Role of disrupted in schizophrenia 1 (DISC1) in stress-induced prefrontal cognitive dysfunction

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Recent genetic studies have linked mental illness to alterations in disrupted in schizophrenia 1 (DISC1), a multifunctional scaffolding protein that regulates cyclic adenosine monophosphate (cAMP) signaling via interactions with phosphodiesterase 4 (PDE4). High levels of cAMP during stress exposure impair function of the prefrontal cortex (PFC), a region gravely afflicted in mental illness. As stress can aggravate mental illness, genetic insults to DISC1 may worsen symptoms by increasing cAMP levels. The current study examined whether viral knockdown (KD) of the Disc1 gene in rat PFC increases susceptibility to stress-induced PFC dysfunction. Rats were trained in a spatial working memory task before receiving infusions of (a) an active viral construct that knocked down Disc1 in PFC (DISC1 KD group), (b) a ‘scrambled’ construct that had no effect on Disc1 (Scrambled group), or (c) an active construct that reduced DISC1 expression dorsal to PFC (Anatomical Control group). Data were compared with an unoperated Control group. Cognitive performance was assessed following mild restraint stress that had no effect on normal animals. DISC1 KD rats were impaired by 1 h restraint stress, whereas Scrambled, Control, and Anatomical Control groups were unaffected. Thus, knocking down Disc1 in PFC reduced the threshold for stress-induced cognitive dysfunction, possibly through disinhibited cAMP signaling at neuronal network synapses. These findings may explain why patients with DISC1 mutations may be especially vulnerable to the effects of stress.

**Translational Psychiatry (2013) 3, e328; doi:10.1038/tp.2013.104; published online 3 December 2013**

**Keywords:** disrupted in schizophrenia 1; prefrontal cortex; schizophrenia; stress; working memory.

INTRODUCTION

Psychiatric disorders such as schizophrenia involve profound dysfunction of the prefrontal cortex (PFC).1–6 The PFC uses working memory to provide top-down modulation of behavior, thought and affect7–9, and its function is weakened by exposure to even mild, uncontrollable stress.10 Psychiatric symptoms are often precipitated or worsened by stress,11–14 causing descent from cognitive coherence to debilitating illness. Thus, it is critical to understand the molecular influences that modulate PFC function during stress in order to develop intelligent medications for psychiatric disorders. Unfortunately, there are currently few pharmacological treatments that ameliorate PFC cognitive deficits, and the challenge of developing effective cognitive enhancers is compounded by the unique neurochemical needs of PFC.

The cellular circuitry underlying spatial working memory has been identified by Goldman-Rakic.7 Spatial working memory is maintained by networks of pyramidal cells that interconnect at dendritic spines in layer III of dorsolateral PFC (Figure 1a). Recent excitation via N-methyl-D-aspartate receptor synapses maintains persistent firing across a delay period when a spatial position is held in working memory.7–15 The efficacy of these network connections is dynamically modulated at spines by intracellular mechanisms collectively termed, dynamic network connectivity16–17 (Figure 1b). Generation of cyclic adenosine monophosphate (cAMP) increases the open state of nearby potassium channels in dendritic spines, for example, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels,18,19 and reduces network firing. Although this mechanism allows for rapid flexibility in sculpting the contents of working memory, it is also vulnerable to a variety of genetic and environmental insults, for example, in psychiatric disorders and during stress. In particular, increased release of catecholamines in PFC during even mild stress exposure increases cAMP signaling, which reduces PFC neuronal firing and impairs working memory.11

