ORIGINAL ARTICLE

DISC1–ATF4 transcripitional repression complex: dual regulation of the cAMP-PDE4 cascade by DISC1

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Disrupted-In-Schizophrenia 1 (DISC1), a risk factor for major mental illnesses, has been studied extensively in the context of neurodevelopment. However, the role of DISC1 in neuronal signaling, particularly in conjunction with intracellular cascades that occur in response to dopamine, a neurotransmitter implicated in numerous psychiatric disorders, remains elusive. Previous studies suggest that DISC1 interacts with numerous proteins that impact neuronal function, including activating transcription factor 4 (ATF4). In this study, we identify a novel DISC1 and ATF4 binding region in the genomic locus of phosphodiesterase 4D (PDE4D), a gene implicated in psychiatric disorders. We found that the loss of function of either DISC1 or ATF4 increases PDE4D9 transcription, and that the association of DISC1 with the PDE4D9 locus requires ATF4. We also show that PDE4D9 is increased by D1-type dopamine receptor dopaminergic stimulation. We demonstrate that the mechanism for this increase is due to DISC1 dissociation from the PDE4D locus in mouse brain. We further characterize the interaction of DISC1 with ATF4 to show that it is regulated via protein kinase A-mediated phosphorylation of DISC1 serine-58. Our results suggest that the release of DISC1-mediated transcriptional repression of PDE4D9 acts as feedback inhibition to regulate dopaminergic signaling. Furthermore, as DISC1 loss-of-function leads to a specific increase in PDE4D9, PDE4D9 itself may represent an attractive target for therapeutic approaches in psychiatric disorders.

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INTRODUCTION

After the initial report on Disrupted-In-Schizophrenia 1 (DISC1) in a large Scottish pedigree,1 the significance of this gene has been extensively studied in human genetics and neurobiology.2,3 Although the involvement of this gene in any specific psychiatric illness categorized by current diagnostic manuals has been debated,4 DISC1 mediates many aspects of neurodevelopment and cellular signaling relevant to neuropsychiatric disorders.

Studies utilizing various mouse models have implicated DISC1 as an important regulator of brain development with roles in neurogenesis,5,6 progenitor proliferation,5,7 and changes in dendritic arborization, migration as well as the integration of cortical and hippocampal neurons.8–13 These functions may be mediated by protein–protein interactions between DISC1 and its binding partners, which include NDEL1,14,15 GSK3β1,5, BBS1/4,12,16 Girdin13,17 and Kalirin-7.18 Accordingly, animal models with genetic disruption of DISC1 display behavioral deficits, including deficits in prepulse inhibition, latent inhibition, spatial and working memory, sociability and increased immobile time in the forced swim test.19 Furthermore, Niwa et al.20 reported that transient deficits in neurodevelopment due to DISC1 deficits lead to adult behavioral changes after adolescent brain maturation.

In addition to important roles of DISC1 in neurodevelopment, DISC1 has a crucial function in neuronal signaling. Millar and co-workers21–25 reported intriguing evidence that DISC1 binds with several isoforms of a cyclic adenosine monophosphate (cAMP)-specific, rolipram-sensitive family of phosphodiesterases, referred to as phosphodiesterase 4D (PDE4s), and that it regulates the enzymatic activity of these proteins via direct protein–protein interaction. There are four PDE4 genes that constitute this family of phosphodiesterases. Each of these four genes encodes an isoform, and each isoform has a number of variants, each distinguished by their unique N-terminal regions. These N-terminal regions confer unique functional roles to each PDE4 variant by targeting interaction to specific protein complexes to influence local cAMP levels in spatially constrained signaling processes.26,27 PDE4s break down cAMP, thereby terminating classical G_{s}\text{a}_{i} protein-coupled receptor signaling (G_{s}\text{a}_{i}Sig), a well-studied pathway downstream of the neurotransmitter dopamine, which is heavily implicated in psychiatric disorders. Amphetamine administration, which increases dopamine levels, is used as a pharmacological model for schizophrenia in model animals. First-generation antipsychotics are known to target the dopamine D2 receptor, which is known to antagonize G_{s}\text{a}_{i}Sig. Mice with PDE4D loss-of-function exhibit an antidepressive phenotype, that is, decreased immobility time in the forced swim test28–30 and increased neurogenesis,31,32 phenotypes opposite to those observed in DISC1 mutant mice.31,32 PDE4 inhibition also enhances dopaminergic signaling downstream of dopamine D1 receptor,33 which potentially explains the observation that DISC1 mutant mice have an altered response to methamphetamine.34
The involvement of DISC1 in gene transcription was initially suggested by its association with activating transcription factors 4 and 5 (ATF4 and ATF5), basic-region-leucine zipper domain-containing transcription factors of the CREB/ATF family.\textsuperscript{35} This finding has been substantiated by further mechanistic studies showing that DISC1 represses ATF4-mediated gene transcription.\textsuperscript{36,37} ATF4 (also known as CREB2) regulates many biological processes, from hematopoiesis and osteoblast differentiation to neuronal progenitor proliferation, synaptic plasticity, learning and memory, and behavior.\textsuperscript{38} Of particular interest is the regulation of ATF4 by dopamine stimulation. Specifically, ATF4 levels increase in response to dopaminergic stimulation.\textsuperscript{39} However, very little is known about the effects of DISC1 dysfunction on the transcriptional targets of ATF4 in the brain.

