# Regulation of *N*-Methyl-D-Aspartate Receptors by Disrupted-in-Schizophrenia-1

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**Background:** Genetic studies have implicated disrupted-in-schizophrenia-1 (DISC1) as a risk factor for a wide range of mental conditions, including schizophrenia. Because *N*-methyl-D-aspartate receptor (NMDAR) dysfunction has been strongly linked to the pathophysiology of these conditions, we examined whether the NMDAR is a potential target of DISC1.

**Methods:** DISC1 was knocked down with a small inference RNA. NMDAR-mediated currents were recorded and NMDAR expression was measured.

**Results:** We found that cellular knockdown of DISC1 significantly increased NMDAR currents in cortical cultures, which were accompanied by an increase in the expression of NMDAR subunit, GluN2A. NMDAR-mediated synaptic response in prefrontal cortical pyramidal neurons was also increased by DISC1 knockdown in vivo. The effect of DISC1 knockdown on NMDAR currents in cortical cultures was blocked by protein kinase A (PKA) inhibitor, occluded by PKA activator, and prevented by phosphodiesterase 4 inhibitor. Knockdown of DISC1 caused a significant increase of cyclic adenosine monophosphate response element-binding protein (CREB) activity. Inhibiting CREB prevented the DISC1 deficiency-induced increase of NMDAR currents and GluN2A clusters.

**Conclusions:** Our results suggest that DISC1 exerts an important impact on NMDAR expression and function through a phosphodiesterase 4/PKA/CREB-dependent mechanism, which provides a potential molecular basis for the role of DISC1 in influencing NMDAR-dependent cognitive and emotional processes.

**Key Words:** CREB, DISC1, GluN2A, NMDA receptors, PKA, schizophrenia

Because the disrupted-in-schizophrenia 1 (*DISC1*) gene was identified at the breakpoint of a balanced t(1;11) translocation that segregates with major mental illnesses in a large Scottish pedigree (1), this molecule has been extensively studied as a promising molecular lead for schizophrenia and mood disorders (2). Animal models that perturb DISC1 have shown endophenotypes relevant to schizophrenia and depression (3–10). Furthermore, human brain imaging studies have found that common polymorphisms in the DISC1 gene are associated with the dysfunction of various critical brain circuits relevant to major mental illnesses (2).

To understand how DISC1 regulates neuronal functions, we need to determine the potential targets of DISC1 that are important in modulating human cognition and emotion. The complexity of DISC1 biology makes this task challenging (2). Many DISC1 transcripts are now known, and their function is poorly understood at present (11). In addition, DISC1 is located in multiple subcellular domains, such as mitochondria, nucleus, and synapse, and the role of different variants in these regions is poorly understood (12,13). Previous studies have started to reveal the function of DISC1 in the developing brain, including neuronal migration, neurite outgrowth, and neurogenesis (14–17). From the network of protein–protein interactions around DISC1, DISC1 has also been implicated in processes of gene transcription, intracellular transport, and synaptic activity (18,19). Although DISC1 may be intimately linked to synapse function, its role in regulating synaptic proteins is just being understood, and there are many gaps in our knowledge (20,21).

Convergent findings suggest that dysfunction of glutamatergic transmission, particularly aberrant *N*-methyl-D-aspartate receptor (NMDAR) signaling, is a core pathology of mental disorders (22–24). In this study, we investigated whether DISC1 plays a role in regulating NMDARs. Understanding the synaptic functions of DISC1 may reveal important mechanistic insights into schizophrenia and related mental disorders (2).

## Methods and Materials

### Primary Neuronal Culture

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of the State University of New York at Buffalo. Rat cortical cultures were prepared as previously described (25,26). Briefly, frontal cortex was dissected from Sprague-Dawley rat embryos (E18), and cells were dissociated using trypsin and tituated through a Pasteur pipette. The neurons were plated on coverslips coated with poly-L-lysine in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, New York) with 10% fetal calf serum at a density of $1 \times 10^5$ cells/cm$^2$. When neurons attached to the coverslip within 24 hours, the medium was changed to neurobasal media (Invitrogen) with B27 supplement. Neurons were maintained for 2-3 weeks before being used for recordings.

### Small Interfering RNA

To knockdown the expression of DISC1 in cultured neurons, we used a short-hairpin RNA (shRNA) against DISC1 tagged with

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Received Jan 31, 2013; revised Jun 12, 2013; accepted Jun 19, 2013.

0006-3223/$36.00
http://dx.doi.org/10.1016/j.biopsych.2013.06.009

enhanced green fluorescent protein (Dharmacon RNA Technologies, Lafayette, Colorado), which has been shown to be a strong suppressor of DISC1 (15,20). Cultured cortical neurons (12–14 days in vitro) were transfected with a control (scrambled) shRNA or with DISC1 shRNA using the Lipofectamine 2000 method. To suppress cyclic adenosine monophosphate response element-binding protein (CREB) expression or function, we transfected cultures with CREB-1 small interfering (si)RNA (Santa Cruz Biotechnology, Santa Cruz, California) or dominant negative CREB (DN-CREB; a gift from Dr. David Ginty at Johns Hopkins University). Neurons were used for experiments 2 days after transfection.

### Antibody Production

Rat DISC1 (C-terminal amino acids 598–854) was cloned into pET15b expressed in *Escherichia coli* BL21 and purified similarly to what was described before for human DISC1 (598–854) (27). See Methods in Supplement 1 for details.

### Lentiviral Production and Lentivirus Infection In Vitro and In Vivo

See Methods in Supplement 1 for details of DISC1 shRNA lentiviral production and infection.

### Western Blotting

See Methods in Supplement 1 for details

### Electrophysiologic Recordings

Recordings of whole-cell ion channel currents in cultured neurons (14–17 days in vitro) used standard voltage-clamp techniques (25,28,31). See Supplementary Methods in Supplement 1 for details.

### Biochemical Measurement of Surface Proteins

Surface NMDA receptors were detected as described previously (28,32,33). See Supplementary Methods in Supplement 1 for details.

### Immunocytochemical Staining

See Supplementary Methods in Supplement 1 for details.

### Statistics

All data are expressed as the mean ± SEM. Experiments with two groups were analyzed statistically using unpaired Student *t* tests. Experiments with more than two groups were subjected to one-way analysis of variance (ANOVA), followed by post hoc Tukey tests.

## Results

### Knockdown of DISC1 Induces an Increase of NMDAR-Mediated Currents In Vitro

Previous studies indicate that the chromosomal translocation causes a reduction of DISC1 expression (34), so we took a strategy to reduce DISC1 levels using RNA interference (similar to a haploinsufficient DISC1 model) and examined its influences on NMDARs receptors. As shown in Figure 1A, the expression of endogenous DISC1, which was recognized by anti-DISC1 440 as three major bands, were markedly diminished in neuronal cultures infected with DISC1 shRNA lentivirus (band 1a: .65 ± .07 of control; band 1b: .64 ± .08 of control; band 1c: .71 ± .08 of control, $n = 8$, $p < .01$, *t* test), similar to previous reports (20,21,29,30).