Genetic insults associated with mental illnesses often affect stress-induced signaling pathways in PFC, and may explain why patients are especially vulnerable to the effects of stress.5,11 A major genetic risk factor is the disrupted in schizophrenia 1 (DISC1) gene. Initially discovered to have a balanced chromosomal translocation mutation in a Scottish pedigree with a high incidence of psychiatric disorders,20–22 it has since been found to be a susceptibility gene for a variety of psychiatric disorders in populations worldwide.21,23–31 DISC1 protein acts as a scaffold for many interacting molecules, for example, phosphodiesterase 4 (PDE4),32–34 and is involved in a variety of cellular functions such as intracellular signaling, neurodevelopment, and synaptogenesis.33,24,35–42 DISC1 regulates intracellular levels of cAMP under conditions of high cAMP production through its interactions with PDE4 enzymes23,33 (Figure 1b). In primate PFC, DISC1 is colocalized with HCN1 channels in layer III dendritic spines19 (Figure 1c), and is thus in an ideal location to modulate recurrent PFC networks. That...
is, DISC1 may anchor PDE4 to the correct subcellular location and act like a ‘molecular brake’ to restore normal cAMP levels following stress exposure, thus maintaining PFC network connectivity. Conversely, loss of DISC1 function in PFC would likely prevent proper PDE4 function, leading to a dysregulated build-up of cAMP in spines, excessive opening of HCN channels, and network dissociation. Consistent with this hypothesis, inhibition of PDE4 in PFC weakens network firing via opening of HCN channels in primates engaged in a working memory task, and impairs working memory in mice with DISC1 knockdown (DISC1 KD; \( N = 12 \), Control \( N = 8 \), Scrambled \( N = 8 \), Control \( N = 12 \), and Anatomical Control \( N = 7 \) groups).

### MATERIALS AND METHODS

All procedures were approved by the Yale Institutional Animal Care and Use Committee.

**Subjects**

Forty-seven young, male Sprague–Dawley rats (2 months old at the beginning of the study; Taconic, Germantown, NY, USA) were housed individually under standard laboratory conditions. They were kept on a 12 h light/dark cycle, and behavioral experiments were conducted during the light phase. Highly palatable rewards (chocolate chips) were used during the experiments to minimize the need for dietary regulation. Water was provided ad libitum, and animals were fed 12–16 g of autoclaved rat chow (Purina Mills, Gray Summit, MO, USA) immediately following testing. They were weighed weekly, and weights were maintained at 400–450 g. The rats were habituated to all procedures, and tested by a single experimenter who was blind to experimental conditions.

### Delayed alternation spatial working memory task

Rats were trained individually in a delayed alternation spatial working memory task in a T-shaped maze, as previously described. Further details are provided in the Supplementary Methods.

Once trained, rats were screened for normal sensitivity to stress exposure by assaying their response to 3 mg kg–1 of the pharmacological stressor, FG7142 (Tocris, Ellisville, MO, USA). This dose had no effect in most adult, male rats in a pilot study. Eleven rats that showed impaired performance, and thus inferred to have an exaggerated stress response, were removed from the study. The 36 remaining rats were assigned to the DISC1 knockdown (DISC1 KD; \( N = 9 \)), Scrambled (\( N = 8 \)), Control (\( N = 12 \)), and Anatomical Control (\( N = 7 \)) groups.

### Production of viral constructs

We initially generated five viral constructs designed to knock down Disc1 expression, Disc1-short hairpin-expressing RNA1–5 (Disc1-shRNA1–5). Briefly, a shRNA was designed to target a sequence within exon 1 of rat Disc1. The promotor, hairpin sequence, and terminator sequences were ligated in an adenovirus-associated virus (AAV) plasmid. Viral constructs were then produced in HEK293 cells by transfecting them with pDG plasmids (kindly provided by Drs Mark Kay and Dirk Grimm, Stanford University, CA). Western blots verified effective KD of Disc1 expression in HEK293 cells by two of the constructs (Figure 2a); of these, Disc1-shRNA1 was
Viral constructs knocked down disrupted in schizophrenia 1 (DISC1) expression in rat prefrontal cortex (PFC). (a) Western blots verified that the Disc1–short hairpin-expressing RNA1 (shRNA1) and Disc1–shRNA2 constructs effectively knocked down expression of full-length rat DISC1 in HEK293 cells in two separate trials (Experiments 1 and 2). Disc1–shRNA1 was subsequently used in the behavioral experiments. (b) Rats received viral infusions in PFC (AP–3.2 mm; ML ± 0.75 mm; DV–4.2 mm) (1) or dorsal to PFC in cingulate cortex area 1 (Cg1)/secondary motor cortex (M2) (Cg1/M2; AP–3.2 mm; ML ± 0.75 mm; DV–2.0 mm) (2) (left, middle), and DISC1 labeling in these regions was compared with that in primary motor cortex (M1). Shaded areas indicate regions compared for stereology (right). Small rectangles represent regions compared for optical densitometry and % area of DISC1 labeling (right); actual sites and orientation varied from field to field.

