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Disrupted-in-Schizophrenia 1 regulates the processing of reelin in the perinatal cortex

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Abstract

Disrupted in Schizophrenia 1 (*DISC1*) is a prominent gene in mental illness research, encoding a scaffold protein known to be of importance in the developing cerebral cortex. Reelin is a critical extracellular protein for development and lamination of the prenatal cortex and which has also been independently implicated in mental illness. Regulation of reelin activity occurs through processing by the metalloproteinases ADAMTS-4 and ADAMTS-5. Through cross-breeding of heterozygous transgenic *DISC1* mice with heterozygous *reeler* mice, which have reduced reelin, pups heterozygous for both phenotypes were generated. From these, we determine that transgenic DISC1 leads to a reduction in the processing of reelin, with implications for its downstream signalling element Dab1. An effect of DISC1 on reelin processing was confirmed *in vitro*, and revealed that intracellular DISC1 affects ADAMTS-4 protein, which in turn is exported and affects processing of extracellular reelin. In transgenic rat cortical cultures, an effect of DISC1 on reelin processing could also be seen specifically in early, immature neurons, but was lost on calretinin and reelin-positive mature neurons, suggesting cell-type specificity. DISC1 therefore acts upstream of reelin in the perinatal cerebral cortex in a cell type/time specific manner, leading to regulation of its activity through altered proteolytic cleavage. Thus a functional link is demonstrated between two proteins, each of independent importance for both cortical development and associated cognitive functions leading to behavioural maladaptation and mental illness.

Keywords

ADAMTS-4, Cerebral cortex, DISC1, Reelin, Transgenic mouse, Transgenic rat

1. Introduction

Disrupted in Schizophrenia 1 (DISC1) is a long standing candidate gene for major mental illness, and in particular schizophrenia, bipolar disorder and major depression. The *DISC1* gene was initially identified in a large family in which it was disrupted by a chromosomal translocation linked to these three conditions (Millar et al., 2000, Thomson et al., 2016). It was reinforced as a gene pertaining to schizophrenia and other conditions through genetic association studies in specific populations (reviewed in Chubb et al., 2008, Bradshaw and Porteous, 2012, Thomson et al., 2013) and via a separate mutation in another family (Sachs et al., 2005), although no single SNP or haplotype has been associated with one of these conditions globally. Functionally, DISC1 is an intracellular scaffold protein established to play a role in cortical development, impacting on both migration of cortical neurons (Kamiya et al., 2005) and cortical progenitor proliferation (Mao et al., 2009). Protein pathology of the non-mutant DISC1 protein has also been associated with mental illness (Leliveld et al., 2008, Ottis et al., 2011) and behavioural disorders (Trossbach et al., 2016).

Reelin meanwhile is a large extracellular protein, originally identified as being deleted in the *reeler* mouse mutant (D'Arcangelo et al., 1995). Reelin is highly expressed during forebrain development by Cajal-Retzius cells (Ogawa et al., 1995), a specialized form of early-born neuron, as well as by some other cell types including postnatal GABAergic interneurons (Pesold et al., 1998). Functionally, reelin is vital for layer formation in the developing cortex, as a result of its ability to modulate the polarity, process orientation and migration of cortical neurons via assorted signalling pathways (reviewed in Förster, 2014, Sekine et al., 2014). Shortly after its identification, reduced levels of reelin were noted in schizophrenia patients (Impagnatiello et al., 1998), with specific species of the reelin protein later shown to be deregulated in this condition, bipolar disorder and depression (Fatemi et al., 2001). Similar results have also been reported since by others, while a considerable number of genetic association studies also associated it with schizophrenia and autism spectrum disorders (reviewed in Ishii et al., 2016). Negative genetic association findings to mental illness have

also been reported, although one positive finding was at the genome-wide level (Shifman et al., 2008). Additionally, mice heterozygous for the *reeler* mutation display phenotypes reminiscent of aspects of schizophrenia (Tueting et al., 1999, Rogers et al., 2013, Schmitt et al., 2013).

Given the roles of these two proteins in both cortical development and the pathogenesis of mental illness, we sought to discern whether they operated in a common pathway. Such links between DISC1 and reelin have been hypothesised previously (Deutsch et al., 2010, Bader et al., 2012), and the two proteins share several common pathway elements, which are known to include LIS1, GSK3β, Akt and APP (Assadi et al., 2003, Ohkubo et al., 2003, Brandon et al., 2004, Hashimoto et al., 2006, Jossin and Goffinet, 2007, Hoe et al., 2009, Mao et al., 2009, Young-Pearse et al., 2010). Our results, however show, for the first time a direct functional link between DISC1 and reelin, with DISC1 found to alter the post-translational regulation of reelin by proteolytic cleavage.

