Dose-dependent expression of claudin-5 is a modifying factor in schizophrenia

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Schizophrenia is a neurodevelopmental disorder that affects up to 1% of the general population. Various genes show associations with schizophrenia and a very weak nominal association with the tight junction protein, claudin-5, has previously been identified. Claudin-5 is expressed in endothelial cells forming part of the blood-brain barrier (BBB). Furthermore, schizophrenia occurs in 30% of individuals with 22q11 deletion syndrome (22q11DS), a population who are haploinsufficient for the claudin-5 gene. Here, we show that a variant in the claudin-5 gene is weakly associated with schizophrenia in 22q11DS, leading to 75% less claudin-5 being expressed in endothelial cells. We also show that targeted adeno-associated virus-mediated suppression of claudin-5 in the mouse brain results in localized BBB disruption and behavioural changes. Using an inducible ‘knockdown’ mouse model, we further link claudin-5 suppression with psychosis through a distinct behavioural phenotype showing impairments in learning and memory, anxiety-like behaviour and sensorimotor gating. In addition, these animals develop seizures and die after 3–4 weeks of claudin-5 suppression, reinforcing the crucial role of claudin-5 in normal neurological function. Finally, we show that anti-psychotic medications dose-dependently increase claudin-5 expression in vitro and in vivo while aberrant, discontinuous expression of claudin-5 in the brains of schizophrenic patients post mortem was observed compared to age-matched controls. Together, these data suggest that BBB disruption may be a modifying factor in the development of schizophrenia and that drugs directly targeting the BBB may offer new therapeutic opportunities for treating this disorder.

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INTRODUCTION

Schizophrenia is a brain disorder that affects ~1% of the population and ~1.5 million people are newly diagnosed each year globally.1 It is characterized by delusions (fixed and false beliefs), hallucinations (visual and auditory), and disorganized thinking and speech that begin in early adulthood and continues through life.2 Symptoms can lead to abnormal social behaviours, depression and anxiety, and may lead to the 10% incidence of schizophrenia.3,4 Little is known about its cause, however, much attention has focused on the role of neurochemistry and aberrant neural connectivity in the brains of subjects.5,6

The disease has a strong genetic component to it, with twin studies suggesting up to 80% heritability of the condition. Many genetic studies have identified linkage to chromosome 22, suggesting this region harbours major susceptible loci for schizophrenia.7–9 Intriguingly, individuals with the chromosomal abnormality 22q11 deletion syndrome (22q11DS) have a 30-fold increased lifetime risk of developing schizophrenia and other neuropsychiatric-related conditions due to microdeletions at the chromosomal region 22q11.21.10–12 22q11DS occurs in ~1 in 4000 live births. Patients with 22q11DS display a distinctive set of developmental defects that can include cardiac abnormalities, intellectual disabilities and distinctive craniofacial patterning.13 The condition is characterized genetically by microdeletions within chromosome 22 and these deletions can be up to 3 Mb in size, which can comprise up to 40 genes. The deletions occur in only one copy of the 22q11 region of chromosome 22, leaving individuals essentially haploinsufficient for the genes within that region. Functional genetic studies associated with 22q11DS patients have previously focused on elucidating the role of individual genes within the deleted region in the hope that genetic predisposition to schizophrenia can be identified. One major component of the so-called blood-brain barrier (BBB), the gene claudin-5, is located within chromosome 22q11.21.

Of the numerous biological barriers throughout the body, the BBB is one of a few highly selective and tightly regulated barriers, reflecting the brain’s critical roles in cognitive function.14 The BBB is essential in regulating the exchange of ions and nutrients between the blood and brain and vice versa, while also protecting
dose-dependent expression of claudin-5
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MATERIALS AND METHODS

All studies carried out in the Smurfit Institute of Genetics in Trinity College Dublin (TCD) adhere to the principles laid out by the internal ethics committee at TCD and all relevant national licences were obtained prior to commencement of all studies. All mice were bred on-site in the specific pathogen-free unit at the Smurfit Institute of Genetics in TCD.