more effective, and was thus used for the behavioral experiments. In addition, a ‘scrambled’ control construct (scrDisc1-shRNA) was designed to target a sequence that was not homologous to any known mammalian gene. Further details are provided in the Supplementary Methods.

Viral infusion surgery

The DISC1 KD, Scrambled, Control, and Anatomical Control groups were counterbalanced by baseline cognitive ability to ensure that preoperative performance was equal between the groups. Surgery was performed for the DISC1 KD, Scrambled, and Anatomical Control rats under Equithesin (pentobarbital-chloral hydrate, 4.32 mg g⁻¹) anesthesia using aseptic methods.

DISC1 KD rats received infusions of the active construct to KD Disc1 expression in PFC, whereas Scrambled rats received the ‘scrambled’ construct in PFC (Figure 2b). Guide cannulae were directed immediately dorsal to PFC (AP–3.2 mm; ML ± 0.75 mm; DV–4.2 mm), and infusion needles reached DV–4.5 mm. Two microliters of viral constructs were infused bilaterally into PFC at 0.25 μl min⁻¹, and the cannulae were left in place for 5 min following the infusions. Pilot studies determined the appropriate parameters for the viral infusion procedure to ensure that only PFC was affected.

Rats in the Anatomical Control group received viral infusions in a region just dorsal to PFC that covered anterior cingulate cortex area 1 and motor area M2 (Cg1/M2) (Figure 2b). Guide cannulae were directed dorsal to PFC (AP–3.2 mm; ML± 0.75 mm; DV–1.7 mm), and infusion needles reached DV–2.0 mm.

The viral constructs were allowed to express for 20 days following surgery before further behavioral data were collected. The Control group received no viral infusions. Testers were blind to group assignments.

One-hour restraint stress

Following viral expression, the rats were re-trained in the delayed alternation task. After reaching a stable baseline, they underwent 1 h restraint stress immediately before testing. Previous studies confirmed that 2 h, but not 1 h, of restraint stress impaired working memory performance in control rats. Thus, we investigated whether 1 h would be sufficient to impair DISC1 KD rats, but not Scrambled, Control, or Anatomical Control rats.

Restraint stress was performed by placing the rat in a restraint device (Harvard Apparatus, Holliston, MA, USA) in a separate room from the usual testing room. This procedure was done within 20–200 days following the viral infusion surgery. Infusions were less effective outside this period, likely because the viral constructs required enough time to express, but not enough for possible compensatory mechanisms to develop in the brain, for example, upregulation of PDE4. Rats were monitored continuously during the restraint period to ensure that there was no undue distress.

Behavioral data analysis

Cognitive performance without stress was compared before and after the viral infusion surgery, as well as between groups. Cognitive performance was defined as a product of the delay and mean pre- or postsurgery score. For the presurgery cognitive performance, scores from three consecutive testing days just prior to surgery were used. For the postsurgery cognitive performance, scores from three consecutive testing days 20–30 days after surgery were used, when the rats’ performance appeared to have stabilized. If the rats underwent 1 h restraint stress during this time, the scores from three consecutive testing days just prior to the restraint stress were used. The pre- and postsurgery cognitive scores were compared using paired t-tests within each group. We also compared the difference
between the pre and postsurgery cognitive scores between groups using a one-way analysis of variance (ANOVA) with a between-subjects factor of group (Control, Scrambled, DISC1 KD, Anatomical Control).

Working memory performance before and after 1 h restraint stress was analyzed using a 2-way mixed-design ANOVA with a between-subjects factor of group (Control, Scrambled, DISC1 KD, Anatomical Control) and a within-subjects factor of stress (baseline, stress). User-defined contrasts were then performed to compare stress scores for DISC1 KD vs Control, Scrambled and Anatomical Control groups, and baseline vs stress for each group.