2. Materials and methods

2.1 Animals

All animal work was approved by the LANUV (State Agency for Nature, Environment and Consumer Protection), North Rhine-Westphalia, Germany. A transgenic rat line expressing human DISC1 under the Syrian hamster *PrP* promoter, on a Sprague-Dawley background, has been described in detail previously (Trossbach et al., 2016). The same cosmid system and technique was used to introduce human DISC1 under the same promoter into a C57BL/6N mouse. The presence of the transgene in a stable founder line was confirmed at both the transcript and protein level within the brain (Fig. 1A-B). Heterozygotes were generated by crossing the transgenic mouse with wild type C57BL6/N mice. As the mouse experiments were performed using heterozygous *DISC1* mice crossed with heterozygous *reeler* mice, the ensuing litter included wild type pups which were used as controls for their transgenic littermates. Pups with both the *DISC1* and *reeler* alleles were not allowed to grow past one week of age. For the generation of brain homogenates, animals were sacrificed by decapitation and the whole brain rapidly extracted and flash-frozen in liquid nitrogen. Tail samples were taken for genotyping. Frozen brains were homogenized to a final concentration of 7.5% (w/v) in 50 mM HEPES pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 100 mM KAc, 1% Triton X-100, containing protease and phosphatase inhibitor cocktails and DNaseI. Homogenates were incubated with gentle spinning at 4°C for 2 hours and then mixed with 2x SDS loading buffer.

2.2. Antibodies

The 20E12 monoclonal antibody was raised against a recombinant fragment of mouse reelin (amino acids 24-500) using previously described protocols (Korth et al., 1999). The final antibody was shown to be specific for reelin (Fig. 1C-E) and was used at x500 dilution for western blot (WB) and x300 for immunocytochemistry (ICC). Anti-DISC1 monoclonal antibody 14F2 (x500 for WB), anti-DISC1 polyclonal antibody FFD5 (x300 for ICC), and polyclonal anti-ADAMTS-4 (x500 for WB) have been described previously (Leliveld et al., 2008, Ottis et al.,

2011, Hisanaga et al., 2012), the latter being a gift from Prof. Mitsuharu Hattori (Nagoya City University, Japan). Commercial antibodies were used against α-actin (Sigma-Aldrich, #A2066, x5000 for WB), Akt (Cell Signaling Technology, #2920 and #4060, x1000 each for WB), calretinin (GeneTex, #GTX103261, x300 for ICC), cofilin (Santa Cruz Biotechnology #sc-12912-R, x200 for WB, and Cell Signaling Technology #3318, x1000 for WB), Dab1 (Merck Millipore, #AB5840, x1000 for WB), EBF2 (antibodies-online, #bs-11740R, x100 for ICC), Erk (Cell Signaling Technology, #4379 and #9107, x1000 each for WB), GSK3β (Cell Signaling Technology #5558 and BD Transduction Laboratories #610201, x1000 each for WB) reelin (Merck Millipore, #MAB5364, x1000 for WB) and α-tubulin (Sigma-Aldrich, #T9026, x2000 for WB).

2.3 Cell and neuronal culture

HEK293 (human kidney derived) cells expressing recombinant reelin or GFP as a control (Förster et al., 2002) were a gift from Dr. Eckart Förster (Centre for Molecular Neurobiology Hamburg, Germany) and were grown in D-MEM with 5% foetal calf serum, penicillin and streptomycin. SH-SY5Y (human neuroblastoma) cells with inducible DISC1 expression were described previously (Trossbach et al., 2016) and were cultured in D-MEM/F-12 with 5% foetal calf serum, non-essential amino acid solution supplement, penicillin and streptomycin. Primary neurons were prepared from the cortices of embryonic day 18 rats, seeded onto poly-L-ornithine coated plates and grown in Neurobasal Medium, supplemented with 2% B-27, 2mM GlutaMAX, penicillin and streptomycin. All cell culture media and supplements from Thermo Fischer Scientific.

HEK293 cells with inducible ADAMTS-4 expression were generated using a lentiviral tetracycline-controlled expression system, consisting of lentiviral expression vectors pLenti CMVtight Hygro DEST and pLenti CMV rtTA3 Blast (gifts from Dr. Eric Campeau (Campeau et al., 2009), Addgene plasmids #26433 & #26429). Full-length human ADAMTS-4 with a C-terminal V5-tag was cloned into pLenti CMVtight Hygro DEST by Gateway cloning (Thermo Fisher Scientific). Viral particles were produced for both constructs in 293FT cells using a third-

generation lentiviral packaging system (Dull et al., 1998). HEK293 cells were then infected sequentially with lentiviral particles expressing rtTA3 or ADAMTS-4, with cells cultured to stability after each infection using blasticidin or hygromycin respectively for selection. Double-stable cells were maintained in D-MEM with 10% foetal calf serum, 1% sodium pyruvate, penicillin, streptomycin, blasticidin and hygromycin.

Cell lysates were prepared in PBS / 1% Triton X-100 / 20 mM $MgCl_2$ containing protease and phosphatase inhibitors and DNaseI. For epigallocatechin gallate (EGCG) experiments, cells were treated with 2 µl/ml doxycycline, 150 µM EGCG (based on experiments by Krstic et al., 2012) and/or vehicle for 18 hours. For protein over-expression experiments, cells were transfected using Lipofecamtine 2000, according to manufacturers' instructions, with pRK5-DISC1 (a gift from Dr. Akira Sawa, John Hopkins Medical School, Baltimore, USA) and/or pCMV-Sport6-ADAMTS-4 (a gift from Prof. Mitsuharu Hattori, Nagoya City University, Japan).

2.4 Western blotting and quantification

Western blotting was performed according to standard protocols. Membranes were blocked for 1 hour in PBS / 0.05% Tween / 5% milk powder, and then antibodies applied in PBS / 0.05% Tween for 1-16 hours. After washing of membranes in the same buffer, IRDye secondary antibodies (LI-COR Biotechnology) were applied at a dilution of x30 000 for 1 hour in PBS / 0.05% Tween. After washing, proteins were visualized using an Odyssey Clx infrared imaging system (LI-COR Biotechnology) and quantified using this systems accompanying software. All protein levels were normalised to actin or tubulin (as shown in the corresponding blot) as a loading control, except for when directly comparing the ratio between two species of the same protein, or when examining protein in cell media..

