Stimulation of Synaptic Vesicle Exocytosis by the Mental Disease Gene DISC1 is Mediated by N-Type Voltage-Gated Calcium Channels

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Lesions and mutations of the DISC1 (Disrupted-in-schizophrenia-1) gene have been linked to major depression, schizophrenia, bipolar disorder and autism, but the influence of DISC1 on synaptic transmission remains poorly understood. Using two independent genetic approaches—RNAi and a DISC1 KO mouse—we examined the impact of DISC1 on the synaptic vesicle (SV) cycle by population imaging of the synaptic tracer vGpH in hippocampal neurons. DISC1 loss-of-function resulted in a marked decrease in SV exocytic rates during neuronal stimulation and was associated with reduced Ca$^{2+}$ transients at nerve terminals. Impaired SV release was efficiently rescued by elevation of extracellular Ca$^{2+}$, hinting at a link between DISC1 and voltage-gated Ca$^{2+}$ channels. Accordingly, blockade of N-type Cav2.2 channels mimics and occludes the effect of DISC1 inactivation on SV exocytosis, and overexpression of DISC1 in a heterologous system increases Cav2.2 currents. Collectively, these results show that DISC1-dependent enhancement of SV exocytosis is mediated by Cav2.2 and point to aberrant glutamate release as a probable endophenotype of major psychiatric disorders.

Keywords: DISC1, hippocampus, glutamate, psychiatric disorders, schizophrenia, neurotransmitter, synaptic vesicle release

INTRODUCTION

Genome-wide association studies and exome sequencing efforts have led to the identification of hundreds of variants associated with psychiatric disorders (International Schizophrenia et al., 2009; Moskvina et al., 2009; Glessner et al., 2010; Fromer et al., 2014; Schizophrenia Working Group of the Psychiatric Genomics, 2014), confirming the complex polygenic nature of these diseases. These genomic data also revealed a significant overlap in genes or gene networks associated with distinct mental illnesses (Cross-Disorder Group of the Psychiatric Genomics et al., 2013), suggesting a common genetic and perhaps circuitry basis for major psychiatric disorders. However, the synaptic and circuitry defects underlying these disorders remain poorly defined, hindering the development of therapeutic solutions.
DISC1 is the prototypical example of a gene associated with several major psychiatric disorders. It was discovered in a Scottish family at the site of a balanced chromosomal translocation that strongly segregates with major depression, schizophrenia and bipolar disorder (St Clair et al., 1990; Millar et al., 2000, 2001). The high penetrance (~70%) of this translocation for mental illness supports a causal link between this genetic lesion and major psychiatric conditions (Chubb et al., 2008). DISC1 variants (haplotypes, single nucleotide polymorphisms and copy number variations) have since been independently associated with depression, schizophrenia, bipolar disorders and autism spectrum disorders (Ekelund et al., 2001, 2004; Sachs et al., 2005; Kilpinen et al., 2008). Thus, DISC1 is a major susceptibility factor for mental illness and a relevant genetic entry point to identify core endophenotypes implicated in neuropsychiatric disorders.

The translocation breakpoint in this Scottish family is located in the C-terminal portion of DISC1 and results in overall reduced expression of the full-length transcript and protein (Millar et al., 2005), suggesting that haploinsufficiency is the main mechanism by which this chromosomal alteration confers risk to disease. Alternatively, it has been proposed that a C-terminal truncated form of DISC1 is expressed from the translocated allele and may be pathogenic (Hikida et al., 2007; Pletnikov et al., 2008), although expression of the truncated DISC1 protein in translocation carriers remains to be demonstrated. Consistent with a disease mechanism based on DISC1 loss-of-function, DISC1 expression is also attenuated in human induced pluripotent stem (iPS) cells derived from members of a family with a DISC1 frame-shift mutation that co-segregates with major psychiatric disorders (Wen et al., 2014).

The identification of a large DISC1 interactome consisting of proteins belonging to different ontologic families suggests broad functions of DISC1 in nerve cells. Accordingly, DISC1 has been implicated in multiple aspects of neuronal and brain development, including neurogenesis (Clapcote et al., 2007; Shen et al., 2008; Mao et al., 2009; Singh et al., 2010; Lee et al., 2011), neuronal migration (Kamiya et al., 2005; Duan et al., 2007; Pletnikov et al., 2008; Mao et al., 2009; Singh et al., 2010; Lee et al., 2011). Even though DISC1 interacts with several signaling proteins known to regulate synaptic functions, relatively little is known about functions of DISC1 at the synapse. In particular, the impact of DISC1 on neurotransmitter release remains largely unexplored, despite the fact that aberrant dopamine and glutamate neurotransmission is a probable cause of schizophrenia and other mood disorders (Howes et al., 2015).