Finally, errors in individual trials following 1 h restraint stress were examined, for example, incorrectly returning to an arm following a correct choice in ‘Win-stay’ trials, and perseverating further by repeatedly choosing the same arm in the T-maze. In addition, in ‘time-out’ or omitted trials, the rats failed to make a choice for 2 min, which could indicate an inability to make a choice or ‘freezing,’ a severe stress response. The number of such error trials following stress was compared between the DISC1 KD and Control groups using Wilcoxon Signed-Rank tests. Statistical analyses were performed using SPSS (IBM Corporation, Armonk, NY, USA).

DISC1 immunohistochemistry

After completion of behavioral testing, viral injection sites were verified with DISC1 immunohistochemistry. For quantitative assessments, we primarily focused on DISC1 expression in dendrites, as we were interested in its role in modulating the connectivity of PFC networks at dendritic spines. To analyze DISC1 expression in PFC, we used stereology, optical densitometry, and % area of DISC1 labeling. Data collection was performed with an Axioskop microscope (Carl Zeiss, Thornwood, NY, USA) interfacing with a Dell personal computer via a Microfire camera (Optronics, Goleta, CA, USA). Further details are provided in the Supplementary Methods.

Quantification of DISC1 staining using isotropic virtual planes-based stereology

The total length and length density of DISC1-stained dendrites were quantified in the regions of interest using StereoInvestigator (MicroBright-Field, Williston, VT, USA). Specifically, the Isotropic Virtual Plane probe was used in a systematic random sampling scheme. To avoid over or under-sampling, 4 sections from each cortical area were analyzed for each rat, chosen based on the quality of tissue. This number was statistically sufficient according to the low coefficients of error (< 0.05).

The length density in PFC was normalized to that in motor cortex within each group, and compared using a 1-way ANOVA with a between-subjects factor of group (Control, Scrambled, DISC1 KD, Anatomical Control). User-defined contrasts compared DISC1 KD vs Control, Scrambled and Anatomical Control groups. We visually verified that the active viral construct knocked down DISC1 in Cg1/M2 in the Anatomical Control group. Statistical analyses were performed using SPSS.

RESULTS

Basal working memory performance following KD of Disc1 in PFC

To minimize stress under basal conditions, rats were fully adapted to all conditions prior to infusion of viral constructs. When pre- and postsurgery cognitive scores were compared within each group using paired t-tests, there were no significant differences (DISC1 KD: $P = 0.76$; Scrambled: $P = 0.35$; Control: $P = 0.20$; Anatomical Control: $P = 0.34$). When the difference between the pre- and postsurgery cognitive scores were compared between groups with a one-way ANOVA, there were no significant differences (F(3,32) = 0.41, $P = 0.75$).

Stress-induced cognitive dysfunction following KD of Disc1 in PFC

One-hour restraint stress impaired working memory performance in DISC1 KD rats, but not Scrambled, Control, or Anatomical Control rats (Figure 3). These results agreed with a previous finding that 1 h restraint stress did not impair working memory in control rats.10 A two-way ANOVA revealed a trend toward a significant main effect of stress (F(1,32) = 3.917, $P = 0.056$), a non-significant main effect of group (F(3,32) = 0.877, $P = 0.463$), and a non-significant interaction between group and stress (F(3,32) = 2.016, $P = 0.131$). User-defined contrasts revealed that 1 h restraint stress significantly impaired performance relative to baseline in DISC1 KD rats ($P = 0.045$), but not in Scrambled ($P = 0.869$), Control ($P = 0.916$), or Anatomical Control ($P = 0.334$) rats. DISC1 KD exacerbated stress-induced working memory deficits in rats tested shortly after transfection (mean of 50% correct), as well as those tested >100 days after transfection (mean of 56.7% correct), consistent with a stable reduction in DISC1 expression over this time period (see below). The normal performance of the Anatomical Control rats, in which DISC1 was reduced dorsal to PFC in Cg1/M2, verified the importance of DISC1 within PFC and not nearby areas for the regulation of working memory.