To further confirm the effectiveness of DISC1 knockdown, we used a newly developed DISC1 antibody, anti-rat (r)DISC1 C-term. The specificity of this DISC1 antibody was first examined with brain lysates from DISC1 knockout rats, which possess a 20-bp deletion within exon 5 of DISC1, resulting in an early stop codon in exon 6 (Product No. TGRA3640; SAGE Labs, Sigma, St. Louis, Missouri). As shown in Figure S1 in Supplement 1, the 3 bands detected with anti-rDISC1 C-term in wild-type rats were largely lost in DISC1 knockout rats. When anti-rDISC1 C-term was used to measure DISC1 expression in rat primary cultures infected with DISC1 shRNA lentivirus, the three bands were also significantly diminished (Figure 1B; band 2a: .60 ± .09 of control; band 2b: .52 ± .07 of control; band 2c: .40 ± .08 of control, $n = 5$, $p < .01$, *t* test).

In DISC1 shRNA-transfected cortical cultures, the whole-cell NMDAR current density (picoamperes per picofarad [pA/pF]) was significantly increased (Figure 1C,D, control shRNA: 21.1 ± 2.1, $n = 13$; DISC1 shRNA: 30.3 ± 3.1, $n = 15$, $p < .05$, ANOVA). To rule out the possibility of "off-target" effects of the DISC1 shRNA, we performed control experiments in which we transfected neurons with a DISC1 rescue construct ($DISC1^R$) that is insensitive to the shRNA (20). As shown in Figure 1C and D, the enhancing effect of DISC1 shRNA on NMDAR current density was prevented by $DISC1^R$ (23.0 ± 1.8, $n = 15$; Figure 1C), suggesting that the finding is due to the selective knockdown of DISC1.

The changes in NMDAR channel properties were also investigated. As shown in Figure 1C and D, NMDAR current charge transfer (μC) was significantly increased in DISC1 shRNA-transfected neurons (control shRNA: 1.8 ± .2, $n = 14$; DISC1 shRNA: 2.5 ± .2, $n = 14$, $p < .05$, *t* test), and NMDAR current decay time constant (msec) was slightly decreased (control shRNA: 703.5 ± 45.3, $n = 14$; DISC1 shRNA: 624.4 ± 39.3, $n = 14$, $p > .05$, *t* test; Figure 1D).

To determine which subpopulations of NMDARs were targeted by DISC1 shRNA, we applied the selective GluN2B inhibitor ifenprodil (10 μmol/L). As shown in Figure 1E and F, ifenprodil caused a similar reduction of the whole-cell NMDAR current amplitudes in both groups (control shRNA: 68.6 ± 1.5%, $n = 10$; DISC1 shRNA: 63.4 ± 1.2%, $n = 12$, $p > .05$, *t* test). The current density (pA/pF) mediated by GluN2A component (ifenprodil-insensitive) was markedly increased in neurons infected with DISC1 shRNA lentivirus (control shRNA: 8.6 ± 1.1, $n = 10$; DISC1 shRNA: 12.9 ± .8, $n = 12$, $p < .01$, *t* test), whereas the current density (pA/pF) mediated by GluN2B component (ifenprodil-sensitive) was largely unchanged (control shRNA: 18.3 ± 1.8, $n = 10$; DISC1 shRNA: 22.3 ± 1.0, $n = 12$, $p > .05$, *t* test). These results suggest that the enhanced NMDAR response induced by DISC1 knockdown is mainly mediated by GluN2A subunits.

### Knockdown of DISC1 Induces an Increase of NMDAR-Excitatory Postsynaptic Currents In Vivo

There are certain experimental limitations with these observations—namely, the changes seen with in vitro DISC1 knockdown may be a phenomenon specific to neuronal cultures, and the NMDA-elicited whole-cell current is mediated by both synaptic and extrasynaptic NMDARs. It is necessary to know whether similar changes also happen at the level of synaptic NMDAR responses with in vivo DISC1 knockdown. To address this, we performed stereotaxic injections of DISC1 shRNA lentivirus into the rat medial prefrontal cortex (PFC) (28). The in vivo knockdown effectiveness was confirmed with anti-DISC1 440 (Figure 2A; band 1a: .46 ± .06 of control; band

