N-methyl-D-aspartate receptor dysfunction in the prefrontal cortex of stroke-prone spontaneously hypertensive rat/Ezo as a rat model of attention deficit/hyperactivity disorder

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Abstract
Aim: We previously reported that stroke-prone spontaneously hypertensive rat/Ezo (SHRSP/Ezo) has high validity as an attention deficit/hyperactivity disorder (AD/HD) animal model, based on its behavioral phenotypes, such as inattention, hyperactivity, and impulsivity. Fronto-cortical dysfunction is implicated in the pathogenesis of AD/HD. In this study, we investigated prefrontal cortex (PFC) function in SHRSP/Ezo rats by electrophysiological methods and radioreceptor assay.

Methods: We recorded excitatory postsynaptic potential in layer V pyramidal neurons in the PFC by intracellular recording method to assess synaptic plasticity in the form of long-term potentiation (LTP). We also performed N-methyl-D-aspartate acid (NMDA) receptor binding assay in the PFC and hippocampus using radiolabeled NMDA receptor antagonist [3H]MK-801.

Results: Theta-burst stimulation induced LTP in the PFC of genetic control, WKY/Ezo, whereas failed to induce LTP in that of SHRSP/Ezo. The Kd value of [3H]MK-801 binding for NMDA receptors in the PFC of SHRSP/Ezo was higher than in the WKY/Ezo. Neither the Bmax nor Kd of [3H]MK-801 binding in the SHRSP/Ezo hippocampus was significantly different to WKY/Ezo.

Conclusion: These results suggest that the AD/HD animal model SHRSP/Ezo has NMDA receptor dysfunction in the PFC.

KEYWORDS
attention deficit/hyperactivity disorder, long-term potentiation, NMDA receptor, prefrontal cortex

1 | INTRODUCTION

Attention deficit/hyperactivity disorder (AD/HD) is a mild developmental disorder, affecting 5%-10% of children worldwide and sometimes persisting through adolescence into adulthood.3 AD/HD was originally considered a “attention deficit and disruptive behavior disorders” in the Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV),2 although it is now categorized as a “neurodevelopmental disorder” by DSM-V.3 AD/HD has 3 distinct aspects, inattention, hyperactivity, and impulsivity. Methylphenidate4 and the noradrenaline reuptake inhibitor atomoxetine5 are used to treat AD/HD.
A variety of animal models of AD/HD have been developed, including 6-hydroxydopamine injection dopaminergic neuron-lesioned rats and dopamine transporter knockout (KO) mice. Furthermore, the spontaneously hypertensive rat (SHR) has been widely used as an AD/HD animal model. SHRs exhibit an AD/HD-like phenotype when compared with their genetic controls, Wistar-Kyoto (WKY) rats. We also reported previously that the stroke-prone spontaneously hypertensive rat/Ezo (SHRSP/Ezo), which is one of the SHR strains, shows behavioral features of AD/HD, such as inattention in the Y-maze task, hyperactivity in the open field test, and impulsiveness due to hypo-anxiety in the elevated plus-maze test. These abnormal behaviors of SHRSP/Ezo are partially ameliorated by AD/HD treatment drugs, such as methylphenidate and some monoamine reuptake inhibitors.

It has been reported that AD/HD patients have fronto-cortical dysfunction, known as hypofrontality. In the animal study, regional lesions of the prefrontal cortex (PFC), a brain region corresponding to the primate dorsolateral PFC implicated in cognitive function, can induce impulsive deficits. Moreover, synaptic plasticity in the PFC, particularly the long-term potentiation (LTP) of excitatory synapses, is considered to be an electrophysiological measure of cognitive function. LTP in the PFC is N-methyl-D-aspartate (NMDA) receptor dependent at the postsynapse membrane, and dopamine is one of monoaminergic modulators of synaptic plasticity. We reported that the atypical antipsychotic clozapine, which ameliorates the cognitive dysfunction in schizophrenia, enhances LTP via the dopamine D1 receptor in the PFC of rats. Thus, assessment of synaptic plasticity in the PFC of rats can be useful approach to obtain pathophysiological and pharmacological knowledge about cognitive dysfunction.