Trial-by-trial error types following 1 h restraint stress

A number of analyses were performed to determine if there were qualitative differences in the patterns of response between baseline and stress conditions in each group. Specifically, the following variables were examined: the maximum number of errors to one side, the number of win–stay trials, and the number of omitted trials. The first two measures are signs of perseverative responding, whereas the third measure can be a reflection of freezing behavior characteristic of a more severe stress response.

No significant differences were observed in any of these measures between stress and non-stress conditions for any group. These findings suggest that the impaired performance following stress in the DISC1 KD group was mainly due to quantitative differences in the number of correct trials, rather than to qualitative changes in any particular type of error, and are consistent with the very mild stressor used in the study.

DISC1 immunohistochemistry

Following the behavioral experiments, we confirmed whether the viral constructs successfully knocked down DISC1 expression in PFC using immunohistochemistry. Figure 4 shows sample images of DISC1 labeling in the PFC and motor cortex in each group. For comparison, sample images of Cg1/M2 in the Anatomical Control and Control groups are shown in Supplementary Figure S1, and verification of the DISC1 antibody in DISC1 knockout rats (SAGE Labs, St Louis, MO, USA) is shown in Supplementary Figure S4. Analysis using stereology, optical densitometry, and % area of DISC1 labeling revealed that DISC1 expression was reduced in PFC in the DISC1 KD group relative to motor cortex, as well as to PFC in
Quantification of DISC1 labeling using isotropic virtual plane-based stereology

Length density of DISC1-labeled dendrites was measured in PFC relative to motor cortex, and compared between groups. A one-way ANOVA revealed a significant main effect of group ($F(3,12) = 13.535, P < 0.0005$). User-defined contrasts revealed that the DISC1 KD group showed reduced length density in PFC relative to the Control ($P = 0.003$), Scrambled ($P = 0.001$), and Anatomical Control ($P = 0.001$) groups (Figure 5). DISC1 KD was still effective 100–200 days after the viral infusions, as these animals had no DISC1 in PFC but maintained DISC1 in nearby motor cortex (Supplementary Figure S3).

**DISCUSSION**

Summary of findings

In this study, we have demonstrated that reducing DISC1 function in PFC increased vulnerability to the impairing effects of acute stress on working memory performance. Under non-stress conditions, KD of Disc1 had no significant effect on working memory. However, rats that received an active viral construct that knocked down Disc1 in PFC were significantly impaired by 1 h restraint stress, a very mild stressor that had no effect on cognitive performance in control animals. Rats that received a construct with a ‘scrambled’ sequence or received the active construct dorsal to PFC were also unaffected by this very mild stress. Thus, reduction of DISC1 expression in the adult rat PFC increased susceptibility to stress-induced PFC dysfunction. Koike et al. and Kvajo et al. have shown that mice with a truncation mutation in Disc1 showed impaired performance in a challenging test of working memory. Our study adds to their findings by investigating the effects of a DISC1 lesion restricted to PFC in adult animals, free from developmental effects of the Disc1 mutation. These data suggest that mutations that reduce DISC1 function may continue to impair PFC cognitive function in the adult cortex during stress exposure.

Potential mechanisms

DISC1 serves as a scaffolding protein for a large number of proteins, including interactions with a variety of PDE4s to regulate cAMP levels. Thus, it is possible that KD of DISC1 aggravates stress-induced cognitive deficits through dysregulation of cAMP signaling. Both physiological and behavioral studies have shown that acute stress impairs PFC network firing and working memory performance via increased cAMP–HCN channel signaling. Although in vitro tissue culture studies have shown that elevation of intracellular cAMP levels causes the release of PDE4D3 and PDE4C2 isoforms from DISC1, it does not affect DISC1 binding to PDE4A1 and PDE4A5 isoforms under these conditions. DISC1 interactions with PDE4s and cAMP signaling appear to be heterogeneous.

