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ORIGINAL ARTICLE

NMDA receptor regulates migration of newly generated neurons in the adult hippocampus via *Disrupted-In-Schizophrenia 1* (*DISC1*)

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Abstract

In the mammalian brain, new neurons are continuously generated throughout life in the dentate gyrus (DG) of the hippocampus. Previous studies have established that newborn neurons migrate a short distance to be integrated into a pre-existing neuronal circuit in the hippocampus. How the migration of newborn neurons is governed by extracellular signals, however, has not been fully understood. Here, we report that NMDA receptor (NMDA-R)-mediated signaling is essential for the proper migration and positioning of newborn neurons in the DG. An intraperitoneal injection of the NMDA-R antagonists, memantine, or 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) into adult male mice caused the aberrant positioning of newborn neurons, resulting in the overextension of their migration in the DG. Interestingly, we revealed that the administration of NMDA-R antagonists leads to a decrease in the expression of *Disrupted-In-Schizophrenia 1* (*DISC1*), a candidate susceptibility gene for major psychiatric disorders such as schizophrenia, which is also known as a critical regulator of neuronal migration in the DG. Furthermore, the overextended migration of newborn neurons induced by the NMDA-R antagonists was significantly rescued by exogenous expression of *DISC1*. Collectively, these results suggest that the NMDA-R signaling pathway governs the migration of newborn neurons via the regulation of *DISC1* expression in the DG.

Keywords: *DISC1*, hippocampus, migration, neurogenesis, NMDA receptor.

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The generation of new neurons, known as neurogenesis, persists throughout life in the DG of the hippocampus (Seki and Arai 1993, 1995; Kuhn *et al.* 1996; Ming and Song 2005; Namba *et al.* 2005). Recent studies have shown that newly generated neurons are associated with synaptic plasticity and cognitive functions, such as learning and memory, as well as some psychiatric diseases, including depression and schizophrenia (Ming and Song 2005; Wojtowicz *et al.* 2008; DeCarolis and Eisch 2010).

Neurogenesis is a complex, multi-step process that involves progenitor cell proliferation, neuronal differentiation, migration, and the integration of newly generated neurons into pre-existing neuronal circuits; these steps are thought to be properly regulated by several factors. In the adult hippocampus, cumulative evidence has shown that

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Abbreviations used: BrdU, 5-bromo-2-deoxyuridine; BSA, bovine serum albumin; B.W., body weight; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; CPP, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; DG, dentate gyrus; *DISC1*, *Disrupted-In-Schizophrenia 1*; GCL, granule cell layer; GFP, green fluorescent protein; i.p., intraperitoneally; LIS1, lissencephaly-1; MK-801, (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5-10-imine hydrogen maleate; NDEL, NudE-like; PBS, phosphate-buffered saline; PFA, paraformaldehyde; SDS, sodium dodecyl sulfate; SGZ, subgranular zone.

neurotransmitters, including glutamate and GABA, affect neurogenesis. The administration of NMDA-R antagonists, such as (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5-10-imine hydrogen maleate (MK-801), CGP37849, and memantine, stimulates progenitor cell proliferation at least 2 days after the drug administration, which may lead to subsequent neuronal production within the following 7–9 days (Cameron *et al.* 1995; Nacher *et al.* 2001; Nacher and McEwen 2006; Maekawa *et al.* 2009; Namba *et al.* 2009). Although the activation of NMDA-R or GABA-R signaling by a specific agonist accelerates neuronal differentiation of progenitor cells, the administration of NMDA-R or GABA-R antagonist inhibits *in vitro* or *in vivo* neuronal differentiation (Kitayama *et al.* 2004; Tozuka *et al.* 2005). In addition, the integration of newly generated neurons into adult hippocampal circuits is also regulated by NMDA-R signaling (Tashiro *et al.* 2006). Thus, the neurotransmitters have crucial modulatory effects on multiple steps of adult neurogenesis, but the effect of neurotransmitters on neuronal migration in the adult hippocampus remains to be addressed. Since the inhibition of NMDA-R or GABA-R signaling in the embryonic neocortex results in unusual neuronal migration (Behar *et al.* 1999; Hirasawa *et al.* 2003; Manent and Represa 2007; Denter *et al.* 2010; Uchino *et al.* 2010), neurotransmitters appear to serve as important extracellular signals for neuronal migration (Heng *et al.* 2007).

Recently, several lines of study have identified various types of intracellular signals involved in neuronal migration. For example, *DISC1* is thought to regulate neuronal migration in the postnatal hippocampus, since the knockdown of *DISC1* expression leads to an abnormal overextension of the migration of newly generated neurons (Duan *et al.* 2007; Kvajo *et al.* 2008; Enomoto *et al.* 2009; Kim *et al.* 2009). *DISC1* was originally identified as a candidate susceptibility gene for major psychiatric disorders based on studies of a chromosomal translocation found in a large Scottish family with a high frequency of schizophrenia, bipolar disorder, and major depression (Millar *et al.* 2000; Chubb *et al.* 2008) and is involved in cAMP signaling, neurite elongation, and cargo trafficking in addition to neuronal migration (Kamiya *et al.* 2005; Millar *et al.* 2005; Shinoda *et al.* 2007; Taya *et al.* 2007). Furthermore, DISC1 cooperates with its binding partners, including lissencephaly-1 (LIS1), NudE-like (NDEL), and Girdin, to regulate neuronal migration (Ozeki *et al.* 2003; Brandon *et al.* 2004; Taya *et al.* 2007; Enomoto *et al.* 2009; Kim *et al.* 2009). *LIS1* was initially identified as a responsible gene for type-1 lissencephaly (Reiner *et al.* 1993; Hattori *et al.* 1994), and its mutation leads to defects in neuronal migration during embryonic and postnatal neurogenesis (Hirotsune *et al.* 1998; Wang and Baraban 2007). NDEL1 was identified as a LIS1-binding protein (Niethammer *et al.* 2000; Sasaki *et al.* 2000). The NDEL1/LIS1/DISC1 complex regulates cytoplasmic dynein and controls proper neuronal migration in embryonic and adult brains (Kamiya *et al.* 2005; Duan *et al.* 2007). Girdin, an actin-binding protein, serves as an Akt substrate and stabilizes actin stress fibers to prevent cell migration (Enomoto *et al.* 2009). Recent studies have demonstrated that Girdin cooperates with DISC1 and regulates the migration of newly generated neurons in the postnatal hippocampus (Enomoto *et al.* 2009; Kim *et al.* 2009). Thus, DISC1 and its interacting protein complex play important roles in neuronal migration during hippocampal neurogenesis.

