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

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Abstract

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

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

Keywords

Schizophrenia, NMDA receptor, GluN1, Animal model, Methamphetamine

Abbreviations

CNS, Central nervous system; HPC, Hippocampus; METH, Methamphetamine; NAc, Nucleus accumbens; NMDA-R, *N*-methyl-D-aspartate receptor; PBS, Phosphate-buffered saline; PFC, Prefrontal cortex; PPI, Prepulse inhibition; qRT-PCR, quantitative reverse transcription polymerase chain reaction; Sal, Saline; s.c., Subcutaneous injection; ST, Striatum; TBST, Tris-buffered saline containing 0.5 % Tween 20

1. Introduction

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

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

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

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

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

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

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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 *et al*., 2006a)	+	+
Delayed increases in glutamate levels in the NAc and PFC (Ito *et al*., 2006b; Abekawa *et al*., 2008)	−	+
Development of behavioral cross-sensitization to MK-801 (Ito *et al*., 2006b; Abekawa *et al*., 2008)	−	+
PPI deficit (Abekawa *et al*., 2008)	−	+
Apoptosis in the PFC (Abekawa *et al*., 2008)	−	+

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

114 Prepulse inhibition

115

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

2. Materials and methods

2.1. Animals

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

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

2.2. Drugs and administration schedules

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

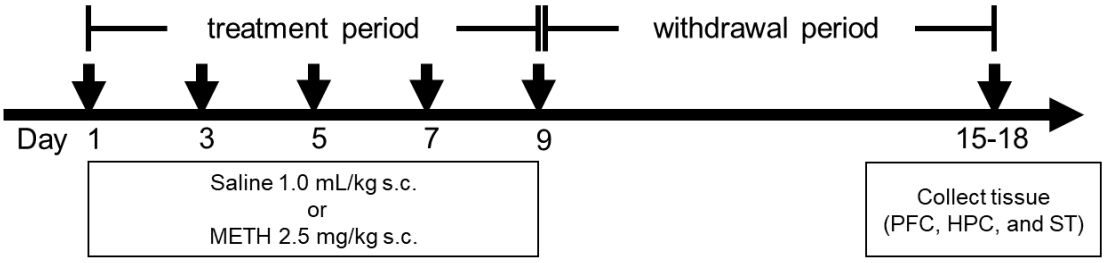

Fig. 1 Schema of the experimental timeline.

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

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

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

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

Total RNA was extracted from the PFC, HPC, and ST of the right hemisphere using an SV Total RNA Isolation System (Z3105, Promega, USA). The cDNA was obtained by reverse transcription using ReverTra Ace ® qPCR RT Master Mix (Toyobo 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 ΔΔCT method for
175 qRT-PCR. *Actb*, the gene encoding β-actin levels were measured as the internal loading
176 control. Relative gene expression was calculated using the 2-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 × protease inhibitor cocktail
182 (cOmplete: Sigma-Aldrich, USA). The homogenate was centrifuged at 1200 ×*g* for 10
183 min at 4 °C. The supernatant was transferred to a new tube and centrifuged at 15,000 ×*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 ×

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

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

2.5. Statistical analyses

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

3. Results

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

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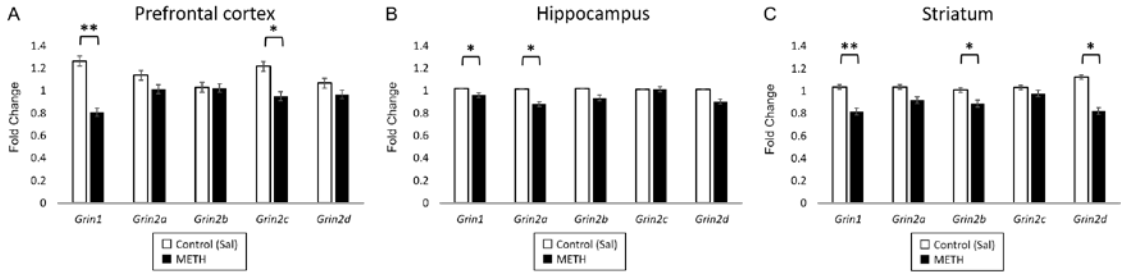

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228 Fig. 2 Gene expression levels of NMDA-R subunits in various brain regions following

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 ± *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).

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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).

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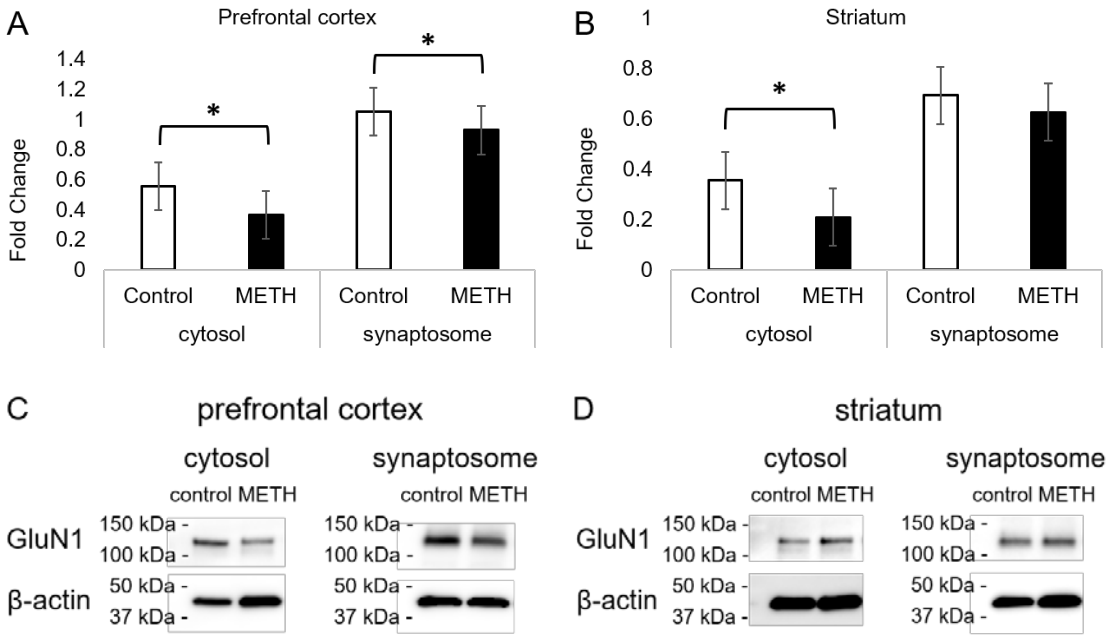

Fig. 3 Changes in protein expression levels of NMDA-R subunits in cellular fractions of tissue from various brain regions following repeated METH administration.

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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_2 receptor antagonists) (Ito *et al.*, 2006b; Abekawa *et al.*, 2008), besides

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

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

5. Conclusion

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

Contributors

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

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Ethical statement

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

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2020.109984.

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