**Alterations of GABAergic and dopaminergic systems in mutant mice with
disruption of exons 2 and 3 of the *Disc1* gene**

Tsuyoshi Nakai^{1*}, Taku Nagai^{1*}, Rui Wang¹, Shinnosuke Yamada¹, Keisuke
Kuroda², Kozo Kaibuchi², Kiyofumi Yamada^{1†}

¹Department of Neuropsychopharmacology and Hospital Pharmacy, ²Department
of Cell Pharmacology, Nagoya University Graduate School of Medicine, 65
Tsuruma-cho, Showa-ku, Nagoya 466-8560, Japan

*Contributed equally to this work

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[†]Address for correspondence:

Kiyofumi Yamada, Ph.D.

Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya
University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya
466-8560, Japan

Tel.: +81-52-744-2674

Fax: +81-52-744-2979

E-mail: kyamada@med.nagoya-u.ac.jp

Abstract

Disrupted-in-schizophrenia-1 (*DISC1*) has been widely associated with several psychiatric disorders, including schizophrenia, mood disorders and autism. We previously reported that a deficiency of *DISC1* may induce low anxiety and/or high impulsivity in mice with disruption of exons 2 and 3 of the *Disc1* gene (*Disc1*^{Δ2-3/Δ2-3}). It remains unclear, however, if deficiency of *DISC1* leads to specific alterations in distinct neuronal systems. In the present study, to understand the role of *DISC1* in γ -aminobutyric acid (GABA) interneurons and mesocorticolimbic dopaminergic (DAergic) neurons, we investigated the number of parvalbumin (PV)-positive interneurons, methamphetamine (METH)-induced DA release and the expression levels of GABA_A, DA transporter (DAT) and DA receptors in wild-type (*Disc1*^{+/+}) and *Disc1*^{Δ2-3/Δ2-3} mice. Female *Disc1*^{Δ2-3/Δ2-3} mice showed a significant reduction of PV-positive interneurons in the hippocampus, while no apparent changes were observed in mRNA expression levels of GABA_A receptor subunits. METH-induced DA release was significantly potentiated in the nucleus accumbens (NAc) of female *Disc1*^{Δ2-3/Δ2-3} mice, although there were no significant differences in the expression levels of DAT. Furthermore, the expression levels of DA receptor mRNA were upregulated in the NAc of female *Disc1*^{Δ2-3/Δ2-3} mice. Male *Disc1*^{Δ2-3/Δ2-3} mice showed no apparent differences in all experiments. *DISC1* may play a critical role in gender-specific developmental alteration in GABAergic inhibitory interneurons and DAergic neurons.

Abbreviations

DISC1, disrupted-in-schizophrenia-1; *Disc1*^{Δ2-3/Δ2-3}, disruption of exons 2 and 3 of the *Disc1* gene; GABA, γ -aminobutyric acid; DA, dopamine; PV, parvalbumin; METH, methamphetamine; DAT, dopamine transporter; *Disc1*^{+/+}, wild type; NAc, nucleus accumbens

Keywords

50 *Disc1*; dopamine release; methamphetamine; parvalbumin-positive interneuron;
51 schizophrenia

1. Introduction

The activity of neuronal networks is regulated by the balance of the excitatory and inhibitory effects of neurotransmitters. Transient dysfunction of neurotransmission affects mental condition from mood swings to aggression and anxiety, whereas long-lasting disturbance of these systems leads to psychiatric illnesses such as mood disorders, schizophrenia (SZ) and autism spectrum disorders (ASD). Neurochemical, histological, genetic and brain imaging studies in psychiatric illnesses suggest the involvement of dopaminergic (DAergic), GABAergic and glutamatergic neurons in the pathophysiology of these diseases (Jones et al., 2011; Mouri et al., 2013; Nestler and Hyman, 2010).

It is well known that GABAergic interneurons, representing approximately 20–30% of neocortical neurons, play a critical role in controlling cell excitability, spike timing, synchrony and oscillatory activity in the mammalian central nervous system. Dysfunction of GABAergic neurotransmission has been implicated in various neuropsychiatric diseases. Psychotic symptoms in SZ have been found to correlate with reduced GABAergic inhibition in the medial temporal region (Busatto et al., 1997). Structural and functional changes in GABAergic inhibitory circuits were reported in ASD and SZ (Chattopadhyaya and Cristo, 2012). These GABAergic alternations are mainly observed in parvalbumin (PV)-expressing interneurons (Gogolla et al., 2009; Lewis and González-Burgos, 2008). Postmortem brain studies revealed significant reductions of glutamate decarboxylase (Fatemi et al., 2002; Yip et al., 2007) and alterations in GABA receptors in ASD (Collins et al., 2006; Fatemi et al., 2009; Oblak et al., 2010). Reduction of the PV-expressing neurons and their dysfunction have been reported in the hippocampus and prefrontal cortex of patients with SZ (Hashimoto et al., 2003; Inan et al., 2013; Konradi et al., 2011). Likewise, in mutant mice with inactivation of PV-expressing neurons, spatial working memory impairment has been observed (Korotkova et al., 2010). Interestingly, when excitatory input is ablated in approximately 50% of cortical interneurons during postnatal development, mutant mice exhibit SZ-related behaviors and loss of PV-expressing cells (Belforte et al., 2010).

