ ; Fig. 1d).

Next, we examined the involvement of  $\alpha_4\beta_2$ -nAChRs in the DRN in ketamine-induced 5-HT release in the mPFC. A microinjection of an  $\alpha_4\beta_2$ -nAChR antagonist, DH $\beta$ E (10 nmol), into the DRN before s.c. ketamine administration significantly attenuated ketamine-induced 5-HT release in the mPFC ( $F_{1,7} = 5.95$ ,  $p < 0.05$ ; Fig. 2a). Consistently, a microinjection of  $\alpha_4\beta_2$ -nAChR agonist, RJR-2403 (0.5 nmol), into the DRN significantly increased 5-HT levels in the mPFC to approximately 170% of the baseline. Significant increases were observed at 20 min after the microinjection (Fig. 2b).

## Discussion

In the present study using *in vivo* microdialysis with local injection of drugs into the DRN, we showed that blockade of AMPARs, especially  $\text{Ca}^{2+}$ -permeable AMPARs, and  $\alpha_4\beta_2$ -nAChRs in the DRN attenuated the ketamine-induced prefrontal 5-HT release. By contrast, direct blockade of NMDA receptors in the DRN by ketamine had no effect on prefrontal 5-HT levels. These results suggest that ketamine induces prefrontal 5-HT release through the activation of  $\text{Ca}^{2+}$ -permeable AMPA receptors and  $\alpha_4\beta_2$ -nAChRs in the DRN. The present data corroborated the sufficiency of DRN-AMPA stimulation for 5-HT



**Fig. 2.** Systemic administration of ketamine increased 5-HT levels in the medial prefrontal cortex (mPFC) through activation of  $\alpha_4\beta_2$ -nicotinic acetylcholine receptors (nAChRs) in the dorsal raphe nucleus (DRN). Extracellular 5-HT levels in the mPFC were continuously measured for 120 min before and after drug administration by *in vivo* microdialysis. (a) Saline or an  $\alpha_4\beta_2$ -nAChR antagonist, DH $\beta$ E (10 nmol), was microinjected into the DRN from -10 to -5 min (bar). Then, ketamine (25 mg/kg) was administered subcutaneously (s.c.) at 0 min (arrow).  $^{##}p < 0.01$  main effect in two-way analysis of variance (ANOVA) vs. DRN-saline group,  $^{***}p < 0.001$ ,  $^{*}p < 0.05$  vs. DRN-saline group (*post-hoc* test).  $n = 4-5$ . Basal values for 5-HT concentrations were  $0.67 \pm 0.16$  nM (DRN-saline) and  $0.61 \pm 0.16$  nM (DRN-DH $\beta$ E). (b) Extracellular 5-HT levels in the mPFC were continuously measured for 120 min by microdialysis before and after local administration of into the DRN. Saline or an  $\alpha_4\beta_2$ -nAChR agonist, RJR-2403 (0.5 nmol), was microinjected into the DRN from 0 to 5 min (bar).  $^{***}p < 0.001$  vs. DRN-saline group (*post-hoc* test).  $n = 3$ . Basal values for 5-HT concentrations were  $1.40 \pm 0.13$  nM (DRN-saline) and  $1.18 \pm 0.14$  nM (DRN-RJR2403).

release in the mPFC and the necessity of them in ketamine-induced 5-HT release in the mPFC. Consistent with the present findings, several lines of evidence suggest that AMPARs and NMDARs in the DRN regulate activity of 5-HT neurons (Gartside et al., 2007). However, it is unclear whether systemic ketamine administration increased glutamate levels in the DRN. In this context, it is noteworthy that subanesthetic dose of ketamine enhances glutamate release in the mPFC through blockade of NMDARs on GABA interneurons (Moghaddam et al., 1997; Homayoun and Moghaddam, 2007).