Genotyping of patients for SNP rs10314

Ethical approval for human studies was obtained through the Royal Victoria Eye and Ear Hospital. Informed consent was obtained from all subjects. DNA of 100 ng from patients was amplified by PCR in a volume of 50 μl using 1× reaction buffer, 200 μM each of dNTPs, 0.2 μM of forward and reverse primers, and 1.25 units of Taq polymerase under the following conditions: 95 °C 5 min; (95 °C 1 min; 58 °C 1 min; 72 °C 1 min)×34; 72 °C 5 min; 4 °C hold. This produced an amplification product of 177+199+227 and 199+404 for the g and c alleles, respectively. The primer sequences for the RT-PCR experiments were supplied by Sigma-Aldrich (Wicklow, Ireland) and were as follows: claudin-5 left, 5′-TTCCTTTCTAGTCGACAGTGCTG-3′, and right, 5′-GCAGTTTTGGCGACTCTAAG-3′; β-actin left, 5′-TACCACACATGTGCCATTACAG-3′; and right, 5′-CAGGAGGACCTGCTTGCACCTA-3′. Relative gene expression levels were measured using the comparative Ct method (ΔΔCt). Expression levels of target genes were normalized to the housekeeping gene β-actin.

Cell culture and transfection

Human embryonic kidney cells (HEK293) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum in a 5% CO2 incubator at 37 °C. One day before transfection, HEK293 cells were seeded on 12-well plates (2.5 × 105 cells per well). The next day, 500 ng of plasmid containing wild-type or rs10314 claudin-5 CDNA was transfected per well using Lipectofectamine 2000 (Invitrogen, Dublin, Ireland). Mouse brain endothelial cells (Bend.3, American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum and 2 mM sodium pyruvate in a 5% CO2 incubator at 37 °C. Bend.3 cells were seeded on 12-well plates (2.5 × 105 cells per well) and 100 ng/ml claudin-5 short hairpin RNA (shRNA) was transfected per well using Lipectofectamine 2000. RNA was extracted from HEK293 cells and Bend.3 cells with the E.Z.N.A. Total RNA Kit 1 (Omega biotek, Norcross, GA, USA) according to the manufacturer’s instructions. Proteins were isolated with lysis buffer (62.5 mM Tris, 2% SDS, 10 mM dithiothreitol, 10 μl protease inhibitor cocktail/100 ml (Sigma-Aldrich), followed by centrifugation at 12 000 r.p.m. for 20 min at 4 °C and supernatant was removed for claudin-5 protein analysis.

Adeno-associated virus production

shRNAs designed to target transcripts derived from mouse claudin-5 were incorporated into adeno-associated virus (AAV)-2/9 vectors. shRNA was cloned into the pShINGLE-15S-shRNA (Clontech, Mountain View, CA, USA) vector. The plasmid incorporating the inducible system with claudin-5 shRNA was digested with BsrBI and BsrGI, and ligated into the Not1 site of the plasmid pAAV-MCS, such as to incorporate left and right AAV-inverted terminal repeats. (L-ITR and R-ITR). AAV-2/9 was then generated using a triple transfection system in a stably transfected HEK-293 cell line for the generation of high-titre viruses (Vector Biolabs, Malvern, PA, USA).

Injection of AAV for claudin-5 suppression in the hippocampus and medial prefrontal cortex

C57/BL6J mice (8–12 weeks old) were anaesthetized using a ketamine/metadomidine mixture administered via intraperitoneal injection and placed in a stereotaxic frame. An incision was made to expose the skull, and burr holes were made using a surgical drill either above the dorsal hippocampus or the medial prefrontal cortex (mPFC). A Hamilton syringe was loaded with an AAV expressing either a shRNA against claudin-5 or a non-targeting (NT) control, and the needle was slowly lowered into the dorsal hippocampus: (co-ordinates: A/P = +1.75 mm; M/L = ± 1.55 mm; D/V = ± 1.75 mm, or mPFC: (co-ordinates: A/P = +1.19 mm; M/L = ± 0.4 mm; D/V = ± 2.5 mm). AAV solution of 2.0 μl was then injected at a rate of 0.5 μl per min, and once complete, the needle was left in place for 5 min before repeating the procedure in the other hemisphere. Anaesthesia was reversed with an intraperitoneal injection of atipamezole and placed in...
an incubator until recovered. All mice were given 7 days of recovery before introducing doxycycline into their drinking water (2 mg/ml in 2% sucrose solution). Doxycycline treatment was continued for the length of the behavioural experiments, with behavioural testing beginning 14 days after the introduction of doxycycline to drinking water to ensure maximal suppression of claudin-5. Behavioural testing was then performed for ~4 weeks before animals were killed for histological and molecular analysis. In addition to mice used for behavioural experiments (see below), a small cohort was injected with an AAV-expressing green fluorescent protein to visualize the extent of AAV localization following injection.