In this study, we found that the administration of an NMDA-R antagonist in adult mice leads to the overextension of the migration of newly generated neurons in the hippocampus and reduced the expression of *DISC1* in the DG. The overextended migration caused by the NMDA-R antagonist was partially rescued by the lentiviral-mediated exogenous expression of DISC1. These findings suggest that NMDA-R signaling regulates neuronal migration by controlling *DISC1* expression.

Materials and methods

The animals used in this study were 3-month-old male and embryonic day (E) 15.5 pregnant C57BL6/J mice (Clea Japan Inc., Tokyo, Japan). All experiments in the result section were performed in 3-month-old male mice. All experimental procedures were approved by The Animal Care and Use Committee of the National Institute of Neuroscience and Nagoya University.

Animals and drug administration

Mice were injected intraperitoneally (i.p.) with MK-801 [1 mg/kg body weight (B.W.); Wako, Osaka, Japan], CPP (10 mg/kg B.W.; Tocris Cookson, Bristol, UK), memantine (10, 30, or 50 mg/kg B.W.; Sigma, St. Louis, MO, USA), methamphetamine (2 mg/kg B.W.; Dainippon Pharmaceuticals Ltd, Osaka, Japan), or D-cycloserine (30mg/kg B.W.; Sigma). Control mice were injected i.p. with the same volume of 0.9% saline (Ohtsuka Pharmaceuticals, Tokyo, Japan). For the 5-bromo-2-deoxyuridine (BrdU; Sigma)-labeling experiment, the mice were injected i.p. with 75 mg/kg B.W. of BrdU from day 9 to day 7 before the memantine injection. The mice were then killed at 2 days after the memantine, MK-801, or CPP-injection.

Tissue preparation

After the mice were deeply anesthetized with sodium pentobarbital (Kyoritsu Pharmaceuticals, Tokyo, Japan), the mice were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). The brains were immersion-fixed at 4°C overnight in the same fixative. After washing in PBS, the brains were successively equilibrated in 10% and 20% sucrose in PBS. Finally, the brains were embedded in Tissue-Tek optimal cutting temperature compound (Sakura, Tokyo, Japan), and frozen in liquid nitrogen (Seki *et al.* 2007).

Immunohistochemistry

Immunohistochemistry was performed using a floating method as described previously (Namba *et al.* 2009). Briefly, the frozen brains were sliced into 40-μm sections using a cryostat (CM-3000; Leica, Nussloch, Germany). After washing in PBS, the sections were incubated at 4°C overnight with rabbit polyclonal anti-green fluorescent protein (GFP) antibody (1:200; Medical & Biological Laboratories, Nagoya, Japan), goat polyclonal anti-doublecortin (Dcx) antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-Neuronal nuclei (NeuN) antibody (1:200; Millipore, Temecula, CA, USA), and/or rabbit polyclonal anti-NDEL1 antibody (1:200; Abcam, Cambridge, MA, USA) in PBS containing 1% bovine serum albumin (BSA), then incubated at room temperature (20–25°C) for 1–2 h with Alexa488-conjugated anti-rabbit IgG (1:400; Invitrogen, Carlsbad, CA, USA), Cy5-conjugated anti-mouse IgG (1:200; Jackson, West Grove, PA, USA) and/or Cy3 or Cy5-conjugated anti-goat IgG antibody (1:200; Jackson) in PBS containing 1% BSA. After washing in PBS, the sections were mounted on a glass slide and examined for fluorescent signals using a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan). For immunestaining with anti-BrdU antibody, the sections were incubated in 2N HCl at 37°C for 35 min, and then washed in PBS. The sections were then incubated with rat monoclonal anti-BrdU antibody (1:400; MorphoSys UK Ltd., Oxford, UK) at 4°C overnight in PBS containing 1% BSA. After washing in PBS, they were then incubated for 1–2 h in PBS containing 1% BSA plus Cy3-conjugated anti-rat IgG antibody (1:200; Jackson) between 20–25°C.

Reverse transcription–PCR

Total RNA extraction, cDNA synthesis, and real-time PCR analysis were performed as described previously (Namba *et al.* 2010) using RNeasy Plus Mini kit (QIAGEN, Germantown, ML, USA), Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA) and the SYBR green labeling system (SYBR Premix Ex Taq 2; Takara, Shiga, Japan), and the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), respectively. The primers and sizes of the PCR products are listed in Table 1. The thermocycler conditions were as follows: 5 s at 95°C, 10 s at 55°C, and 30 s at 72°C for 40 cycles. The data were analyzed using the delta–delta C_t method with glyceraldehydes-3-phosphate dehydrogenase as an internal control (Kodomari *et al.* 2009; Namba *et al.* 2010).

Immunoblot analysis

Lysate was prepared from the dissected dentate gyri at 2 days after the injection of memantine or saline as described previously (Namba *et al.* 2010). Briefly, the dissected dentate gyri were homogenized in lysis buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)]. After removing the nuclei and debris by centrifugation (2000 *g*, 4°C, 10 min), the protein concentration of the supernatant was determined using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA).

The proteins were subjected to immunoblotting with rabbit polyclonal anti-pan Ca^{2+}/calmodulin-dependent kinase II (CaMKII) antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA) or rabbit polyclonal anti-phospho CaMKII antibody (1:1000; Cell Signaling Technology). Blots were visualized using a chemiluminescence detection system (ECL-prime; Amersham Biosciences, Piscataway, NJ, USA). The membrane was re-probed with anti-β actin antibody (1:1000; Sigma) after incubation with Stripping buffer [62.5 mM Tris-HCl (pH 7.5), 2% SDS, 100 mM 2-mercaptoethanol] at 60°C for 30 min.

Infection of lentivirus into the dentate gyrus

Lentivirus carrying GFP-tagged mouse *DISC1* cDNA (pFUW-GFP-mDISC1) (Duan *et al.* 2007) was prepared using the ViraPower Lentiviral expression system (Invitrogen) according to the manufacturer's instructions. The lentivirus-GFP-*DISC1* (1 μL/injection) was stereotaxically injected into the dentate gyrus (anteroposterior, 2.5 mm; lateral, 2.0 mm; ventral, 3.0 mm from bregma, respectively) as described previously (Seki *et al.* 2007; Namba *et al.* 2010).