Furthermore, the DRN is innervated by glutamatergic neurons from many brain regions such as PFC, lateral habenula and hypothalamus (Amargos-Bosch et al., 2007). Thus, it is possible that the indirect activation of glutamatergic neurons in these regions might induce glutamate release in the DRN, resulting in 5-HT release in the mPFC. Since the DRN contains GABA interneurons which suppress 5-HT neuronal activities, it is postulated that blockade of NMDA receptors on GABA interneurons by ketamine locally may disinhibit 5-HT neurons in the DRN, resulting in 5-HT release (Boothman et al., 2006). On the contrary, the present study revealed that intra-DRN injection of ketamine had no effect, but rather decreased prefrontal 5-HT levels. In rat organotypic raphe slice culture (Nagayasu et al., 2010), we found that acute treatment with ketamine (1–100  $\mu$ M) decreased the extracellular 5-HT levels (Supplementary Methods and Figure 1), consistent with the previous electrophysiological study in which the application of ketamine to the DRN reduced basal 5-HT neuronal firing rate (McCardle and Gartside, 2012). Taken together, it is suggested that ketamine enhances glutamatergic innervations of the DRN from other brain regions such as mPFC, resulting in activation of 5-HT neurons in the DRN. Consistent with this hypothesis, bilateral perfusion of ketamine into the mPFC is reported to enhance 5-HT release (Amargos-Bosch et al., 2006; Lopez-Gil et al., 2012). Measurement of glutamate release in the DRN following local injection of ketamine into the mPFC would be beneficial.

In addition to the activity of glutamatergic neurons themselves, glutamate release from presynaptic terminals can be enhanced by the activation of presynaptic nAChRs (McGehee et al., 1995). In the DRN, this is caused by the activation of  $\alpha_4\beta_2$ -nAChRs, but not  $\alpha_7$  nAChRs and results in the increase of 5-HT neuronal firing (Garduno et al., 2012). In line with these reports, the present data demonstrated that ketamine-induced 5-HT release in the mPFC requires  $\alpha_4\beta_2$ -nAChRs in the DRN, and also stimulation of them is sufficient to increase prefrontal 5-HT release. Several reports suggest that systemic administration of ketamine or other NMDAR antagonists increases ACh release in the cortex (Kim et al., 1999; Nelson et al., 2002). Besides, the DRN is innervated by cholinergic neurons from pedunculopontine nucleus and laterodorsal tegmentum (Woolf and Butcher, 1989). Although it is not determined whether systemic ketamine administration increases ACh levels in the DRN, our results suggest that the enhancement of cholinergic neuronal activity in these brain areas might underlie ketamine-induced 5-HT release through presynaptic  $\alpha_4\beta_2$ -nAChRs in the DRN.

Conventional antidepressants such as SSRIs are reported to require several weeks of administration to exert their therapeutic effects, and this delay can be partly explained by potent suppression of 5-HT neuronal activity by 5-HT autoreceptor activation (Gardier et al., 1996). Recently, Challis et al., clearly showed that social defeat stress suppresses the 5-HT neuronal firing through

increased activity of GABAergic interneurons in the DRN (Challis et al., 2013). Therefore, it is possible that acute SSRI treatment may not be effective in depressed animals, due to suppressed firing rate of 5-HT neurons. In this context, the present study indicates that ketamine, with doses effective *in vivo* (Yang et al., 2013), rapidly stimulate 5-HT neuronal activity through AMPARs and  $\alpha_4\beta_2$ -nAChRs unlike acute SSRIs. Taken together with the previous findings that the antidepressant-like effect of ketamine is mediated through 5-HT (Gigliucci et al., 2013), it is possible that increasing the 5-HT neuronal firing is important for rapid antidepressant-like effect *in vivo*, although further behavioral and electrophysiological analyses are needed.

In conclusion, the present study revealed that ketamine increases 5-HT release in the mPFC through the activation of AMPARs and  $\alpha_4\beta_2$ -nAChRs in the DRN. The present findings provide evidence for the importance of dorsal raphe AMPARs and  $\alpha_4\beta_2$ -nAChRs in the effect of ketamine, which may underlie rapid therapeutic efficacy of ketamine.

### Supplementary material

For supplementary material accompanying this paper, visit <http://dx.doi.org/10.1017/S1461145714000649>.

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### Statement of Interest

None.

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