Supplementary materials for "Role of Disrupted in Schizophrenia 1 (DISC1) in Stress-Induced Prefrontal Cognitive Dysfunction" (Gamo et al.)

Supplementary Methods

Delayed alternation spatial working memory task

Rats were trained in the delayed alternation spatial working memory task in a T-shaped maze. Rats began in the start box behind the start gate at the bottom of the 'T'. For the first trial, when the start gate was lifted, they were rewarded for entering either arm at the top of the 'T'. The rats were then picked up and returned to the start box for the duration of the delay period, until the start gate was lifted again. During subsequent trials, rats were rewarded only for entering the arm opposite to the previously entered arm after each delay period. The procedure was repeated for 10 trials per day. The choice point, which was the junction at which the rat turned to either arm, was wiped with 75% ethanol solution after each trial to remove any olfactory cues.

The delay periods were adjusted for each rat such that they were performing at a stable baseline, and ranged from "0" seconds to over a minute. Rats were determined to be performing at a stable baseline if they performed 60, 70 or 80% correct for 3 consecutive testing days before a treatment (but not more than 1 day with 60%). This baseline allowed us to detect either improvement or impairment in performance following a treatment.

Production of viral constructs

Rat brain total RNA from the frontal cortex was purified using Trizol reagent (Invitrogen, Grand Island, NY). 2µg RNA were reverse transcribed using Superscript II (Invitrogen), and the *Disrupted in schizophrenia 1* (*Disc1)* coding region was amplified from the cDNA using Pfu Ultra II polymerase (Stratagene, La Jolla, CA). The PCR product was cloned into a pSTBlue-1 vector (Novagen, San Diego, CA), and subjected to direct sequencing. An error-free clone was subcloned into pAAV-MCS (Stratagene) to create pAAV-DISC1.

5 plasmid DNA constructs were generated to code for short hairpin RNAs (shRNAs) directed against sequences within exon 1 of rat *Disc1*. Vectors containing an H1 promotor for shRNA expression and a cytomegalovirus (CMV) promotor for lacZ expression, flanked by AAV Inverted Terminal Repeat (ITR) sequences (pAAV-lacZ-shRNA), were created to allow viral expression of shRNAs (Disc1-shRNA1-5). A control shRNA viral construct containing a scrambled sequence was also created with an identical nucleotide composition as the Disc1-shRNA1 target sequence, with no homology to any mammalian gene in the Genbank database (scrDisc1-shRNA).

Infectious adenovirus was generated according to a modified version of the methods described by Auricchio et al. (2011)[1]. Briefly, shRNAs were co-transfected with pDG plasmid (kindly provided by Drs. Mark Kay and Dirk Grimm, Stanford University, CA) into Human Embryonic Kidney 293 (HEK293) cells, and virus was purified 48h later by iodixanol gradient centrifugation. Viral titers were measured by transduction of camptothecin pre-treated HEK293

cells, followed by ß-galactosidase staining. This adenoviral technology successfully knocked down HCN1 expression in the rat prefrontal cortex (PFC) in Wang et al. (2007)[2].

Testing of viral constructs using Western blots

HEK293 cells were transfected with control DNA (pSTBlue-1, Novagen), pAAV-DISC1, pAAV-DISC1 and Disc1-shRNA1, or pAAV-DISC1 and Disc1-shRNA2. After 48h, cells were lysed and subjected to SDS-PAGE. Blots were probed with a goat polyclonal anti-DISC1 antibody at 1:200 and then probed with an infrared-labeled anti-goat secondary antibody (Rockland Inc) at 1:5000. Blots were scanned on an Odyssey Imager infrared scanner (Odyssey Imager, LI-COR Biosciences). Both knockdown (KD) constructs effectively knocked down DISC1 expression, especially Disc1-shRNA1 (Figure 2A). Thus, Disc1-shRNA1 was used in the behavioral experiments. This construct targeted the following sequence in exon 1, which is conserved in all known DISC1 isoforms in rodents[3]: AACCTTCTGCTTCTCCGCGTT.