Statistical analysis

To analyze the localization of Dcx+ or BrdU+ cells, more than 100 cells from two to five sections per mouse were analyzed. To avoid double counting, adjacent sections were not used for the cell counting. All of the counting was performed under the confocal laser-scanning microscope (FV1000) with 60× objective. Data were evaluated using a one-way ANOVA followed by a *post hoc* Scheffe *F*-test. All the values were expressed as the mean ± SEM, and p-values < 0.05 were considered significant.

Results

Administration of NMDA-R antagonist causes the overextended migration of newly generated neurons in the adult hippocampus

In the adult hippocampus, neuronal progenitor cells arising from the subgranular zone (SGZ) migrate into the granule

Table 1 Primer sequences for PCR

Name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplification size (bp)	Accession Nos
DISC1-1	CAGTGGGTTTTGGCAAGAAT	CCAAGGGAGAGTTGGATGAA	210	NM_174854.2
DISC1-2	GAGCTCACGGAGGAGATTTG	ACCTTCCAACACTTCCATGC	216	NM_174854.2
GAPDH	GTCATCATCTCCGCCCCTTCTGC	GATGCCTGCTTCACCACCTTCTTG	443	NM_008084
Girdin	GTGTCATTGCAGGAGCAGAA	GACTTCAGGGTGCCATGTTT	287	NM_176841.3
Lis1	GATGACAAGACCCTCCGTGT	GAGCTCAAATGGGGTAACCA	240	NM_013625.3
NDEL1	GGACTCTGCGCGATATCAAT	TTCCACATCCAGTGATCGAG	126	NM_023668.2

cell layer (GCL) and differentiate in mature neurons. Since most newly generated neurons express Dcx (a marker for immature neuron), Dcx+ cells are mainly located in the SGZ and inner part of the GCL. To investigate the effect of NMDA-R antagonist on the migration of newly generated neurons, we first analyzed the localization of Dcx+ immature neurons. Three-month-old male mice were i.p. injected with saline as a control or three types of NMDA-R antagonist: uncompetitive (memantine, 50 mg/kg B.W.), non-competitive (MK-801, 1 mg/kg B.W.), or competitive (CPP, 10 mg/kg B.W.). Two days later, their brains were fixed with 4% PFA for immunohistochemistry using anti-Dcx antibody (Fig. 1a–e). To perform the statistical analysis, we divided the GCL into three parts, that is, the outer third of the GCL, the middle third of the GCL, and the inner third of the GCL and the SGZ, and then counted the Dcx+ cells localized in each layer. In the control mice, most of the Dcx+ cells were located in the inner third of the GCL (97.0 ± 0.6%, $n = 3$), whereas a few Dcx+ cells were found in the middle and outer third of the GCL (outer, 0.0 ± 0.0%; middle, 3.0 ± 0.6%; $n = 3$; Fig. 1b and f). In contrast, the percentages of the Dcx+ cells in the outer and middle thirds of the GCL were significantly higher in the memantine-injected mice (outer, 3.7 ± 0.3%; middle, 12.3 ± 0.3%; $n = 3$). Conversely, the percentage of Dcx+ cells in the SGZ and inner third of the GCL was significantly reduced (84.0 ± 1.8%, $n = 3$; Fig. 1c and f). Similar results were obtained from the mice injected with the other antagonists, CPP and MK-801 (CPP: outer, 1.7 ± 0.3%; middle, 11.7 ± 2.2%; inner, 86.7 ± 1.9%; MK-801: outer, 1.3 ± 0.3%; middle, 8.7 ± 1.8%; inner, 90.0 ± 1.7%; $n = 3$; Fig. 1d–f). On the other hand, the total number of Dcx+ cells was approximately equal among the control and drug-injected mice (Figure S1).

To investigate the dose-dependent effect of NMDA-R antagonist on the localization of the Dcx+ cells, we used memantine because it showed most prominent effect on neuronal positioning (Fig. 1f). Mice were injected with 10, 30, or 50 mg/kg B.W. of memantine and were killed 2 days after the injection (Fig. 1g). At the 10 mg/kg B.W. dose, no significant differences in the localization of the Dcx+ cells, compared with the control mice, were observed (outer, 0.3 ± 0.3%; middle, 5.0 ± 0.6%; inner, 94.7 ± 0.7%; $n = 3$).