2.5 Immunocytochemistry and microscopy

Primary neurons on glass cover slips coated in L-orthinine were fixed with 4% paraformaldehyde in PBS for 10 min and then permeabilised for 30 min with PBS / 0.5% Triton

X-100. Cells were blocked with PBS / 10% goat serum and then incubated sequentially with primary and Alexa Flour secondary antibodies (Thermo Fischer Scientific, used at x500 dilution), washing three times with PBS in-between. Coverslips were mounted onto glass slides with ProLong Gold mounting medium containing DAPI (Thermo Fischer Scientific) and viewed using an LSM-510 confocal microscope (Zeiss).

2.6 Real time PCR

Reverse transcription of RNA samples was carried out using the M-MLV Reverse Transcriptase (H–) Point Mutant (Promega). RT-PCR analysis was performed using a StepOnePlus Real-Time PCR Cycler (Applied Biosystems) and the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Relative expression levels were calculated using StepOnePlus Software 2.0 (Applied Biosystems) with the $2^{-\Delta\Delta C_T}$ method. ADAMTS4 expression levels were normalized twice; first to the housekeeping gene ARF1 and then to the expression levels of untreated controls. The following primer sets were used: ADAMTS4:: 5′ -CCTGACCACTTTGACACAGC-3′ and 5′ -CTGACTGGAGCCCATCATCT′ ; ARF1: 5′ -GACCACGATCCTCTACAAGC-3′ and 5′ -TCCCACACAGTGAAGCTGATG-3′ .

2.7 Statistics

Distribution of genotypes among crosses of transgenic *DISC1* and *reeler* mice were analysed by chi-squared test. Comparisons of protein signals between three or more genotypes or treatments were performed by one-way ANOVA, with Dunnett's multiple comparison test. Comparisons of only two genotypes were performed using Walsh's t-test. All error bars represent standard error of the mean.

3. Results

3.1 Transgenic DISC1 alters the processing of reelin in the perinatal mouse brain

To investigate potential interplay between the DISC1 and reelin proteins during neurodevelopment, *reeler* heterozygous mice were cross-bred with heterozygous mice expressing human DISC1 (Fig. 1A-B, see section 2.1). Heterozygous *reeler* were chosen over homozygous *reeler* mice because *DISC1* mouse models (both knockdown animals and those expressing mutant variants of the protein) show subtle behavioural and cognitive deficits (Lipina and Roder, 2014) which would likely not be detectable on the background of the gross rearrangements in cortical layering associated with the homozygous *reeler* mouse (Ogawa et al., 1995). Genotyping confirmed the presence of all four genotypes (wild type: WT, *DISC1* heterozygote: Dhet, *reeler* heterozygote: Rhet, and animals heterozygous for both: DhetRhet), although the distribution was not Mendelian (Fig. 2A), demonstrating that at least one of the genetic alterations affects viability. The *reeler* phenotype was the more detrimental to viability. Curiously, the DhetRhet genotype was more abundant than the Rhet alone, potentially implying that transgenic DISC1 can partially rescue viability defects due to *reeler*, however with the numbers of animals used, no statistically significant interaction was detected by the χ^2 test ($p = 0.10$).

To probe possible interactions between transgenic DISC1 and reelin in these animals, five litters of pups from such breedings were sacrificed within twelve hours of birth (P0-P0.5) and brain homogenates prepared. Western blotting of the brains showed a reduction in the total level of reelin by approximately 45% could be confirmed in the presence of a *reeler* allele (WT vs. Rhet, Fig. 2B&C, Supplemental Fig. S1A&B), however this loss was unevenly distributed. Three species were detected using our N-terminal derived antibody, consistent with 450 kDa full length reelin, and two known processed fragments (Lambert de Rouvroit et al., 1999) described in the literature as being 350 kDa and 130-170 kDa (which in our gel system runs at 130-140 kDa). In the brain homogenates of animals with the *reeler* allele, signal intensity of the species corresponding to processed reelin decreased, while there was no

significant change in levels of full length (non-processed) reelin. The signalling pathways responsible for processing of reelin may therefore be regulated in order to maintain homeostasis of full length reelin. In contrast, the presence of a *DISC1* transgene led to an increase in full length reelin of 38±11% (WT vs. Dhet, Fig. 2B&C, $p < 0.05$), but not in the total level of all reelin species (full length and processed), with a corresponding decrease in processed reelin. This implied that expression of transgenic DISC1 was inhibiting the processing of reelin in the brains of these animals. The increase in the proportion of reelin which was full length when a *DISC1* allele was present was also statistically significant (Fig. 2D, $p < 0.01$).