DA neurons are necessary for the performance of motor behavior, motivation, working memory and reward system (Girault and Greengard, 2004). DAergic function has a role in different human conditions, including Parkinson's disease, drug addiction, compulsive behavior, attention-deficit/hyperactivity disorder, ASD and SZ (Carlsson, 2001; Dichter et al., 2012; Hyman et al., 2006; Iversen and Iversen, 2007; Swanson et al., 2007). In particular, hypofunction of the mesocortical DAergic system may be related to negative symptoms and cognitive dysfunction, whereas hyperfunction of the mesolimbic DAergic system may be associated with positive symptoms in SZ (Abi-Dargham et al., 2002; Castner et al., 2000; Laviolette, 2007). It has been suggested that increased affinity of DA D₂ receptors (i.e., D₂ receptor supersensitivity) in the nucleus accumbens (NAc) may contribute to psychosis in SZ (Seeman, 2011). Additionally, a positron emission computerized-tomography (PET) study found higher synaptic DA concentrations in the ventral striatum of SZ (Kegeles et al., 2010). In preclinical study, mice lacking the DA transporter (DAT) gene displayed markedly increased levels of DA in the mesolimbic system (Giros et al., 1996), leading to hyperlocomotion and sensorimotor gating deficit (Barr et al., 2004). Overexpression of DA D₂ receptor in the striatum was also associated with abnormal behaviors including working memory impairment and social interaction deficit (Kellendonk et al., 2006). These findings suggest that mesocorticolimbic DA neurons as well as PV-interneurons could have pivotal functions in the development of psychiatric disorders.

Disrupted-in-schizophrenia-1 (*DISC1*) was identified in a large Scottish family by positional cloning of a t(1;11)(q42.1;q14.3) translocation, which is associated with major psychiatric disorders including SZ and mood disorders (Millar et al., 2000; Schumacher et al., 2009; St Clair et al., 1990). Independent linkage and association studies have implicated *DISC1* in other mental disorders, such as affective disorders and ASD of diverse populations (Blackwood et al., 2001; Devon et al., 2001; Ekelund et al., 2001; Hennah et al., 2005, 2009; Hodgkinson et al., 2004; Kilpinen et al., 2008). Accumulating evidence suggests that *DISC1* is a scaffold protein that regulates specific signaling pathways and

interacts with many proteins (Chubb et al., 2008; Enomoto et al., 2009; Kamiya et al., 2005; Kim et al., 2009; Mao et al., 2009; Millar et al., 2005; Shinoda et al., 2007; Singh et al., 2010; Taya et al., 2007), which is associated with neurogenesis, neuronal migration, axon/dendrite formation and synapse formation (Brandon and Sawa, 2011; Hikida et al., 2012).

Recently, Kuroda et al. (2011) have generated mice lacking exons 2 and 3 of the *Disc1* gene (*Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice), which have deficiency of full-length DISC1 protein. The *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice develop normally with no apparent morphological abnormalities in the brain, such as cytoarchitecture and brain volume. The mutant mice exhibit electrophysiological and behavioral abnormalities. A threshold is shifted to up-forward to induce long-term potentiation (LTP) in the dentate gyrus (DG) of *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice. Adult *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice show low anxiety and/or high impulsivity in the elevated plus-maze and cliff-avoidance tests and slight increase in social interaction, but there are no apparent differences during the juvenile period. Emotional abnormalities in *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice are ameliorated by treatment with the atypical antipsychotic clozapine. In addition, gender-specific behavioral abnormalities have been observed in *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice. Female *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice show deficiency in prepulse inhibition and an enhanced response to the indirect DAergic agonist methamphetamine (METH), while male *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice are normal. However, it remains unclear whether defect of DISC1 leads to gender-dependent alteration in DAergic and GABAergic neurons. In order to understand the role of DISC1 in DAergic and GABAergic neurotransmission, we investigated the neurochemical and histological changes in male and female *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice. Our findings suggest that abnormal behaviors in female *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice may be associated with the imbalance of GABAergic and DAergic systems.

2. Materials and Methods

2.1. Animals

Construction of the *Disc1* gene-targeting vector and generation of *Disc1* mutant mice were described previously (Kuroda et al., 2011). Mice homozygous for the *Disc1* mutation (*Disc1*^{Δ2-3/Δ2-3} mice) were produced at the expected mendelian ratio by intercrossing mice heterozygous for the *Disc1* mutation. *Disc1*^{+/+} and *Disc1*^{Δ2-3/Δ2-3} adult mice (20-35 g) were used in all experiments. All of the animal protocols were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine; in addition, the Principles for the Care and Use of Laboratory Animals, which were approved by the Japanese Pharmacological Society, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals were followed.