DISC1 immunohistochemistry

Rats were sacrificed by transcardial perfusion. They were deeply anesthetized with an overdose of Euthasol (pentobarbital sodium + phenytoin sodium, 4.4mg/kg; Virbac Animal Health, Fort Worth, TX) and perfused with phosphate buffer saline (PBS) followed by 4% paraformaldehyde fixative in 0.1M phosphate buffer (PB). The right hemisphere was notched and brains were sectioned coronally at 50µm using a vibratome. Sections were processed free-floating. To minimize background staining, sections were blocked in 3% hydrogen peroxide in 0.1M PBS

(30min. at room temperature, RT). In between procedures, sections were always washed in 0.1M PBS.

Two rabbit polyclonal antibodies, NB110-40773 (lot A1) and NB110-40775 (lot C2) (Novus Biologicals, Littleton, CO), were combined to label DISC1. NB110-40773 targeted an internal region within residues 400-500 in the N-terminal region of rat DISC1, and was shown to stain somata in pilot studies. NB110-40775 targeted an internal region within residues 700-800 in the C-terminal region of mouse DISC1, and was shown to stain dendrites. Thus, combining them was expected to give a more complete picture of DISC1 expression than with either antibody alone. The antibodies were prepared at a concentration of 1:1000 in a solution containing 5% normal goat serum, 2% bovine serum and 0.1% Triton X. The sections were incubated in the primary antibodies for 48h at 4°C.

Next, the sections were incubated in a biotinylated Goat anti-Rabbit secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) at 1:200 in 0.1M PB for 24h at 4°C, and subsequently incubated in a standard Avidin/Biotin Peroxidase Complex (ABC) solution (Vector Laboratories, Burlingame, CA) for 4h at RT.

Finally, the sections were developed using 0.025% 3-3'-diaminobenzidine (DAB) (SK-4100, Vector Laboratories) solution in 1x Tris Buffer. The reaction was started by adding 1µL 30% hydrogen peroxide, and stopped by rinsing the sections in 0.1M PB. The sections were mounted on gelatin-coated slides, preserving left-right orientation. A set of adjacent sections was also stained for Nissl using standard cresyl violet histological procedures. The sections were then

dehydrated in a series of alcohols ending with 100% xylene, and coverslipped with Permount mounting medium.

Quantification of DISC1 staining using isotropic virtual planes-based stereology

This method allowed efficient and accurate estimation of the total length and length density of stained dendrites in PFC and motor cortex. The method is not biased by the thickness, orientation, spatial distribution or staining intensity (as long as they were visible) of the dendrites, nor affected by shrinkage of tissue during the histological procedures[4]. All conditions for successful use of the technique were met, namely, the diameter of stained dendrites was much smaller than the thickness of the sections, dendrites were stained through the thickness of the sections, and thin focal planes were obtained by sampling at high magnification.

Section outlines were drawn at 5x magnification, and the PFC and motor cortex were defined using a rat brain atlas[5]. Optimal stereological parameters were determined from a pilot study using the same materials. The StereoInvestigator® (MicroBrightField Inc., Williston, VT) program randomly placed a 500x500μm grid over the designated region. Then, 60x60x8μm counting frames were automatically placed at the corners of the grid, within which isotropic virtual planes were spaced 30μm apart in a systematic random manner. Intersections between stained dendrites and planes were observed at 100x oil immersion with a Plan Neo-Fluar lens (optical aperture 1.3), and were indicated with markers. Two μm guard zones were observed in each visual field. The program estimated the total stained dendrite lengths and length densities from the number of intersections[4].

Quantification of DISC1 staining using optical densitometry

To verify and complement the stereological results, DISC1 labeling in dendrites was also quantified using optical densitometry. Digital photographs of PFC and motor cortex were taken at 100x oil immersion and converted to 8-bit greyscale (with 0 as black and 255 as white). A "mask" was then created by subtracting the background. The limits of the mask were obtained manually by visually determining the threshold intensity sufficient to maintain only the stained regions, while blacking out the rest of the image. Finally, the brightness of the stained dendrites was measured, and this value was converted to optical density defined as log(brightness).