To determine whether this had a functional effect, the downstream Reelin signalling factor Dab1 was analysed. It was not possible to measure Dab1 phosphorylation in this assay due to the use of flash frozen as opposed to fresh brain tissue (a necessity due to crossbred mouse litters being born on different dates). Therefore the level of total Dab1 was instead assayed, as reelin also influences the degradation of Dab1 (Arnaud et al., 2003). While the presence of a *reeler* allele reduced Dab1 protein levels by 18±5% (WT vs. Rhet, Fig. 2E&F, Supplemental Fig. S1C, $p = 0.056$), this was restored in the presence of a *DISC1* allele (Rhet vs. DhetRhet, Fig. 2E&F, $p < 0.05$). This would be consistent with the DISC1-related restoration of normal levels of specifically full length reelin rescuing the deficit in Dab1 activity, even while levels of total reelin are decreased. Both DISC1 and reelin have previously been reported to affect Akt signalling (Hashimoto et al., 2006, Jossin and Goffinet, 2007), a downstream element of Dab1, and indeed the presence of a *DISC1* allele did increase the phosphorylation of Akt (Fig. 2G&H, Supplemental Fig. S1D), with a similar trend effect of the *reeler* allele, although the effects were not seen to be cumulative. No significant effect was seen however on three other downstream elements of reelin signalling: Erk1, GSK3β or cofilin (Supplemental Fig. S2).

3.2 Intracellular DISC1 can affect extracellular reelin in cell culture through the ADAMTS-4 enzyme

Processing of reelin at both of its major cleavage sites occurs through two enzymes of the A Disintegrin and Metalloproteinase with Thrombospondin motifs family, ADAMTS-4 and ADAMTS-5 (Hisanaga et al., 2012, Krstic et al., 2012). While both proteins could be detected in the P0-P0.5 mouse brain samples, their expression levels were too weak, and the available antibodies too lacking in specificity, to reliably quantify. Therefore, to investigate a potential relationship between DISC1 and ADAMTS in reelin processing, cell media containing freshly prepared recombinant reelin was incubated for 18 hours with transgenic SH-SY5Y neuroblastoma cells that express DISC1 in response to doxycycline (Trossbach et al., 2016), but no detectable endogenous DISC1 protein. Cells were variously treated with doxycycline, EGCG (a selective inhibitor of ADAMTS-4 and 5, Vankemmelbeke et al., 2003) and/or vehicle controls. The ensuing conditioned media was then analysed by western blot (Fig. 3A) showing no effect of DISC1 expression on total reelin levels, unlike EGCG which led to a 50% total reelin reduction (Fig. 3B). Notably however, DISC1 expression led to an increase in the proportion of reelin in the media which was full length, suggesting that DISC1 was inhibiting reelin processing, as in the transgenic mice (Fig. 3C). Furthermore, this effect was not seen using DISC1-expressing cells which had also been treated with EGCG, suggesting that the effect of DISC1 on reelin processing is dependent on ADAMTS-4/5 (Fig. 3C).

It is notable in this experiment that overexpression of intracellular DISC1 is able to affect the processing of recombinant reelin in the cell media, despite the two proteins presumably never coming into direct contact with each other. To determine whether ADAMTS-4 may provide the link between them, HEK293 cells expressing exogenous reelin were transfected for 6 hours with human DISC1 and/or murine ADAMTS-4 and then incubated for 42 hours in fixed volumes of media. As expected, expression of ADAMTS-4 led to an increase in reelin processing in the conditioned media (Fig. 3D&E), whereas this was not seen following expression of both ADAMTS-4 and DISC1. This was, seemingly because the expression of DISC1 led to a dramatic decrease in levels of cellular ADAMTS-4 (Fig. 3D&F), whereas in contrast the expression of ADAMTS-4 had no significant effect on levels of DISC1 protein. Both the levels of ADAMTS-4 within the cell (predominantly the 105 kDa form, retaining its pro-

domain) and extracellular ADAMTS-4 (the known 70kDa active form, Wang et al., 2004) were affected. An equivalent effect of DISC1 on ADAMTS-4 mRNA levels was not seen however (Fig. 3G), suggesting that the DISC1 protein may instead effect the stability or turnover of ADAMTS-4 in the cell. This effect of DISC1 on ADAMTS-4 levels could also be seen in another cellular system: transgenic HEK293 cells which over-express human ADAMTS-4 in response to doxycycline (Fig. 3H, described in section 2.3). In this case, transient over-expression of human DISC1 led to an approximately 50% reduction in levels of both the pro-form and the active, mature form of ADAMTS-4 (Fig. 3H&I).

3.3 Transgenic DISC1 alters reelin processing in a cell type dependent manner in rat cortical cultures

To determine whether the effect of DISC1 on reelin processing was generalizable to other experimental systems, our existing DISC1-transgenic rat model was employed (Trossbach et al., 2016). Primary cortical neuronal cultures were prepared from six E18 transgenic embryos, as well as from six wild type Sprague-Dawley rat embryos ("control embryos"), and seeded onto plates at a consistent density. After 24 hours in vitro, fresh media (without reelin) was added and allowed to condition for 48 hours before western blotting. Levels of full length reelin or the larger of the two processed species were fairly consistent, but too low to accurately quantify. However, the 130 kDa form was approximately 40% lower in media conditioned with transgenic cultures compared to those from control cultures (Fig. 4A,B). No reelin species of 130 kDa or higher could be detected in cell lysates. Therefore, like in the mouse brain homogenates, processing of reelin is decreased in rat cortical cultures in the presence of a transgenic *DISC1* allele. Notably however, media conditioned with neurons instead up to 8 days in vitro did not show this effect (Fig. 4C), suggesting the effect of DISC1 on reelin processing to be developmentally time-dependent. Levels of ADAMTS-4/5 in the cells/media were too low to accurately measure, meaning that it cannot be conclusively demonstrated if the enzymes were responsible for the effect in this system.