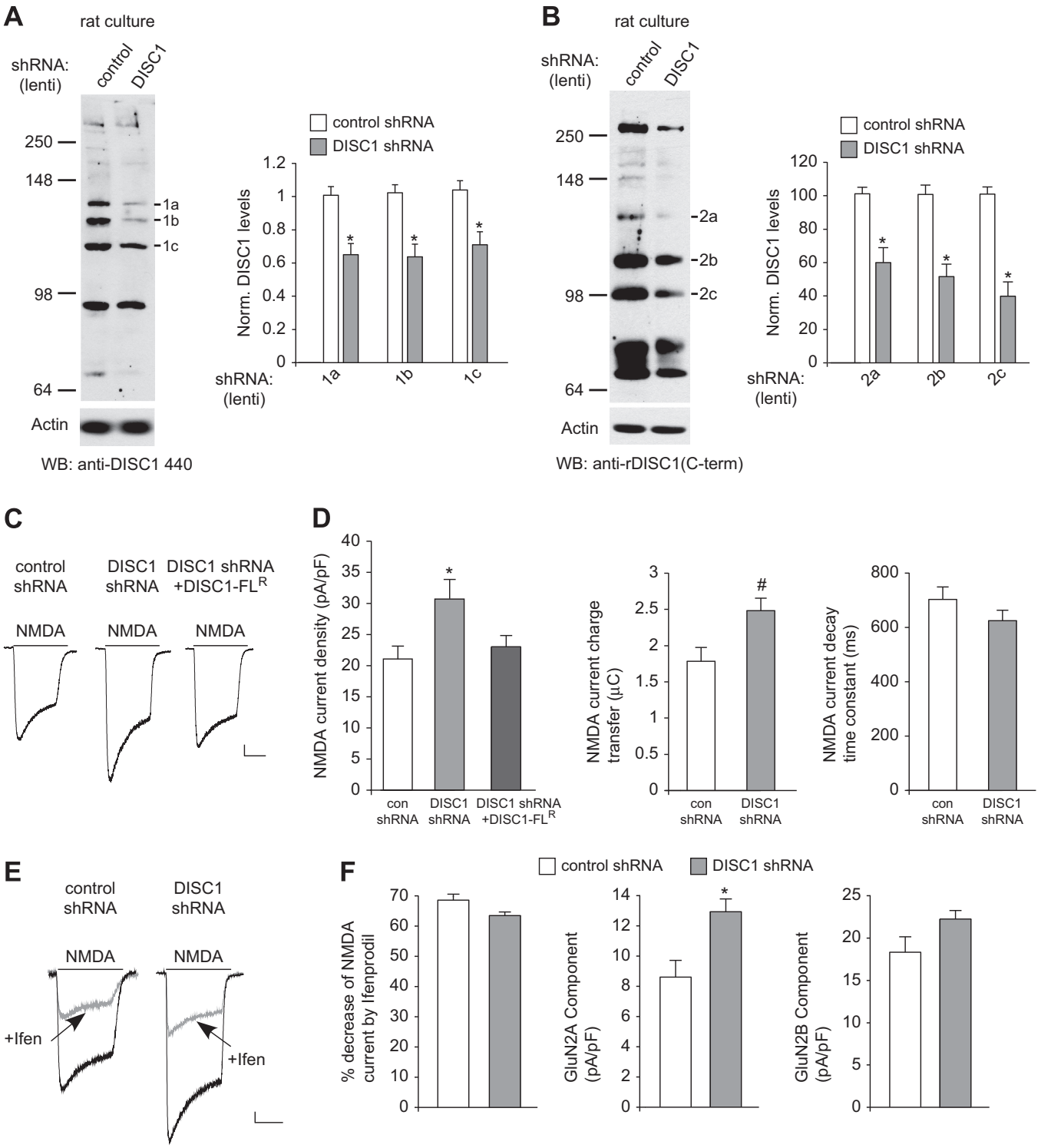


**Figure 1.** Knockdown of disrupted-in-schizophrenia-1 (DISC1) increases *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) current density and synaptic response in vitro. **(A, B)** Immunoblots and quantification analysis of DISC1 (A: anti-DISC1 440; B: anti-rDISC1 C-term) in rat cortical cultures infected with control short-hairpin RNA (shRNA) or DISC1 shRNA lentivirus. $^{*}p < .01$. **(C)** Representative NMDA (100 μM)-elicited current traces in cultured cortical neurons transfected with control shRNA, DISC1 shRNA or DISC1 shRNA plus DISC1-FL$^{R}$, a full-length DISC1 rescue construct that is insensitive to the DISC1 shRNA. Scale bar: 100 picoamperes, 1 sec. **(D)** Cumulative data (mean ± SEM) of NMDAR current density, charge transfer and decay time constant in neuronal cultures with different transfections. $^{*}p < .01$. $^{\#}p < .05$. **(E)** Representative NMDAR current traces in the absence or presence of ifenprodil (10 μmol/L, a GluN2B blocker) in cultured cortical neurons transfected with control shRNA or DISC1 shRNA. Scale bar: 100 picoamperes, 1 sec. **(F)** Cumulative data (mean ± SEM) of GluN2A and GluN2B components in neuronal cultures with different transfections. $^{*}p < .01$. con, control; Norm., normalized; WB, western blot.

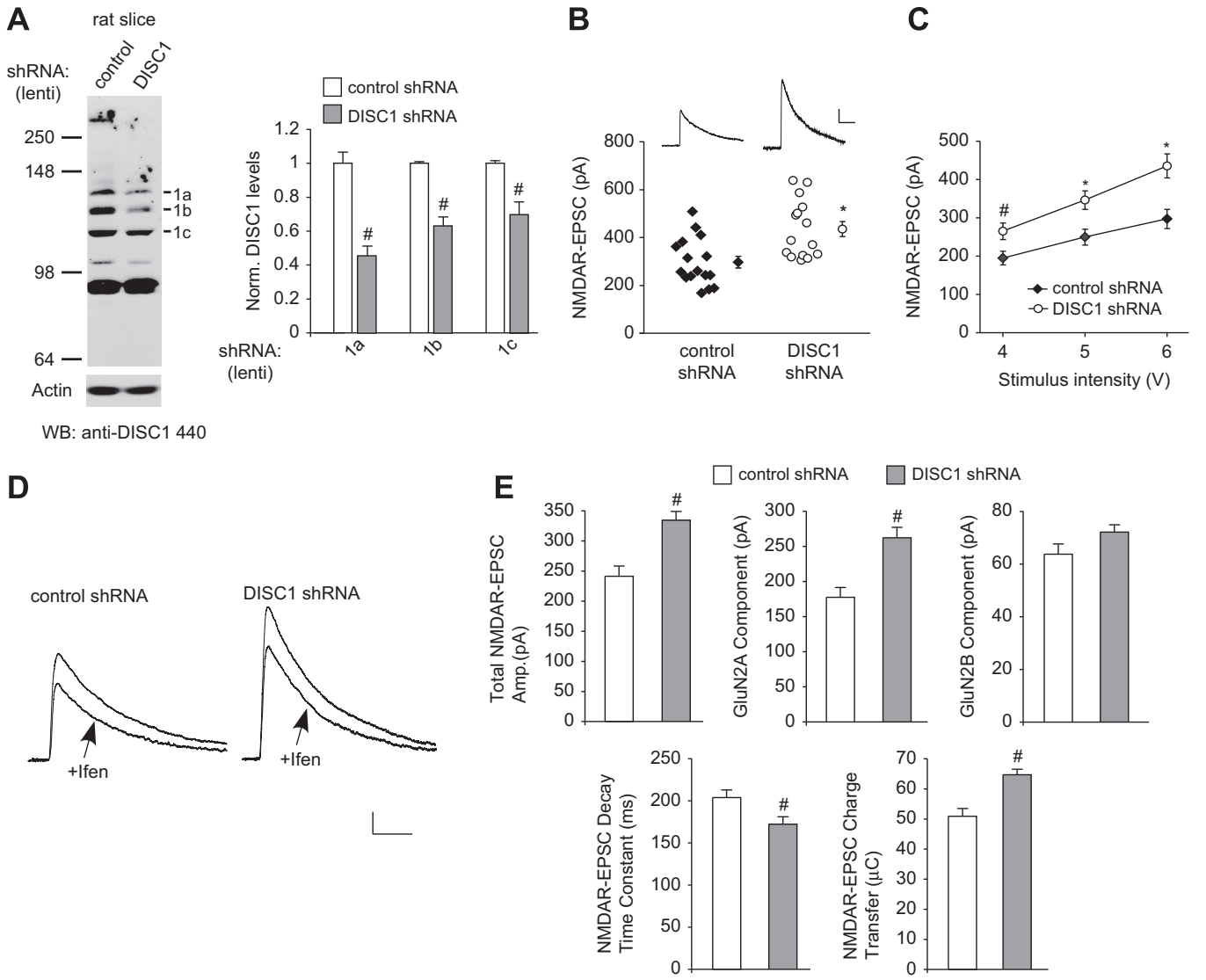


**Figure 2.** Knockdown of disrupted-in-schizophrenia-1 (DISC1) increases *N*-methyl-D-aspartate receptor (NMDAR)-excitatory postsynaptic currents (EPSC) in prefrontal cortex (PFC) pyramidal neurons in vivo. **(A)** Immunoblots and quantification analysis of DISC1 (detected with anti-DISC1 440) in rat PFC slices taken from animals with stereotaxical injections of control short-hairpin RNA (shRNA) or DISC1 shRNA lentivirus. $^{\#}p < .05$. **(B)** Dot plots showing the amplitude of NMDAR-EPSC in control shRNA or DISC1 shRNA lentivirus-infected PFC pyramidal neurons. The average data are also shown. $^{*}p < .01$. Inset: representative NMDAR-EPSC traces. Scale bar: 100 pA, 200 msec. **(C)** Summarized input-output curves of NMDAR-EPSC in pyramidal neurons from rats with the PFC injection of control shRNA or DISC1 shRNA lentivirus. $^{*}p < .01$, $^{\#}p < .05$. **(D)** Representative NMDAR-EPSC traces in the absence or presence of ifenprodil (Ifen; 10 μmol/L) in PFC pyramidal neurons infected with control shRNA or DISC1 shRNA lentivirus. Scale bar: 50 picoamperes, 100 msec. **(E)** Cumulative data (mean ± SEM) of total NMDAR-EPSC amplitude, GluN2A or GluN2B components, decay time constant and charge transfer in neurons with different viral infections. $^{\#}p < .05$.