Immunocytochemistry and immunohistochemistry
HEK293 cells and BEND.3 cells were seeded on 1% fbroblastoid-coated Nuncl Lab-Tek II Chamber Slides (Thermo Scientific, Dublin, Ireland) in Dulbecco's modified Eagle's medium. After plasmid or shRNA transfection, cells were fixed for 10 min at room temperature with ice-cold methanol, washed twice with PBS and incubated with 5% normal goat serum before overnight incubation with polyclonal rabbit anti-claudin-5 (1:100). Cells were then washed twice with PBS and incubated with Cy3-conjugated goat anti-rabbit IgG secondary antibody (1:500; Abcam, Cambridge, UK) for 2 h at room temperature and counterstained with Hoechst 33258 to visualize nuclei.

Mice were killed and the brains quickly removed. One hemisphere was embedded in optimal cutting temperature compound (Tissue, Dublin, Ireland), snap-frozen in liquid nitrogen and stored at ~20 °C prior to slicing on a cryostat. Mouse brain cryosections (12 µm thick) were post-fixed in ice-cold methanol for 10 min at room temperature and washed three times in PBS. Sections were then incubated with 5% normal goat serum before overnight incubation with primary antibodies (rabbit anti-claudin-5 1:100, rabbit anti-ZO-1 1:100 and rabbit anti-occludin 1:100, Life Technologies). Sections were double-stained with isolectin-IB4-Alexa Fluor 488 1:300, Life Technologies, to label vessels. For permeability experiments, sections were incubated with Fluorescein isothiocyanate-conjugated polyclonal rabbit anti-human fibro- ncin 1:100, DAKO, or Cy3-conjugated streptavidin 1:100, Sigma-Aldrich, overnight at 4 °C. Following three washes in PBS, sections were incubated with Cy3-conjugated goat anti-rabbit IgG secondary antibody (1:500; Abcam) for 2 h at room temperature, washed three times with PBS and counterstained with Hoechst 33258 for 30 s at a dilution of 1:10 000 of a stock 1 mg/ml solution to visualize nuclei. Sections were mounted and coverslipped with Aqua Poly/mount (Polysciences, Washington, PA, USA). Sections were imaged with a Zeiss LSM 710 confocal laser scanning microscope (Cambridge, MA, USA). TJ signal intensity was quantified with the region of interest defined by isolectin-IB4 staining. For biotin and fibrocin quantification, the average signal intensity of 10 regions of interest within the blood vessels was normalized to the average signal intensity of 10 regions of interest within the blood vessels. All image analysis was performed in ImageJ (National Institutes of Health, Rockville, MD, USA).

Behavioural analyses
For detailed methodology for behavioural analyses, please see Supplementary Methods.

Primary mouse brain microvascular endothelial cell isolation
Microvessels were isolated from cortical grey matter of C57BL/6J mice by collagenase/disase (Roche) digestion and bovine serum albumin density gradient centrifugation. Purified vessels were seeded onto collagen IV fibroblastoid-coated tissue-culture plates or Corning (Corning, NY, USA) HTS 24-well Transwell polyester inserts (0.4 µm pore size, vessels from five mouse brains per 3.5 mm diameter chamber). Microvessels were grown in DMEM (Lonza, Cambridge, UK) with (5 µg/ml) puromycin during the first 3 days for endothelial cell selection) for 2–3 weeks until their transendothelial electrical resistance values plateaued.

Drug treatments
Haloperidol, lithium chloride and chlorpromazine were purchased from Sigma-Aldrich. Confluent primary mBMEC cells were treated with 0.2, 2 and 20 µM chlorpromazine, or haloperidol diluted in culture medium containing 0.1% DMSO, and 0.1, 1 and 10 µM lithium diluted in culture medium. For in vivo injections, haloperidol and chlorpromazine were diluted in 2.5% polyethylene glycol 400 in saline and 200 µl was injected via tail vein at a dose of 1 mg/kg body weight. Lithium was diluted in saline and injected at a dose of 100 mg/kg body weight. Animals were killed 24 h following injection, and tissues were processed for protein and RNA analysis.