To investigate this effect, cortical neuronal cultures were examined by immunofluorescence. After 1-3 days in vitro, reelin was seen to be strongly expressed by a small population of cells, less than 1% of those in culture, with a distinctive morphology (Fig. 4D). These cells also expressed Ebf2 (Early B-cell Factor 2, Fig. 4E), but were negative for the calretinin (Fig. 4F), which would be consistent with Cajal-Retzius cells. In contrast, such cells could not be seen in cultures after 8-10 days in vitro. Instead at this time point, a much more abundant population of reelin-expressing cells were seen to be present, which were calretinin-positive and with a more classically neuronal morphology (Fig. 4G). The effect of DISC1 on reelin therefore appears to be specific to certain cell types. The presence of human DISC1 in both sets of reelin positive cells, was confirmed in the transgenic cultures (Fig. 4H).

4. Discussion

Despite their established importance for both mental illness and cortical development, no strong functional links between DISC1 and the reelin pathway has previously been established. It has been shown that knockdown or over-expression of either Dab1 or APP leads to mislocalisation of DISC1 (Young-Pearse et al., 2010), indicating DISC1 to lie downstream of them and thus most likely of reelin signalling, however here we demonstrate for the first time that DISC1 also has a function upstream of reelin, through regulating its proteolytic cleavage.

Reelin is naturally processed *in vivo* from a 450 kDa full length protein, with the N-terminal section of the protein, required for its multimerization and signalling through Dab1 (Utsunomiya-Tate et al., 2000, Kohno et al., 2009) existing in 130-170 kDa and 350 kDa processed fragments. In our P0 mouse brain homogenate tests (section 3.1), the loss of a reelin allele (through one copy of the *reeler* mutation) led to the expected approximately 50% decrease in total reelin. Notably however, there was no significant change in the amount of full length (450 kDa) reelin present, suggesting that some cellular feedback mechanisms exist in order to regulate the amount of reelin processing which occurs. DISC1 may well influence such processes as, in the same system, the presence of transgenic DISC1 was shown to lead to a reduction in both the N- and C-terminal cleavage, without altering total reelin levels. It remains to be determined whether this is an effect of the presence of additional functional DISC1, or due to the presence of insoluble aggregated DISC1, which can also be detected in these transgenic mice, as it was in our previously described transgenic rat (Trossbach et al., 2016). Furthermore, as complete brain homogenate was employed, it is not possible to determine if the effect of DISC1 on reelin occurs in specific brain regions, as might be hypothesised based on the decrease of reelin expression in the prefrontal cortex which has been associated with schizophrenia (Habl et al., 2012). Nevertheless, the effect of DISC1 on N-terminal cleavage of reelin could also be confirmed in primary neurons derived specifically from the cortex of rats, as determined by measuring levels of the processed 130 kDa reelin species, although levels of the other processed species were too low to easily detect and quantify. These differential

effects of DISC1 on individual reelin species are of particular interest given it has previously been reported that full length reelin is increased in the blood of schizophrenia patients, whereas one of its major breakdown products (described by the authors as 180 kDa, but seemingly corresponding to the 130 kDa described here) were lowered in patients with bipolar disorder or depression (Fatemi et al., 2001).

ADAMTS-4 is an extracellular protease, originally identified as a processor of cartilage aggrecan (Tortorella et al., 1999). Both ADAMTS-4 and the closely related enzyme ADAMTS-5 are also known to act on reelin, cleaving reelin at two sites yielding the 350 kDa and 130-170 kDa reelin species (Hisanaga et al., 2012, Krstic et al., 2012). Notably, it was ADAMTS-4, which plays the more prominent role in reelin processing (Krstic et al., 2012), which was affected by over-expression of DISC1 (section 3.2). Furthermore, in cell culture, DISC1 was shown to affect the processing of extracellular reelin, except following the inactivation of ADAMTS-4/5 with EGCG, suggesting an ADAMTS enzyme to be the mechanism by which this effect is relayed out of the cell. In concordance with this, expression of DISC1 was shown to modulate levels of ADAMTS-4 both within and outside the cell, suggesting DISC1 to have an effect on ADAMTS-4 prior to it becoming exported. The subsequent effect of ADAMTS-4 on reelin can be assumed to occur outside of the cell, in this case in the cell media.

In cortical cultures from E18 rats (section 3.3), the effect of DISC1 was detectable in the first 3 days *in vitro*, while reelin was expressed solely by EBF-positive cells with a distinctive morphology, but not following the emergence of Reelin-expressing cells resembling interneurons, demonstrating a cell-type specific effect. We have interpreted these early cells to most likely be Cajal-Retzius cells based on their expression timing and the presence of reelin and Ebf2, but not calretinin, although in the absence of positional data, this designation must be treated as provisional (Anstötz et al., 2014). Decline in Cajal-Retzius cell numbers occurs principally in rats at around two weeks after birth, as a result of caspase 3-dependent apoptosis, although loss of reelin expression occurs prior to cell death (Chowdhury et al., 2010, Anstötz et al., 2014, Anstötz et al., 2016). If these reelin and EBF2-positive, but calretinin-negative cells are indeed Cajal-Retzius cells, then the slight differences in timing likely reflect

the lack of localization-specific signalling in cell culture compared to the highly ordered cerebral cortex. The absence of cells positive for both reelin and calretinin in the P0-P2 rat cortex has also been reported previously (Martinez-Galan et al., 2014).