2.2. Immunohistochemistry for PV

Disc1^{+/+} and *Disc1*^{Δ2-3/Δ2-3} mice were deeply anesthetized with diethyl ether, and transcardially perfused with ice-cold 0.1 M phosphate buffer (PB, pH 7.4) followed by 4% paraformaldehyde (PFA) in PB. Their brains were removed and post-fixed in the same fixative overnight. Then, the brains were cryoprotected using 20% and 30% sucrose each day at 4°C, embedded in Tissue-Tek O.C.T. compound (Sakura Finetech, Tokyo, Japan) and then stored at -80°C. The brains were cut into 50 μm coronal sections on a cryostat (CM3000; Leica Microsystems GmbH, Wetzlar, Germany) and the free-floating sections were used for PV immunohistochemistry. The sections were collected according to the brain atlas (Franklin and Paxinos, 1997): medial prefrontal cortex (mPFC), between +1.54 and +1.98 from the bregma; and hippocampus, between -1.34 and -2.06 from the bregma. They were incubated with blocking solution containing 5% normal goat serum and 0.3% Triton X-100 in PBS for 30 min, and then incubated with mouse anti-PV antibody (1:200, Swant, Bellinzona, Switzerland) for 48 h at 4°C with constant shaking. Sections were incubated with peroxidase-labeled polymer at room temperature for 1 h, and the reaction was visualized using 3,3'-diaminobenzidine (EnVision Kit, Dako, Tokyo, Japan).

2.3. Quantitative analysis of PV immunohistochemistry

To quantify the number of PV-positive cells in each brain region, three images were acquired with a Keyence Biozero microscope (BZ-8000; Keyence, Osaka, Japan). The acquisition parameters were kept the same for all images. Both right and left hemispheres of 3-5 different sections for each selected area, located according to the atlas of Franklin and Paxinos (1997), were examined in order to count the PV-positive cells. The region of interest (ROI) was defined using the imaging software WinRoof (Mitani Corp., Fukui, Japan) as follows: 1200 × 700 μm in the mPFC, 1200 × 700 μm in the CA1 subregion, 800 × 900 μm in the CA3 subregion and 500 × 1500 μm in DG of the hippocampus. The areas of PV-positive cells were measured using the software after subtraction of the threshold value. The threshold was automatically measured at a minimum level using this software in the first image and was kept constant across all images (Niwa et al., 2010). The PV-positive cells were counted three times with different ROI in one section, which was performed in an individual blinded manner. This procedure resulted in a total of 4 determinations of the number of PV-positive cells within a specified area of each brain. The average of the 4 determinations was used for statistical analysis.

2.4. *In vivo* microdialysis

Mice were anesthetized with sodium pentobarbital [50 mg/kg, intraperitoneal injection (i.p.)] and a guide cannula (AG-6, Eicom Corp., Kyoto, Japan) was implanted in the NAc (+1.5 mm anteroposterior, +0.8 mm mediolateral from the bregma, 4.0 mm dorsoventral from the skull) according to the mouse brain atlas (Franklin and Paxinos, 1997). On recovery from the surgery, a dialysis probe (A-I-6-01; membrane length 1 mm, Eicom Corp.) was inserted through the guide cannula, and perfused with artificial cerebrospinal fluid (aCSF; 147 mmol/l NaCl, 4 mmol/l KCl and 2.3 mmol/l CaCl₂) at a flow rate of 1.0 μl/min. The outflow fractions were collected every 20 min. After the collection of baseline fractions, the mouse was administered METH (2 mg/kg, i.p., Dainippon Sumitomo Pharma, Osaka, Japan). DA levels in the dialysates were analyzed using a high-performance liquid chromatography (HPLC) system (HTEC-500, Eicom Corp.) equipped with an

191 electrochemical detector (Nagai et al., 2010).

193 **2.5. Western blotting analysis**

194 *Disc1*^{+/+} and *Disc1*^{Δ2-3/Δ2-3} mice were decapitated and their brains were removed. The NAc
195 was rapidly dissected out on ice and sonicated in 100 μL of RIPA buffer [20 mM Tris-HCl
196 (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium
197 deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1
198 μg/ml leupeptin, 1 mM PMSF and Complete protease inhibitor cocktail (Roche,
199 Indianapolis, IN)]. Lysates from the NAc were cleared by centrifugation at 15,000 x g for
200 20 min. The protein concentration was determined using a DC Protein Assay Kit (Bio-Rad
201 Laboratories, Hercules, CA), and protein was boiled for 5 min at 95°C in sample buffer
202 [0.0625 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 5% glycerol, 0.002%
203 bromophenol blue and 5% 2-mercaptoethanol], applied to 8% SDS-polyacrylamide gel and
204 subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA).
205 The membranes were blocked with Blocking One-P (Nacalai Tesque, Inc., Kyoto, Japan) at
206 room temperature for 1 h and incubated with a primary antibody at 4°C overnight. Then,
207 membranes were washed with Tris-buffered saline-Tween 20 [TBS-T: 10 mM Tris-HCl (pH
208 7.4), 100 mM NaCl and 1% Tween-20] for 30 min each. After incubation with a
209 horseradish peroxidase-conjugated secondary antibody for 1 h, the immune complex was
210 detected using ECL Prime Western blotting detection reagents (GE Healthcare, Chalfont St.
211 Giles, Buckinghamshire, UK). The intensity of the bands on the membranes was
212 analyzed by densitometry (Atto Instruments, Tokyo, Japan). All the data from Western
213 blotting are expressed as relative fold change in expression with respect to the control.
214 The primary and secondary antibodies were diluted with Can-Get-Signal Solutions 1 and 2
215 (Toyobo Co., Ltd., Osaka, Japan), respectively, to enhance antibody-antigen binding.
216 Primary antibodies used were as follows: a rat anti-DAT (1:2500; Millipore) and a goat
217 anti-β-actin antibody (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA). The
218 secondary antibodies used were horseradish peroxidase-conjugated anti-rat (1:5000;