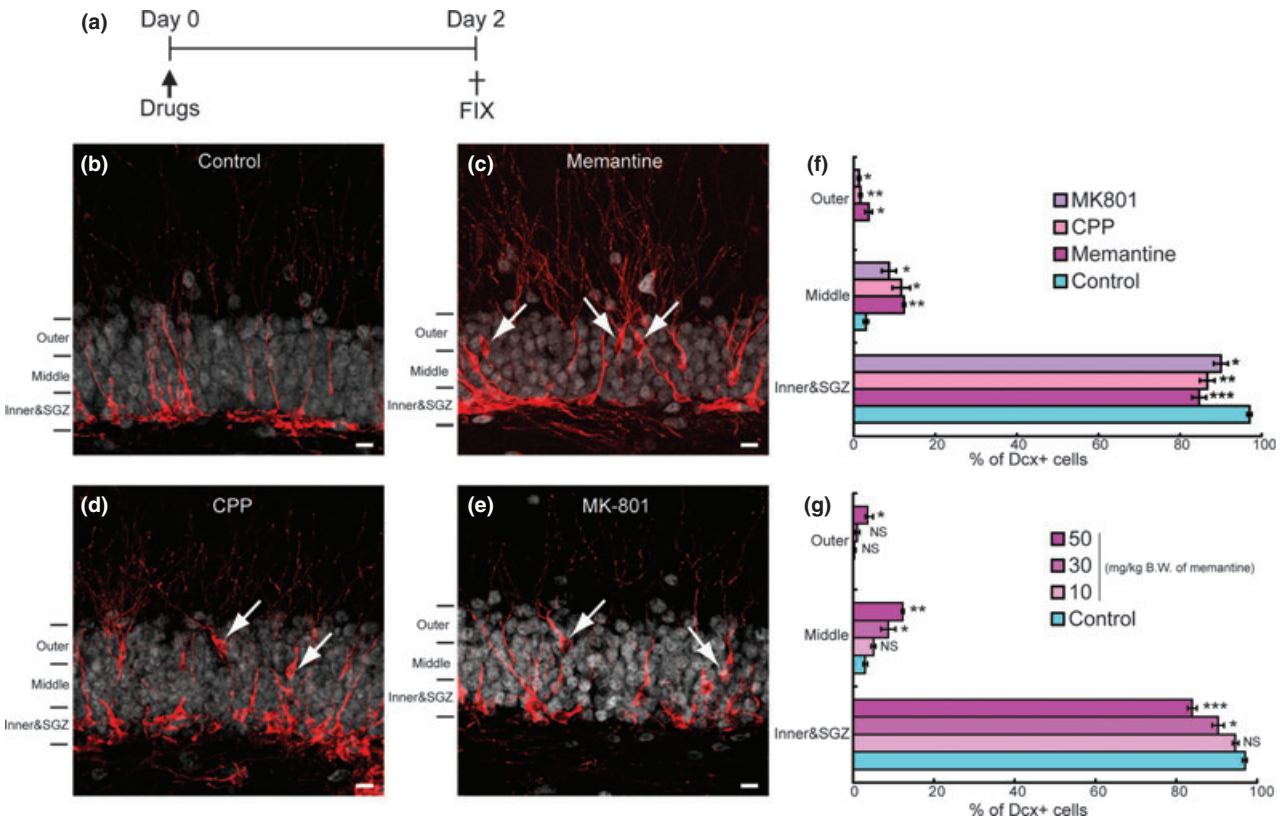

Fig. 1 Administration of NMDA-R antagonist results in the aberrant positioning of Dcx+ immature neurons. (a) Schematic illustration of the experimental design. (b–e) Representative immunohistochemical images of the Dcx+ cells (red) and the NeuN+ cells (white) in control mice (b) and mice injected with memantine (c), CPP (d), or MK-801 (e). (f) Quantitative analysis of the location of the Dcx+ cells in the GCL. (g) Dose-dependent effect of memantine on the positioning of the Dcx+ cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS, not significant. Scale bars = 10 μm.

In contrast, at dose of 30 and 50 mg/kg B.W., the percentage of Dcx+ cells in the middle third of the GCL increased significantly, whereas those in the SGZ and the inner third of the GCL decreased significantly (30 mg/kg: outer, 1.0 ± 0.6%; middle, 8.7 ± 1.9%; inner, 90.3 ± 1.5%; $n = 3$; 50 mg/kg: outer, 3.7 ± 0.3%; middle, 12.3 ± 0.3%; inner, 84.0 ± 1.8%; $n = 3$), indicating that the effect of memantine on the localization of the Dcx+ cells was in a dose-dependent manner.

Since previous studies have shown that some NMDA-R antagonists stimulate dopamine release and act as a dopamine receptor agonist (Imperato *et al.* 1990; Spanagel *et al.* 1994), we examined the involvement of dopaminergic signal in cellular migration. Here, we used methamphetamine as a dopamine receptor agonist at a low dose (2 mg/kg B.W.; Adachi *et al.* 2001; Shoblock *et al.* 2003), because the high dose of methamphetamine (e.g. more than 20 mg/kg B.W.) induced neurotoxicity (Fukumura *et al.* 1998). Mice were injected with methamphetamine (2 mg/kg B.W.) and fixed 2 days after the injection. In contrast to the NMDA-R antagonist, methamphetamine did not affect the localization of the Dcx+ cells (Fig. 2), suggesting that a dopaminergic signal was not involved in the migration of newly generated neurons in the adult hippocampus.

Previous evidence demonstrated that the aberrant activation of NMDA-R elicits epileptic seizure in rats (Mares and Velisek 1992). We therefore examined the effect of D-cycloserine, an NMDA-R agonist (Hood *et al.* 1989), on the localization of the Dcx+ cells. Mice were injected with D-cycloserine (30 mg/kg B.W.) (Yang *et al.* 2010) and their brains were fixed 2 days later. D-Cycloserine did not affect the localization of the Dcx+ cells (Fig. 3) suggesting that activation of NMDA-R did not affect the migration of newly generated neurons in the adult hippocampus.

To examine whether the aberrant localization of the Dcx+ cells was caused by the overextended migration of newly generated cells, we next performed a BrdU pulse-labeling experiment. The mice were injected with BrdU from day 9 to day 7 before the memantine-injection; 2 days later, their brains were fixed with 4% PFA for immunohistochemistry with anti-BrdU antibody (Fig. 4a–c). In the memantine-injected mice, the percentages of BrdU+ cells in the outer and middle thirds of the GCL increased significantly, whereas those in the SGZ and the inner third of the GCL decreased significantly ($n = 3$; Fig. 4b–d). We further examined the effect of memantine on neuronal differentiation and maturation using an anti-NeuN antibody (a mature neuron marker) and an anti-Dcx antibody (an immature neuron marker). The administration of NMDA-R antagonist did not affect the rate of neuronal differentiation and maturation ($n = 3$; Fig. 5) suggesting that the aberrant localization of the BrdU+ cells was not caused by the abnormal neuronal differentiation and maturation of newly generated cells. These results show that the administration of an NMDA-R antagonist causes the overextended migration of newly generated neurons in the adult hippocampus.

Administration of NMDA-R antagonist reduces the expression of *DISC1* mRNA in the adult dentate gyrus

Since recent studies have shown that DISC1 plays an important role in the proper migration of newly generated neurons in the adult hippocampus (Duan *et al.* 2007), we next examined the expression of *DISC1* in drug-injected mice using a quantitative RT-PCR method. Three-month-old