Immunoelectron microscopy data indicate that DISC1 appears to be crucial for the proper localization of PDE4s within PFC neurons, anchoring them to precise subcellular locations. Thus, loss of DISC1 would lead to spatially dysregulated cAMP signaling. For example, PDE4A and DISC1 are both found near HCN channels in spines (Figure 1c), and unanchoring of PDE4A may lead to excessive cAMP opening of HCN channels and reduced PFC network firing. (We were not able to investigate the role of HCN channels in the current study, as these experiments would require cannulation procedures that are incompatible with restraint stress.)
DISC1 also anchors PDE4A to the spine apparatus (endoplasmic reticulum), where it is positioned to regulate feedforward cAMP–calcium signaling.19 Unanchoring of PDE4A from this key location may lead to increased calcium and cAMP–protein kinase A signaling, which may reduce neuronal firing by opening SK and KCNQ potassium channels, and closing canonical transient receptor potential channels. KCNQ channels are localized on PFC spines,17 are opened by protein kinase A signaling,57 and have been shown to interact with human DISC1 in vitro.18 Physiological recordings have also shown that DISC1 KD in rat PFC hyperpolarizes layer V pyramidal neurons and reduces their firing, at least in part, through cAMP-induced reductions in transient receptor potential channels currents and increases in calcium-activated SK currents (El Hassar & Yeckel, unpublished data). Thus, dysregulated DISC1–cAMP signaling can lead to a number of changes that reduce PFC neuronal excitability. Furthermore, loss of DISC1 may impair energy regulation by unanchoring PDE4B from mitochondria in PFC dendrites;19 this mechanism may be particularly problematic under conditions of stress exposure.

Finally, although the current study focused on the contribution of DISC1 to working memory in the adult PFC, DISC1 likely also affects PFC function via developmental changes in the PFC circuitry. Disc1-mutant mouse models show altered dendritic structure and spine density in PFC pyramidal cells.41,42 Furthermore, many models show reduced immunoreactivity for parvalbumin-positive interneurons in PFC.43 Such DISC1-related changes would likely affect working memory performance both during development and as adults.

Role of various DISC1 isoforms

The DISC1 KD virus used here was designed to target all known rodent DISC1 isoforms, and thus our results likely reflected reduction or absence of total DISC1 protein in PFC. This manipulation is not identical to the translocation mutation observed in the Scottish pedigree in which DISC1 was initially discovered.20–22 However, our results are still relevant, as the translocation mutation has been proposed to result in ‘hoplaining’ sufficiency’ due to a loss of the whole protein,32 or in the expression of a truncated protein with dominant-negative function.28,56,62,63 Thus, these families may have a ‘functional KO’ of DISC1 actions.

Relevance to psychiatric disorders

The current findings may extend to a range of psychiatric disorders associated with dysregulated cAMP signaling, where patients often show precipitation or exacerbation of symptoms with stress.11–14 For example, schizophrenia is associated with a number of alterations that either reduce the regulation of cAMP signaling, or aggravate the induction of cAMP-calcium signaling.17 DISC1 may normally protect PFC cognitive function under mild stress by acting as a molecular ‘brake’ on stress-induced increases in cAMP. However, patients with insulins to DISC1 may show increased sensitivity to the effects of stress due to impaired interactions with PDE4 and excessive build-up of cAMP, which in turn may weaken network synapses. Understanding these intracellular pathways in PFC may help us to develop rational strategies for novel psychiatric treatments and cognitive enhancers.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This research was supported by NINDS T32 grant NS07224 to NJS, and a NARSAD Distinguished Investigator Award, PO1 grant AG03004 and 1RL1AA017536 within US4RR024350 to AFTA. We would like to thank Drs Mark Kay and Dirk Grimm (Stanford University, CA) for the AAV viral constructs. This work has been presented in abstract form at the DISC1 2010 meeting in Edinburgh, Scotland in September 2010; Society for Neuroscience Meeting in San Diego, CA in November 2010; New York Academy of Sciences Advancing Drug Discovery for Schizophrenia meeting in New York, NY in March 2011; and the Biological Psychiatry meeting in Philadelphia, PA in May 2012.

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