Santa Cruz Biotechnology) and anti-goat antibody (1:8000; Santa Cruz Biotechnology).

2.6. Real-time reverse transcription (RT)-PCR

Disc1^{+/+} and *Disc1*^{Δ2-3/Δ2-3} mice were decapitated and their brains were removed. The mPFC, hippocampus, striatum and NAc were immediately dissected on ice. The total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). Residual DNA was removed using TURBO DNase-free Kit (Invitrogen, Carlsbad, CA, USA). The isolated RNA was converted into cDNA using Super Script III First-Strand Synthesis System (Invitrogen) for RT-PCR. Quantitative real-time RT-PCR was performed using a 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) in a reaction mixture with a volume of 25 µl containing 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems), 0.5-1.0 µg of cDNA and 0.5 µM primers for each of the target genes. All reactions were carried out in duplicate with or without a template control, as well as RT samples. PCR cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and then 60 cycles of 95 °C for 15 s and 60 °C for 1 min. The primers used are shown in Table 1. Data were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference housekeeping gene.

2.7. Statistical analysis

All data are expressed as the mean ± S.E. For immunohistochemistry, Western blotting and RT-PCR analysis, Student's *t*-test was used for comparing two sets of data. For *in vivo* microdialysis, two-way analysis of variance (ANOVA) with repeated measures was used followed by Bonferroni *post hoc* tests when *F* ratios were significant (*p*<0.05).

3. Results

3.1. Disturbance of PV interneurons in *Disc1*^{Δ2-3/Δ2-3} mice

To examine the alterations of the GABAergic system in *Disc1*^{Δ2-3/Δ2-3} mice, we counted the number of PV-positive cells in the hippocampus and mPFC of *Disc1*^{+/+} and *Disc1*^{Δ2-3/Δ2-3} mice. In the hippocampus, PV-positive cells were found in CA1, CA3 and DG of the hippocampus in both *Disc1*^{+/+} and *Disc1*^{Δ2-3/Δ2-3} mice (Fig. 1A). Most of the PV-positive cells were found in the CA1 region followed by the CA3 region and DG in both *Disc1*^{+/+} and *Disc1*^{Δ2-3/Δ2-3} mice, and the distributions of PV-positive cells in each subregion were similar in the two genotypes (Fig. 1A-C). Female *Disc1*^{Δ2-3/Δ2-3} mice had a significant reduction of the total number of PV-positive cells in the hippocampus compared with female *Disc1*^{+/+} mice ($p < 0.05$, Fig. 1C), while no significant difference was observed in male mice (Fig. 1B). This reduction in female mice was likely due to the changes in the CA3 and DG regions of the hippocampus (Fig. 1C). In the mPFC, a uniform distribution of PV-positive cells was seen in layers II-VI of *Disc1*^{+/+} and *Disc1*^{Δ2-3/Δ2-3} mice, with no apparent differences among the different genotypes and genders of the mice (data not shown).

3.2. METH-induced DA release and DAT expression levels in *Disc1*^{Δ2-3/Δ2-3} mice

Our previous report demonstrated that METH-induced hyperactivity is significantly potentiated in *Disc1*^{Δ2-3/Δ2-3} mice, especially females, compared with that in *Disc1*^{+/+} mice. To clarify whether potentiation of METH-induced hyperactivity in *Disc1*^{Δ2-3/Δ2-3} mice is associated with alteration of DAergic neurotransmission in the NAc, we examined METH-induced DA release by *in vivo* microdialysis. The results indicated that basal levels of DA in the NAc of *Disc1*^{Δ2-3/Δ2-3} male and female mice did not differ from those in *Disc1*^{+/+} mice (*Disc1*^{+/+} male, 3.2 ± 0.4 pg/fraction, $n=8$; *Disc1*^{+/+} female, 3.1 ± 0.4 pg/fraction, $n=6$; *Disc1*^{Δ2-3/Δ2-3} male, 2.2 ± 0.3 pg/fraction, $n=10$; *Disc1*^{Δ2-3/Δ2-3} female, 2.4 ± 0.4 pg/fraction, $n=6$). Two-way repeated measures ANOVA revealed significant effects of genotype in