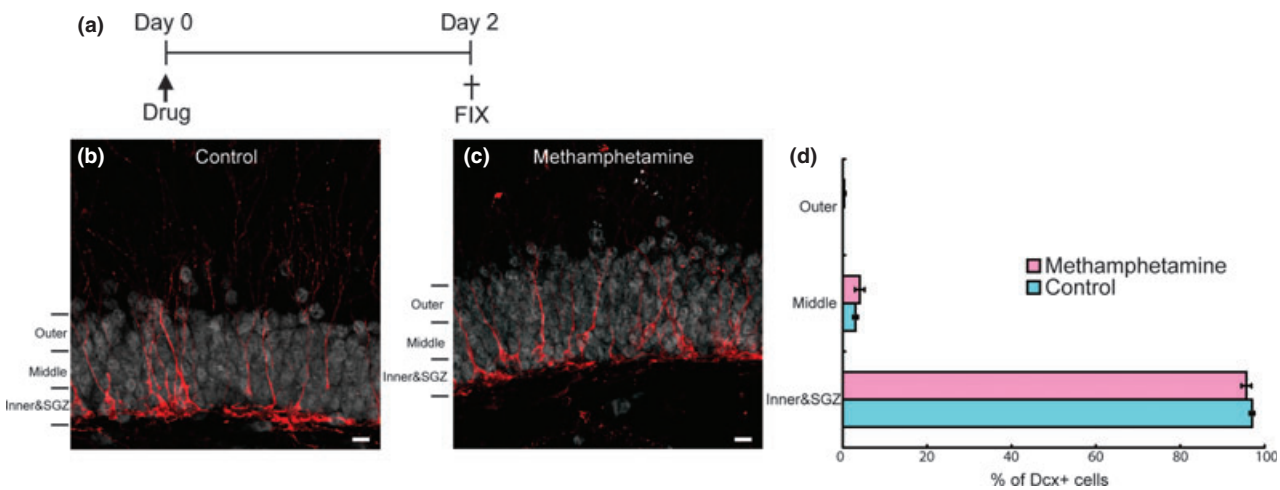

Fig. 2 Administration of methamphetamine does not affect the positioning of Dcx+ immature neurons. (a) Schematic illustration of the experimental design. (b and c) Representative immunohistochemical images of the Dcx+ cells (red) and the NeuN+ cells (white) in the control (b) or methamphetamine-injected mice (c). (d) Quantitative analysis of the location of the BrdU+ cells in the GCL. $n = 3$. Scale bars = 10 μm.

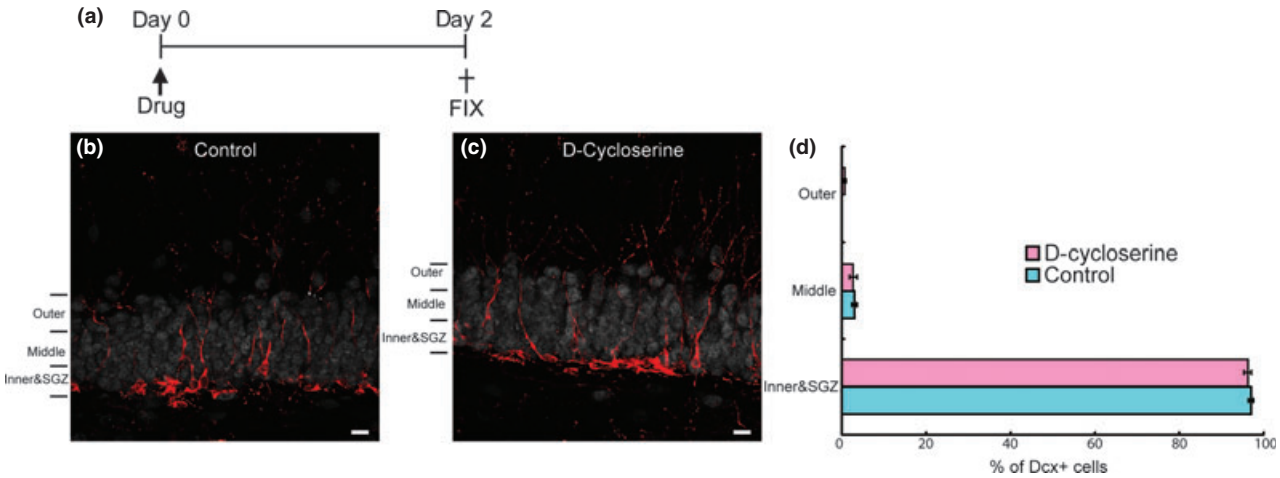

Fig. 3 Administration of D-cycloserine does not affect the positioning of Dcx+ immature neurons. (a) Schematic illustration of the experimental design. (b and c) Representative immunohistochemical images of the Dcx+ cells (red) and the NeuN+ cells (white) in the control (b) or methamphetamine-injected mice (c). (d) Quantitative analysis of the location of the BrdU+ cells in the GCL. $n = 3$. Scale bars = 10 μm.

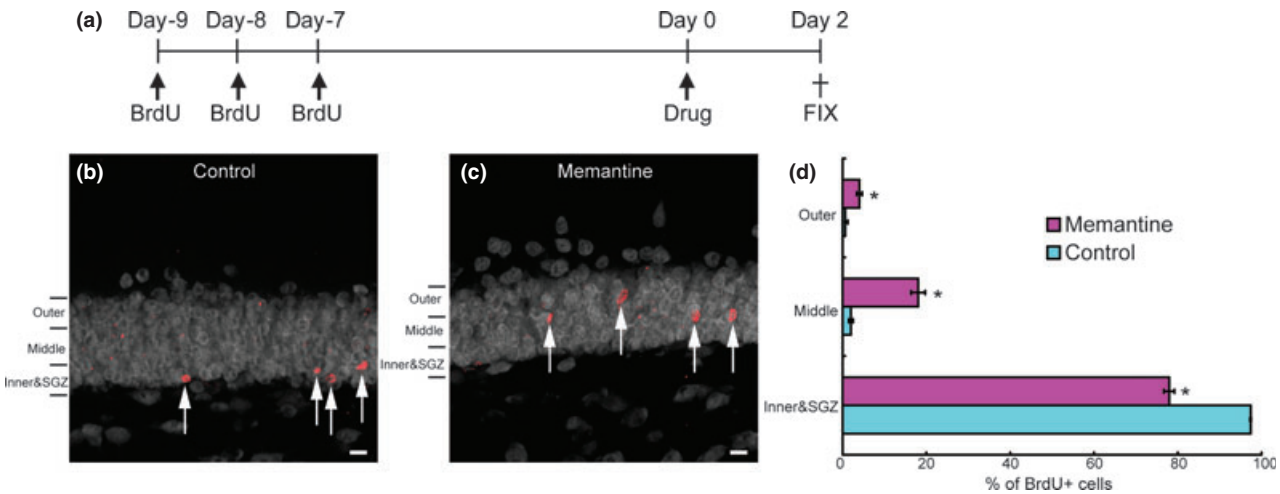

Fig. 4 Administration of NMDA-R antagonist causes overextended migration of newly generated neurons. (a) Schematic illustration of the experimental design. (b and c) Representative immunostaining images of the BrdU+ cells (red) and the NeuN+ cells (white) in the control (b) or memantine-injected mice (c). Arrows indicate the BrdU+ cells. Scale bars = 10 μm. (d) Quantitative analysis of the location of the BrdU+ cells in the GCL. $^*p < 0.05$.