Generation of inducible claudin-5 knockout mice
Mice were generated by standard blastocyst injection at Charles River Laboratories (Mayo, Ireland) using their strain VAF/EliteTM health standards. A doxycycline-inducible claudin-5 shRNA (160 variant) was inserted at the Col1a1 locus on chromosome 11. In addition, a CAG-lox-stop-lox-rTA3-IRESmKate2 (CL2R3K) allele was knocked in at the endogenous Ros26 locus on chromosome 6. This gene utilizes the endogenous Ros26 promoter to drive expression of the reverse tetracycline-controlled transactivator (M2rtTA), once mice are crossed to a Cre-recombinase expressing mouse.23–27 Mice were maintained as homozygous for the claudin-5 shRNA-containing gene and the rTA gene. When required for experiments, mice were crossed to transgenic Tie-2-Cre expressing animals and Cre-negative mice were used as littermate controls.

For behavioural experiments, all mice (Cre-positive and Cre-negative) were given doxycycline in their drinking water (2 mg/ml in 2% sucrose solution) and kept on doxycycline for the length of the behavioural experiments. Behavioural testing began 14 days after the introduction of doxycycline to drinking water to ensure maximal suppression of claudin-5. Behavioural testing was then performed for ~4 weeks before animals were killed for histological and molecular analysis.

Magnetic resonance imaging
BBB integrity was assessed in vivo via magnetic resonance imaging (MRI), using a dedicated small rodent 7 T MRI system located at TCD (www.neuroscience.tcd.ie/technologies/mri.php). Anaesthetized mice were physically monitored (electrocardiogram, respiration and temperature) and placed on an MRI-compatible support cradle, with a built-in system for maintaining the animal's body temperature at ~37 °C. The cradle was then positioned within the MRI scanner. Accurate positioning was ensured by acquiring an initial rapid pilot image, which was then used to ensure the correct geometry was scanned in all subsequent MRI experiments. Upon insertion into the MRI scanner, high-resolution anatomical images of the brain were acquired (100 µm in-plane and 500 µm through-plane spatial resolution). To visualize brain damage and lesion volumes, high-resolution images were acquired using Rapid Acquisition with Relaxation Enhancement (RARE) 2-D sequence with a RARE factor of 8 and an echo time resulting in an effective time of 42.2 ms (with a flip angle of 180°). With an acquisition matrix of 128 × 128 and a field of view of 1.8 × 1.8 cm2, the pixel resolution was 0.141 mm per pixel. In the coronal plane, 15 slices, each measuring 0.25 mm in thickness were acquired. Repetition time was 7274.2 ms, and four averages were used for a total measuring time of 7 min 45 s.

Compromises of the BBB were then visualized in high-resolution T1-weighted MR images (resolution, 0.156 × 0.156 × 5 mm3; field of view: 20 × 20 × 17.9 mm3; matrix: 128 × 128 × 30; TR/TE: 500/2.7 ms; flip angle: 30°; number of images: 3; acquisition time: 12 s). The segmented vessels were drawn and signal intensity changes were measured as mean pixel intensity and normalized to the signal intensity prior to injection of Gad-DTPA.

Electroretinography analysis of mice
Cre-positive C57/160 mice were dark-adapted overnight and prepared for electroretinography under dim red light. Pupillary dilation was carried out by instillation of 1% cyclopentolate and 2.5% phenylephrine. Animals were anesthetized by intraperitoneal (i.p.) injection of ketamine (2.08 mg per 15 g body weight) and xylazine (0.21 mg per 15 g body weight). Standardized flashes of light were presented to the mouse in a Ganzfeld bowl to ensure uniform retinal illumination. The electroretinography responses were recorded simultaneously from both eyes by means of gold wire electrodes (Roland Consult, Brandenburg, Germany) using Vidisic (Dr Mann Pharma, Berlin, Germany) as a conducting agent and to maintain corneal hydration.