female mice [genotype, $F(1, 10)=6.76$, $p<0.05$; time after the METH injection, $F(8, 80)=16.68$, $p<0.01$; time after the METH injection x genotype interaction, $F(8, 80)=0.53$, $p=0.83$, Fig. 2B], but not in male mice [genotype, $F(1, 16)=0.19$, $p=0.67$; time after the METH injection, $F(8, 128)=16.79$, $p<0.01$; time after the METH injection x genotype interaction, $F(8, 128)=0.66$, $p=0.7232$, Fig. 2A]. METH-induced DA release in the NAc was significantly potentiated in *Disc1* ^{$\Delta 2-3/\Delta 2-3$} female mice compared with that in *Disc1*^{+/+} female mice ($p<0.05$, Fig. 2B), while there was no significant difference in male mice (Fig. 2A). To determine whether the differences in METH-induced DA release were due to DAT expression, we further analyzed the protein level of DAT in the NAc of *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice. There were no significant differences in DAT expression levels of the NAc of *Disc1* ^{$\Delta 2-3/\Delta 2-3$} and *Disc1*^{+/+} mice (Fig. 3).

3.3. mRNA expressions of GABA_A and DA receptors in *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice

We examined the mRNA expressions of GABA_A receptor subunits and DA receptor D₁ and D₂ subtypes in the mPFC, hippocampus, striatum and NAc of *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice. Expression levels of DA D₁ and D₂ receptor mRNA were significantly increased in the NAc of female *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice compared with those in *Disc1*^{+/+} mice ($p<0.05$ for D₁ receptor, $p<0.01$ for D₂ receptor, Fig. 3B). Similarly, DA D₂ receptor mRNA level in the NAc of male *Disc1* ^{$\Delta 2-3/\Delta 2-3$} mice was increased compared with the level in male *Disc1*^{+/+} mice, but the change was not statistically significant (Fig. 3A). There was no difference at all in the DA receptor D₁ subtype mRNA level between male *Disc1* ^{$\Delta 2-3/\Delta 2-3$} and *Disc1*^{+/+} mice (Fig. 3A). Furthermore, no differences were evident in DA D₁ and D₂ receptor mRNA levels in other brain areas, including the mPFC, hippocampus and striatum (data not shown). Regarding GABA_A receptor subunit expression, there were no significant differences between *Disc1* ^{$\Delta 2-3/\Delta 2-3$} and *Disc1*^{+/+} mice in the mPFC and hippocampus, in neither male nor female mice (Table 2).

4. Discussion

The main findings of the present study are that deficiency of DISC1 leads to anatomical and neurochemical abnormalities in PV-positive GABA inhibitory interneurons and the functional alternations of DAergic neurons, which may be associated with behavioral phenotypes in *Disc1*^{Δ2-3/Δ2-3} mice.

We sought to determine whether a deletion of exons 2 and 3 of the *Disc1* gene would alter PV-positive interneuron density and GABA_A receptor expression in *Disc1*^{Δ2-3/Δ2-3} mice because one of the most prominent cellular features of SZ that has emerged from postmortem studies is a consistent reduction of PV neurons (Lewis et al., 2005). In the present study, we detected a significant reduction of PV-positive interneurons in the hippocampus of *Disc1*^{Δ2-3/Δ2-3} mice, which was mainly observed in the CA3 and DG regions, but not in the CA1 region, of the hippocampus. These results are consistent with previous findings reported for other *Disc1* mutant mice (Ayhan et al., 2011; Hikida et al., 2007; Lee et al., 2013; Niwa et al., 2010; Shen et al., 2008). In the hippocampus, fast-spiking interneurons, which express PV, mediate rapid and precisely timed inhibition and therefore play a particularly important role in controlling the discharge of granule cells (Jonas et al., 2004). It is possible that the deficiency of PV-positive cells in the hippocampus leads to the impairments of LTP and abnormality of fear memory in *Disc1*^{Δ2-3/Δ2-3} mice (Kuroda et al., 2011).

The number of PV-interneurons was normal in the mPFC of *Disc1*^{Δ2-3/Δ2-3}, although mice with knockdown or L100P mutation of DISC1 have impaired interneuron migration and positioning in the cortex (Lee et al., 2013; Streinecke et al., 2012). It has been demonstrated that DISC1 was preferentially expressed in the hippocampal, cortical, cerebellar and olfactory neurons in the brain, and the signals in pyramidal cells of CA1-3 and granule cells of DG in the hippocampus were more potent at the postnatal stage than in adulthood (Austin et al., 2003, 2004; Honda et al., 2004; Kuroda et al., 2011; Ma et al., 2002; Miyoshi et al., 2003). In addition to pyramidal neurons, DISC1 expression has been detected in medial ganglionic eminence-derived cortical interneurons (Streinecke et al., 2012). Detailed analysis is required in this regard because the causes and functional

consequences of these abnormalities are yet to be determined.