In this study, we investigated the role of ATF4 and DISC1 in transcriptional repression and how this functional interaction impacts dopaminergic signaling. We find that ATF4 and DISC1 act together in a transcriptional repressor complex to regulate specifically the transcription of PDE4D9, 1 of 11 known PDE4D variants.\textsuperscript{40} PDE4D9 is a long variant of PDE4D that is specifically the transcription of MAPKAPK2,\textsuperscript{40,41} for further details refer to Lynex \textit{et al.}\textsuperscript{42} We further demonstrate that this repressor activity is decreased by dopamine via G\(\alpha\)s,G\(\alpha\)i, which activates PKA and promotes the phosphorylation of DISC1 at S58. Our findings illustrate that the DISC1–ATF4 interaction regulates a transcriptional feedback loop that responds to, and modulates, dopaminergic neurotransmission and provide mechanistic insights into how the perturbation of this complex may lead to aberrant signaling that may manifest as symptomatology associated with various psychiatric illnesses.

**MATERIALS AND METHODS**

**Mice**

C57Bl6J (C57) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Pregnant Swiss-Webster (SW) mice were obtained from Taconic (Hudson, NY, USA) after confirming that the colony did not contain the 25 bp deletion within DISC1 found in some SW lines.\textsuperscript{43} ATF4 mice have been described previously.\textsuperscript{44} All mice were kept in a reverse-light cycle and food and water was provided ad libitum. All procedures were performed in accordance with the CAC of MIT.

**Generation of antibodies and plasmids**

A table of antibodies and plasmids used in this study that have been previously described is provided as Supplementary Material Tables 1 and 2. A table of antibodies and plasmids used in this study that have been previously described is provided as Supplementary Material Tables 1 and 2.

**Production of pLentilox3.7 ATF4 shRNA**

ATF4 and DISC1 short hairpin RNA (shRNA) were cloned into the pLentilox 3.7 vector (Addgene, Cambridge, MA, USA; plasmid 11795) as described previously.\textsuperscript{45} Briefly, complementarity-5’ phosphorylated oligonucleotides encoding anti-ATF4, DISC1 and PDE4D9 shRNA (sequences provided in Supplementary Material Table 4) were annealed, digested with Xhol and ligated into the pLentilox 3.7 vector that had been digested with Hpal and Xhol. Proper insertion and orientation of the sequence downstream of the U6 promoter was confirmed using the sequencing primer 5’-CATGTCGAGGGAAGAATAGTAGAC-3’. To create DISC1 shRNA and scr-shRNA mCherry, the GFP cDNA sequence contained within pLentilox3.7 was excised and replaced with the mCherry cDNA sequence, obtained by amplification using PCR -mCherry (Addgene; plasmid 31165).

**Chromatin immunoprecipitation**

Chromatin immunoprecipitations (ChIPs) were carried out from hippocampal or cortical tissue from adult (12-week-old) C57Bl6J, or ATF4\textsuperscript{46} mice. pLentilox3.7 constructs described previously were transfected into C57Bl6J background, homozygous for all mouse sequences, according to the manufacturer’s protocol for the EZ-MagnaChIP kit (Millipore, Temecula, CA, USA). A measure of 1 µl of eluted purified DNA was used for each analysis condition.

**Amphetamine treatment**

Animals were injected intraperitoneally with 0.35 mg kg\textsuperscript{-1} 3,4-diamphetamine sulfate (Tocris Bioscience, Bristol, UK) resuspended in 0.9% saline at 70 ng µl\textsuperscript{-1}, or with saline, and then placed in a novel cage for 2 or 4 h for observation.