Given the preferential expression of DISC1 in the hippocampus and the involvement of this brain structure in cognition and psychiatric disorders (Chubb et al., 2008), we set out to determine the impact of DISC1 on synaptic vesicle (SV) cycling in hippocampal neurons. We used two independent genetic strategies to alter DISC1 expression—RNAi and a DISC1 KO mouse—and imaged SV cycling and Ca\(^{2+}\) dynamics in large synapse populations. We show that DISC1 elevates synaptic Ca\(^{2+}\) signals and boosts SV exocytosis at glutamatergic terminals. Our results further indicate that N-type voltage-gated Ca\(^{2+}\) channels (VGCCs) mediate the stimulatory effect of DISC1 on SV release. These findings identify a central role of DISC1 in neurotransmitter release and provide new insights on the biological basis of synaptic dysfunction in major psychiatric disorders.

**MATERIALS AND METHODS**

### DNA, shRNA Constructs, Lentiviruses and Antibodies

The pCAGGs vGlut1-pHluorin (vGpH) and pCAGGs Synaptophysin-GCaMP3 (SyGC3) constructs were gifts from R. Edwards (UCSF; Voglmaier et al., 2006) and S. Voglmaier (UCSF; Li et al., 2011). The pFUGW (Addgene #14883) shRNA-expressing lentiviral vector was modified to express mCherry (pFUmChW). The shRNA targeting sequences are as follows: (1) Scramble 5’GGAGGACAGCGTGAATTAC3’ (Kamiya et al., 2005); (2) DISC1-E 5’GGGTACATGGAAGACAG3’ (exon 2; Duan et al., 2007); and (3) DISC1-A 5’GGAAAGG GCTAGAGATGTTC3’ (exon 9) designed with Block-it shRNA from Invitrogen. pFUGW scramble shRNA was a gift from A. Sawa (Johns Hopkins). The DISC1 shRNAs constructs were cloned by introducing double-stranded DNA oligos into lentiviral vector pII3.7 (Addgene #11795) using the HpaI and XhoI sites. DNA fragments containing the U6 promoter and shRNAs were then PCR amplified and cloned into pFUmChW using the PacI site. The human DISC1 gene L variant (NCBI Refseq NM018662.2) was PCR amplified from pCMV6-XL5 DISC1-tGFP (Origene) and cloned into pIRES2-DsRed-Express (Clontech) using NheI and SmaI sites. All constructs were sequenced before use. Lentiviral particles expressing pFUmChW shRNAs were prepared as described in Tiscornia et al. (2006). The DNA constructs used for whole-cell patch clamp recording are as follows: Cav2.1 (generated in T. W. Soong’s lab), Cav2.2 (Addgene #26568), GFP-β2, and αδ1 (kindly provided by T. Snutch, UBC). The rabbit polyclonal Abs against Cav2.1 (#ACC-001) and Cav2.2 (#ACC-002) were from Alomone Labs. The polyclonal Ab against the C-terminus of mouse DISC1 was previously described (Kuroda et al., 2011). The polyclonal Ab against human DISC1 (ab59017) and monoclonal Ab against bassoon were from Abcam (ab82958).

### Mouse Lines, Primary Neuron Cultures and Transfection Protocols

DISC1 (Δ2–3) mice (C57BL/6JmsSlc) have been described before Kuroda et al. (2011). Heterozygous DISC1\(^{wt/Δ2–3}\) mice were crossed to each other to obtain DISC1\(^{wt/wt}\) and DISC1\(^{Δ2–3/Δ2–3}\) lines from which hippocampal neurons were prepared from E17/E18 embryos, according to published procedures using papain (Worthington) digestion (Huettner and Baughman, 1986). Primary rat hippocampal neurons were prepared from E18 embryos according to Kaech and
channels and N-type Ca\textsuperscript{2+} sucrose in PBS and permeabilized with 100 ng/ml Digitonin coverslips were fixed in 4% paraformaldehyde with 4% For immunofluorescence studies, neurons grown on glass Time-lapse confocal imaging was performed on an inverted Eclipse TE2000-E microscope (Nikon, USA), mounted with a spinning-disk confocal scan head (CSU-10; Yokogawa, Singapore). Institutional Animal Care and Use Committee (IACUC) of All animal procedures were approved by the SingHealth Institutional Animal Care and Use Committee (IACUC) of Singapore.