The GABA_A receptor is an ionotropic receptor that passes chloride ions through its pore. Little is known about the relationship between DISC1 and GABA_A receptors, although interplay between DISC1 and GABA signaling regulates the development of newborn dentate granule neurons during adult neurogenesis (Kim et al., 2012). The mRNA expression of GABA_A receptor subunits examined in the present study did not differ between *Disc1*^{Δ2-3/Δ2-3} and *Disc1*^{+/+} mice in the mPFC and hippocampus. It is therefore unlikely that deletion of *Disc1* affects GABA_A receptor expression, at least at the transcriptional level, although DISC1 may be involved in the signaling of other GABA_A receptor subunits and GABA_B receptor.

The NAc is a crucial node in the neuronal circuit receiving glutamatergic inputs from the PFC, hippocampus and basolateral amygdala and DAergic projections from the ventral tegmental area (Guillin et al., 2007). The increase in sustained DA activity in the NAc may be associated with the emergence of psychotic experiences. In previous reports, PET imaging studies indicated the relationship between alterations of D₁ receptor availability and cognitive functions in SZ (Abi-Dargham et al., 2002; Karlsson et al., 2002; Okubo et al., 1997). It has been proposed that abnormality of the DAergic system in SZ induces perturbation of the information flow within the circuit via the NAc.

Our previous report demonstrated that METH-induced hyperactivity is significantly potentiated in *Disc1*^{Δ2-3/Δ2-3} mice, while the contents of DA and its metabolites in the brain are comparable to those in *Disc1*^{+/+} mice (Kuroda et al., 2011). In the present study, we demonstrated that METH-induced increase in extracellular DA levels in the NAc of female *Disc1*^{Δ2-3/Δ2-3} mice was significantly higher than the response in female *Disc1*^{+/+} mice. There were no significant changes in the DAT expression level of the NAc between *Disc1*^{+/+} and *Disc1*^{Δ2-3/Δ2-3} mice, suggesting that potentiation of METH-induced DA release in female *Disc1*^{Δ2-3/Δ2-3} mice is not due to the alterations of DAT expression level in the NAc. Our observation is consistent with previous findings in other *Disc1* mutant (Jaaro-Peled et al., 2013; Lipina et al., 2010) and knockdown mice (Niwa et al., 2010).

The expression levels of DA D₁ and D₂ receptor mRNA were significantly increased in the NAc of female *Disc1*^{Δ2-3/Δ2-3} mice compared with those in *Disc1*^{+/+} mice. Activation of DA D₁ receptors leads to an increase in excitability and promotes transition to the up-state via increases in NMDA receptor, L-type calcium channel and sodium channel current (Surmeier et al., 2010). Medium spiny neurons expressing DA D₁ receptor in the NAc project to GABA interneurons in the VTA, and its activation can drive the disinhibition of VTA DA neurons (Bocklisch et al., 2013). In contrast, DA D₂ receptors are found on the terminals of DA neurons and post-synaptically on medium spiny neurons, both of which are known to inhibit DA transmission (Ford, 2014). Therefore, deficiency of *Disc1* may lead to the enhancement of METH-induced DA release through postsynaptic DA D₁ receptors, but not through the presynaptic DAT or DA D₂ receptors.

The interplay between the reduction of PV-positive interneuron density in the hippocampus and potentiation of METH-induced DA release in the NAc of *Disc1*^{Δ2-3/Δ2-3} mice remains to be determined. Disinhibition of the hippocampal circuits may contribute to psychological abnormalities because hippocampal hyperactivity leads to dysregulation of the mesolimbic DAergic system (Lodge and Grace, 2011). It has been reported that hippocampal stimulation results in an increase in DA neuronal activity and DA release through polysynaptic projection involving a glutamatergic input to the NAc, which then increases GABAergic neuronal activity projecting to the ventral pallidum (Floresco et al., 2003; Legault et al., 2000). This increase in GABA activity decreases tonic ventral pallidum activity, resulting in disinhibition of DA neuronal activity in the VTA (Lodge and Grace, 2011). Recently, transplantation of interneuron precursors in adult hippocampus has been shown to ameliorate DAergic abnormalities in cyclin D2-deficient mice, a mouse model of hippocampal disinhibition (Gilani et al., 2014). Therefore, the reduction of PV-positive interneurons in the hippocampus may be involved in the hyperresponsiveness to METH in *Disc1*^{Δ2-3/Δ2-3} mice.

Clinical studies have demonstrated clear gender differences in the peak age of onset, antipsychotic reactivity, symptoms, cognition performance and academic/social

premorbid impairment in patients with SZ (Allen et al., 2013; Brébion et al., 2013; Leung and Chue, 2000). In the present study, we demonstrated that female, but not male, *Disc1*^{Δ2-3/Δ2-3} mice exhibited a significant reduction of PV-positive interneurons in the hippocampus, exacerbated METH-induced DA release and increases in DA D₁ and D₂ receptor mRNA levels in the NAc, which may somewhat explain the gender-dependent behavioral phenotypes of *Disc1*^{Δ2-3/Δ2-3} mice (Kuroda et al., 2011). Gender-dependent abnormal behaviors have also been reported in other DISC1 mutant models (Ayhan et al., 2011; Pletnikov et al., 2008; Pogorelov et al., 2012). There are many potential factors that may cause abnormalities in female mice. For instance, preclinical and clinical studies have suggested that estrogen and brain-derived neurotrophic factor (BDNF) are involved in the pathophysiology of SZ (Häfner et al., 1991; Pillai, 2008), and that they play a crucial role in development, function and synaptic plasticity of the hippocampus (Fester et al., 2011; Friedman et al., 1991; Rune et al., 2006).