**Primary neuronal cultures**

Dorsal hippocampal cultures were prepared from embryonic E15–16 SW mice as described previously.\textsuperscript{47} Experiments were performed on 10–17 days in vitro (DIV) primary cultures. Dissociated rat cortical cultures were prepared as described previously.\textsuperscript{48}

**Production and titration of virus**

Lentiviral particles were made as described previously.\textsuperscript{5} Briefly, pMD.2G and pCMVdeltaR8.2 (Addgene; plasmids 12259 and 12263) and pLentilox3.7 constructs described previously were transfected into 90% confluent HEK-293T cells at a ratio of 18:6:5 to generate lentiviral particles. Lentiviral particles were made as described previously.\textsuperscript{5} Briefly, pMD.2G and pCMVdeltaR8.2 (Addgene; plasmids 12259 and 12263) and pLentilox3.7 constructs described previously were transfected into 90% confluent HEK-293T cells at a ratio of 18:6:5 to generate lentiviral particles. Lentiviral particles were made as described previously.\textsuperscript{5} Briefly, pMD.2G and pCMVdeltaR8.2 (Addgene; plasmids 12259 and 12263) and pLentilox3.7 constructs described previously were transfected into 90% confluent HEK-293T cells at a ratio of 18:6:5 to generate lentiviral particles. Lentiviral particles were made as described previously.\textsuperscript{5} Briefly, pMD.2G and pCMVdeltaR8.2 (Addgene; plasmids 12259 and 12263) and pLentilox3.7 constructs described previously were transfected into 90% confluent HEK-293T cells at a ratio of 18:6:5 to generate lentiviral particles. Lentiviral particles were made as described previously.\textsuperscript{5} Briefly, pMD.2G and pCMVdeltaR8.2 (Addgene; plasmids 12259 and 12263) and pLentilox3.7 constructs described previously were transfected into 90% confluent HEK-293T cells at a ratio of 18:6:5 to generate lentiviral particles. Lentiviral particles were made as described previously.\textsuperscript{5} Briefly, pMD.2G and pCMVdeltaR8.2 (Addgene; plasmids 12259 and 12263) and pLentilox3.7 constructs described previously were transfected into 90% confluent HEK-293T cells at a ratio of 18:6:5 to generate lentiviral particles. Lentiviral particles were made as described previously.\textsuperscript{5} Briefly, pMD.2G and pCMVdeltaR8.2 (Addgene; plasmids 12259 and 12263) and pLentilox3.7 constructs described previously were transfected into 90% confluent HEK-293T cells at a ratio of 18:6:5 to generate lentiviral particles.
Luciferase activity assay

CAD or N2A cells were plated at a density of 2.5 \times 10^5 cells per ml in 24-well plates one day before transfection with a plasmid expressing Renilla luciferase under the control of a human thymidine kinase 1 promoter (pBLCAT3; Promega, Madison, WI, USA), and Firefly luciferase within the pGL3 backbone under the control of numerous promoters and enhancers, as described below, at a ratio of 1:10. For assays comparing ATF4 and DISC1 knockdown, cells were also transduced 2 h after transfection, with lentivirus (multiplicity of infection = 5) expressing shRNA constructs for DISC1 or a mouse U6 promoter. Assays were conducted 2 days after transfection/viral transduction using the dual-luciferase reporter assay kit (Promega) and luminescence was detected using the Spectramax-L luminescence microplate reader (Molecular Devices, Sunnyvale, CA, USA), and analyzed as described previously.7

Immunoblot analysis

Unless otherwise indicated, at the end of the indicated treatments, cells were lysed in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) containing protease and phosphatase inhibitors, and boiled after dilution in SDS sample buffer (2% SDS, 0.6 M dithiothreitol, 62.5 mM Tris (pH 6.8), 10% glycerol, 0.0025% bromophenol blue). Equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis and western blot analysis using the indicated antibodies at the concentrations included in Supplementary Table 3. For protein extraction from whole tissue, the forebrain or hippocampus was dissected and homogenized in RIPA buffer. Lysates were spun at 13,000 r.p.m. for 15 min, after which supernatants were removed and analyzed for protein concentration (Bio-Rad Protein Assay, Hercules, CA, USA). SDS buffer was added to equal amounts of protein and immunoblot analysis was performed using 25–200 µg of protein per well using the 1.5 mm gel thickness Mini-Protein Tetra (Bio-Rad).

Co-immunoprecipitation

HEK293T or HeLa cells were transfected with various constructs using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, cells were lysed with IP buffer (0.4% Triton X-100, 200 mM NaCl, 50 mM Tris pH 7.5), RIPA buffer or hypotonic buffer (0.2% NP40, 5% glycerol, 0.5 mM MgCl2, 50 mM NaCl, 50 mM Tris pH 7.5) containing protease and phosphatase inhibitors. Equal amounts of lysates were incubated with antibody-conjugated beads (Sigma, Santa Cruz Biotechnology, Dallas, TX, USA) in IP buffer overnight at 4°C, and then washed three times in IP buffer.