Based on these findings, we hypothesized that SHRSP/Ezo as an AD/HD animal model has some degree of fronto-cortical incompetence. Therefore, we assessed excitatory synaptic function in the PFC of SHRSP/Ezo using intracellular recording method and functional analysis of NMDA receptor by competitive protein-binding method.

2 MATERIAL AND METHODS

2.1 Animals

Male SHRSP/Ezo and WKY/Ezo (genetic control) rats were bred in our laboratory. Rats were housed in a room with a 12-h light-dark cycle under constant temperature (21 ± 2°C). All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee of Health Sciences University of Hokkaido.

2.2 Electrophysiology

2.2.1 Slice preparation

At 6 weeks old, each rat was anesthetized with urethane (U2500; Sigma-Aldrich, 1 g/kg, intraperitoneal administration) and perfused transcardially with 50 mL of an ice-cold cutting solution (mmol/L: sucrose 228; glucose 30; KCl 3; NaHCO3 26; MgCl2 10; CaCl2 0.5; 2.2.2 Intracellular recording

Pyramidal neurons in layer V of the PFC were visually identified using an upright microscope (BX51WI) equipped with an infrared CCD camera system (Hamamatsu Photonics, Japan). Recording pipettes were made from glass capillaries (GD 1.5; Narishige, Japan) using a Flaming/Brown type puller (P-97; Sutter Instruments, USA) and connected to the head stage of a patch clamp amplifier (Axopatch 200B; Molecular Devices, Sunnyvale, CA, USA). The resistance of the patch pipette was 3-6 MΩ when filled with the standard intracellular solution. The composition of the pipette solution was (in mmol/L): K-gluconate 140, NaCl 8, HEPES 10, EGTA 0.4, ATP-2Na 3, GTP-2Na 0.2, and lidocaine N-ethyl bromide (QX314; Sigma-Aldrich) 10, pH 7.3. QX314 is generally used for inhibition of action potential generation to accurate assessment of Excitatory postsynaptic potential (EPSP) amplitude. The signal was digitized by an A/D converter (Digidata 1420A; Molecular Devices) and stored on a computer using pClamp 10 software (Molecular Devices). Capacitance compensation and bridge-balance adjustment were made simultaneously. Signals from only 1 neuron were recorded in each slice. After intracellular recording from layer V pyramidal neurons held at membrane potential of ~70 mV, recording mode was switched to current-clamp mode. Data were obtained using standard intracellular recording in current-clamp mode so as not to disturb membrane depolarization. Excitatory postsynaptic potential was evoked by single electrical pulse (200 µs duration) applied to layer II/III of the PFC slice at 0.03 Hz through a bipolar Teflon-coated tungsten electrode (outer diameter 125 µm). The evoked potential was inhibited by 1 µmol/L tetrodotoxin (206-11071; Wako Pure Chemical Industries, Ltd.). After recording baseline, theta-burst frequency stimulation (4 shocks at 100 Hz, repeated 10 times at 5 Hz) was applied 5 times with a 1-minute interval as the high-frequency stimulation protocol. Isolated forebrains were transferred to the cutting solution within 1 minute. Brain slices were prepared as described previously according to the Paxinos and Watson brain atlas. Acute coronal slices (300 µm thickness) including the PFC region were prepared using a vibrating microslicer (5000 mz; Campden Instruments Ltd, Loughborough, UK). Preparations were allowed to recover for 1 hour in artificial cerebrospinal fluid (aCSF) at room temperature (20-25°C). The composition of the aCSF was (in mmol/L): NaCl 124, KCl 1.8, NaHCO3 26, KH2PO4 1.2, MgCl2 1.3, CaCl2 2.5, and glucose 10, pH 7.35, and it was aerated with 95% O2 and 5% CO2. Each slice was transferred to a recording chamber on the stage of an upright microscope (BX51WI; Olympus, Japan) and superfused with aCSF (30°C) at a rate of 1.2 mL/min. Bicuculline (3 µmol/L; 026-14291; Wako Pure Chemical Industries, Ltd.) was included in the aCSF to inhibit GABAergic response throughout the experiment. Moreover, we added low concentration of dopamine (3 µmol/L; Sigma-Aldrich) to aCSF during recording from PFC neurons, because appropriate concentration of tonic/background dopamine is important to induction of LTP in rat PFC under physiological condition.