In this paper, it has been demonstrated that an established mental illness-related protein involved in neurodevelopment, DISC1, and a major neurodevelopmental protein implicated in mental illness, reelin, exist in a common pathway in the perinatal cortex within two different transgenic rodent models, with DISC1 inhibiting the processing of reelin by ADAMTS-4/5. Common areas of function of the two proteins, particularly with regard to their individually identified roles in neuronal migration are therefore of considerable interest when considered in light of the neurodevelopmental hypothesis of schizophrenia (Murray and Lewis, 1987, Weinberger, 1987, Fatemi and Folsom, 2009). Further analysis of the consequences of DISC1 on reelin would thus reveal more regarding their common neurodevelopmental functions, with potential implications for the early development of pathological features that influence mental illness in later life.

5. Author disclosure

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5.2 Contributors

N.J.B. and C.K. designed the work, analysed data and drafted the manuscript. N.J.B., S.V.T. and S.Wa. performed the experiments. S.V.T. generated the novel DISC1-transgenic mice and managed animal experiments. S.K. and I.P. generated the novel anti-reelin monoclonal antibody. S.Wa. and S.We. generated the novel ADAMTS-4-expressing cells. All authors contributed to and have approved the final manuscript.

5.3 Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Figures

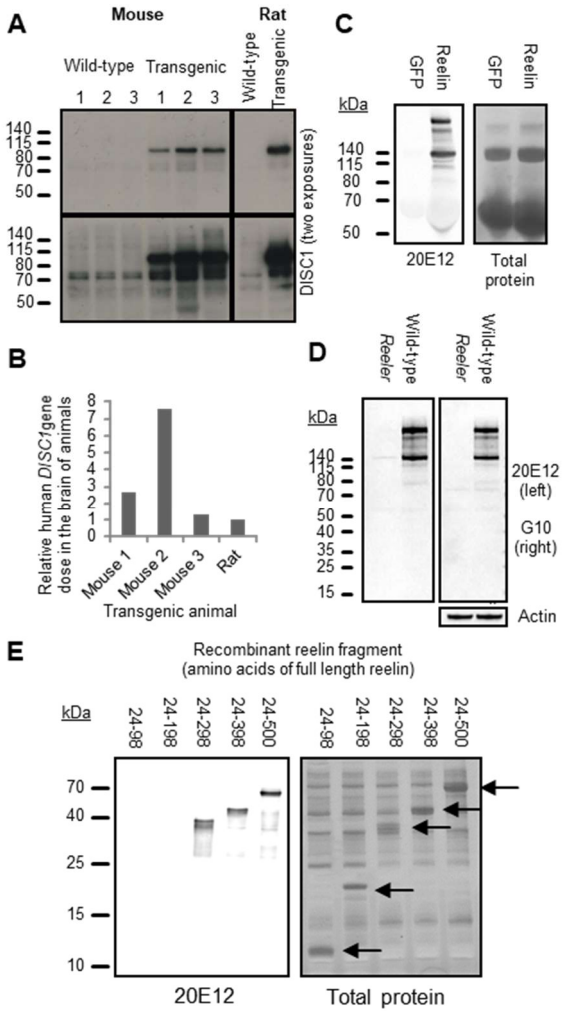

Figure 1. (**A**) Western blot showing the expression of DISC1 in three transgenic mice and three litter mate controls. Samples from the previously established DISC1 rat are shown for comparison. The DISC1 antibody, 14F2, was described previously (Ottis et al., 2011) and is specific for human DISC1 over rodent DISC1 (Trossbach et al., 2016). (**B**) Quantitative PCR of the level of human *DISC1* in the genomic DNA of the transgenic animals from part A. All wild-type animals were negative for human *DISC1*. (**C**) The novel anti-reelin monoclonal antibody 20E12 tested on media which had been incubated with HEK293 cells exogenously expressing either GFP or reelin. (**D**) Protein species detected by both 20E12 and the established G10 antibody in the brain homogenates of P4 wild-type or *reeler* mice. (**E**) Epitope mapping of the 20E12 antibody using different recombinant protein fragments of mouse reelin. Protein species representing recombinant reelin are indicated by arrows.