1b: .62 ±.06 of control; band 1c: .70 ± .07 of control, $n = 4$, $p < .05$, *t* test).

We then examined the impact of DISC1 knockdown in vivo on NMDAR-mediated excitatory postsynaptic currents (EPSC) in PFC slices. As shown in Figure 2B, the amplitude of NMDAR-EPSC evoked by the same stimulus was significantly bigger in PFC pyramidal neurons with DISC1 knockdown (control shRNA: 297.2 ± 24.7 pA, $n = 16$; DISC1 shRNA: 435.5 ± 31.2 pA, $n = 15$; $p < .01$, *t* test). DISC1 knockdown also caused a substantial increase of the input/output curves of NMDAR-EPSC induced by a series of stimulus intensities (Figure 2C; 35%–45% increase, $p < .01$, ANOVA, $n = 15$–16 per group). The amplitudes of GluN2A-mediated NMDAR-EPSC (ifenprodil-insensitive) were significantly increased in PFC pyramidal neurons infected with DISC1 shRNA lentivirus (Figure 2D,E; control shRNA: 177.5 ± 13.8, $n = 8$; DISC1 shRNA: 262.2 ± 14.7, $n = 9$, $p < .05$, *t* test), whereas GluN2B-mediated NMDAR-EPSC (ifenprodil-sensitive) was largely unchanged (control shRNA: 63.8 ± 3.8, $n = 8$; DISC1 shRNA: 72.2 ± 2.7, $n = 9$; $p > .05$, *t* test). In addition, a significant reduction of the decay time constant (control shRNA: 203.1 ± 8.9 msec, $n = 8$; DISC1 shRNA: 172.2 ± 8.7 msec, $n = 9$; $p < .05$, *t* test) and a significant increase of the transfer charge (control shRNA: 50.9 ± 2.6 μC, $n = 8$; DISC1 shRNA: 64.7 ± 1.8 μC, $n = 9$; $p < .05$, *t* test) of NMDAR-EPSC were observed in neurons with DISC1 knockdown. It suggests that the enhanced synaptic

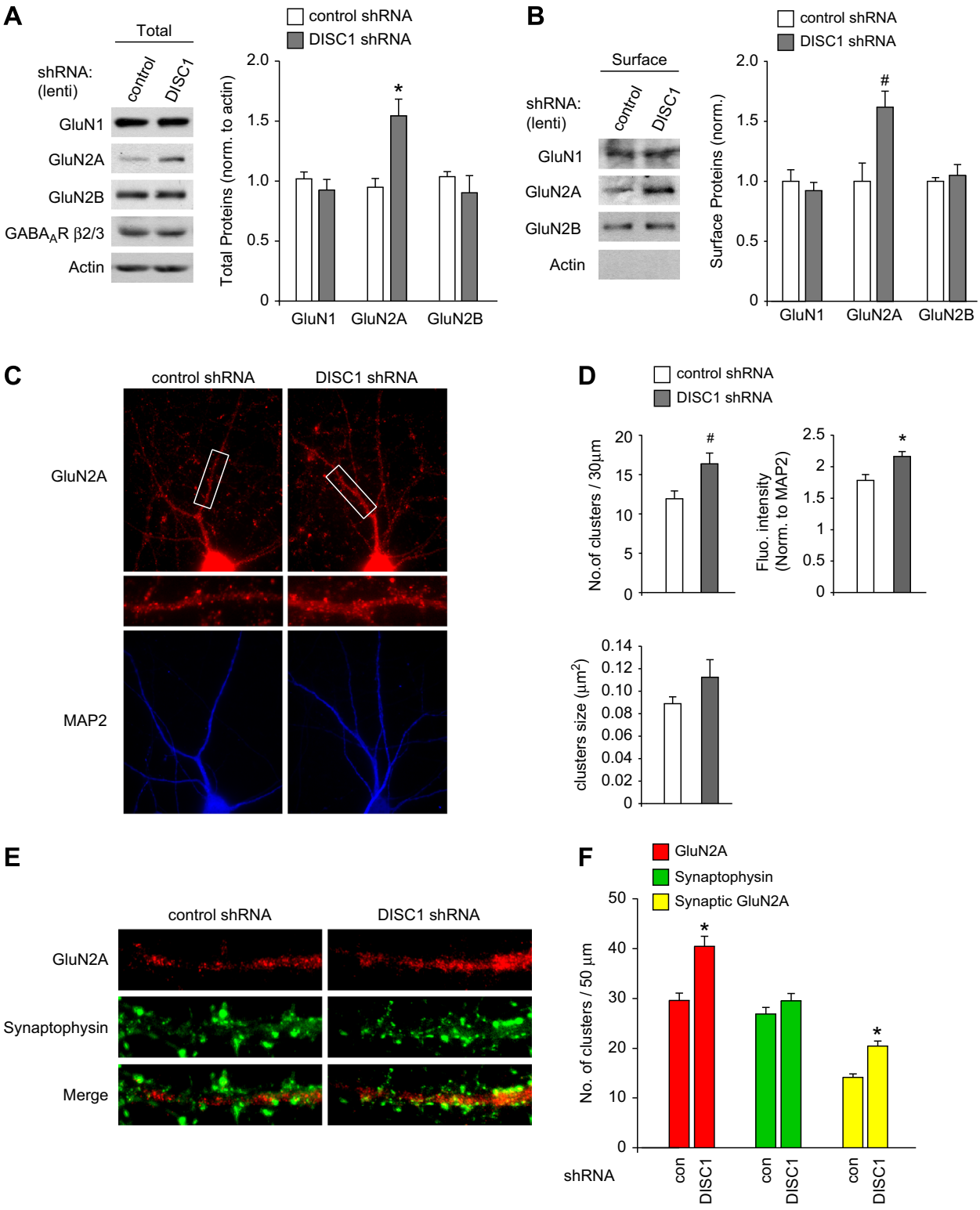


**Figure 3.** Knockdown of disrupted-in-schizophrenia-1 (DISC1) increases *N*-methyl-D-aspartate receptor (NMDAR) subunit GluN2A expression. **(A)** Immunoblots and quantification analysis of GluN1, GluN2A, GluN2B, gamma-aminobutyric acid-$_A$R β2/3, and actin in cultured cortical neurons infected with control short-hairpin RNA (shRNA) or DISC1 shRNA lentivirus. *$p < .01$. **(B)** Immunoblots and quantification analysis of surface GluN1, GluN2A, and GluN2B in cultured cortical neurons infected with control shRNA or DISC1 shRNA lentivirus. Actin was used as a control. *$p < .05$. **(C)** Immunocytochemical images of NMDAR GluN2A subunits and MAP2 in cortical cultures transfected with a control shRNA or DISC1 shRNA. **(D)** Quantitative analysis of GluN2A clusters (density, intensity, size) along the dendrites in control shRNA or DISC1 shRNA-transfected neurons. *$p < .01$, $^{\#}p < .05$. Immunocytochemical images **(E)** and quantitative analysis **(F)** of synaptic GluN2A clusters (synaptophysin colocalized, yellow puncta) in cortical cultures transfected with control shRNA or DISC1 shRNA. *$p < .01$. Fluo., fluorescence; Norm., normalized.

