



Title	Changes in subunit composition of NMDA receptors in animal models of schizophrenia by repeated administration of methamphetamine
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1 Changes in subunit composition of NMDA receptors in animal models of  
2 schizophrenia by repeated administration of methamphetamine

3

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19 **Abstract**

20 The dopamine and glutamate hypotheses reflect only some of the pathophysiological  
21 changes associated with schizophrenia. We have proposed a new “comprehensive  
22 progressive pathophysiology model” based on the “dopamine to glutamate hypothesis.”  
23 Repeated administration of methamphetamine (METH) at a dose of 2.5 mg/kg in rats has  
24 been used to assess dynamic changes in the pathophysiology of schizophrenia. Previous  
25 use of this model suggested *N*-methyl-D-aspartate receptor (NMDA-R) dysfunction, but  
26 the mechanism could only be inferred from limited, indirect observations. In the present  
27 study, we used this model to investigate changes in the expression of NMDA-R subunits.  
28 Repeated administration of METH significantly decreased the gene expression levels of  
29 glutamate ionotropic receptor NMDA type subunit (*Grin*) subtypes *Grin1* and *Grin2c* in  
30 the prefrontal cortex (PFC), *Grin1* and *Grin2a* in the hippocampus (HPC), and *Grin1*,  
31 *Grin2b*, and *Grin2d* in the striatum (ST). We observed a significant difference in *Grin1*  
32 expression between the PFC and ST. Furthermore, repeated administration of METH  
33 significantly decreased the protein expression of GluN1 in both cytosolic and  
34 synaptosomal fractions isolated from the PFC, and significantly decreased the protein  
35 expression of GluN1 in the cytosolic fraction, but not the synaptosomal fraction from the  
36 ST. These regional differences may be due to variations in the synthesis of GluN1 or

37 intracellular trafficking events in each area of the brain. Considering that knockdown of  
38 *Grin1* in mice affects vulnerability to develop schizophrenia, these results suggest that  
39 this model reflects some of the pathophysiological changes of schizophrenia, combining  
40 both the dopamine and glutamate hypotheses.

41

42

#### 43 **Keywords**

44 Schizophrenia, NMDA receptor, GluN1, Animal model, Methamphetamine

45

46

#### 47 **Abbreviations**

48 CNS, Central nervous system; HPC, Hippocampus; METH, Methamphetamine; NAc,

49 Nucleus accumbens; NMDA-R, *N*-methyl-D-aspartate receptor; PBS, Phosphate-buffered

50 saline; PFC, Prefrontal cortex; PPI, Prepulse inhibition; qRT-PCR, quantitative reverse

51 transcription polymerase chain reaction; Sal, Saline; s.c., Subcutaneous injection; ST,

52 Striatum; TBST, Tris-buffered saline containing 0.5 % Tween 20

53

54

55        **1. Introduction**

56

57        Schizophrenia is a mental illness that affects approximately 1% of the general  
58        population, regardless of sex, race, or nationality. The onset of schizophrenia typically  
59        occurs in the late teens to thirties, manifesting as a variety of symptoms, most of which  
60        are chronic in nature (Carlsson *et al.*, 1997; Lewis and Lieberman, 2000; Van Os. *et*  
61        *al.*, 2010). The precise etiology of schizophrenia remains controversial, but there is  
62        consensus that it is a multifactorial neurodevelopmental disorder that is influenced by  
63        both genetic and environmental factors. The pathology of schizophrenia progresses  
64        from hyperactivity of dopaminergic systems, which was originally posited by the  
65        dopamine hypothesis, to *N*-methyl-D-aspartate receptor (NMDA-R) dysfunction due to  
66        changes in expression or composition of NMDA-R subunits (Olney and Farber, 1995;  
67        Goff and Coyle, 2001).

68

69        NMDA-Rs, which are widely distributed throughout the central nervous system  
70        (CNS), are essential mediators of synaptic transmission and neuronal plasticity. NMDA-  
71        Rs are tetrameric receptors composed of two essential GluN1 subunits along with two  
72        GluN2 or GluN3 subunits, which have four (GluN2A - GluN2D) and two subtypes

73 (GluN3A and GluN3B), respectively. These NMDA-R subtypes differ in their molecular  
74 (subunit) composition, which is plastic and changes during development and in response  
75 to alterations in neuronal activity (Cull-Candy and Leszkiewicz, 2004; Traynelis *et al.*,  
76 2010; Paoletti *et al.*, 2013). The protein name mirrors the gene name, with just the two-  
77 letter code difference (i.e., Grin1 translates to GluN1, Grin2a translates to GluN2A).