Immunoprecipitations were performed using equal amounts of protein and by rocking the lysates with the 1 µg of the indicated antibodies in IP buffer overnight at 4°C, or by incubation with the indicated antibodies overnight at 4°C, followed by incubation with Protein A and Protein G agarose beads for 1 h, and then washed three times in IP buffer, RIPA buffer or hypotonic buffer before eluting in SDS buffer.

In vitro kinase-IP assay of GST-ATF4 and GFP-mDISC1

GST-ATF4 protein (0.5 µg) was re-suspended in 250 µl PKA kinase reaction buffer (New England Biolabs) supplemented with 200 µM ATP. HEK293T cells were transfected with GFP-mDISC1T15 and lysed 24 h after transfection in IP buffer (0.4% Triton X-100, 200 mM NaCl, 50 mM Tris (pH 7.5)) with phosphatase and protease inhibitors. Following centrifugation to remove cellular debris, GFP-DISC1mT1 was isolated by affinity purification using 50 µg of mouse (B2) anti-GFP-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. The beads were washed four times with IP buffer, and then two times with PBS; for the last wash, the beads were re-suspended in 5 ml PBS with protease inhibitor, and 1.2 ml of the resuspended PBS beads was aliquoted into each of four Eppendorf tubes. The PBS was removed from the GFP-agarose beads and 50 µl of the GST-ATF4 in PKA kinase buffer (50 mM Tris-HCl, 10 mM MgCl2, 200 µM ATP (pH 7.5)) was added to each reaction condition. In all, 2500U of PKA, 2500U of calf intestinal alkaline phosphatase or 0.5 µg bovine serum albumin was added into the reaction mixture and allowed to incubate at room temperature for 7 min before washing five times with IP buffer. For the GFP-only input, the beads were washed following the addition of bovine serum albumin in PKA kinase reaction buffer without GST-ATF4. Input (2%) is shown for GST-ATF4.

Immunocytochemistry of primary neurons and HeLa cells

Immunolabeling of primary neurons and HeLa cells was performed as described previously.4 When used, okadaic acid (0.5 µM; EMD Millipore, Billerica, MA, USA) was added 2 h before cells were fixed. The percentage of DISC1-positive cells was quantified by dividing the number of cells showing nuclear DISC1 immunoreactivity by the number of 4′,6-diamidino-2-phenylindole-labeled cells.

Data analysis

All data were analyzed using the Prism software using statistical tests indicated (GraphPad Software, La Jolla, CA, USA).

RESULTS

DISC1 binds to the PDE4D gene locus in an ATF4-dependent manner

We identified an ATF4 binding site within the genomic locus encoding the PDE4D gene in the course of an unbiased ChIP screen that was conducted to identify ATF4 genomic target loci in vivo in the developing brain (manuscript in preparation). Binding to the locus on PDE4D, along with several other identified loci, was confirmed by ChIP-PCR (Supplementary Figure 1). Given that DISC1 regulates ATF4 transcriptional activity in vitro36 and lacks a DNA-binding domain, we hypothesized that DISC1 associated with the PDE4D gene locus in an ATF4-dependent manner. To test this hypothesis, we conducted ChIP-PCR using a polyclonal DISC1 antibody that recognizes the N terminus of DISC1 with dorsal hippocampal samples from 8-week-old wild-type (ATF4+/−/−), ATF4 heterozygous (ATF4+/−/−) and ATF4 knock-out (ATF4−/−/−) littermate mice. We observed that the association of DISC1 to the PDE4D locus was nearly absent in ATF4−/−/− mice, and severely disrupted in the ATF4−/−/− littermates (Figure 1a). These data indicate that the association of DISC1 to the PDE4D locus requires the presence of ATF4.

Given that the PDE4D and DISC1 proteins have been shown to interact directly,22,23,25 we felt that PDE4D was an interesting ATF4 target. Our ChIP data indicate that ATF4 binds to a region of the PDE4D locus that is 5′ to the common catalytic domain, and downstream of some, but not all, of the unique PDE4D variant exons.26,28 The location of the ATF4 and DISC1 binding site on the PDE4D locus led us to hypothesize that ATF4 and DISC1 regulate the transcription of some, but not all, of the unique PDE4D variant exons.26,28 We chose to characterize the transcription profile of the various PDE4D transcripts by ATF4 and DISC1, we used shRNA to knock down these proteins while measuring the mRNA levels of PDE4D variants using semiquantitative PCR. DIV 14 cultured hippocampal neurons were transduced at DIV 4 with lentivirus carrying control scrambled (Scr), ATF4 or DISC1 shRNA-expressing lentivirus.3 Using semiquantitative reverse transcription-PCR,19,30 an approach used in the past to detect changes in differential expression of the unique variants, we observed a marked increase specifically in the levels of the PDE4D9 transcript upon both ATF4 and DISC1 shRNA-mediated knockdown (Figure 1b). This increase in PDE4D9 mRNA was confirmed with in situ hybridization in wild-type and ATF4−/−/− mice. Using an antisense probe targeting the unique N terminus of PDE4D9 on E15 brain sections, we observed a