The average optical density of 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. Image processing and data acquisition were done blindly with ImageJ (National Institutes of Health, Bethesda, MD), and statistical analyses were performed using SPSS.

Quantification of DISC1 labeling using % area of DISC1 labeling

To further verify and complement the stereological and optical density results, the average % area labeled with DISC1 was measured in PFC and motor cortex. While optical density measures were based on a gradient of gray values from 0 to 255, the data here were strictly

binary. Also, as opposed to isotropic virtual planes in which only dendritic profiles were considered, signal from occasionally stained somata and other cellular regions were included here. Area estimates were determined based on pixels being positive or negative for signal content using the same photographs that were used for optical density measures. The % area of signal for PFC was normalized to that in motor cortex, and compared using a 1-way ANOVA with a between-subjects factor of group (Control, DISC1 KD, Scrambled, Anatomical Control). User-defined contrasts compared DISC1 KD vs. Control, Scrambled and Anatomical Control groups. Image processing and data acquisition were done blindly with ImageJ, and statistical analyses were performed using SPSS.

Verification of DISC1 antibodies

An additional set of immunohistochemical tests was performed to verify the specificity of the NB110-40773 and NB110-40775 DISC1 antibodies. PFC sections from two DISC1 knock-out (KO) rats, which contained a deletion in exon 2 of *Disc1*, and two wild-type (WT) control rats (SAGE Labs, St. Louis, MO) were stained with the two antibodies, as described above. As knocking down *Disc1* using shRNA constructs may not affect all DISC1 protein in the region, the DISC1 KO rats provide a more rigorous way to test the antibodies.

Supplementary Results

Quantification of DISC1 staining using optical densitometry

Optical density of DISC1-labeled dendrites was measured in PFC relative to motor cortex, and compared between groups. A 1-way ANOVA revealed a significant main effect of group ($F[3, 11] = 18.36$, $p = 0.00014$). User-defined contrasts revealed that the DISC1 KD group showed reduced optical density in PFC relative to the Control ($p = 0.0045$), Scrambled ($p < 0.0005$) and Anatomical Control ($p = 0.0031$) groups (Supplementary Figure S2A).

Quantification of DISC1 labeling using % area of DISC1 labeling

Percent area of DISC1 labeling in PFC relative to motor cortex was compared between groups. A 1-way ANOVA revealed a significant main effect of group ($F[3, 11] = 10.37$, $p = 0.0016$). User-defined contrasts revealed that the DISC1 KD group showed reduced % area of signal in PFC relative to the Control ($p = 0.022$), Scrambled ($p = 0.0044$) and Anatomical Control ($p = 0.0010$) groups (Supplementary Figure S2B).

Comparison of rats that underwent 1h restraint stress earlier and later following viral infusions

To address whether the DISC1 KD viral infusions were still effective when performed up to 200 days after surgery, we compared the stress response and DISC1 immunohistochemistry in PFC between those rats that underwent restraint stress within 20-100 days following surgery and those that underwent stress within 100-200 days following surgery. These two groups showed similar mean stress responses (50% correct in the former group vs. 56.7% correct in the latter group). In those rats for which DISC1 immunohistochemistry was quantified, the latter group showed significant reductions in DISC1 staining in PFC (0 length density for a rat that

underwent stress 176 days after viral infusion, Supplementary Figure S3; 0.00444 length density for another rat that underwent stress 197 days after viral infusion), compared to motor cortex in DISC1 KD rats (average length density of 1.3488) and PFC in Scrambled (average length density of 0.811) and Control (average length density of 1.046) rats. These similarities suggest that the viral infusions were effective even after more than 100 days following surgery.

Verification of DISC1 antibodies

When the NB110-40773 and NB110-40775 DISC1 antibodies were tested in PFC sections from DISC1 KO rats, the NB110-40773 antibody showed some off-target staining, while the NB110-40775 antibody showed specificity to DISC1. The NB110-40775 antibody revealed dendritic staining in PFC of control animals from the original study and of WT control rats purchased from SAGE Labs, but showed little PFC staining in DISC1 KD and DISC1 KO rats (Figure S4).