male mice were injected with saline as a control, memantine (50 mg/kg B.W.), CPP (10 mg/kg B.W.), methamphetamine (2 mg/kg B.W.), or D-cycloserine (30 mg/kg B.W.); 1 day later, the total RNA was prepared from the DG. So far, two mouse *DISC1* mRNA isoforms have been identified: one isoform consists of 13 exons and the other lacks exon 9. We then designed two specific primer sets for RT-PCR. One primer set (*DISC1-1*) was used for the amplification of exon 2 and the other (*DISC1-2*) was used for the amplification of exons 9–10. A significant reduction in *DISC1* mRNA expression was observed in the memantine-injected and CPP-injected mice but not in the methamphetamine-injected mice and D-cycloserine-injected mice using both primers sets ($n = 3$; Fig. 6a and b). We further examined the dose-dependent effect of memantine on *DISC1* mRNA expression, and found that *DISC1* mRNA expression was reduced in a dose-dependent manner ($n = 3$; Fig. 6c). These results indicate that the administration of the NMDA-R antagonist reduced *DISC1* mRNA expression in the adult DG.

Since previous studies have shown that NDEL1, Girdin, and LIS1, which are molecules that interact with DISC1, are also important for the migration of hippocampal granule cells (Duan *et al.* 2007; Wang and Baraban 2007; Enomoto *et al.* 2009; Kim *et al.* 2009), we then investigated the expression of *NDEL1*, *Girdin*, and *LIS1* mRNAs in memantine (50 mg/kg B.W.)-injected mice using a quantitative RT-PCR method.

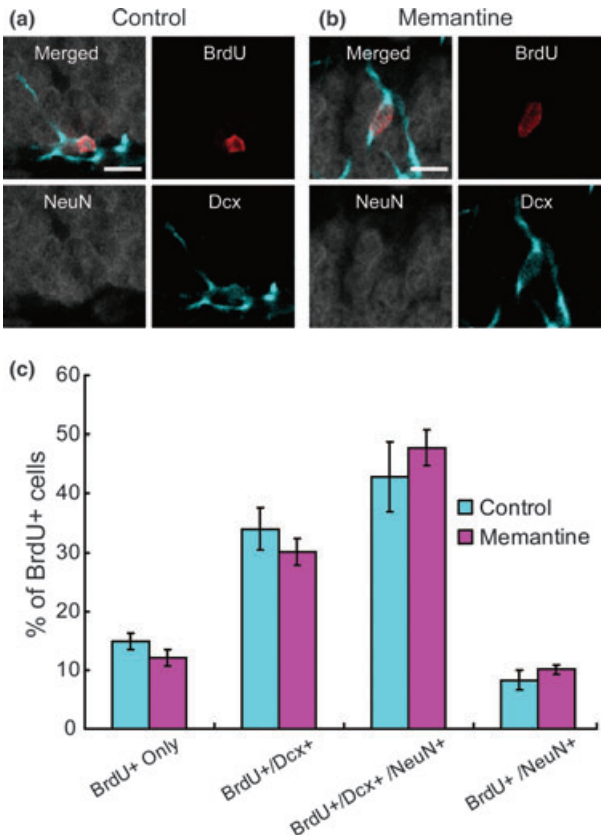

Fig. 5 Administration of NMDA-R antagonist does not affect the rate of neuronal differentiation and maturation. (a and b) Representative immunostaining images of BrdU+ cells (red), NeuN+ cells (white), and Dcx+ cells (blue) in the control (a) or memantine-injected mice (b). Scale bars = 10 μm. (c) Quantitative analysis of the percentage of BrdU+ cells, BrdU+/Dcx+ cells, BrdU+/Dcx+/NeuN+ cells, and BrdU+/NeuN+ cells.

The result showed that the expression levels of each of these three genes were almost comparable between control and memantine-injected mice ($n = 3$; Fig. 6d) suggesting that NMDA-R-mediated regulation of neuronal migration does not involve the transcription of these genes.

Exogenous expression of *DISC1* partially rescued the overextended migration of Dcx+ cells caused by the administration of NMDA-R antagonist

To investigate whether the overextended migration of newly generated neurons in NMDA-R antagonist-injected mice is attributable to the reduction in *DISC1* expression, we next performed *in vivo* rescue experiments using DISC1-expressing lentivirus. We injected lentivirus that encodes for DISC1 fused with GFP (GFP-DISC1) into the DG of 3-month-old mice at 7 days following saline or memantine (50 mg/kg B.W.) injection and prepared brain sections 2 days after the drug injection (Fig. 7a). In the saline-injected mice, no differences in the localization of the Dcx+ cells were seen between the lentivirus-infected (GFP+) (Fig. 7b and c) and non-infected (GFP-) cells (Fig. 7b and d) suggesting that exogenous expression of GFP-DISC1 itself had no effect on the migration of the newly generated cells, consistent with the previous study (Duan *et al.* 2007). In addition, the exogenous expression of GFP-DISC1 appeared not to affect the expression of NDEL1 (Figure S2). In the memantine-injected mice, the aberrant positioning of Dcx+ cells was partially rescued by the exogenous expression of GFP-DISC1 (Fig. 7e and f), whereas the non-infected (GFP-) cells remained in the outer or middle third of the GCL (Fig. 7e and g). The statistical analysis indicated that the overextended migration of the Dcx+ cells caused by memantine administration was partially rescued by the lentivirus-mediated expression of DISC1 (Fig. 7h). These findings suggest that DISC1 is involved in the neuronal migration regulated by NMDA-R signaling.