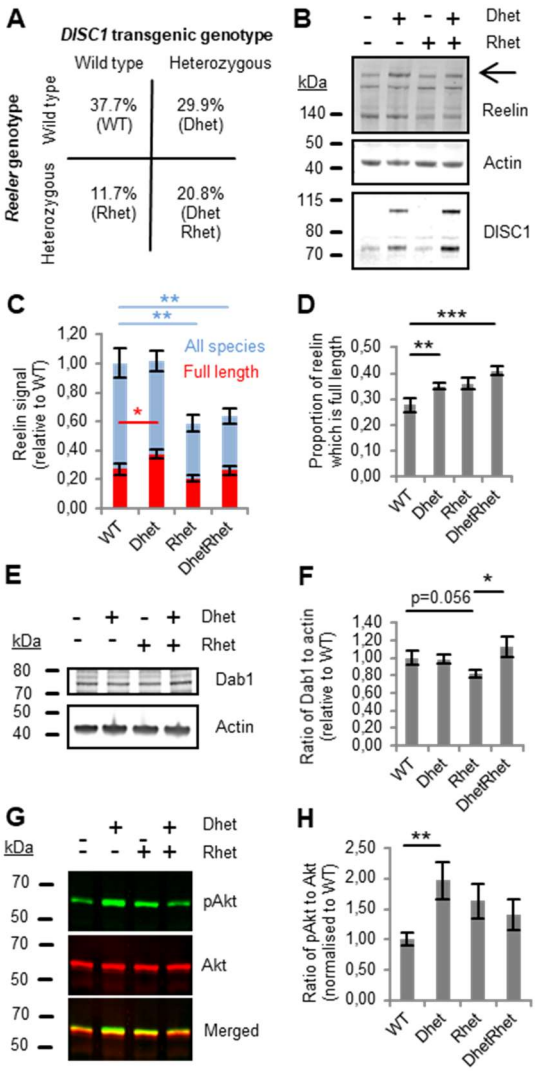

Figure 2. (**A**) Distribution of genotypes of 77 pups from crossing of *DISC1* and *reeler* heterozygous mice, surviving until birth. The distribution was not Mendelian (χ^2 p-value = 0.009). There was no statistically significant interaction between *DISC1* and *reeler* genotypes, however the results were suggestive enough that this might be seen in a larger sample (χ^2 p-value = 0.10). (**B**) Representative blots of full length reelin (arrow) and its processed forms in P0 mouse brain homogenates. In total 44 brains were prepared in this way, with post-mortem genotyping revealing them to consist of 13 WT brains, 17 Dhet, 6 Rhet and 8 DhetRhet. Full data in supplemental Fig. S1A&B. The 70 kDa DISC1 species likely represents a physiological breakdown product of human DISC1 which has been described previously (James et al., 2004), including in post mortem human brain tissue (Leliveld et al., 2008) and the DISC1 transgenic rat (Trossbach et al., 2016). For a review, see Soares et al. (2011). (**C**)

Quantification of reelin levels in homogenates from all of these brains, showing both total reelin (full length and processed combined, light grey) and full length reelin alone (dark grey). (**D**) The effect of a *DISC1* allele on the proportion of reelin present which is full length. (**E**) Representative blot of Dab1 levels, compared with actin, in the brain homogenates of crossed heterozygous *reeler* and *DISC1* mice. Full data in supplemental Fig. S1C. (**F**) Quantification of Dab1 levels. (**G**) Western blot of phosphorylated Akt (serine-473) and total Akt. Full data in supplemental Fig. S1D. (**H**) Quantification of the ratio of phosphorylated to total Akt. WT: Wild-type, Dhet: *DISC1* transgenic heterozygote, Rhet: *Reeler* heterozygote, DhetRhet: Heterozygous for both the *DISC1* transgene and *reeler,* *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