NMDAR response induced by DISC1 knockdown is mediated by GluN2A subunits.

### Knockdown of DISC1 Increases the Level of Total and Surface GluN2A Subunits

The enhancement of NMDAR responses by DISC1 knockdown could result from increased NMDAR expression or surface delivery/stability. Thus, we examined NMDAR subunits in neurons with DISC1 knockdown. As shown in Figure 3A and B, the total and surface levels of GluN2A were significantly elevated in PFC cultures infected with DISC1 shRNA lentivirus (total GluN2A: 1.7 ± .2-fold of control; $n = 5$, $p < .01$, *t* test; surface GluN2A: 1.6 ± .1 fold of control; $n = 6$, $p < .05$, *t* test). The levels of GluN1, GluN2B, or gamma-aminobutyric acid-$_A$R β2/3 subunits were largely unchanged (Figure 3A,B).

Immunocytochemical studies (Figure 3C,D) also indicated that neurons transfected with DISC1 shRNA showed a significant increase in the GluN2A cluster density (number of clusters/30 μm; control shRNA: 11.9 ± 1.0, $n = 26$; DISC1 shRNA: 16.4 ± 1.4, $n = 22$, $p < .05$, *t* test) and GluN2A cluster intensity (control shRNA: 1.8 ± .1, $n = 26$; DISC1 shRNA: 2.2 ± .1, $n = 22$, $p < .01$, *t* test). The size (μm$^2$) of GluN2A clusters was not significantly affected by DISC1 shRNA (control shRNA: .09 ± .01, $n = 26$; DISC1 shRNA: .1 ± .02, $n = 22$, $p > .05$, *t* test).

To provide more direct evidence on DISC1 regulation of NMDARs at synapses, we measured synaptic NMDAR clusters, as indicated by GluN2A colocalized with the synaptic marker synaptophysin. As shown in Figure 3E and F, in DISC1 shRNA-transfected neurons, a significant increase of synaptic GluN2A (yellow puncta) cluster density (number of clusters/50 μm) was observed (control shRNA: 14.1 ± 1.6, $n = 36$; DISC1 shRNA: 20.4 ± 1.7, $n = 26$, $p < .01$, *t* test), whereas synaptophysin clusters were not altered (control shRNA: 26.9 ± 1.9, $n = 36$; DISC1 shRNA: 29.5 ± 2.2, $n = 26$, $p > .05$, *t* test). The increased GluN2A synaptic expression could underlie the increased NMDAR response by DISC1 knockdown.

Finally, to test whether DISC1 regulation of NMDARs is via a direct physical interaction, we performed coimmunoprecipitation experiments. We did not find any DISC1 coprecipitating with NMDA NR1 subunits from rat cortical slices (Figure S2 in Supplement 1).

### DISC1 Regulation of NMDARs Is Dependent on a Phosphodiesterase 4/Protein Kinase A/CREB-Dependent Pathway

Next we examined the potential mechanism underlying DISC1 regulation of NMDARs. It has been shown that DISC1 interacts with phosphodiesterase 4 (PDE4) isoforms (34), an enzyme that inactivates cyclic adenosine monophosphate (cAMP) and orchestrates downstream signaling via cAMP effectors such as protein kinase A (PKA) (35). We hypothesize that DISC1 knockdown may change cAMP-PKA signaling via PDE4 modification.

To test this, we first examined the involvement of PKA in DISC1 regulation of NMDARs. Cultured neurons were treated with a specific PKA inhibitor or PKA activator during DISC1 shRNA transfection (48 hours). As shown in Figure 4A and B, DISC1 shRNA failed to increase NMDAR current density (pA/pF) in the presence of the PKA inhibitor PKI (.2 μmol/L; control shRNA: 22.5 ± 2.0, $n = 10$; DISC1 shRNA: 20.9 ± 1.9, $n = 10$, $p > .05$, ANOVA), which was significantly different from untreated neurons (control shRNA: 20.7 ± 1.3, $n = 20$; DISC1 shRNA: 27.7 ± 1.8, $n = 23$, $p < .05$, ANOVA). Treatment with the PKA activator 8-cpt-cAMP (50 μmol/L) induced a significant increase of NMDAR current density (control shRNA: 23.7 ± 1.6, $n = 30$; control shRNA + cAMP: 30.9 ± 2.5, $n = 22$, $p < .01$, ANOVA), and occluded the enhancing effect of DISC1 shRNA (31.2 ± 2.6, $n = 17$). Moreover, in

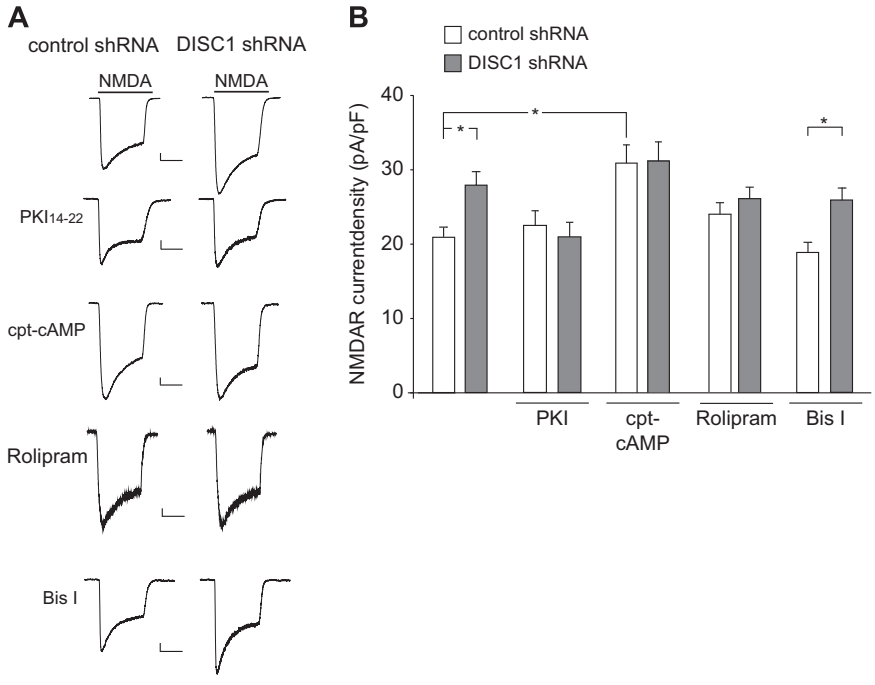


**Figure 4.** Protein kinase A (PKA) activation is required for disrupted-in-schizophrenia-1 (DISC1) regulation of *N*-methyl-D-aspartate receptors (NMDARs). **(A)** Representative whole-cell NMDAR current traces in cultured cortical neurons transfected with a control short-hairpin RNA (shRNA) and DISC1 shRNA in the absence or presence of PKI (.2 μmol/L, a PKA inhibitor), 8-cpt-cyclic adenosine monophosphate (cpt-cAMP; 50 μmol/L, a PKA activator), rolipram (.1 μmol/L, a phosphodiesterase 4 inhibitor), and bisindolylmaleimide I (Bis I, .5 μmol/L, a protein kinase C inhibitor). Scale bar: 100 picoamperes, 1 sec. **(B)** Cumulative data (mean ± SEM) of NMDAR current density in transfected neurons with different treatments. *$p < .05$.

the presence of rolipram (.1 μmol/L, 48 hours), a selective inhibitor of PDE4, particularly the PDE4B subtype (36,37), DISC1 shRNA failed to enhance NMDAR current density (control shRNA: 23.7 ± 1.6, $n = 12$; DISC1 shRNA: 26.5 ± 1.5, $n = 9$, $p > .05$, ANOVA).