78

79 Postmortem analyses of brains taken from patients with schizophrenia have reported  
80 reduced expression of glutamate ionotropic receptor NMDA type subunit (Grin) subtypes  
81 GRIN1 and GRIN2C in the prefrontal cortex (PFC) (Akbarian *et al.*, 1996; Weickert *et*  
82 *al.*, 2013; Catts *et al.*, 2016), and GRIN1 and GRIN2A in the hippocampus (HPC) (Gao  
83 *et al.*, 2000; Law and Deakin, 2001); these genes encode three specific subunits of  
84 NMDA-Rs , GluN1, GluN2C, and GluN2A respectively. A meta-analysis has determined  
85 effect sizes for changes in mRNA and protein expression levels of the essential GluN1  
86 subunit in the PFC in schizophrenia. In schizophrenic patients, compared to unaffected  
87 controls, the pooled effect size was  $-0.64$  (95% confidence interval:  $-1.08$  to  $-0.20$ ) and  
88  $-0.44$  (95% confidence interval:  $-0.80$  to  $-0.07$ ) for reductions in GluN1 mRNA and  
89 protein expression, respectively(Catts *et al.*, 2016).

90

91 We previously reported that repeated administration of methamphetamine (METH),  
92 which increases dopamine levels in the nucleus accumbens (NAc) at a dose of 2.5 mg/kg,  
93 but not at 1.0 mg/kg, also increased glutamate levels in the medial prefrontal cortex  
94 (mPFC) and the NAc (Ito *et al.*, 2006a). At this dose, repeated administration of METH  
95 results in the following: (1) development of behavioral cross-sensitization to MK -801 (a  
96 non-competitive NMDA-R antagonist) (Ito *et al.*, 2006a); (2) prepulse inhibition (PPI)  
97 deficit (Abekawa *et al.*, 2008), which is an indicator of cognitive dysfunction; and (3)  
98 induction of apoptosis in the PFC (Abekawa *et al.*, 2008), indicating brain atrophy. In  
99 addition, administration of atypical antipsychotics and mood stabilizers attenuates some  
100 or all of these changes (Ito *et al.*, 2006b; Abekawa *et al.*, 2008; Nakato *et al.*, 2010;  
101 Abekawa *et al.*, 2011; Nakato *et al.*, 2011).

102

103 Based on these pathophysiological changes induced by repeated METH  
104 administration, we proposed a new “comprehensive progressive pathophysiology model”  
105 based on the “dopamine to glutamate hypothesis” (Abekawa *et al.*, 2012). This model  
106 mimics the dysfunction of NMDA-Rs in schizophrenia. However, in our previous study,  
107 the precise molecular mechanisms could not be determined because changes could only  
108 be inferred from indirect evidence (Table 1).

109

110

111 Table 1. Electrophysiological, molecular, and behavioral changes caused by repeated

112 administration of METH.

	1.0 mg/kg	2.5 mg/kg
Development of behavioral sensitization (Ito <i>et al.</i> , 2006a)	+	+
Delayed increases in glutamate levels in the NAc and PFC (Ito <i>et al.</i> , 2006b; Abekawa <i>et al.</i> , 2008)	–	+
Development of behavioral cross-sensitization to MK-801 (Ito <i>et al.</i> , 2006b; Abekawa <i>et al.</i> , 2008)	–	+
PPI deficit (Abekawa <i>et al.</i> , 2008)	–	+
Apoptosis in the PFC (Abekawa <i>et al.</i> , 2008)	–	+

113 METH, Methamphetamine; NAc, Nucleus accumbens; PFC, Prefrontal cortex; PPI,

114 Prepulse inhibition

115



116

117       Using this model, we aimed to assess changes in both the gene and protein expression  
118 levels of NMDA-R subunits, including *Grin1*, *Grin2a*, *Grin2b*, *Grin2c*, and *Grin2d* in the  
119 PFC, the HPC, and the striatum (ST) to more precisely elucidate the molecular  
120 mechanisms driving changes in receptor functionality that mediate behavior.