Discussion

NMDA-R signaling plays important roles not only in excitatory neuronal transmission but also in neurogenesis (Nacher and McEwen 2006; Heng *et al.* 2007). Cumulative studies have demonstrated that treatment with an NMDA-R antagonist enhances the proliferation of progenitor cells and increases the number of newly generated neurons in the adult hippocampus (Cameron *et al.* 1995; Nacher *et al.* 2001; Maekawa *et al.* 2009; Namba *et al.* 2009). In addition, the integration and the survival of newly generated neurons are also regulated by NMDA-R activity (Tashiro *et al.* 2006). However, the involvement of NMDA-R signaling in neuronal migration, which is an important step in adult hippocampal neurogenesis, remains unclear. In this study, we showed that the inhibition of NMDA-R activity using specific antagonists resulted in the aberrant positioning of Dcx-positive immature neurons in the adult hippocampus. A BrdU-labeling experiment suggested that this aberrant positioning was caused by the overextended migration of newly generated neurons. These findings suggest that NMDA-R signaling is involved in neuronal migration in addition to its role in the proliferation of progenitor cells and the integration and survival of newly generated neurons in the adult hippocampus.

DISC1 has been largely studied as a key regulator of adult neurogenesis. The expression of *DISC1* in the adult brain is restricted to two types of neurons: hippocampal dentate granule neurons and interneurons of the olfactory bulb (Austin *et al.* 2004). These neurons are continuously produced throughout life. A recent study demonstrated that the down-regulation of *DISC1* using the retrovirus-mediated expression of DISC1-small hairpin RNA in the adult hippocampus led to accelerated neuronal integration, resulting in aberrant morphological development and the mispositioning of newly generated granule neurons (Duan *et al.*

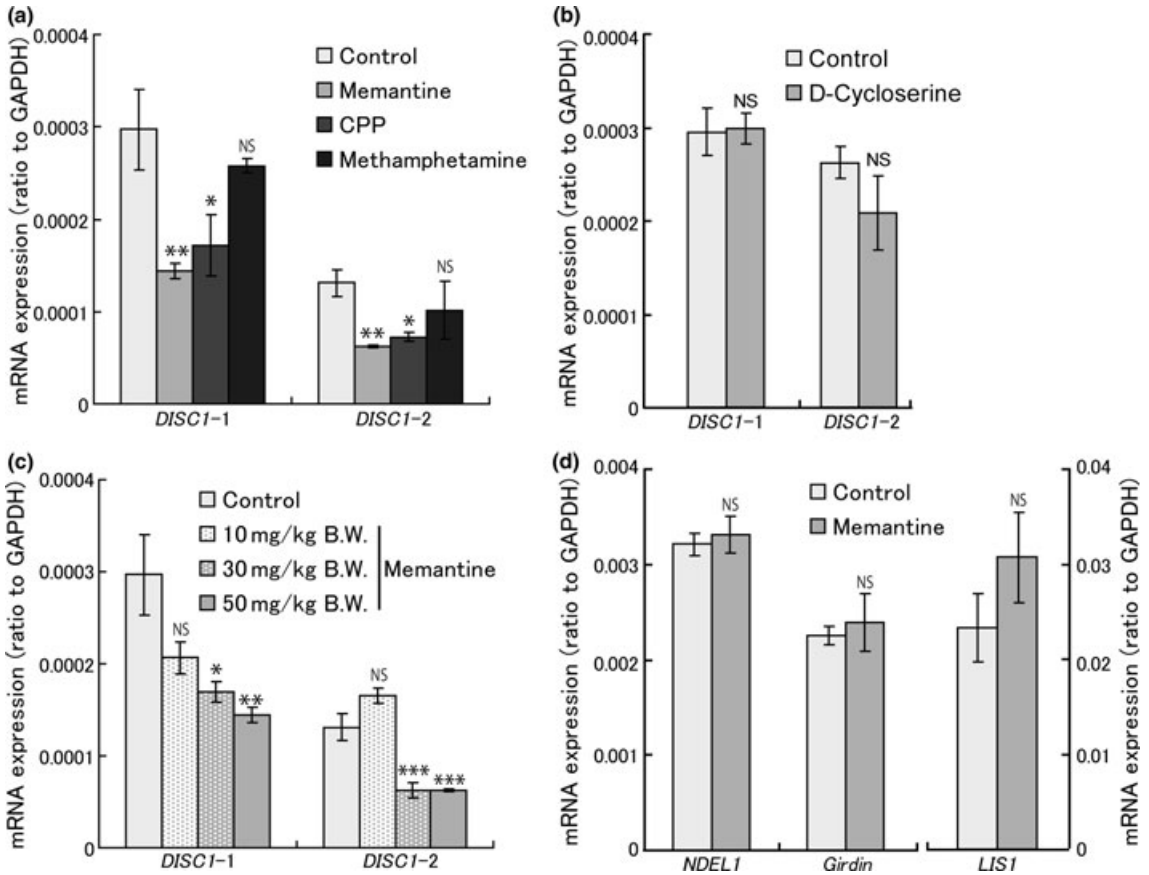

Fig. 6 Administration of NMDA-R antagonists reduces the *DISC1* mRNA expression but does not affect *NDEL1*, *Girdin*, and *LIS1* mRNA expressions in the adult dentate gyrus. (a) Quantitative analysis of the *DISC1* mRNA expression in control or memantine-, CPP-, or methamphetamine-injected mice. (b) Quantitative analysis of the *DISC1* mRNA expression in control or D-cycloserine-injected mice. (c) Dose-dependent effect of memantine on *DISC1* mRNA expression. (d) Quantitative analysis of *NDEL1*, *Girdin*, and *LIS1* mRNA expressions in control or memantine-injected mice. The ratio of mRNA expression was evaluated using GAPDH as an internal control and real-time PCR. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. NS, not significant.

2007). Since the overextended migration in memantine-treated mice was very similar to the mispositioning caused by the down-regulation of *DISC1*, we examined the expression of *DISC1* in memantine-treated mice and found a reduction in *DISC1* expression. This finding suggests that DISC1 is critical for the positioning of newly generated neurons and that its expression is regulated by NMDA-R signaling in the adult hippocampus.