Live-Cell Confocal Imaging and Immunofluorescence

Time-lapse confocal imaging was performed on an inverted Eclipse TE2000-E microscope (Nikon, USA), mounted with a spinning-disk confocal scan head (CSU-10; Yokogawa, Japan), and equipped with a temperature controlled (36.5°C) stage and an autofocusing system (PFS; Nikon). Images were acquired with an Orca-Flash 4.0 CCD camera (Hamamatsu Photonics, Japan) controlled by MetaMorph 7.8.6 (Molecular Devices, CA, USA) at 0.5 Hz or 20 Hz. Samples were imaged using a 60x (NA 1.4) objective in Tyrode’s buffer (150 mM NaCl, 2.5 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 6 mM glucose, 25 mM HEPES, pH 7.4) supplemented with 25 µM 6-cyano-7-nitroquinoxaline-2,3-dione/CNQX (Tocris Bioscience) and 50 µM D,L-2-amino-5-phosphonovaleric acid/AP5 (Tocris Bioscience). Coverslips were mounted in an RC-21BRFS chamber (Warner Instruments, USA) equipped with platinum wire electrodes. Field stimulation was induced by a square pulse stimulator (Grass Technologies, USA) and monitored by an oscilloscope (TDS210, Tektronix, USA). Trains of action potentials (APs) were generated by applying 20 V pulses (1 ms duration) at 10 or 20 Hz. Our typical stimulation paradigm for vGpH measurements involved two consecutive trains of 300 APs at 10 Hz, separated by ~5 min to allow synapses to recover. vGpH responses between the first and second stimulation were highly reproducible (data not shown). For measuring SV exocytic rates, the second stimulation was preceded (30 s earlier) by the addition of Bafilomycin A1 (Baf; AG scientific, USA) 0.5 µM. To normalize for total expression of vGpH (Figure 1B), we added on DIV2, at a MOI (multiplicity of infection) of 1–3. All animal procedures were approved by the SingHealth Institutional Animal Care and Use Committee (IACUC) of Singapore.

Immunochemical procedures

Cultured primary neurons or HEK293T cells were washed with ice-cold PBS and lysed with RIPA buffer (10 mM Tris-HCl pH = 7.2, 150 mM NaCl, 1% Triton-X-100, 0.1% SDS, 5 mM EDTA, 0.25% Na-deoxycholate) supplemented with Complete protease inhibitor and PhosphoStop phosphatase inhibitor (Roche). For analysis of hippocampal tissue, the
hippocampi from P10 mice were harvested and homogenized in RIPA buffer using a Dounce tissue homogenizer. Lysates were cleared by centrifugation and boiled in Laemmli sample buffer. Equal amount of total proteins were loaded. Samples were then analyzed by SDS-PAGE, transferred onto nitrocellulose membranes, probed with appropriate primary and HRP-labeled secondary antibodies and revealed by enhanced chemiluminescence.

Whole-Cell Patch Clamp Recordings of Cav2.1 and Cav2.2 Currents
Cav2.1 or Cav2.2, the auxiliary subunits (GFP-β2a and α2δ1) and DISC1 were transiently transfected in HEK 293 cells using the calcium phosphate method (Huang et al., 2012). Whole-cell patch-clamp recordings were performed within 36–72 h after transfection. The external solution contained (in mM) 10 HEPES, 140 TEA-MeSO3 and 5 BaCl2 (pH 7.4, 300–310 mOsm). The glass pipette solution was backfilled with pipette solution (in mM) 10 HEPES, 5 CsCl, 138 Cs-MeSO3, 0.5 EGTA, 1 MgCl2, 2 mg/ml MgATP (pH 7.3, 290–300 mOsm). HEK 293 cells were held at −90 mV using the Axopatch 700B amplifier (Axon Instruments). The series resistance for all recordings was less than 5 MΩ; 70–80% compensation on serial resistance and cell membrane capacitance were applied. A P/4 protocol was used to subtract leakage current. All recordings were obtained with an Axon Digidata 1440A data acquisition system, sampled at 5–50 kHz and low pass-filtered at 1 kHz or 6 kHz. The I-V curves were obtained from 10 mV voltage-steps ranging from −50 to 40 mV and fitted with a modified Boltzmann equation:

$$I = G_{\text{max}} (E_{\text{rev}} - V) \left( 1 + e^{\frac{V_{1/2\text{act}} - V}{k_{\text{act}}}} \right)$$

where, $I =$ current density (in pA/pF), $G_{\text{max}} =$ maximum conductance (in nS/pF), $E_{\text{rev}} =$ reversal potential, $V =$ measured potential, $V_{1/2\text{act}} =$ midpoint voltage for current activation, and $k_{\text{act}} =$ the slope factor.

We used a tail protocol to measure current density; cells were depolarized using 10 mV voltage steps, from −60 to 60 mV. Following depolarization, tail currents were evoked with a 10 ms pulse at −50 mV. The data were fitted with single Boltzmann equation:

$$I = I_{\text{max}} + (I_{\text{max}} - I_{\text{min}}) \left( 1 + e^{\frac{V_{1/2\text{inact}} - V}{k_{\text{inact}}}} \right)$$

where, $I_{\text{max}}$ and $I_{\text{min}} =$ maximal and minimal current respectively, $V_{1/2\text{inact}} =$ the half-maximal voltage for current inactivation, $k_{\text{inact}} =$ slope of inactivation curve.