121

122

## 123       2. Materials and methods

124

### 125       2.1. Animals

126       Seven-week-old male Sprague–Dawley rats (Sankyo Labo Service Corporation,  
127 Inc., Japan), weighing 210–230 g at the start of the experiment, were housed in plastic  
128 cages with dimensions of 30 × 25 × 18 cm, with a wire mesh top and sawdust bedding  
129 (two rats / cage). The colony room was under controlled lighting (lights on from 7:00  
130 A.M. to 7:00 P.M.), temperature ( $23 \pm 1$  °C), and humidity ( $50 \pm 10\%$ ). Animals were  
131 allowed free access to standard laboratory chow and tap water. Animals were handled  
132 daily for at least four days before the start of the experiment and were tested only once in  
133 each experiment. All experiments were ethically approved by the Animal Research

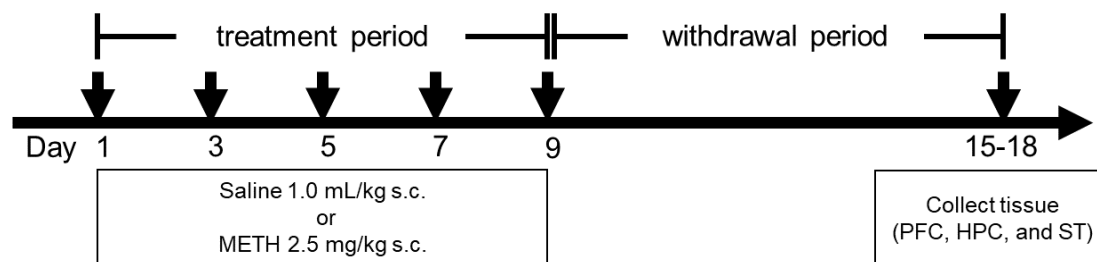
134 Committee of Hokkaido University (permission number 13–0137, 17–0086) and  
135 performed in accordance with ARRIVE guidelines, the Guide for the Care and Use of  
136 Laboratory Animals of Hokkaido University, and the guidelines established by the US  
137 National Institutes of Health guide for the care and use of laboratory animals (NIH  
138 Publications No. 8023, revised 1978).

139

## 140 2.2. Drugs and administration schedules

141 For this study, METH (Dainippon Sumitomo Pharma Co., Ltd., Japan) was  
142 dissolved in sterile physiological saline and injected subcutaneously at a volume of 1.0  
143 mL/kg at a dose of 2.5 mg/kg. The control group was subcutaneously administered 1.0  
144 mL/kg of saline. Injections were repeated five times on alternating days (treatment period).  
145 To avoid acute, confounding effects of having the drug on-board during testing, we  
146 allowed time for a sufficient withdrawal (wash-out) period (6–9 days), then rats were  
147 anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg), decapitated, and the  
148 PFC, HPC, and ST were collected (the experimental timeline is shown in Fig. 1). The  
149 METH dose (2.5 mg/kg) was selected based on the optimal manifestation of  
150 electrophysiological, molecular, and behavioral changes we observed in our previous  
151 study (Table 1).

152



153

154 Fig. 1 Schema of the experimental timeline.

155 Rats were injected with saline or METH five times, followed by a 6–9 days  
156 withdrawal period before tissue collection.

157 METH, methamphetamine; PFC, prefrontal cortex; HPC, hippocampus; ST,  
158 striatum; s.c., subcutaneous injection.

159

### 160 2.3. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

161 Immediately after tissue collection, the right hemisphere of each brain was  
162 rapidly immersed in RNAlater™ Stabilization Solution (Thermo Fisher Scientific, USA),  
163 maintained at 4 °C for two days, then stored at –80°C until use.