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substantial increase in PDE4D9 transcripts in ATF4/C0/C0 compared with wild-type embryonic brains (Figure 1c). Consistent with the observed increases in PDE4D9 mRNA, PDE4D9 protein levels were also significantly increased in dorsal hippocampal brain lysates from 8-week-old ATF4þ/C0 mice compared with wild-type (ATF4þ/þ) littermates (Figure 1d). We stereotactically injected lentivirus-expressing DISC1 shRNA into the 8-week-old mouse hippocampus, and observed that PDE4D9 protein levels were significantly higher following this in vivo shRNA-mediated DISC1 knockdown compared with control shRNA (Figure 1e). Collectively, these results indicate that the loss of ATF4 or DISC1 expression specifically increases PDE4D9 mRNA. These findings are consistent with the notion that ATF4 and DISC1 function in a transcriptional repressor complex at the PDE4D9 gene locus to repress specifically transcription of the PDE4D9 isoform. Collectively, these results suggest that ATF4 is necessary for the recruitment of DISC1 to the PDE4D9 gene locus, and that these two factors together regulate the expression of the PDE4D9 variant.

ATF4 and DISC1 bind the regulatory region of PDE4D9 and suppress its transcription

PDE4 promoters do not have TATA boxes and other features that help in its identification. To determine whether the increase in PDE4D9 mRNA and protein that follows DISC1/ATF4 knockdown results from increased transcriptional activity, we tested the effects of ATF4 and DISC1 knockdown in luciferase-based reporter assays using putative PDE4D9 promoter and enhancer regions, an approach used successfully to identify promoter regions from other PDE4 isoforms. We cloned the 1000 bp upstream of the PDE4D9 coding region and placed this putative promoter region upstream of the luciferase cDNA in the pGL3 vector (Figures 2a and b). Compared with the baseline luciferase expression from pGL3 alone, which lacks a promoter, we observed significant luciferase activity in the presence of the 1000 bp fragment (4D9pro-Luc; Figure 2b), suggesting that this region of the PDE4D9 locus likely contains the PDE4D9 promoter. Next, we assayed whether the presence of the ATF/DISC1 binding site of the PDE4D9 locus affects the transcriptional activity of the putative PDE4D9 promoter. The ATF4/DISC1 binding site (450 bp) was divided into four fragments (EnhA–D), each of which was placed immediately upstream of the putative PDE4D9 promoter. The presence of fragments EnhA, EnhB and EnhC did not significantly affect PDE4D9 promoter-driven luciferase activity. However, fragment EnhD markedly increased the activity of the putative PDE4D9 promoter (Figures 2a and b), marking it as a potential enhancer region in the PDE4D9 locus.

Upon close examination of the sequence of EnhD, we identified the sequence GGGTGCAAT, which has homology to known non-palindromic ATF4/C/EBP binding motifs, consistent with our
findings that it may represent an ATF4 binding region (Figure 2c). To test whether this sequence is responsible for the transcription-enhancing ability of fragment EnhD, we repeated the luciferase promoter assays using a mutated and wild-type EnhD fragment in the presence of shRNA targeting ATF4, DISC1 or both. The knockdown of ATF4 and DISC1 had no significant effect on the luciferase activity of the 4D9proLuc vector, which expresses the putative PDE4D9 promoter alone, compared with control shRNA. However, in the presence of the EnhD fragment in the 4D9proLuc vector, the knockdown of DISC1 led to an increase in luciferase activity in transfected cells. This increase was also observed in cells transfected with both ATF4 and DISC1 shRNA,