The involvement of NMDA-R signaling in neurogenesis during embryonic development has also been studied. Recent studies have demonstrated that NMDA-R antagonists, including D(-)-2-amino-5-phosphonopentanoic acid (APV), MK-801, Cerestat, and memantine, increase the proliferation of neuronal stem/progenitor cells (Hirasawa *et al.* 2003; Jin *et al.* 2006; Uchino *et al.* 2010). Furthermore, treatment with an NMDA-R antagonist caused the abnormal migration of pyramidal neuron in the embryonic neocortex (Behar *et al.* 1999; Hirasawa *et al.* 2003; Uchino *et al.* 2010). Our recent study showed that the inhibition of NMDA-R prevented changes from a multipolar to bipolar morphology in migrating immature neurons and delayed neurite extension in the direction of the leading process and axon-like processes (Uchino *et al.* 2010). Interestingly, Kamiya *et al.* (2005) recently reported that small hairpin RNA-mediated suppression of DISC1 or the expression of its dominant-negative mutation in E14.5 embryos caused retarded migration and morphological defects, including neurite outgrowth in the developing neocortex. DISC1 is broadly expressed in various brain regions, including the ventricular zone during embryonic development (Austin *et al.* 2004). We further observed that prenatal treatment with an NMDA-R antagonist reduced the expression of *DISC1* in the developing neocortex (Figure S3). These results indicate that the expression of DISC1, which is associated with neuronal migration and morphological maturation including neurite outgrowth, is regulated by NMDA-R signaling not only in the adult hippocampus, but also during neocortical development.

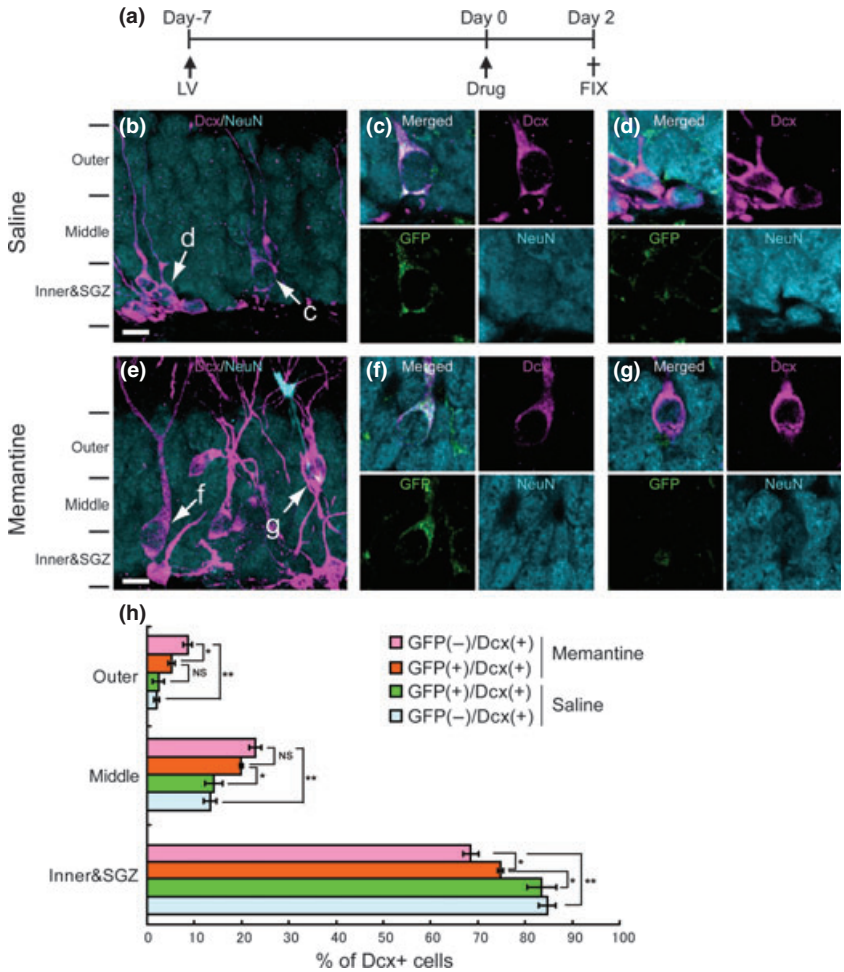

Fig. 7 The aberrant positioning of Dcx+ cell caused by memantine is partially rescued by lentiviral-mediated DISC1 expression. (a) Schematic illustration of the experimental design. (b–g) Representative immunostaining images of the Dcx+ cells (red), the GFP+ cells (green), and the NeuN+ cells (blue) in the control (b–d) or memantine-injected mice (e–g). Scale bars = 10 μm. (h) Quantitative analysis of the location of the infected or non-infected Dcx+ cells in the GCL. $^{*}p < 0.05$, $^{**}p < 0.01$. NS, not significant.

The present results provide the new notion that DISC1 acts downstream of NMDA-R signaling. However, the aberrant positioning caused by NMDA-R antagonist was partially, but not completely, rescued by the lentiviral-mediated expression of *DISC1*. Considering that recent studies have shown that NMDA-R antagonist treatment caused changes in expression and activity of various molecules (O'Donnell *et al.* 2003; Paulson *et al.* 2003; Namba *et al.* 2010; Uchino *et al.* 2010), this result raises the possibility that other molecules associated with NMDA-R signaling may be involved in neuronal migration. One intriguing molecule is CaMKII, which is a major downstream mediator of NMDA-R signaling. Interestingly, in the adult DG of mice heterozygous for CaMKII deficiency, an increased number of immature neurons and their aberrant positioning were observed (Yamasaki *et al.* 2008). In this study, we examined the expression of CaMKII and its autophosphorylation in memantine-treated mice, which revealed no prominent changes in both the expression and autophosphorylation of CaMKII compared with control mice (Figure S4). However, the data do not necessarily exclude the contribution of CaMKII signaling in transducing the effects of memantine administration, since it is known that the modification of NMDA-R signaling leads to the changes of CaMKII interaction with other proteins without the alteration of its expression levels (Dhavan *et al.* 2002). Further studies are needed to determine how NMDA-R signaling regulates neuronal migration and DISC1 expression.

In summary, we found that the inhibition of NMDA-R signaling causes the overextended migration of newly generated neurons in the adult hippocampus, indicating that NMDA-R regulates the migration of newly generated neurons in addition to the proliferation of progenitor cells, and the survival and integration of newly generated neurons. Taken together with the role of NMDA-R in excitatory neuronal transmission, these findings suggest that NMDA-R plays an important role in the formation and maintenance of hippocampal neuronal network followed by neurogenesis.

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Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. Administration of NMDA-R antagonist does not affect the total number of Dcx+ immature neurons.

Figure S2. Exogenous expression of GFP-DISC1 does not affect the expression of NDEL1.

Figure S3. Administration of memantine reduces *DISC1* mRNA expression in the embryonic neocortex.

Figure S4. Administration of NMDA-R antagonist does not affect the CaMKII expression and its phosphorylation.

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