Because protein kinase C (PKC) has been shown to enhance NMDAR-mediated current (38), we also tested its role in DISC1 regulation of NMDARs. As shown in Figure 4A and B, in the presence of the specific PKC inhibitor bisindolylmaleimide I (.5 μmol/L, 48 hours), the enhancing effect of DISC1 knockdown on NMDAR current density (pA/pF) was intact (control shRNA: 18.8 ± 1.4, $n = 22$; DISC1 shRNA: 25.5 ± 1.6, $n = 19$, $p < .05$, ANOVA). Taken together, these results suggest that DISC1 shRNA increases NMDAR currents via a mechanism at least partially dependent on elevated PKA activity.

To find out whether the DISC1 knockdown-induced increase of NMDAR currents is due to PKA phosphorylation of NMDAR subunits, we examined phospho-$^{S897}$GluN1 levels (39). As shown in Figure S3 in Supplement 1, pGluN1 levels were largely unchanged in neuronal cultures infected with DISC1 shRNA lentivirus (1.0 ± .2-fold of control, $n = 8$, $p > .05$, $t$ test).

One of the key downstream targets of PKA is CREB, which is phosphorylated at residue serine-133 (S133) by multiple protein kinases, including PKA and $Ca^{2+}$/calmodulin-dependent protein kinases (40). After CREB is phosphorylated, it is translocated from the cytosol to the nucleus, binding to the cAMP-response element on the promoter region of many target genes to modulate their transcription. Our Western blot assays indicate that the level of $^{Ser133}$phospho-CREB (active form of CREB) was significantly increased in PFC cultures infected with DISC1 shRNA lentivirus (Figure 5A; 2.6 ± .2-fold of control, $n = 3$, $p < .01$, $t$ test). To further test the impact of DISC1 knockdown on CREB activation, immunocytochemical experiments were performed in cultured PFC neurons transfected with DISC1 shRNA. As shown in Figure 5B, the nuclear phosphor-CREB staining intensity was significantly increased in DISC1 shRNA-transfected neurons (control shRNA: 2.5 ± .1, $n = 27$; DISC1 shRNA: 3.3 ± .2, $n = 45$, $p < .01$, $t$ test), suggesting that DISC1 knockdown indeed induced CREB activation and nuclear translocation.

We further investigated the signaling molecules involved in DISC1 regulation of CREB activity. As shown in Figure 5C and D, PKI treatment (.2 μmol/L, 48 hours) decreased pSer133-CREB level and blocked the enhancing effect of DISC1 shRNA lentivirus (PKI + control shRNA: .5 ± .06 fold of control, $n = 11$, PKI + DISC1 shRNA: .6 ± .05-fold of control, $n = 9$), and treatment with the ERK inhibitor, PD98059 (20 μmol/L, 48 hours), was ineffective (PD98059+control shRNA: .9 ± .04 fold of control, $n = 15$; PD98059 + DISC1 shRNA: 2.1 ± .14-fold of control, $n = 6$, $p < .001$, ANOVA). Treatment with 8-cpt-cAMP (50 μmol/L, 48 hours) also increased the pCREB level (Figure S4 in Supplement 1; 1.6 ± .1-fold of control; $n = 5$, $p < .01$, $t$ test). CREB levels were largely unchanged by these treatments. These results suggest that DISC1 knockdown induces the upregulation of CREB activity via a PKA-dependent mechanism.

To directly test the role of CREB in DISC1 regulation of NMDARs, we inhibited CREB function by either knocking down its expression (Figure 6A) or by transfecting a DN-CREB construct (S133A mutation). As shown in Figure 6B and C, the enhancing effect of DISC1 shRNA on NMDAR current density (pA/pF) was lost in the presence of CREB siRNA (control shRNA+CREB siRNA: 20.1 ± 1.5, $n = 17$; DISC1 shRNA+CREB siRNA: 19.3 ± 1.1, $n = 15$, $p > .05$, ANOVA), or DN-CREB (control shRNA+DN-CREB: 22.5 ± 2.3, $n = 11$; DISC1 shRNA+DN-CREB: 22.1 ± 1.4, $n = 15$, $p > .05$, ANOVA), which was significantly different from neurons without CREB inhibition (control shRNA: 22.4 ± 3.2, $n = 7$; DISC1 shRNA: 33.5 ± 4.1, $n = 7$, $p < .05$, ANOVA). Immunocytochemical studies (Figure 6D,E) also indicated that DN-CREB blocked the effect of DISC1 shRNA on GluN2A cluster density (control shRNA: 11.4 ± .7, $n = 21$; DISC1 shRNA+DN-CREB: 9.7 ± .7, $n = 23$, $p > .05$, $t$ test) and GluN2A cluster intensity (control shRNA: 1.8 ±.1, $n = 21$; DISC1 shRNA+DN-CREB: 1.7 ± .1, $n = 23$, $p > .05$, $t$ test). The size ($\mu m^2$) of GluN2A clusters was not significantly affected by DISC1 knockdown (control shRNA: .089 ± .01, $n = 21$; DISC1 shRNA+DN-CREB: .091 ± .01, $n = 23$, $p > .05$, $t$ test). Taken together, these results suggest that DISC1 depletion drives an enhanced NMDAR response, at least in part via a mechanism depending on PKA/CREB activation.

Finally, we examined the potential downstream target of CREB involved in DISC1 regulation of NMDARs. BDNF is a possible candidate, because CREB activation induces the increased expression of BDNF, which is essential for neuronal maturation (41). However, no significant increase was observed on BDNF expression by DISC1 knockdown (Figure S5 in Supplement 1; .9 ± .1-fold of control, $n = 8$, $p > .05$, $t$ test).

## Discussion

Since the *DISC1* gene was identified, extensive molecular, cell biological, animal model, and human genotype-phenotype studies have been conducted to address multiple roles and mechanisms of DISC1 in the brain (9,42–44). Because DISC1 is thought to drive a range of endophenotypes that underlie major mental conditions (45), elucidating the precise biological functions of DISC1 has become an intensely studied topic (2). In recent years, the possible role of DISC1 in regulating synapse formation and function has gained critical experimental support. Our own work has shown that DISC1 plays a critical role in regulating excitatory synaptic function through the molecules kalirin and TNIK (20,21). Building on these studies, we have now gone on to show that DISC1 regulates NMDAR function. Our results suggest that loss of DISC1 in vitro leads to increased NMDAR current densities in cortical cultures. In vivo knockdown of DISC1 also results in potentiated NMDAR synaptic responses in layer V pyramidal neurons from prefrontal cortical slices. Whether this effect of DISC1 knockdown on NMDARs is universal in all cortical regions/layers/neuronal types awaits further investigation. Given the critical role of NMDAR in various mental disorders (22–24,46,47), our results could provide a potential molecular mechanism underlying the behavioral phenotypes seen in animals and humans with DISC1 genetic variations.