Figure 2. The activating transcription factor 4–Disrupted-In-Schizophrenia 1 (ATF4–DISC1) binding region acts as a transcriptional repressor to endogenous PDE4D9 promoter. (a) Schematic of PDE4D genetic loci. (b) Schematic of constructs used to study the transcriptional effects of the ATF4–DISC1 binding region. (c) Placement of the 1000 bp region immediately upstream of PDE4D9 coding region in front of the luciferase (Luc) gene upregulates luciferase activity, indicating that this region acts as a promoter. Fragment D significantly altered luciferase activity relative to the others (n = 5, analysis of variance (ANOVA) P < 0.0001, *Dunnett’s test P < 0.05 for comparison of Luc to all others, ***Tukey’s test P < 0.001 for EnhD–PDE4D9 compared with other fragments, PDE4D9 promoter only). (d) A putative CRE/CEBP binding site was identified within enhancer fragment D (EnhD). The site was mutated to eliminate sequence similarity to CRE/CEBP binding site. (e) Comparison of the effects of control, ATF4 or DISC1 short hairpin RNA (shRNA) combinations on luciferase activity on EnhD–PDE4D9. The presence of EnhD upstream of the 4D9 promoter is critical for the dramatic elevation in signal seen after ATF4, DISC1 shRNA expression (n = 5, ANOVA P < 0.0001, ***Tukey’s test P < 0.001 for comparisons between EnhD–PDE4D9 DISC1 shRNA and DISC1/ATF4 shRNA compared with all others). NS, nonsignificant; Scr, scrambled.
but not in cells expressing shRNA against ATF4 alone. When the putative ATF4 binding region was mutated in the EnhD fragment (mutEnhD 4D9proLuc), none of the shRNAs significantly affected the luciferase activity (Figure 2d), indicating a loss of enhancer-like activity. Taken together, these results indicate that the ATF4/DISC1 binding site acts as an enhancer region to regulate PDE4D9 transcription, and that the binding of the DISC1 protein, via ATF4, to this region acts to repress PDE4D9 promoter activity. The fact that ATF4 knockdown alone does not significantly affect luciferase expression suggests that the requirement for ATF4 binding may not be captured in this construct. For example, in the genome, ATF4 binding may serve the purpose of bringing the enhancer region in close proximity with the promoter, a position that is re-created in our luciferase construct.

Signaling via the dopamine D1 receptor modulates the expression of PDE4D9

As mentioned previously, PDE4s act to terminate Gαs-protein-coupled receptor signaling (Gαs,ρ) downstream of dopamine D1 receptor activation, and the loss of PDE4 expression in mice has an antidepressant effect, while overactivation of the D1 receptor via amphetamine administration models aspects of schizophrenia in animals. Thus, alterations in the levels of the PDE4D9 variant may play an important role in neuronal dopaminergic signaling pathways. To address whether the regulation of PDE4D9 by ATF4/DISC1 is influenced by extracellular signaling, such as neurotransmitters, we treated cultured hippocampal neurons with potassium chloride (KCl, 55 mM), which induces generalized depolarization, baclofen (100 μM), which activates the GABA_B receptor, or prostaglandin E2 (10 mM), which binds the prostaglandin E2 receptor and reportedly increases phosphodiesterase activity in other systems. Interestingly, none of these agents influenced PDE4D9 levels in DIV 14 cultured hippocampal neurons (Figure 3a and Supplementary Figure 3). However, dopamine (100 μM) as well as isoproterenol (100 μM), β-adrenergic receptor agonist, significantly increased the level of PDE4D9 mRNA (Figure 3a). We also observed an increase in PDE4D1, 2 and 5, with dopamine and isoproterenol treatment, consistent with past work demonstrating that elevated CAMP levels increase these transcripts. This suggests that agonists of D1-type dopamine receptors, β-adrenergic receptors or Gs路过 coupled, GPCR stimulation increase PDE4D9 transcription.

We then tested the effects of dopaminergic signaling on PDE4D9 mRNA levels by using quantitative PCR in DIV 14 primary hippocampal neurons treated with vehicle, dopamine (10 μM), the D1-type receptor agonist SKF81297 (6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-[1,4]benzazepine) (3 μM) and the D2-type receptor agonist quinpirole (10 μM). We found that treatment with dopamine and SKF81297, but not quinpirole, increased PDE4D9 mRNA levels, indicating that activation specifically of the dopamine D1-type receptor increases PDE4D9 transcription in neurons (Figure 3b). Furthermore, acute amphetamine treatment, which increases both noradrenergic and dopaminergic signaling, at a dose shown to induce hyperactivity (0.35 mg·kg冬1) in 8-week-old wild-type mice increased both mRNA and protein levels of PDE4D9 in the hippocampus (Figures 3c and d). Taken together, these results indicate that PDE4D9 transcription is regulated by dopamine via D1-type receptor signaling, and that neuropharmacologically relevant doses of amphetamine increase PDE4D9 transcription in behaving animals.