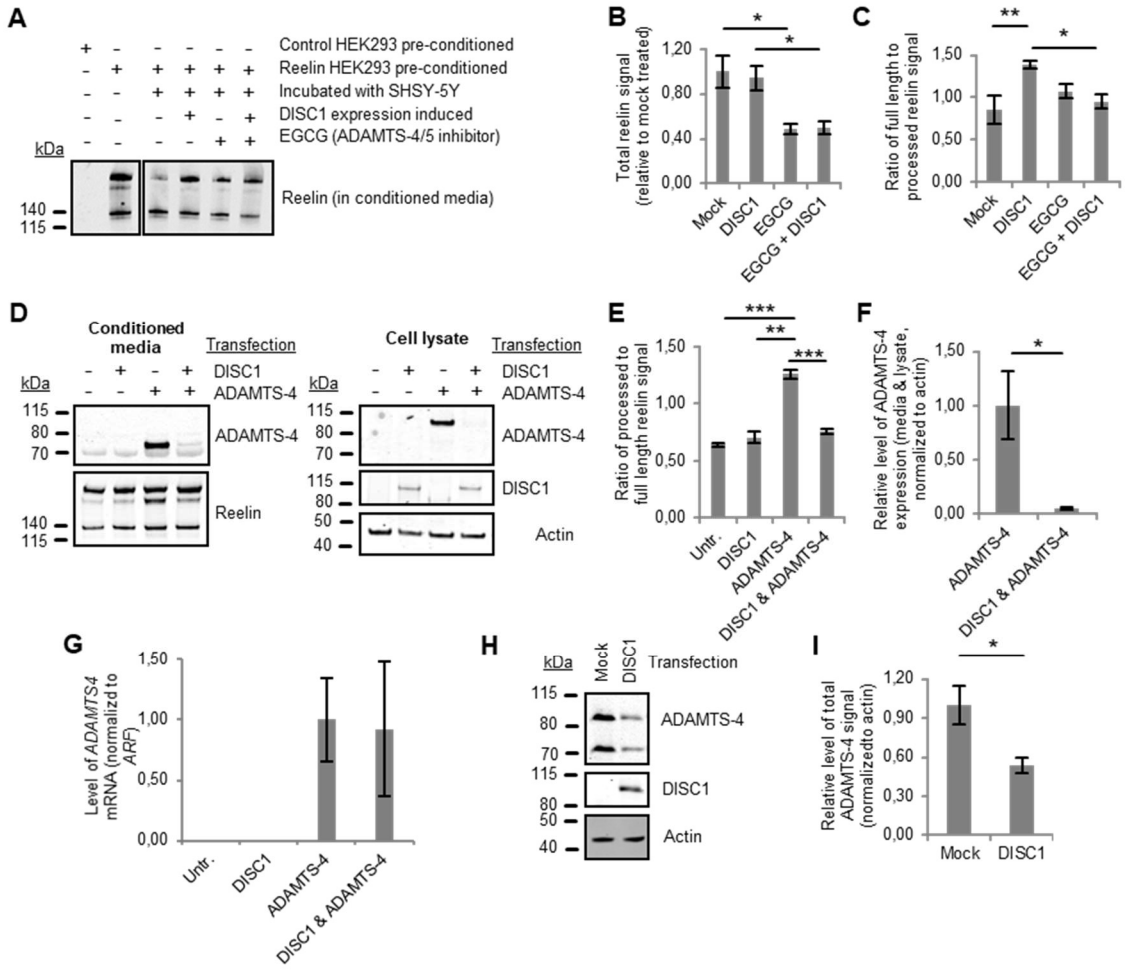

Figure 3. (**A**) Western blot of recombinant reelin media which had been incubated for 18 hours with SH-SY5Y cells, in the presence of absence of doxycycline-driven recombinant DISC1 expression and/or the ADAMTS inhibitor EGCG. Samples of recombinant reelin media and control media, which were incubated alongside the experiments but in the absence of cells, are shown for comparison. (**B**) Quantification of mean total reelin in the samples. (**C**) Quantification of the ratio of full length to processed reelin in this cell media. (**D**) Western blots of cell lysates and conditioned media from HEK293 cells expressing reelin, further transfected with DISC1 and/or ADAMTS-4. Note that the size discrepancy between the ADAMTS-4 seen in the media and in the cell lysate is due to the latter still containing its N-terminal pro domain, which is cleaved off prior to being exported from the cell. (**E**) Quantification of the ratio of full length to processed reelin in this cell media. (**F**) Quantification of mean total ADAMTS-4 in the samples in which it had been expressed, with or without DISC1. (**G**) Real time PCR results (n = 3) of cDNA from equivalent HEK293 cells transfected with DISC1 and ADAMTS-4 in the

same manner, shows no effect (**H**) Western blot of lysates from transgenic HEK293 cells which over-express ADAMTS-4, with or without transient transfection with DISC1. In this case, both the pro-domain containing ADAMTS-4 species and the active cleaved variant can be seen in the cell lysate. (**I**) Quantification of total ADAMTS-4 levels (both species), after normalisation to actin. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

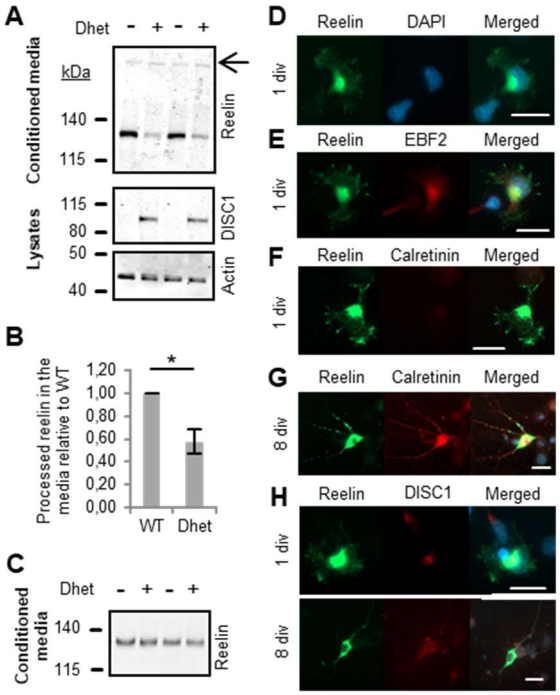

Figure 4. (**A**) Lower levels of processed reelin are seen in media incubated for 48 hours (24-72 hours in vitro) with cortical cultures from DISC1 transgenic rat embryos than from controls. Full length reelin (indicated by an arrow) is dimly visible but does not vary. Transgenic DISC1 could be detected by Western blotting in the cell lysate. (**B**) Quantification of four independent tests, each comprising six wild-type and six transgenic internal replicates. (**C**) No change in levels of processed reelin in media from neurons at 8 days in vitro (div). The higher order band is not detectable. (**D**) Presence of a subpopulation of reelin expressing cells in cultures after 1 day in vitro. (**E**) These cells also express Ebf2. (**F**) These cells are negative for calretinin (image taken using the same capture settings as part G). (**G**) By 8 days in vitro these are no longer visible, but calretinin and reelin-positive interneurons are apparent. (**H**) Both reelin-positive cell populations contain transgenic DISC1, detected using human DISC1 specific antibody FFD5. Scale bars represent 20μm.

Abbreviations

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
APP	Amyloid precursor protein
Dab1	Disabled 1
div	Days in vitro
Dhet	*DISC1* transgenic heterozygote
DhetRhet	*DISC1* transgenic heterozygote/*reeler* heterozygote hybrid
DISC1	Disrupted in schizophrenia 1
Ebf2	Early B-cell factor 2
Erk	Extracellular signal-regulated kinase
EGCG	Epigallocatechin gallate
GSK3	Glycogen synthase kinase 3
ICC	Immunocytochemistry
LIS1	Lissencephaly 1 (protein, encoded by *PAFAH1B1*)
Rhet	*Reeler* heterozygote
WB	Western blot
WT	Wild type