164 Total RNA was extracted from the PFC, HPC, and ST of the right hemisphere  
165 using an SV Total RNA Isolation System (Z3105, Promega, USA). The cDNA was  
166 obtained by reverse transcription using ReverTra Ace® qPCR RT Master Mix (Toyobo  
167 Co., Ltd., Japan). PCR was performed using Thunderbird SYBR qPCR mix (Toyobo Co.,

168 Ltd., Japan); each sample was measured in triplicate. The amplification parameters were:  
169 40 cycles of denaturing at 95 °C for 15 s and annealing and extension at 60 °C for 30 s.  
170 For each cycle, the fluorescent emission of SYBR green was quantified for each sample  
171 and used to calculate the threshold cycle numbers (Ct). The reaction conditions for each  
172 primer set for *Grin1*, *Grin2a*, *Grin2b*, *Grin2c*, *Grin2d*, and *Actb* genes were selected  
173 based on previous studies, which are shown in Supplementary Table 1.

174 The expression level of each gene was determined using the  $\Delta\Delta\text{CT}$  method for  
175 qRT-PCR. *Actb*, the gene encoding  $\beta$ -actin levels were measured as the internal loading  
176 control. Relative gene expression was calculated using the 2- $\text{ddCt}$  method.

177

#### 178 2.4. Western blotting

179 Immediately after tissue collection, the left hemisphere of each brain was rapidly  
180 homogenized with a glass homogenizer using an ice-cold synaptosome isolation reagent  
181 (Syn-PER: Thermo Fisher Scientific, USA) containing 1  $\times$  protease inhibitor cocktail  
182 (cOmplete: Sigma-Aldrich, USA). The homogenate was centrifuged at 1200  $\times g$  for 10  
183 min at 4 °C. The supernatant was transferred to a new tube and centrifuged at 15,000  $\times g$   
184 for 20 min at 4 °C. After centrifugation, the supernatant was stored as the cytosolic  
185 fraction at  $-80$  °C until protein analysis. The remaining pellet was resuspended in 1  $\times$

186 phosphate-buffered saline (PBS; pH 7.4, catalog number: 048–29,805, FUJIFILM Wako  
187 Pure Chemical Corporation, Japan) and stored as the synaptosomal fraction at –80 °C  
188 until protein analysis.

189           To quantify protein expression in the cellular fractions from each brain region,  
190 10 µg of the cytoplasmic fraction and 2 µg of the synaptosome fraction were subjected to  
191 gel electrophoresis at a constant voltage of 200 V for 30 min (4–15% Mini-PROTEAN®  
192 TGX™: Bio-Rad), then transferred at a constant voltage of 100 V for 1 h to Amersham  
193 Hybond polyvinylidene fluoride (PVDF) membranes (10,600,057, GE Healthcare Life  
194 Science, USA) with a pore size of 0.2 µm in a Tris-glycine transfer buffer (25 mM tris  
195 base, 192 mM glycine, 20% methanol). The samples were blocked for 1 h with 2% skim  
196 milk in TBST (Tris-buffered saline containing 0.5% Tween 20) at room temperature, then  
197 incubated overnight at 4 °C with the following primary antibodies: β-actin (1:1000  
198 dilution; mouse monoclonal, 010–27,841, FUJIFILM Wako Pure Chemical Corporation,  
199 Japan) and GluN1 (1:500 dilution; rabbit monoclonal, ab109182, Abcam, USA). After  
200 washes with TBST (3 × 10 min), the blots were incubated for 1 h at room temperature  
201 with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit  
202 IgG or goat anti-mouse IgG; 1: 5000, Amersham Biosciences, UK), followed by washes  
203 with TBST (3 × 10 min). The samples were incubated with ImmunoStar LD (FUJIFILM

204 Wako Pure Chemical Industries, Japan), a luminescent substrate, and images were  
205 captured using ImageQuant LAS 4000 (GE Healthcare Life Science, USA). The NIH  
206 ImageJ software was used to quantify the protein bands by densitometry.  $\beta$ -actin was used  
207 as the loading control.

208

### 209 2.5. Statistical analyses

210 JMP<sup>®</sup> Pro 14.0.0 (SAS Institute Inc., Cary, North Carolina, USA) software was used  
211 for statistical analyses. Group means of data quantified from qRT-PCR and Western  
212 blotting to assess mRNA and protein expression changes were compared using the  
213 Wilcoxon rank-sum test. Significance level was set as 0.05. Data are presented as  
214 mean  $\pm$  standard error of the mean (*SEM*).