The functional NMDAR complex is composed of two GluN1 and two GluN2A/B subunits. The GluN1 subunit is normally present in excess, so the determining factor for channel abundance is the GluN2 subunit (48). The DISC1 knockdown-induced enhancement of NMDAR-mediated current is accompanied by a selective increase of GluN2A protein expression and GluN2A synaptic clusters, indicating that DISC1 deficiency leads to an increased number of GluN1/GluN2A channels, which consequently elevates NMDAR responses. This is the first report of such an effect, but there have been previous reports implicating a role in NMDAR regulation by DISC1. For example, DISC1 exon 2 and 3 knockout mice were shown to display a modified LTP response, suggestive of altered NMDAR function (49).

Our data show that the increased NMDAR current densities in DISC1 deficient cells are caused by the CREB-dependent

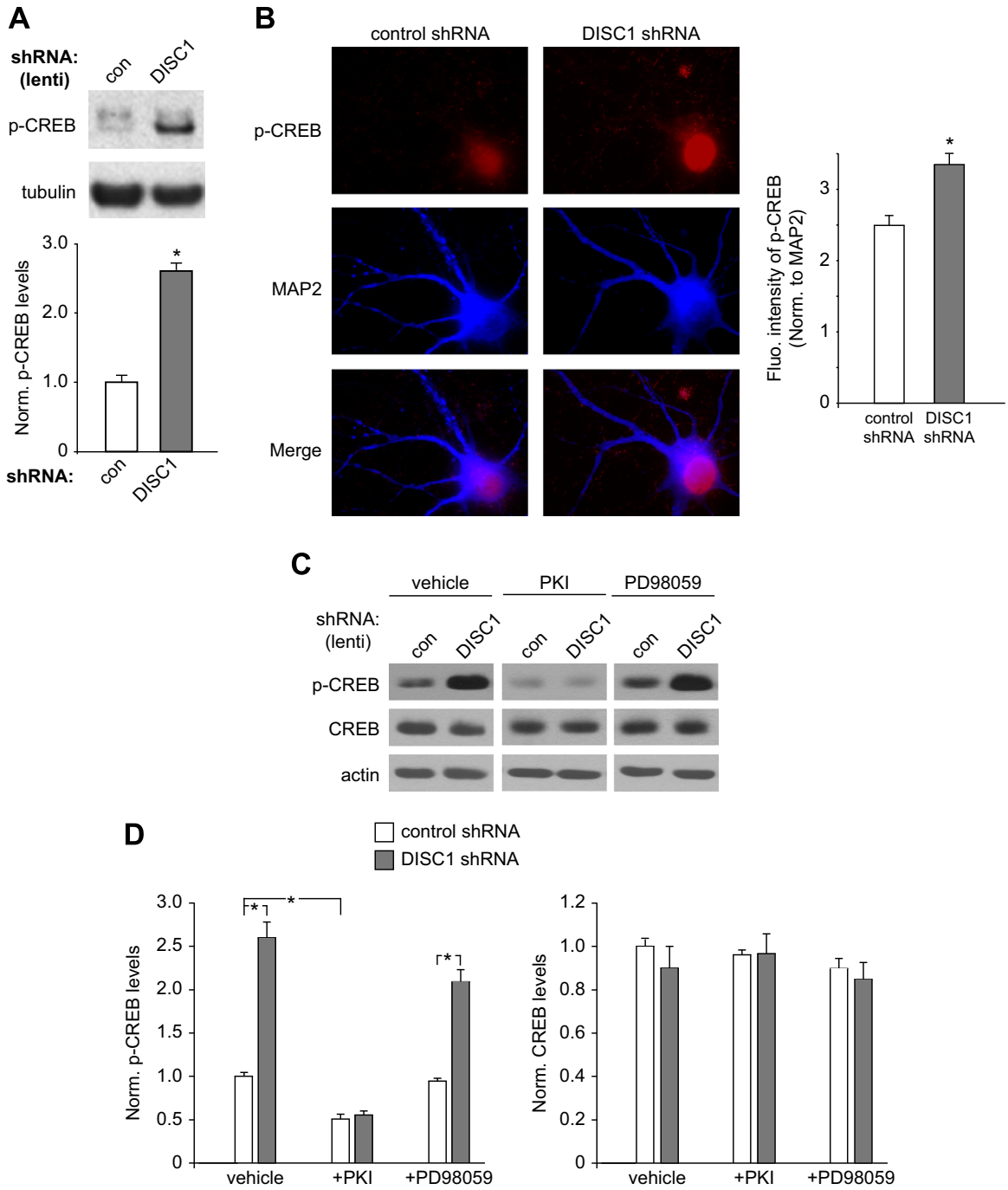


**Figure 5.** Disrupted-in-schizophrenia-1 (DISC1) knockdown induces an increase of cyclic adenosine monophosphate response element-binding protein (CREB) activity. **(A)** Immunoblots and quantification showing the level of phosphor-CREB in cortical cultures infected with control short-hairpin RNA (shRNA) or DISC1 shRNA lentivirus. Tubulin was used as a control. *$p < .01$. **(B)** Immunocytochemical images and quantitative analysis of p-CREB in prefrontal cortex cultures transfected with a control shRNA or DISC1 shRNA. Microtubule-associated protein 2 (MAP2) was costained. *$p < .01$. **(C, D)** Immunoblots and quantification showing the level of p-CREB and CREB in cortical cultures infected with control shRNA or DISC1 shRNA lentivirus in the presence of vehicle, PKI (.2 μmol/L) or PD98059 (20 μmol/L, an extracellular signal-regulated kinase inhibitor). *$p < .001$. con, control; Fluo., fluorescence; Norm., normalized.

elevation of GluN2A expression. Putative cAMP response elements have been found in the promoter of GluN2A (50), and the activity-dependent developmental increases in GluN2A is mediated by a PKA/CREB pathway (51). It is known that GluN2A and GluN2B, which have distinct synaptic localizations and channel kinetics, play different roles in synaptic plasticity (52,53). A recent report showed that forebrain-specific overexpression of GluN2A led to deficits in certain forms of long-term depression and long-term memory (54). Thus, the DISC1 knockdown-induced selective increase in GluN2A could lead to aberrant NMDAR-dependent synaptic plasticity and cognitive processes.