Statistics
The experimental design of this study implies that data collected at individual synaptic terminals are clustered according to neuron preparations and imaged fields. Galbraith et al. (2010) demonstrated that such clustering can adversely affect statistical inference when not accounted for. In order to
probe for clustering effects (i.e., intra-field correlations), we tested whether bouton responses significantly vary from field to field. For this, we conducted Wald tests of the null hypothesis of no difference in mean outcome across fields within each condition, which revealed ($p$-value < 0.0001) strong intra-field correlation. To account for these correlations, we performed our statistical analysis in the framework of linear mixed models (Laird and Ware, 1982) with normally-distributed random field effects and preparation fixed effects. In experiments involving one genetically-modified condition and one control group, we tested the null hypothesis of no difference between the mean outcome of the groups via 2-sample $t$-tests. In experiments involving two genetically-modified conditions and one control group, we tested the null hypothesis of no difference between the mean outcome of each group and the control group jointly using Wald tests. All tests were performed at the 5% level of statistical significance and carried out using the statistical software Stata version 13.2. Because our statistical approach (linear mixed model) is not a standard practice when analyzing synaptic properties, we compared the $p$-values obtained with our method with those measured by the more common field averaging approach, where the information of a field is collapsed to a single independent observation by taking the mean of bouton responses. Both methods are valid and gave comparable results (Supplementary Table T1), although the field averaging approach is less statistically efficient as it diminishes the information that can be obtained from the data by reducing individual measurements in a field to one observation (Galbraith et al., 2010).

**RESULTS**

**DISC1 Loss-of-Function Slows Down SV Cycling**

We opted for an imaging approach based on the synaptic tracer vGpH to explore the impact of DISC1 on the SV cycle. vGpH consists of the pH-sensitive GFP variant pHluorin (Miesenbock et al., 1998) fused to the SV-resident glutamate transporter vGlut1. pHluorin, which faces the acidic lumen of SVs, undergoes a ∼20-fold increase in fluorescence intensity when exposed to the neutral pH of the extracellular milieu after membrane fusion. (Sankaranarayanan et al., 2000). Following glutamate discharge and vGpH re-uptake, SVs are rapidly re-acidified and vGpH fluorescence is quenched. This property has made vGpH a valuable tool to monitor both exo- and endocytosis of SVs at single synapses.

Due to the inherent variability in the properties of individual synaptic boutons (Ariel et al., 2013) we measured SV cycling in large synapse populations. For this, we developed an image analysis algorithm that identifies all responding boutons in a given field (Figures 1A,B), and imaged 16–18 fields from at six independent rat hippocampal neuron cultures, yielding close to a thousand synaptic boutons for each condition. We employed this algorithm to monitor SV cycling in response to a stimulation paradigm that consists of two consecutive trains of APs (Fernandez-Alfonso and Ryan, 2004; Burrone et al., 2006). The amplitude of the first vGpH transient is governed by the relative rates of SV exo- and endocytosis during stimulation (Figure 1C). To separate the contributions of exo- and endocytosis, we blocked SV re-acidification with the vacuolar H$^+$ ATPase inhibitor Bafilomycin (Baf) during the second stimulation, which allows selective measurement of SV exocytosis (Figure 1C). To account for cell-to-cell variation in vGpH expression, we normalized each trace based on the vGpH signal measured after NH$_4$Cl addition (Figure 1C). The endocytic component of SV cycling was then computed by subtracting the first vGpH transient from the vGpH response after Baf treatment (Figure 1D). Synaptic traces with baseline drifts between the first and second stimulation or after Baf addition were excluded from the analysis (see “Materials and Methods” Section). Exocytosis largely dominates at the onset of stimulation, while endocytosis kicks in towards the end of the AP train. Exo- and endocytic rates were finally obtained by linear fitting of the exo- and endo traces (Figure 1D). Exocytic rates are about three times higher than endocytic rates under this stimulation protocol. To evaluate the variability of bouton responses across fields and neuron preparations, we compiled exocytic rates from six independent experiments (Supplementary Figure S1). Statistical analysis of these responses revealed substantial field to field variation (see “Materials and Methods” Section), implying that the information collected from each bouton does not constitute an independent measurement. To compare synaptic responses in different fields and conditions we used a statistical approach that accounts for correlations within fields and variations in neuron preparations (see “Materials and Methods” Section and Supplementary Table T1).

We then silenced the DISC1 gene using a published shRNA sequence (shRNA-E), targeting exon 2 (Duan et al., 2007) and a new shRNA sequence (shRNA-A) targeting exon 9. These two shRNAs target regions of the DISC1 gene that are fully conserved in rodents and are thus expected to silence rat and mouse DISC1 with the same efficiency. To probe the effect of these shRNAs on DISC1 protein levels, we used an antibody raised against the C-terminus of mouse DISC1, which has previously been validated in a DISC1 KO mouse (Kuroda et al., 2011). In our hands, this antibody poorly reacts with rat DISC1 (not shown). In mouse hippocampal neurons, however, it detects one major protein at ∼100 kDa corresponding to the predicted full length DISC1 (Kuroda et al., 2011) and which is substantially reduced in both shRNA-E and -A transduced cells (Figure 2A).