215

### 216 3. Results

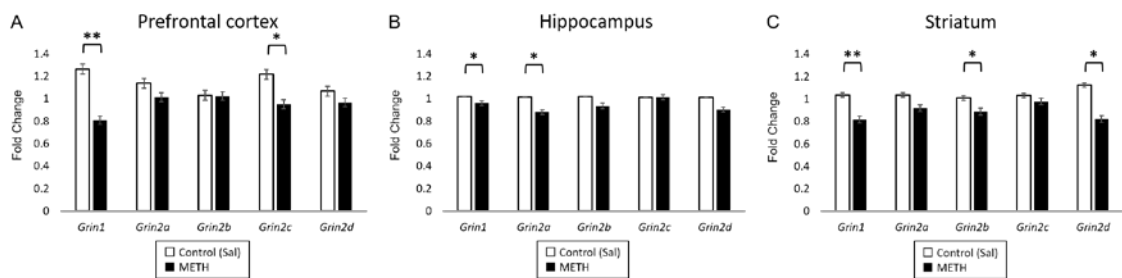
217

218 The RNA expression levels of *Grin1*, *Grin2a*, *Grin2b*, *Grin2c*, and *Grin2d* in the PFC,  
219 HPC, and ST were quantified by qRT-PCR. Repeated administration of METH  
220 significantly decreased gene expression levels of *Grin1* ( $Z = 3.17$ ,  $p = .0015$ ) and *Grin2c*  
221 ( $Z = 2.31$ ,  $p = .0207$ ) in the PFC (Fig.2A), *Grin1* ( $Z = 2.15$ ,  $p = .0315$ ) and *Grin2a* ( $Z =$

222 2.67,  $p = .0076$ ) in the HPC (Fig.2B), and *Grin1* ( $Z = 2.95$ ,  $p = .0032$ ), *Grin2b* ( $Z = 2.55$ ,  
 223  $p = .0108$ ), and *Grin2d* ( $Z = 2.09$ ,  $p = .0364$ ) in the ST (Fig.2C). The expression of *Grin1*  
 224 was significantly different in the PFC compared to the ST.

225

226



227

228 Fig. 2 Gene expression levels of NMDA-R subunits in various brain regions following  
 229 repeated METH administration.

230 Repeated administration of 2.5 mg/kg METH significantly decreased expression  
 231 levels of *Grin1* and *Grin2c* in the PFC (A), *Grin1* and *Grin2a* in the HPC (B), *Grin1*,  
 232 *Grin2b*, and *Grin2d* in the ST (C).

233 Data are shown as mean  $\pm$  SEM. \* $p < .05$ , \*\* $p < .0033$ . NMDA-R, *N*-methyl-D-  
 234 aspartate receptor; METH, methamphetamine; PFC, medial prefrontal cortex; HPC,  
 235 hippocampus; ST, striatum; qRT-PCR, quantitative reverse transcription polymerase  
 236 chain reaction.

237 ( $N = 30$  rats/group. Data were compared by Wilcoxon rank-sum tests with a

238 Bonferroni correction).

239

240

241 In order to validate this finding, we further analyzed the protein expression levels  
242 of GluN1 by western blotting in the cytosolic and synaptosomal fractions of the PFC and  
243 the ST, which were significantly decreased even after Bonferroni correction.