Dopamine and amphetamine regulate the association of DISC1 with the PDE4D9 enhancer region

To examine the role of DISC1 and ATF4 in the regulation of PDE4D9 by D1-coupled signaling, we performed ChIP in hippocampal tissue from mice treated acutely either with amphetamine (0.35 mg·kg冬1) or saline. DISC1 occupancy of the putative PDE4D9 enhancer region was significantly lower at both 2 and 4 h following amphetamine administration compared with controls (Figure 4a). Interestingly, we also observed a detectable, but nonsignificant, increase in ATF4 occupancy at this locus with amphetamine treatment (Figure 4a). Taken together, these results demonstrate that DISC1, but not ATF4, dissociates from the PDE4D9 enhancer region upon dopaminergic stimulation of D1-type receptors, thereby de-repressing PDE4D9 transcription in an activity-dependent manner.

PKA phosphorylation impairs the interaction of DISC1 and ATF4

ATF4 and DISC1 have both been shown to be phosphorylated by PKA, following phosphorylation by PKA, ATF4 switches from being a repressor to being an activator. Given the importance of PKA in regulating both ATF4 and DISC1 function, we examined whether PKA phosphorylation affects the DISC1–ATF4 interaction. To this end, full-length GFP-tagged DISC1 and GST-tagged ATF4 proteins were purified and subjected to an in vitro binding assay. We found that the interaction of DISC1 with ATF4 was substantially diminished in the presence of the catalytic domain of PKA compared with non-transfected controls, and that the addition of alkaline phosphatase increased the DISC1–ATF4 interaction (Figure 4b). Thus, PKA-mediated phosphorylation of DISC1, ATF4 or both disrupts their interaction.

We then sought to determine how the PKA-mediated phosphorylation of DISC1 impacts its function as a transcriptional repressor. When primary rat neurons were treated with forskolin, a potent PKA activator, the abundance of nuclear DISC1 decreased dramatically compared with the DMSO control (Figure 4c). Taken together, these results demonstrate that PKA activation leads to a change in affinity between DISC1 and ATF4, leading to changes in DISC1 localization and occupancy of DISC1 at the PDE4D enhancer region.

The phosphorylation of DISC1 S58 mediates ATF4–DISC1 interaction and DISC1 nuclear localization

Previous work has shown that serine-58 (S58) of DISC1 is phosphorylated by PKA and that this residue is proximal to a nuclear localization signal. Thus, we tested whether the PKA-mediated phosphorylation of this residue is responsible for the altered subcellular distribution of DISC1 following PKA activation. HeLa cells were transfected with vectors expressing wild-type or an S58A mutant of DISC1, and treated with okadaic acid, another PKA enhancer. We observed that PKA activation reduced the nuclear distribution of wild-type DISC1, and that this effect was significantly smaller for the S58A mutant, indicating that phosphorylation at this site affects the subcellular distribution of DISC1 (Figure 5a). Indeed, the basal localization of the S58A DISC1 mutant was primarily nuclear, whereas the phosphomimetic mutant, S58E, appeared mainly in the cytosol (Figure 5b). In addition, phosphorylation of S58 appears to regulate the interaction between DISC1 and ATF4, which increases in the presence of S58A, and decreases with S58E, compared with wild-type DISC1 (Figure 5c).

PDE4D9 is a cytosolic protein that colocalizes with, and binds to, DISC1 but not ATF4

Previous studies have demonstrated that DISC1 binds to, and affects, the activity of several PDE4 isoforms. These interactions are also regulated by PKA and raise the possibility that PDE4D9 itself may form a complex with DISC1 and ATF4. To address whether this is the case, we conducted immunoprecipitation studies between PDE4D9, DISC1 and ATF4. PDE4D9 interacts with DISC1, and this interaction decreases with dopamine treatment; conversely, we did not observe any interaction between PDE4D9 and ATF4 (Supplementary Figure 4). To address the subcellular
localization of PDE4D9 in relation, C-terminal-myc-tagged PDE4D9 was overexpressed in DIV 10 hippocampal neurons. PDE4D9 is cytosolic, whereas ATF4 is nuclear. DISC1 expression is seen in both the nucleus and the cytoplasm. No gross changes were seen in localization of PDE4D9 with dopamine treatment (Supplementary Figure 4).

DISCUSSION

Since its discovery more than a decade ago, extensive study has revealed multiple roles for DISC1 in neurodevelopment. Many of these roles are implicated in etiologies of neuropsychiatric disorders of neurodevelopmental origin, including neurogenesis, neuronal migration and integration.3,8,12,13,15,17,18 In contrast, the main finding of this study was the elucidation of a novel role for DISC1 in intracellular signaling involving dopaminergic neurotransmission. This newly described function of DISC1 may underlie important pathophysiologicals of various neuropsychiatric disorders. Given that molecules involved in these pathologies may represent attractive targets for drug discovery, our findings have significance in the translational aspects of clinical medicine. Furthermore, through this study, we also delineate a novel mechanism involving the regulation of PDE4 at two levels, that is, at the protein level (interaction with DISC1, shown previously22,25) and at the transcriptional level (mediated by DISC1–ATF4, this study).