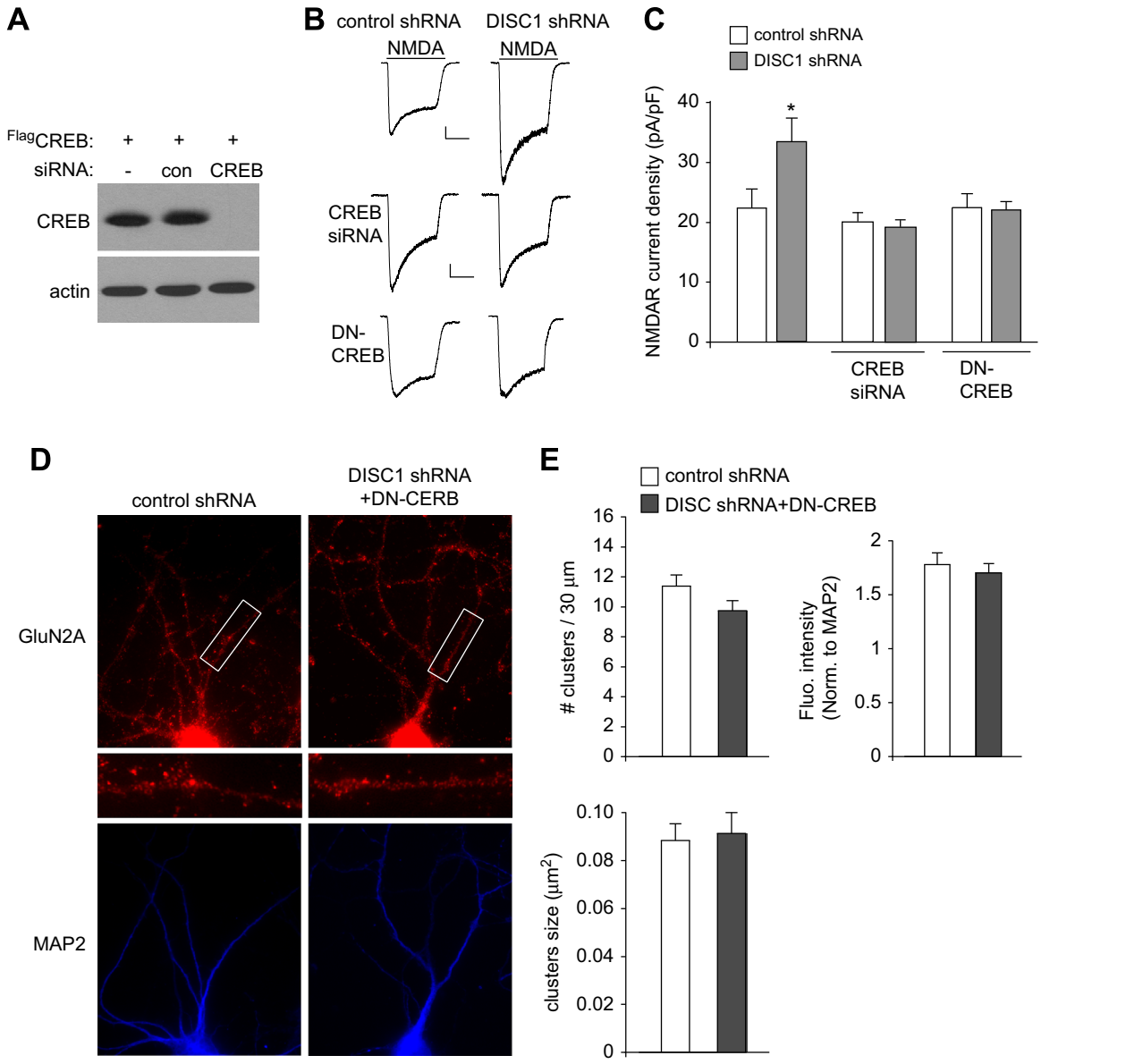


**Figure 6.** Inhibiting cyclic adenosine monophosphate response element-binding protein (CREB) blocks disrupted-in-schizophrenia-1 (DISC1) regulation of *N*-methyl-D-aspartate (NMDA) receptors (NMDARs). **(A)** Western blots in HEK293 cells transfected with FLAG-tagged CREB in the absence or presence of a control small interfering (si)RNA or CREB siRNA. **(B)** Representative whole-cell NMDAR current traces in cultured cortical neurons transfected with a control short-hairpin RNA (shRNA) or DISC1 shRNA in the absence or presence of CREB siRNA or dominant negative (DN)-CREB. Scale bar: 100 picoamperes, 1 sec. **(C)** Cumulative data (mean ± SEM) showing NMDAR current density in neurons with different transfections. $*p < .05$. **(D)** Immunocytochemical images of GluN2A subunits and MAP2 in cortical cultures transfected with a control shRNA or cotransfected DN-CREB with DISC1 shRNA. **(E)** Quantitative analysis of GluN2A clusters (density, intensity, size) along the dendrites in transfected neurons. Fluo., fluorescence; Norm., normalized.

DISC1 has been shown to interact with many proteins, and its interactome has provided a powerful framework to understand DISC1 function from initially an in silico perspective to confirmation in cellular and in vivo contexts (2,18). One of the critical binding partners is the cAMP phosphodiesterase PDE4 that hydrolyses cAMP specifically and downregulates cAMP-dependent pathways including PKA (34,55,56) and the transcription factor ATF4/CREB2 that controls gene expression (19). DISC1 was thought to sequester PDE4B in resting cells and release it in an activated state in response to elevated cAMP (34). PDE4 isoforms other than PDE4B can also be sequestered by DISC1, and these are not dynamically released, probably because they bind in different fashions to DISC1 (56), which is consistent with the hypothesis that targeted PDE4 family proteins are involved in the control of spatially defined signaling complexes (35). Previous studies have shown that *DISC1* mutation (truncation) leads to the reduction of PDE4B expression and elevated cAMP-PKA signaling (57). Mice with mutant DISC1 proteins, which exhibit reduced binding of DISC1 to PDE4B, have lower PDE4B activity (4). Consistently, our data suggest that DISC1 knockdown results in the upregulated PKA-CREB signaling, which is likely via the inhibition of PDE4 function.

There may be additional complexity to the DISC1-NMDA interaction, however. Recently DISC1 has been shown to bind and regulate serine-racemase in astrocytes (58). The primary function of serine-racemase is to catalyze the conversion of

L-serine to D-serine. D-serine is thought to the obligatory coagonist of the NMDAR in most brain regions. This could potentially have some role in our recording experiments. In addition, it has been reported that there is a loss of DISC1 from the synapse in GluN1 knockdown mice (29). It is clear there is a complex but critical partnership between the NMDAR and DISC1. The role we have carved out for the GluN2A subunit could be critical in this.

## Conclusion

In summary, we have revealed how DISC1 regulates the NMDAR, a key synaptic target involved in cognitive and emotional processes under normal and pathologic conditions. Our results could help to understand the synaptic functions of DISC1 in neurons and may clarify the role of DISC1 in schizophrenia and related psychiatric disorders (2,59–61).

*This work was supported by National Institutes of Health grants to ZY (Grant Nos. MH-084233 and MH-101690) and AS (Grant Nos. MH-094268 and MH-069853), and NEURON-ERANET DISCover/ Grant No. BMBF 01EW1003 to CK. AH-T is currently affiliated with the Center for Disease Biology and Integrative Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan. NJB is currently affiliated with AstraZeneca Neuroscience iMED, Cambridge, Massachusetts.*

*We thank Xiaoqing Chen for her excellent technical support.*

*All authors report no biomedical financial interests or potential conflicts of interest.*

*Supplementary material cited in this article is available online at http://dx.doi.org/10.1016/j.biopsych.2013.06.009.*

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