244

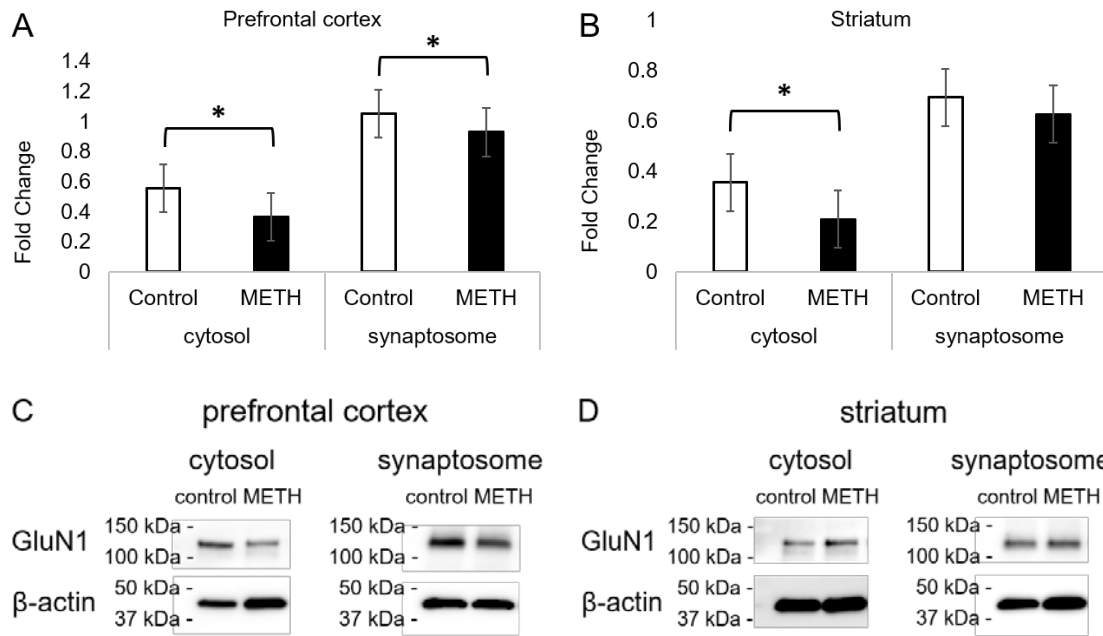
245 Repeated administration of METH significantly decreased the protein  
246 expression levels of GluN1 in both the cytosolic ( $Z = 2.11, p = .0351$ ) and synaptosomal  
247 ( $Z = 1.97, p = .0488$ ) fractions of tissue from the PFC (Fig.3A, C). Similarly, protein  
248 expression levels of GluN1 levels were also decreased in the cytosolic fractions ( $Z = 2.57,$   
249  $p = .0102$ ) but not the synaptosomal fractions ( $Z = 0.78, p = .4357$ ) of tissue from the ST  
250 (Fig.3B, D).

251

252

253





254

255

256 Fig. 3 Changes in protein expression levels of NMDA-R subunits in cellular fractions of  
 257 tissue from various brain regions following repeated METH administration.

258 Repeated administration of METH significantly decreased the protein  
 259 expression level of GluN1 in the cytosolic and synaptosomal fractions of the PFC (A, C),  
 260 and the cytosolic fraction of the ST (B, D).

261 Data are expressed as the mean  $\pm$  SEM. \* $p < .05$ . NMDA-R, *N*-methyl-D-  
 262 aspartate receptor; METH, methamphetamine; PFC, prefrontal cortex; ST, striatum.

263 ( $N = 11-12$  rats/group; statistical comparisons were performed using the Wilcoxon  
 264 rank-sum test).

265

266

267 4. Discussion

268

269 The results of this study suggest a decrease in population of NMDA-R subtype  
270 GluN1/2C or GluN1/2A/2C in the PFC, GluN1/2A in the HPC, and GluN1/2B, GluN1/2D,  
271 and GluN1/2B/2D in the ST. In accordance with our previous studies, we have determined  
272 the mechanism through which METH induces changes in NMDA-R expression and  
273 subunit composition. Dopamine is released following administration of METH, followed  
274 by glutamate release, which reduces levels of the GluN1 subunit, ultimately leading to  
275 decreased expression of functional NMDA-Rs. These alterations in gene expression of  
276 NMDA-R subunits are associated with the development of behavioral cross-sensitization  
277 to MK-801, PPI deficit, and apoptosis within the PFC (Table 1).