Supplementary References

1. Auricchio, A., Hildinger, M., O'Connor, E., Gao, G. P. & Wilson, J. M. Isolation of highly infectious and pure adeno-associated virus type 2 vectors with a single-step gravity-flow column. *Hum. Gene Ther.* **12**, 71-76 (2001).

2. Wang, M. *et al*. Alpha2A-adrenoceptors strengthen working memory networks by inhibiting cAMP-HCN channel signaling in prefrontal cortex. *Cell* **129**, 397-410 (2007).

3. Nakata, K. et al. DISC1 splice variants are upregulated in schizophrenia and associated with risk polymorphisms. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 15873-8 (2009).

4. Larsen, J. O., Gundersen, H. J. & Nielsen, J. Global spatial sampling with isotropic virtual planes: estimators of length density and total length in thick, arbitrarily orientated sections. *J. Microsc.* **191**, 238-248 (1998).

5. Paxinos G., W. C. in *The Rat Brain in Stereotaxic Coordinates* (Academic Press, New York, 1982).

Supplementary Figures

Figure S1. Examples of photographs taken from Cg1/M2 and motor cortex in Control and Anatomical Control rats. Actual photograph site and orientation varied from field to field. Scale bar applies to both images.

Figure S2. DISC1 KD virus reduced DISC1 expression in PFC in DISC1 KD rats.

A. Optical density of DISC1-labeled dendrites was measured in PFC and normalized to that in motor cortex. The optical density in PFC was reduced in the DISC1 KD group relative to that in the Control (*p = 0.0045), Scrambled (*p < 0.0005) and Anatomical Control (*p = 0.0031) groups.

B. Percent area of DISC1 labeling was measured in PFC and normalized to that in motor cortex. The % area of signal in PFC was reduced in the DISC1 KD group relative that in the Control (*p = 0.022), Scrambled (*p = 0.0044) and Anatomical Control (*p = 0.0010) groups.

Figure S3. DISC1 staining was reduced in PFC in a DISC1 KD rat that underwent restraint stress 176 days after viral infusion (length density = 0; bottom) relative to PFC in a Control rat (top), suggesting that viral KD of DISC1 was effective up to approximately 200 days following infusion. Scale bar applies to both images.

Figure S4. The specificity of the DISC1 NB110-40775 antibody (Novus Biologicals, Littleton, CO) was verified in DISC1 knock-out (KO) rats containing a deletion of exon 2 in *Disc1* (SAGE Labs, St. Louis, MO). Examples of dendritic staining in medial PFC (shown in inset) are shown for control rats from the original study (upper-left panel) and for wild-type (WT) control rats purchased from SAGE Labs (lower-left panel). The DISC1 knock-down (KD) rats from the original study (upper-right panel), in which *Disc1* was knocked down in medial PFC, and DISC1 KO rats (lower-right panel) showed little dendritic staining. Scale bar applies to all images.

Supplementary Tables

Quantitative estimates of DISC1-stained dendrites in PFC and motor cortex

		PFC			Motor cortex		
		Lv ($1/\mu m^2$)	Log(brightness)	% area of signal	Lv ($1/\mu m^2$)	Log(brightness)	% area of signal
Control	Mean	0.1394	1.29733	15.86958	0.3390	1.550247	21.16792
	SEM	0.0188	0.10771	2.811968	0.0897	0.032231	1.631942
Scrambled	Mean	0.1082	1.514592	17.265	0.2238	1.380893	16.73625
	SEM	0.0209	0.046796	1.606759	0.0769	0.042773	1.569491
DISC1 KD	Mean	0.0080	0.584932	4.054167	0.1798	1.568422	17.47833
	SEM	0.0038	0.088204	0.674235	0.0302	0.103544	3.301278
Anatomical control	Mean	0.0675	1.353804	15.19	0.1323	1.331147	15.46125
	SEM	0.0090	0.05226	1.438222	0.0261	0.069335	1.648165

Table S1. Quantitative estimates of DISC1-stained signals in PFC and motor cortex for each group, according to stereology (Lv), optical densitometry (log[brightness]) and % area of DISC1 labeling.