authorization.
and applied to the same electrode. Synaptic plasticity was evaluated by measuring EPSP amplitude as an index of synaptic efficacy. A sustained change of over 20% increase in EPSP amplitude for at least 30 minutes after burst stimulation was considered to reflect LTP.\(^{20,23}\) The post-theta-burst EPSP amplitude ratio (A/B) of each neuron was calculated using the EPSP amplitude after theta-burst (A) divided by the EPSP amplitude before theta-burst (B). Area under the curve (AUC) \((\% \times \text{minute} \times 10^{-4})\) was calculated 40 minutes after burst stimulation to evaluate the ensemble effect of EPSP.

### 2.3 Radioreceptor assay

Six-week-old SHRSP/Ezo and WKY/Ezo rats were anesthetized by inhalation of isoflurane (099-06571; Wako Pure Chemical Industries, Ltd., Japan) and immediately decapitated. The whole brain was put on the ice, and the PFC and hippocampus dissected out. The tissue was homogenized in ice-cold 5 mmol/L Tris buffer (0.32 mol/L sucrose, 1 mmol/L EDTA, pH 7.4) containing a proteinase inhibitor (Complete MINI tablet, Roche, Germany) using a Polytron homogenizer (PT10-35, Kinematica GmH, Littau, Switzerland) at full speed with 3 consecutive 30 seconds bursts before centrifugation at 1000 g for 10 minutes at 4°C. Subsequently, the supernatant was recovered and centrifuged at 48 000 g for 20 minutes at 4°C 3 times in the same buffer (Avanti HP-30; Beckman Coulter Inc., Pasadena, CA, USA). The final membrane pellet was resuspended in 5 mmol/L Tris-HCl buffer without proteinase inhibitor. The protein concentrations of samples were determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories Ltd., Hercules, CA, USA) using bovine serum albumin as the standard. The membrane tissue was diluted to 50 μg proteins/assay tube and incubated in Tris-HCl buffer at 30°C for 1 hour, containing 0.1-80 nmol/L \[^{3}H\]labeled MK-801 (ART0661; American Radiolabeled Chemicals Inc., Saint Louis, MO, USA) in the absence or presence of 1 μmol/L MK-801 (M107; Sigma-Aldrich) in a final volume of 500 μL. The reaction was terminated by rapid filtration through glass fiber filters (1822-024; Whatman GF/C, Maidstone, Kent, UK). The radioactivity trapped on filters was measured using a liquid scintillation counter (LC-6100; Aloka, Japan). Radioactivity was applied at 0.9135-93; Nacalai Tesque, Japan). Specific binding was determined by subtracting nonspecific binding from total binding. Radioactivity was also analyzed as the mean ± SEM. The variance was not significantly different between the groups, we performed Student’s t test. Data with significantly different variances were analyzed using Mann-Whitney U tests. All statistical procedures were conducted using IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armonk, NY, USA). The alpha level was set at 5%.

### 3 RESULTS

#### 3.1 Electrophysiology

First, we carried out intracellular recording using whole-cell patch clamp method to investigate the functional differences between the PFC of WKY/Ezo and SHRSP/Ezo. Membrane capacitance was not different between WKY/Ezo \((n = 7, 76.30 \pm 0.59 \text{ pF})\) and SHRSP/Ezo \((n = 8, 73.86 \pm 1.43 \text{ pF})\) \((t = 1.58, \text{not significant: ns})\). Input resistance was 156.71 ± 2.36 MΩ and 153.43 ± 2.75 MΩ in WKY/Ezo \((n = 7)\) and SHRSP/Ezo \((n = 8)\), respectively, and there was not significant between each rat \((t = 0.38, df = 13, \text{ns})\).

In general, membrane capacitance and input resistance reflect both the cell size and the density of the resting ion channels on the membrane.\(^{24,25}\) Thus, these data suggest that there is no difference of morphological characteristics of PFC neurons in layer V between WKY/Ezo and SHRSP/Ezo.