278

279 In the model used in this study, there was a decrease in the expression of GluN1 and  
280 decreased intracellular trafficking events that regulate NMDA-R expression (Fig.3A, B).  
281 After NMDA-Rs are synthesized in and exported from the endoplasmic reticulum, the  
282 receptors travel through the Golgi apparatus before being inserted into vesicles and  
283 directly trafficked to the plasma membrane or into dendrites. Alternatively, vesicles may  
284 be trafficked to dendritic Golgi outposts before reaching the cell membrane or to the

285 synapse (Horak *et al.*, 2014). Two molecules genetically linked to schizophrenia,  
286 neuregulin and serine–threonine phosphatase PP2B (also known as calcineurin) regulate  
287 these NMDA-R trafficking events. In a hypothetical model, activation of the ErbB4  
288 receptor by neuregulin suppresses tyrosine phosphorylation of the GluN2A subunit,  
289 which promotes NMDA-R internalization. In another model, PP2B dephosphorylates and  
290 activates striatal-enriched tyrosine phosphatase (STEP), which, in turn, dephosphorylates  
291 tyrosine residues on GluN1 and/or GluN2 subunits to promote NMDA-R internalization  
292 (Lau *et al.*, 2007).

293

294           In this study, in the PFC, the protein expression level of GluN1 decreased in both  
295 the cytosolic and synaptosomal fractions following repeated METH administration, while  
296 in the ST, the protein expression level of GluN1 was significantly decreased in the  
297 cytosolic fraction, but there was no significant difference in expression in the  
298 synaptosomal fraction. These regional differences may be due to variable expression of  
299 functional NMDA-Rs, which is regulated by intracellular receptor trafficking (Ladépêche  
300 *et al.*, 2014). Depending on the number of NMDA-R subunits containing GluN1 and  
301 differences in intracellular trafficking mechanisms, the changes in NMDA-R expression  
302 may be differentially delayed across brain regions. It will be necessary to further assess

303 NMDA-R expression and associated intracellular trafficking in each region of the brain.

304

305           The METH models are considered limited, as they do not fully reflect the  
306 negative symptoms and cognitive impairment associated with the development of  
307 schizophrenia (Jones *et al.*, 2011; Marcotte *et al.*, 2001). There is also a theory that purely  
308 dopaminergic models may be unlikely to lead to a great improvement in efficacy or safety  
309 of antipsychotic drugs (Steeds *et al.*, 2015). In addition, pharmacological models using  
310 NMDA-R antagonists have been widely used but these typically produce only transient  
311 changes in behavior and brain function (Featherstone *et al.*, 2015). Because NMDA-R  
312 antagonists induce hypo-functionality of the receptor throughout the brain, these animal  
313 models cannot selectively target NMDA-Rs in specific neural circuits; thus, these models  
314 may fail to precisely elucidate the mechanisms resulting in the pathophysiology of  
315 schizophrenia (Olney *et al.*, 1999). At present, models mimicking the dysfunction of both  
316 presynaptic dopamine release and NMDA-R functionality may provide the best tools to  
317 explore the molecular, cellular, and behavioral aspects of schizophrenia (Howes *et al.*,  
318 2015). While pharmacological models may never be able to accurately mimic all aspects  
319 of such a complex condition as schizophrenia, they may still be able to provide valuable  
320 insight into the neurobiological mechanisms underlying specific symptom domains

321 (Curran *et al.*, 2009). The METH model is a classical pharmacological model, but there  
322 are many points that need to be re-evaluated because multiple hypotheses are included  
323 depending on the protocol, as in this study.

324

325 *Grin1* knockdown mice or GluN2A and GluN2B mutant mice are also  
326 commonly used animal models of schizophrenia (Lee *et al.*, 2019). GluN1-mediated  
327 deficits in either pyramidal or GABAergic neurons could cause an imbalance in neuronal  
328 excitation and inhibition in cortical neural circuitry, leading to development of behavioral  
329 phenotypes that mimic symptoms of schizophrenia. Due to overlapping roles of GluN2A  
330 and GluN2B subunits in learning and memory (Sakimura *et al.*, 1995; Kiyama *et al.*,  
331 1998; Moriya *et al.*, 2000), GluN2A and GluN2B mutant mice would serve as great  
332 models to study the pathophysiology of cognitive changes associated with the  
333 development of schizophrenia. Heterozygous (GluN1 +/-) mice exhibit a 30% reduction  
334 in GluN1 receptor expression, and the current study suggests that these mice may be  
335 among the most sensitive models of increased vulnerability to schizophrenia  
336 (Featherstone *et al.*, 2015).