Next, we probed the impact of these shRNAs on the SV cycle in rat hippocampal neurons. We initially chose to work with rat, rather than mouse neurons, because we can harvest substantially more cells from a rat embryonic litter. vGpH signals in shRNA-E and -A expressing neurons display a slower rise and lower amplitude during the first and second stimulation, relative to control cells expressing a scramble shRNA (Figure 2B). Analysis of exocytosis shows...
substantial synapse-to-synapse variation in all conditions, but reveals a marked decrease in exocytic rates (Figures 2C,D) and amplitudes (Figure 2C) in both shRNA-E and -A expressing neurons. DISC1 silencing also resulted in slightly slower SV endocytosis, although this effect did not reach statistical significance (Figures 2E,F).

Reduced amplitude of the exocytic response prompted us to test whether DISC1 also regulates the size of the total releasable pool. For this, we applied a stimulus strong enough (1200 APs at 10 Hz) to maximally deplete releasable SVs from presynaptic terminals (Ariel and Ryan, 2010). Under this stimulation regime, vGpH responses reached the same plateau in DISC1-silenced and control neurons, albeit with different kinetics, arguing against an effect on the total releasable pool but confirming the stimulatory function of DISC1 in SV exocytosis (Figure 2G).

To rule out the possibility of RNAi off-target effects—shRNA-E has recently been suggested to inhibit neuron migration in the developing cortex independently of DISC1 (Tsuboi et al., 2015)—we examined SV cycling in a DISC1 KO mouse that lacks exons 2 and 3 of the DISC1 gene (Kuroda et al., 2011). Homozygous DISC1Δ2–3/Δ2–3 mice show no detectable levels of the major isoform of DISC1 (Figure 3A). Hippocampal neurons derived from DISC1Δ2–3/Δ2–3 mice display SV cycling defects that are remarkably similar to those observed with RNAi. The rates and amplitudes of exocytic responses were substantially reduced relative to wt cells (Figures 3B–D), while endocytic rates were marginally, but not significantly diminished (Figures 3E,F). Together, these results show that both genetic ablation and RNAi knockdown of DISC1 selectively disrupts SV exocytosis at glutamatergic synapses with no detectable impact on the total releasable pool.

**DISC1 Stimulates Ca\(^{2+}\) Influx and Regulates Cav2.2-Dependent SV Release**

Because SV exocytosis is initiated by Ca\(^{2+}\) influx through VGCCs, we measured evoked Ca\(^{2+}\) transients at presynaptic terminals using the SV-targeted Ca\(^{2+}\) sensor SyGC3 (Li et al., 2011). Ca\(^{2+}\) signals evoked by 300 APs (10 Hz) or 200 APs (20 Hz) show a rapid rise and partial decay during the stimulus (Figures 4A,B,D,E), as observed by others (Li et al., 2011). These Ca\(^{2+}\) transients were significantly reduced in shRNA-E and -A expressing neurons, under the same stimulation conditions used for vGpH measurements (Figures 4A,C), or in response to a higher frequency stimulus (Figure 4B). Similarly,
DISC1Δ2–3/Δ2–3 neurons show lowered Ca²⁺ transients compared to wt cells (Figures 4D–F). We were concerned that, under such prolonged stimulations, Ca²⁺ concentration in terminals might reach levels that partially saturate SyGC3. Ca²⁺ signals were therefore also examined in response to shorter stimulations (20 APs, 20 Hz) under conditions well below SyGC3 saturation (Tian et al., 2009; Akerboom et al., 2012). A clear decrease in the amplitude of Ca²⁺ transients was also observed in DISC1-silenced neurons under this stimulation regime (Figure 4G). Together, these Ca²⁺ imaging data hint at a Ca²⁺ influx defect in DISC1-inactivated cells, although abnormal clearance of Ca²⁺ from the presynaptic terminal could also be involved (see “Discussion” Section).

To determine whether reduced Ca²⁺ influx is the primary cause of SV cycling defects, we attempted to rescue SV exocytosis in DISC1-silenced neurons by elevating extracellular Ca²⁺ concentration. Shifting extracellular Ca²⁺ concentration from 2 to 4 mM increases AP-induced Ca²⁺ entry (Ariel and Ryan, 2010) and restored the vGpH response (Figure 4H), in line with a role of DISC1 in facilitating Ca²⁺ influx.