Estrogens modulate several major neurotransmitter systems including DAergic and GABAergic systems (Kritzer and Kohama, 1999; Sanchez et al., 2010; Weiland, 1992). Estradiol benzoate was reported to increase amphetamine-stimulated nigrostriatal DA release (Becker, 1990; Castner et al., 1993). The effect of METH is facilitated by pretreatment with estradiol in female mice (Chen et al., 2003). Estrogen receptor-β colocalizes extensively with PV-labeled interneurons in the hippocampus of rats (Blurton-Jones and Tuszynski, 2002). Estrogen may exert at least some of its effects through changes in BDNF–TrkB signaling. For instance, during the estrous cycle, high levels of estrogen have been associated with decreased BDNF mRNA expression in the hippocampus and PFC (Sun and Alkon, 2006). The phosphorylation level of TrkB is reduced in the striatum of female *Bdnf* heterozygous mice, which have been reported to show a 50% reduction in BDNF expression levels similar to those seen in SZ patients (Weickert et al., 2003). Taken together, gender-dependent abnormalities of *Disc1*^{Δ2-3/Δ2-3} mice may be associated with estrogens and BDNF, but further studies are required to address this issue.

In conclusion, we found a significant reduction of PV-positive interneurons in the hippocampus of female *Disc1*^{Δ2-3/Δ2-3} mice. Significant potentiation of METH-induced DA release was found in the NAc of female *Disc1*^{Δ2-3/Δ2-3} mice, which was accompanied by a significant increase in DA D₁ and D₂ receptor mRNA levels with no changes in DAT protein level in the NAc. Our study suggests that disruption of exons 2 and 3 of the *Disc1* gene leads to imbalance of GABAergic and DAergic systems in female mice.

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Figure Legends

Fig. 1 Changes in PV-positive interneurons in *Disc1*^{Δ2-3/Δ2-3} mice. **A**, Representative
photographs showing PV-positive cells in the hippocampus. Scale bar: 200 μm. **B** and **C**,
Changes in the number of PV-positive interneurons in the hippocampus of male (B) and
female (C) mice. Total indicates the sum of PV-positive interneurons in CA1, CA3 and
dentate gyrus (DG) subregions. Values represent the mean ± S.E. (male *Disc1*^{+/+}, n=4;
male *Disc1*^{Δ2-3/Δ2-3}, n=4; female *Disc1*^{+/+}, n=4; female *Disc1*^{Δ2-3/Δ2-3}, n=4). **p*<0.05 compared
with the corresponding *Disc1*^{+/+} mice.

Fig. 2 METH-induced DA release in the NAc of *Disc1*^{Δ2-3/Δ2-3} mice. **A** and **B**, Changes in
METH-induced DA release in the NAc of male (A) and female (B) mice. A dialysis probe
was inserted through the guide cannula and perfused with Ringer's solution at a flow rate
of 1.0 μl/min. Following the collection of three baseline fractions, mice were injected with
METH (2 mg/kg, i.p.) at time 0 min. Values represent the mean ± S.E. (male *Disc1*^{+/+}, n=8;
male *Disc1*^{Δ2-3/Δ2-3}, n=10; female *Disc1*^{+/+}, n=6; female *Disc1*^{Δ2-3/Δ2-3}, n=6). **p*<0.05
compared with the corresponding *Disc1*^{+/+} mice.

Fig. 3 DAT expression in the NAc of *Disc1*^{Δ2-3/Δ2-3} mice. **A** and **B**, Changes in DAT level in the NAc of male (A) and female (B) mice. Expression levels of DAT were quantified by Western blotting analysis. β-Actin was used to normalize protein loading. Values represent the mean ± S.E. (n=6 in each group of mice).

Fig. 4 DA receptor subtype mRNA expression in the NAc of *Disc1*^{Δ2-3/Δ2-3} mice. **A** and **B**, Changes in DA D₁ receptor and DA D₂ receptor mRNA levels in the NAc of male (A) and female (B) mice. Expression levels of DA D₁ receptor and DA D₂ receptor mRNA were quantified by real-time RT-PCR using the 2^{-ΔΔCT} method. Each value was normalized to the internal reference housekeeping gene GAPDH. Values represent the mean ± S.E. (n=5 in each group of mice). **p*<0.05 and ***p*<0.01 compared with the corresponding *Disc1*^{+/+} mice.

Fig. 1. (Nakai et al.)

Fig. 2. (Nakai et al.)

A

B

Fig. 3. (Nakai et al.)

A

Male

B

Female

Fig. 4. (Nakai et al.)