Single electrical stimulation in layer II/III of the PFC evoked sharp membrane depolarization with an exponential decay, indicating development of EPSP. We observed typical EPSP waveform before/after theta-burst stimulation (Figure 1A) and assessed the amplitude of EPSP from the baseline. As shown in Figure 1B, preburst baseline was calculated as the average of 6 consecutive amplitudes before the theta-burst stimulation \((WKY/Ezo, n = 7, 4.39%; SHRSP/Ezo, n = 8, 5.24\%)\) and SHRSP/Ezo \((n = 8),\) there was not significant difference between each baseline \((t = 0.03, df = 9, \text{ns})\). Immediately after theta-burst stimulation, LTP induction, significant enhancement of EPSP amplitude, was observed in WKY/Ezo, whereas the EPSP amplitude in SHRSP/Ezo tended to decrease. AUC \((\% \times \text{minute} \times 10^{-4})\) was as follows: WKY/Ezo, 103.80 ± 4.39%; SHRSP/Ezo, 104.07 ± 5.24%, and there was no significant difference between each baseline \((t = 0.03, df = 9, \text{ns})\). Immediately after theta-burst stimulation, LTP induction, significant enhancement of EPSP amplitude, was observed in WKY/Ezo, whereas the EPSP amplitude in SHRSP/Ezo tended to decrease. AUC \((\% \times \text{minute} \times 10^{-4})\) was as follows: WKY/Ezo, 34.60 ± 5.70 \((n = 8)\); SHRSP/Ezo, 11.34 ± 3.52 \((n = 7)\) \((t = 3.13, df = 13, *P < .05)\) compared with SHRSP/Ezo; Figure 1C).

These results indicate that layer V pyramidal neurons in the PFC of WKY/Ezo have facilitating synaptic plasticity, while plasticity in SHRSP/Ezo differs, particularly in regard to burst stimulation inducing long-term depression-like changes in these neurons.

#### 3.2 Radioreceptor assay

The electrophysiological analysis revealed functional dysfunction in the PFC of SHRSP/Ezo. Because LTP in rodent PFC is NMDA receptor-dependent manner,\(^{15}\) we carried out radioreceptor assay focusing NMDA receptor. As shown in Table 1, significant difference was observed in the \(K_d\) values of \[^{3}H\]MK-801 binding between WKY/Ezo \((n = 4, 19.43 \pm 1.69 \text{ nmol/L})\) and SHRSP/Ezo \((n = 4, 31.58 \pm 3.80 \text{ nmol/L})\) \((t = 2.92, df = 6, *P < .05)\). However, there
was no significant difference in the $[^3]H$MK-801 binding $B_{\text{max}}$ values between WKY/Ezo ($n = 4$, $2.61 \pm 0.14$ fmol/mg protein) and SHRSP/Ezo ($n = 4$, $3.26 \pm 0.37$ fmol/mg protein), although that of SHRSP/Ezo showed an increasing tendency compared with WKY/Ezo ($t = 1.65$, $df = 6$, $ns$).

Additionally, we investigated $[^3]H$MK-801 binding to NMDA receptors in the hippocampus, as a comparative brain region. Neither the $B_{\text{max}}$ nor $K_d$ of $[^3]H$MK-801 binding in the hippocampus was significantly different between WKY/Ezo ($n = 4$, $K_d$: $24.21 \pm 4.15$ nmol/L; $B_{\text{max}}$: $3.32 \pm 0.24$ fmol/mg protein) and SHRSP/Ezo ($n = 4$, $K_d$: $26.59 \pm 4.41$ nmol/L; $B_{\text{max}}$: $3.20 \pm 0.25$ fmol/mg protein) ($t = 0.34$, $0.35$, respectively, $df = 6$ both, $ns$).

These results suggest that the low affinity of $[^3]H$MK-801 for NMDA receptors in SHRSP/Ezo is region-specific manner, occurring in the PFC but not the hippocampus.

### DISCUSSION

In this study, we assessed PFC function in SHRSP/Ezo, an AD/HD animal model, focusing on excitatory synapses and neural plasticity. Theta-burst stimulation to layer II/III did not induce LTP in layer V...
pyramidal neurons of the PFC in SHRSP/Ezo. Moreover, the $K_d$ (but not the $B_{max}$) value of $[^3H]MK-801$ NMDA receptor binding in the PFC of SHRSP/Ezo was significantly higher than that of WKY/Ezo.