At central synapses, the P/Q-type (Cav2.1) and N-type (Cav2.2) Ca²⁺ channels are the main sources of Ca²⁺ for initiation of SV exocytosis (Catterall et al., 2013). We used channel-specific toxins to determine the contribution of each subtype to SV release in hippocampal neurons. ω-Conotoxin GVIA (an N-type blocker) reduced SV exocytic rates by 73 ± 5% (Figures 5A,B), while ω-Agatoxin TK (a P/Q-type blocker) resulted in a 42 ± 3% decrease in SV exocytic rates (Figures 5C,D), in a good agreement with a recent study (Ariel et al., 2013). Note that in these experiments, exocytic rates were approximated by measuring the initial slope of the vGpH response in the absence of Baf (see Figure 1D). Importantly, blockade of Cav2.2 almost completely occluded the effect of DISC1 silencing on SV exocytosis (Figures 5A,B,E). Blockade of Cav2.1, on the other hand, slightly increased the inhibitory impact of DISC1 knockdown on SV release rates (Figures 5C–E). We conclude from these results that DISC1 selectively stimulates Cav2.2-dependent SV release at hippocampal synapses.

We then asked whether DISC1 regulates Cav2.2 activity by controlling its presynaptic localization and/or abundance. Co-localization studies of endogenous Cav2.2 with the active zone marker bassoon revealed extensive presence of Cav2.2 in synaptic boutons, both in control and DISC1-silenced neurons (Supplementary Figures S2A–C). We found no significant difference between these two groups in the fraction of boutons
FIGURE 4 | DISC1 loss-of-function reduces evoked Ca^{2+} transients at nerve terminals. SyGC3 imaging in rat hippocampal neurons (DIV14–16) in response to two different trains of APs. (A) Average Ca^{2+} transients in neurons expressing scr (1052 boutons, 15 fields, 5 experiments (exps)), DISC1-E (1836 boutons, 9 fields, 3 exps) and -A (754 boutons, 11 fields, 3 exps) shRNAs, in response to 300 APs, 10 Hz. The DISC1-E and DISC-A groups are significantly different than the scr group (p = 0.0057). (B) Average Ca^{2+} transients in neurons expressing scr (861 boutons, 9 fields, 5 exps), DISC1-E (2118 boutons, 7 fields, 3 exps) and -A (1549 boutons, 9 fields, 3 exps) shRNAs, in response to 200 APs, 20 Hz. (C) Cumulative probability of SyGC3 peak intensity from individual boutons corresponding to (A). (D) Average Ca^{2+} transients in DISC1 wt/wt (1090 boutons, 11 fields, 3 exps) and DISC1 Δ2–3/Δ2–3 (727 boutons, 9 fields, 3 exps) neurons, in response to 300 APs, 10 Hz. The DISC1 wt/wt and DISC1 Δ2–3/Δ2–3 groups are statistically different (p = 0.0182). (E) Average Ca^{2+} transients in DISC1 wt/wt (1365 boutons, 9 fields, 3 exps) and DISC1 Δ2–3/Δ2–3 (1233 boutons, 8 fields, 3 exps) neurons, in response to 200 APs, 20 Hz. (F) Cumulative probability of SyGC3 peak intensity from individual boutons corresponding to (D). (G) Average Ca^{2+} transients in neurons expressing scr (290 boutons, 6 fields, 2 exps), and DISC1-E (990 boutons, 6 fields, 2 exps) shRNAs, in response to 20 APs, 20 Hz. The scr and DISC1-E groups are statistically different (p = 0.0038). (H) Average vGpH traces in scr (180 boutons, 5 fields, 2 exps) and DISC1-E (488 boutons, 6 fields, 2 exps) shRNA-expressing neurons during two consecutive trains of APs (300 AP, 10 Hz) in the presence of 2 or 4 mM extracellular Ca^{2+}. We finally examined the effect of DISC1 on Cav2 currents by whole cell patch-clamp recordings. Co-transfection of recombinant DISC1 (Figure 6A) in HEK293T cells together with Cav2.2 and its auxiliary subunits (see “Materials and Methods” Section) resulted in a 27% increase in Cav2.2 peak current density (Figures 6B,C). Likewise, tail current density of Cav2.2 was substantially elevated in DISC1-overexpressing cells (Figures 6D,E). Current density can be