Table 1. Real-time RT-PCR primer sequences

Transcript	Ref. Seq ID	Forward primer	Reverse primer	Amplicon (bp)
GABA _A receptor subunit				
α₁ (GABRA1)	NM_010250	5'-CAAGAGCAGAAGTTGTCTATGAGT-3'	5'-GCACGGCAGATATGTTTGAATAAC-3'	215
α₂ (GABRA2)	NM_008066	5'-GCTACGCTTACACAACCTCAG-3'	5'-GACTGGCCCAGCAAATCATACT-3'	115
α₄ (GABRA4)	NM_010251	5'-AGAACTCAAAGGACGAGAAATTGT-3'	5'-TTCACCTTCTGTAACAGGACCCC-3'	118
β₂ (GABRB2)	NM_08070	5'-AACTACATCTTCTTTGGGAGAGGA-3'	5'-GGTCCATCTTGTTGACATCCAG-3'	106
γ₂ (GABRG2)	NM_008073	5'-ACTTCTGGTGACTATGTGGTGAT-3'	5'-GGCAGGAACAGCATCCTTATTG-3'	147
DA receptor subtype				
D₁ (DRD1a)	NM_010076.3	5'-ATCGTCACTTACACCAGTATCTACAGGA-3'	5'-GGCAGGAACAGCATCCTTATTG-3'	100
D₂ (DRD2)	NM_010077.2	5'-GTCCTGTCCTTCACCATCTCTTG-3'	5'-CGAGACGATGGAGGAGTAGACC-3'	102
GAPDH	NM_008084	5'-CAATGTGTCCGTCGTGGATCT-3'	5'-GTCCTCAGTCTAGCCCAAGATG-3'	124

Table 2. Analysis of GABA_A receptor subunit mRNA expression in the mPFC and hippocampus of *Disc1*^{+/+} and *Disc1*^{Δ2-3/Δ2-3} mice

mRNA fold change	mPFC			Hippocampus		
Transcript	Male + Female	Male	Female	Male + Female	Male	Female
GABA _A receptor subunit						
α₁ (GABRA1)						
<i>DISC1</i> ^{+/+}	1.00 ± 0.27	1.00 ± 0.43	1.00 ± 0.32	1.00 ± 0.39	1.00 ± 0.29	1.00 ± 0.89
<i>DISC1</i> ^{Δ2-3/Δ2-3}	0.80 ± 0.20	0.72 ± 0.51	0.89 ± 0.29	1.30 ± 0.39	0.92 ± 0.34	1.84 ± 0.64
α₂ (GABRA2)						
<i>DISC1</i> ^{+/+}	1.00 ± 0.17	1.00 ± 0.09	1.00 ± 0.37	1.00 ± 0.13	1.00 ± 0.11	1.00 ± 0.22
<i>DISC1</i> ^{Δ2-3/Δ2-3}	1.53 ± 0.35	1.66 ± 0.78	1.40 ± 0.25	1.31 ± 0.48	1.46 ± 1.17	1.18 ± 0.24
α₄ (GABRA4)						
<i>DISC1</i> ^{+/+}	1.00 ± 0.26	1.00 ± 0.27	1.00 ± 0.37	1.00 ± 0.44	1.00 ± 0.67	1.00 ± 0.67
<i>DISC1</i> ^{Δ2-3/Δ2-3}	1.11 ± 0.31	0.70 ± 0.39	0.80 ± 0.31	1.33 ± 0.46	1.34 ± 0.86	1.32 ± 0.39
β₂ (GABRB2)						
<i>DISC1</i> ^{+/+}	1.00 ± 0.44	1.00 ± 0.99	1.00 ± 0.37	1.00 ± 0.19	1.00 ± 0.25	1.00 ± 0.35
<i>DISC1</i> ^{Δ2-3/Δ2-3}	0.75 ± 0.21	0.80 ± 0.68	0.71 ± 0.21	1.19 ± 0.34	0.86 ± 0.44	1.63 ± 0.51
γ₂ (GABRG2)						
<i>DISC1</i> ^{+/+}	1.00 ± 0.15	1.00 ± 0.22	1.00 ± 0.25	1.00 ± 0.16	1.00 ± 0.26	1.00 ± 0.26
<i>DISC1</i> ^{Δ2-3/Δ2-3}	0.98 ± 0.14	0.97 ± 0.40	0.99 ± 0.19	0.87 ± 0.08	0.77 ± 0.11	0.97 ± 0.13

Each value shows relative fold change in transcripts with respect to *Disc1*^{+/+} mice. The transcripts in *Disc1*^{+/+} and *Disc1*^{Δ2-3/Δ2-3} mice were quantified by real-time RT-PCR using the 2^{-ΔΔCT} method, and normalized to the internal reference housekeeping gene GAPDH. Values represent the mean ± S.E. [*Disc1*^{+/+} (n=3-5) and *Disc1*^{Δ2-3/Δ2-3} (n=3-5) in male, *Disc1*^{+/+} (n=3-5) and *Disc1*^{Δ2-3/Δ2-3} (n=3-5) in female, *Disc1*^{+/+} (n=6-10) and *Disc1*^{Δ2-3/Δ2-3} (n=6-10) in male + female].