DISC1 associates with the genomic locus and represses the transcription of an ATF4 transcriptional target

The ATF4 transcription factor contains a basic-leucine-zipper domain and can homodimerize or form heterodimers with many interaction partners including those from the AP-1 and C/EBP family. The ATF4 homo- or heterodimers have different affinities for various DNA binding regions. Previous studies have demonstrated that ATF4 binding of DISC1 is dependent on a leucine-zipper domain contained within DISC1.36 In this paper, we show that the interaction of DISC1 with the PDE4D9 locus is dependent on ATF4; this is consistent with the notion that DISC1 forms a heterodimer with ATF4 to bind to this genomic region, and furthermore that the ATF4/DISC1 heterodimer is repressive to transcription. The role of DISC1’s interactions with other transcriptionally relevant partners is not well-characterized, and further studies in this area would add to our understanding of DISC1 function in transcriptional regulation by providing clues as to the composition of the ATF4–DISC1 repressor complex, potentially identifying novel targets for therapeutic intervention.
Dual regulation of PDE4

PDE4s suppress and terminate cAMP signaling in various distinct subcellular compartments by converting cAMP to AMP. Our finding that cAMP signaling alters the interaction profile of ATF4 and DISC1, thereby increasing PDE4D9 expression, adds to the growing body of literature on the regulation of PDE4s by DISC1. Previous studies have shown that DISC1 regulates some, but not all, gene products of the four PDE4 genes via direct interaction and inhibition in a cAMP activity-dependent manner. Both mechanisms, that is, direct inhibition of activity and transcriptional repression of phosphodiesterases, are regulated by PKA, indicating that these two mechanisms are activated in a feedback mechanism to regulate cAMP signaling in different timeframes, and in specific subcellular regions. The dissociation of PDE4s from DISC1 in response to cAMP signaling reflects an acute feedback, in which increased cAMP signaling induces its own termination signal by releasing PDE4s from sequestration by DISC1. On the other hand, transcriptional induction reflects a longer-term, sustained feedback mechanism, in which increased cAMP signaling increases PDE4D9 levels, leaving the cell poised to shut down cAMP activity. This finding may have therapeutic implications. Further studies exploring the role of PDE4D9 in, for example, amphetamine-induced sleep disturbances, sensitization and withdrawal may be warranted. The recent discovery of differences in DISC1–ATF4 interaction by human DISC1 coding variants also indicates that there may be human coding variants of DISC1 that are likely to elicit differential responses to dopaminergic stimulation at the cellular level, in addition to having baseline differences in DISC1–ATF4 interaction, which may help to explain the observed differences in attention observed in DISC1 coding variants, as norepinephrine and dopamine are implicated in the coupling of attention-related networks.

The DISC1–ATF4 interaction is physiologically regulated by dopamine, via the D1 receptor, as well as noradrenergic signaling, via the b1 and b2 receptors, activate Gz, leading to the activation of adenyl cyclase and increased cAMP levels. High concentration of cAMP activates PKA, and the PDE4s terminate this signaling in different subcellular locations by converting cAMP to AMP. Numerous psychotherapeutic agents impact cAMP levels via secondary actions. The classical antidepressants, monoamine oxidase inhibitors, act by elevating dopamine, serotonin and norepinephrine, while cocaine and amphetamines, abused for their euphoric effects and noted for psychotomimetic actions, act through the excitation of dopaminergic signaling. ATF4-dependent transcription is mediated by several factors, including oxidative/nutrient stress and cAMP-induced PKA activity. Notably, the cocaine-amphetamine response transcript, which is a product of ATF4-mediated transcription, is upregulated in response to cocaine and anphetamine. Our study provides further clues into the pathophysiology of psychiatric phenotypes secondary to the dysregulation of this pathway. An increase in PDE4D9 levels secondary to the activation of cAMP is a form of transcriptional regulation.
PDE4D9. This baseline increase in a phosphodiesterase would be regulated by CREB. Thus, this study adds to the literature of psychiatric disorder pathophysiology. The perturbation of this pathway may disrupt the ability of neurons to respond appropriately to neurotransmitter signaling, ultimately affecting the neuronal circuitry and resulting in abnormal behavioral responses that may manifest as psychiatric disease.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)