Abnormal induction of synaptic plasticity in the PFC of rats contributes to pathological states of executive function. Pharmacological inhibition of the NMDA receptor in the rat PFC induces cognitive dysfunction in behavioral experiments and impairs LTP induction. Blot et al. reported that NMDA receptor blockade led to an aberrant plasticity induction in the PFC, associated with cognitive dysfunction. Our previous data showed that methylphenidate, which ameliorates AD/HD-like behaviors in SHRSP/Ezo, and we preliminarily confirmed that methylphenidate recovered the impairment of LTP in SHRSP/Ezo in vivo electrophysiological experiments via enhancement of dopamine release in the PFC (S. Hiraide, H. Shikanai, K. Iizuka and T. Izumi, unpublished data). Therefore, SHRSP/Ezo can have PFC dysfunction, resulting AD/HD-like phenotypic (behavioral) outcomes, such as inattention and impulsivity.

In the binding assay, the $K_d$ value of $[^3H]MK-801$ NMDA receptor binding in the PFC of SHRSP/Ezo was higher compared with that of WKY/Ezo. The $B_{max}$ value was not significantly different from controls. These data indicate that the affinity of $[^3H]MK-801$ for NMDA receptors in the PFC of SHRSP/Ezo is lower than that in WKY/Ezo, despite there being no difference in the number of NMDA receptors in the PFC. Moreover, neither the $B_{max}$ nor $K_d$ of $[^3H]MK-801$ binding in the hippocampus was significantly different between each type of rats, suggesting that NMDA receptor dysfunction in SHRSP/Ezo is PFC-specific manner. These results are consistent with a recent report that the PFC, but not hippocampus, is likely to be responsible for AD/HD-related behavioral deficits in SHRSP/Ezo. However, Jansen et al. reported that one of the AD/HD animal models, SHR, showed the NMDA receptor subunit dysfunction in the hippocampus. Further experiments are needed to reveal the details of NMDA receptor in the hippocampus of SHRSP/Ezo for the AD/HD pathological study.

The NMDA receptor is composed of a tetramer with subunits from 2 obligatory NR1, and other NR2A, NR2B, NR2C, or NR2D subunits. Genetic changes such as NR2A and NR2B gene polymorphisms have been observed in AD/HD patients. Some animal studies have demonstrated that NR2A and NR2B subunits are critical for the expression of LTP in the cortical as well as hippocampal region. Thus, our present data might reflect the NR2A and/or NR2B subunit dysfunction in the PFC of SHRSP/Ezo, resulting induction of the AD/HD behavior. Further experiments are needed to reveal the molecular details of NMDA receptor in the AD/HD pathological study.

NMDA receptors are activated by glutamate in the presence of the co-agonists D-serine or glycine. These glutamate modulator binding sites have been focused on as potential targets for the treatment of psychiatric disorders. D-serine is the endogenous co-agonist of synaptic NMDA receptors in pyramidal cells in layer V of the PFC. Serine racemase KO mice, which have dramatic reductions in D-serine, exhibit cognitive dysfunction. Our preliminary data show that glycine enhances the affinity of $[^3H]MK-801$ to NMDA receptors in the PFC of SHRSP/Ezo, indicating the refinement effect of glycine (H. Shikanai, N. Oshima and T. Izumi, unpublished data). These data might indicate possible roles of glycine as a cofactor for NMDA receptor-mediated treatment of AD/HD patients. In conclusion, here we have revealed that an AD/HD animal model, SHRSP/Ezo, has impaired cortical excitatory synapse based on NMDA receptor dysfunction. Our findings suggest that fronto-cortical NMDA receptor dysfunction possibly underlies the pathophysiological basis of AD/HD, and these data might indicate new therapeutic targets in AD/HD treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest associated with this manuscript.

DATA REPOSITORY

All relevant data are within the manuscript and in Tables S1-S4.

ANIMAL STUDIES

All experiments were performed with approval of the Institutional Animal Care and Use Committee of Health Sciences University of Hokkaido.

AUTHOR CONTRIBUTIONS

HS and NO designed research. HS, NO, HK, SK, and SH performed research. HS and NO analyzed data. HS, NO, HT, KI, KO, and TI wrote the manuscript.

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SUPPORTING INFORMATION

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