containing Cav2.2, or in the intensity of Cav2.2 staining in presynaptic terminals (Supplementary Figures S2B,C). Nor did we find evidence for altered presynaptic localization and intensity of Cav2.1 (Supplementary Figures S2D–F) or reduced density of presynaptic terminals (Supplementary Figure S2G) in DISC1 knockdown neurons. Thus, synaptic targeting and abundance of Cav2 channels does not seem to be regulated by DISC1.
FIGURE 5 | DISC1 regulates Cav2.2-dependent SV exocytosis. (A) Average vGlutH traces in scr (101 boutons, 6 fields, 2 exps) and DISC1-E (87 boutons, 5 fields, 2 exps) shRNA-expressing neurons during consecutive trains of APs (300 AP, 10 Hz) in the absence or presence of the Cav2.2 blocker ω-Conotoxin GVIA (125 nM). (B) Boxplot of SV exocytic rates before and after ω-Conotoxin GVIA application. Exocytic rates were measured by linear fitting of the first six time points of the vGlutH response. (C) Average vGlutH traces in scr (1294 boutons, 9 fields, 3 exps) and DISC1-E (1282 boutons, 9 fields, 3 exps) shRNA-expressing neurons during consecutive trains of APs (300 AP, 10 Hz) in the absence or presence of the Cav2.1 blocker ω-Agatoxin TK (125 nM). (D) Boxplot of SV exocytic rates before and after ω-Agatoxin TK application. (E) Table showing the percentage of inhibition of exocytosis rate by DISC1 knockdown before and after Cav2.2- or Cav2.1 blockade.

**DISCUSSION**

We used here two independent gene-targeting approaches together with large-scale imaging of presynaptic function to determine the impact of the mental disease gene DISC1 on the glutamate release machinery. Our results show that DISC1 accelerates SV exocytosis and thus enhances presynaptic performance. This boosting effect is mediated by N-type Ca^{2+} channels, establishing the first link between DISC1 and VGCC activity.
FIGURE 6 | DISC1 enhances Cav2.2 and Cav2.1 currents. (A) Western blot showing expression of ectopic (human) DISC1 in HEK293 cells. (B,C) Cav2.2 Current-voltage (I-V) curves for hDISC1-expressing and control cells. (B) Stimulation protocol and individual current responses shown at three different voltages (−30, 0 and 30 mV). (C) Average I-V plots for hDISC1 (peak = 54.2 ± 4.5 pA/pF, 13 cells) and control (peak = 39.2 ± 4.9 pA/pF, 12 cells), p = 0.033. (D-F) Cav2.2 activation curves in response to the tail protocol. (D) Illustration of the tail protocol and individual tail currents measured at −50 mV after three different voltage steps (−20, 0 and 40 mV). (E) Average current density based on tail currents for DISC1 (164.3 ± 7.3 pA/pF, 12 cells) and control (110.5 ± 6.3 pA/pF, 12 cells), *p < 0.001. (F) Normalized activation curve from tail currents showing no significant difference between DISC1 (V_{50}: −6.98 ± 3.43 mV, 12 cells) and control (V_{50}: 0.86 ± 3.62 mV, 12 cells), p = 0.13. (G,H) Cav2.1 Current-voltage (I-V) curves for hDISC1-expressing and control cells. (G) Stimulation protocol and individual current responses shown at three different voltages (−30, 0 and 30 mV). (H) Average I-V plots for hDISC1 (peak = 79.9 ± 8.3 pA/pF, 15 cells) and control (peak = 56.3 ± 7.4 pA/pF, 13 cells), p = 0.046. (I-K) Cav2.1 activation curves in response to the tail protocol. (I) Illustration of the tail protocol and individual tail currents measured at −50 mV after three different voltage steps (−20, 0 and 40 mV). (J) Average current density based on tail currents for DISC1 (156.3 ± 5.7 pA/pF, 25 cells) and control (123.2 ± 7.4 pA/pF, 17 cells), *p = 0.003. (K) Normalized activation curve from tail currents showing no significant difference between DISC1 (V_{50}: −5.39 ± 0.49 mV, 24 cells) and control (V_{50}: −4.41 ± 0.93 mV, 17 cells), p = 0.31.
DISC1 inactivation in hippocampal neurons results in decreased Ca\(^{2+}\) transients at hippocampal terminals in response to AP firing, consistent with a deficit in Ca\(^{2+}\) entry. Because these Ca\(^{2+}\) signals are shaped by both Ca\(^{2+}\) entry and clearance processes, we cannot exclude a role of DISC1 in regulating Ca\(^{2+}\) extrusion or buffering. A stimulatory effect of DISC1 on Ca\(^{2+}\) entry is further supported, however, by its positive impact on Cav2.2 and Cav2.1 currents, the two main VGCCs underlying the initiation of neurotransmission at hippocampal synapses. How DISC1 enhances Cav2 currents is unclear. The lack of an effect on gating suggests that DISC1 promotes surface expression of these Ca\(^{2+}\) channels, although we cannot exclude a role of DISC1 in regulating single-channel conductance or open probability. Our immuno-localization studies revealed no detectable impact of DISC1 on presynaptic abundance of Cav2 channels. This does not rule out, however, the possibility that DISC1 regulates the number of functional Ca\(^{2+}\) channels at the surface of the terminal.