337

338 The model used in this study differs from the typical amphetamine and METH

339 models, which are based solely on the dopamine hypothesis; it is instead a model that  
340 reflects other aspects of the pathology of schizophrenia, combining both the dopamine  
341 and glutamate hypotheses. In studies with different protocols using METH, *Grin1* and  
342 GluN1 were not necessarily decreased (Simões *et al.*, 2007; González *et al.*, 2018), and  
343 in contrast to the results of the present study, GluN2B in the mPFC was decreased  
344 (Lominac *et al.*, 2016). The effect on NMDA-R varies with the dose of METH, and a  
345 protocol similar to that used in this study may be desirable to replicate both the  
346 dopaminergic overactivity and NMDA-R malfunction seen in schizophrenia.

347

348         In our model, we have identified four steps in the pathophysiological  
349 mechanisms driving changes in NMDA-R functionality. First, dopamine is released in the  
350 PFC and the NAc during psychotic episodes, with increased glutamate release in severe  
351 cases (Ito *et al.*, 2006b; Abekawa *et al.*, 2008). Second, increased glutamate release  
352 reduces GluN1 expression and the number of functional NMDA-Rs at the plasma  
353 membrane (Fig. 3). As NMDA-Rs become desensitized and dysfunctional, glutamate  
354 release is eventually reduced (Abekawa *et al.*, 2012). Third, these changes result in a  
355 disease state with increased susceptibility to NMDA-R antagonism (possibly  
356 unresponsive to D<sub>2</sub> receptor antagonists) (Ito *et al.*, 2006b; Abekawa *et al.*, 2008), besides

357 the recognized dysfunction (Abekawa *et al.*, 2008; Abekawa *et al.*, 2012). Finally,  
358 cerebral atrophy occurs when AMPA receptors ( $\alpha$ -amino-3-hydroxy-5-methyl-4-  
359 isoxazole propionic acid receptor) are repeatedly stimulated during the phase of increased  
360 synaptic glutamate release (Abekawa *et al.*, 2008). Peripheral blood levels of D-serine  
361 binding to GluN1 may be the greatest biological marker for diagnosis and treatment of  
362 patients with schizophrenia (Ohnuma and Arai, 2011).

363

364 Although schizophrenia is considered a neurodevelopmental disorder with deficits  
365 occurring during early brain development, the majority of animal studies have been  
366 focused on METH or NMDA-R antagonist-induced changes in adulthood (Harrison *et al.*,  
367 2005; Fatemi and Folsom, 2009; Powell, 2010; Rapoport *et al.*, 2012). Further studies  
368 should address schizophrenia as a neurodevelopmental disorder by administering METH  
369 during pregnancy and in periods leading up to adulthood to examine its impact on  
370 behavior and neural circuitry during development (Lee *et al.*, 2019). Some studies further  
371 point to how re-expression or overexpression of NMDA-R subunits can rescue behavioral  
372 deficits associated with symptoms of schizophrenia, suggesting that enhancing levels of  
373 certain NMDA-R subunits may ameliorate hypo-functionality of the receptor.

374

375 5. Conclusion

376 In conclusion, repeated METH administration induces changes in not only  
377 dopaminergic systems but also glutamatergic systems, and it alters NMDA receptor  
378 function and subunit expression. This suggests that our model reflects some of the  
379 pathophysiological changes of schizophrenia, and may be useful for identifying new  
380 therapeutic agents for the treatment of schizophrenia.

381

382

383 **Contributors**

384 MO, KI, and MK designed the study; MO and MK performed the experiments;  
385 MO analyzed the data and wrote the first draft of the manuscript; all authors contributed  
386 to the interpretation of the data and commented on the manuscript. All authors have  
387 approved the final manuscript.

388

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391

392 **Ethical statement**



393 All experiments were ethically approved by the Animal Research Committee of  
394 Hokkaido University (permission number 13–0137, 17–0086), and performed in  
395 accordance with ARRIVE guidelines, the Guide for the Care and Use of Laboratory  
396 Animals of Hokkaido University, and the guidelines established by the US National  
397 Institutes of Health guide for the care and use of laboratory animals (NIH Publications  
398 No. 8023, revised 1978).

399

#### 400 **Declaration of Competing Interest**

401 The authors declare that there is no conflict of interest.

402

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407

#### 408 **Appendix A. Supplementary data**

409 Supplementary data to this article can be found online at  
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411

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