Although overexpression of DISC1 enhances both Cav2.1 and Cav2.2 currents in a heterologous system, our findings point to a selective influence of DISC1 on Cav2.2-dependent SV exocytosis. How is this selectivity achieved? It is possible that both Cav2.1 and Cav2.2 compete for DISC1 regulation at presynaptic terminals. The dominant contribution of Cav2.2 to SV release probably results (at least in part) from increased levels of Cav2.2 (relative to Cav2.1) at hippocampal terminals (Ariel et al., 2013). Thus, preferential regulation of Cav2.2 by endogenous levels of DISC1 combined with greater abundance of Cav2.2 may explain why DISC1-dependent SV exocytosis appears to be exclusively mediated by Cav2.2. Specificity could also arise, however, from functions of DISC1 downstream of Ca\(^{2+}\) entry. For example, DISC1 could be involved in positioning Cav2.2 in close proximity to the SV release machinery at the active zone, a process that dictates speed and fidelity of neurotransmission. While the role of Ca\(^{2+}\) entry in SV exocytosis is undisputed, its impact on SV endocytosis remains somewhat controversial. Recent evidence suggests, however, that Ca\(^{2+}\) couples rates of SV exo- and endocytosis and optimizes endocytic rates during AP bursts (Armbruster et al., 2013). We observed reduced endocytic rates in both DISC1-silenced and DISC1\(^{Δ2–3/Δ2–3}\) neurons. Although this effect was small and did not reach statistical significance, our results suggest that the influence of DISC1 on Cav2.2 impacts both exo- and endocytosis of SVs at hippocampal terminals.

Our findings extend on two recent studies implicating DISC1 in presynaptic function. In glutamatergic neurons differentiated from human iPS cells and derived from members of a family with a frameshift mutation in DISC1, deficits in SV release were observed after KCl-induced depolarization (Wen et al., 2014). In a separate report, RNAi knockdown of DISC1 in layer 2/3 neocortical neurons increases paired-pulse facilitation and appears to reduce probability of glutamate release (Maher and Loturco, 2012). Collectively, these results clearly identify DISC1 as a positive modulator of glutamate release.

The efficacy of neurotransmitter release determines not only the strength of synaptic excitation, but also dictates various forms of short-term plasticity (Abbott and Regehr, 2004), suggesting broad functions of DISC1 in synaptic computation and neural circuit performance. Although we have not directly measured release probability (P\(_r\)), the impact of DISC1 on Ca\(^{2+}\) entry and SV cycling suggests a positive influence on P\(_r\) (see Maher and Loturco, 2012). Because synaptic facilitation and depression depend largely on the initial P\(_r\) (high P\(_r\) favors depression while low P\(_r\) favors facilitation), our results predict altered short-term plasticity in DISC1-deficient neural circuits. Of interest, abnormal short-term plasticity has been associated with deficits in moment-to-moment information processing and working memory, two hallmarks of schizophrenia (Crabtree and Gogos, 2014). Reduced efficacy in glutamate release may also be relevant for forms of long-term potentiation (LTP) that have clear presynaptic components, such as LTP at the perforant path-granule cell synapse in the dentate gyrus (Errington et al., 1987). Notably, this form of LTP is impaired in DISC1\(^{Δ2–3/Δ2–3}\) mice—a stronger tetanic stimulus is required for the expression of LTP (Kuroda et al., 2011). Our findings suggest that this LTP deficit could be due to a failure of the presynaptic terminal to undergo activity-dependent increase in release probability.

Several genes encoding VGCC subunits, including CACNA1C, CACNB2 and CACNAII have repeatedly been associated with schizophrenia and other psychiatric disorders (Ferreira et al., 2008; Cross-Disorder Group of the Psychiatric Genomics, 2013; Hamshere et al., 2013; Ripke et al., 2013; Schizophrenia Working Group of the Psychiatric Genomics, 2014). Although CACNA1A (Cav2.1) and CACNA1B (Cav2.2) are not typically associated with risk loci, RIM1 (also called RIMS1)—a presynaptic scaffold that regulates density of P/Q- and N-type Ca\(^{2+}\) channels and SV docking at the active zone (Han et al., 2011)—was recently identified as a candidate gene for schizophrenia in the largest GWAS study conducted to date (Schizophrenia Working Group of the Psychiatric Genomics, 2014). Together these findings point to neurotransmitter release as a central process targeted in schizophrenia.

In conclusion, our results shed light on a novel mechanism by which a major susceptibility gene for mental illness enhances the efficacy of glutamate release, and provide further support for a central role of glutamate neurotransmission in schizophrenia and other major mood disorders.

**AUTHOR CONTRIBUTIONS**

WT and JVT performed and analyzed all imaging and biochemical experiments. QL performed and analyzed whole-cell patch-clamp recordings. MB carried out the statistical analysis. KBL made the original observation implicating DISC1 in SV cycling. KKu and KK made the DISC1 Δ2–3 mouse and the DISC1 C-terminus antibody. WT and MF wrote Matlab scripts. TWS and MF supervised the project. MF wrote the article.
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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnsyn.2016.00015/abstract

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