Acoustic prepulse inhibition
Sensorimotor gating was assessed by prepulse inhibition (PPI) of the acoustic startle response. The PPI apparatus consisted of a soundproof PPI chamber with a weighing scale positioned in the centre of the chamber beside a loudspeaker. Mice were maintained in a holding chamber placed on the scale. Each mouse was given 2 days to habituate to the holding chamber and PPI chamber with a constant background white noise
(65 dB). Before the beginning of the experiment, all instruments were calibrated and startle stimulus (71, 77, 83, 100, 110, 120 dB) were set. PPI was divided into three stages:

1. Two minutes habituation with constant background noise of 65 dB.
2. Presentation of prepulse (71, 77, 83 dB) and pulse (100, 110, 120 dB) intensities, to habituate animals to startle stimulus.
3. Ten blocks of random combinations of prepulse alone, pulse alone, prepulse plus pulse and no stimulus trials.

Startle stimulus were presented as 20 ms bursts of white noise within an interstimulus (time between presentation of prepulse and pulse stimuli) interval of 100 ms. Data were averaged across the 10 blocks for each prepulse, prepulse plus pulse and no stimulus trial, and PPI was calculated as:

\[
\%\text{PPI} = 100 \times (\text{pulse} - \text{prepulse plus pulse})/\text{pulse}.
\]

### Human brain sections

Free-floating 60 μm-thick sections of post-mortem human brain tissue from 24 deceased schizophrenia patients and 24 age-matched controls were obtained from the Stanley Medical Research Institute. In addition to information relating to their schizophrenia diagnosis, the data accompanying these samples also included genotype information. Sections were washed in PBS and antigen retrieval was performed by boiling the sections for 2 × 5 min in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Sections were permeabilized with 0.5% Triton X-100; blocked with 5% NGS; and incubated with monoclonal mouse anti-claudin-5 (1:50; Santa Cruz, Dallas, TX, USA) for 48 h at 4 °C. Brain sections were incubated with Cy3-conjugated goat anti-mouse IgG (1:300, Abcam) for 3 h at room temperature and counterstained with Hoechst 33258. Z-stacks were acquired with identical acquisition settings across samples and three-dimensional images were rendered with ImageJ 3D viewer. Staining and microscopy were performed blind to diagnosis. Quantification of claudin-5 staining (intensity and continuity) in these samples was performed using software designed for assessing blood vessel networks (Angiogenesis Analyser plug-in for ImageJ, National Institutes of Health). Briefly, the red channel (claudin-5) from the confocal images (at ×10 magnification) was isolated and was processed to reduce background staining. The software was then set to map the blood vessels present in each section and the total length of the vessels was quantified (number of pixels). This quantified data was analysed based on diagnosis and genotype.

### Statistical analyses

Statistical analysis was performed using Student’s t-test, with significance represented by a P-value of ≤ 0.05. For multiple comparisons, analysis of variance was used with a Tukey-Kramer post-test and significance represented by a P-value of ≤ 0.05. G*Power was used to choose sample size and ensure adequate power for experiments.

### RESULTS

Claudin-5 variant rs10314 weakly associates with schizophrenia in 22q11DS patients and causes decreased protein expression

Individuals with 22q11DS display a distinctive set of developmental defects that can include cardiac abnormalities, intellectual disabilities and distinctive craniofacial patterning (Figure 1a). The condition is characterized genetically by microdeletions within

**Figure 1.** Claudin-5 variant rs10314 causes decreased protein expression. (a) Distinctive craniofacial pattern characteristic of 22q11DS. (b) Chromosomal location of claudin-5 gene on Chr. 22q11.21. (c) Weak association of the claudin-5 variant rs10314 in a population of 67 22q11DS patients (*P* = 0.0388, two-sided *t*-test). (d) Claudin-5 expression in HEK-293 cells expressing normal or rs10314 variant claudin-5. Claudin-5 protein is significantly decreased in rs10314-expressing plasmid 24 h post transfection (**P** < 0.01). (e) Claudin-5 expression in HEK-293 cells expressing wild-type (white arrows) or rs10314-expressing cDNA. (f) Levels of claudin-5 transcript remain unchanged 24, 48, 72 and 96 h post transfection of wild-type and rs10314-expressing cDNA. (g) Analysis of hsa-MiR-3934 and (h) hsa-MiR-125a-3p binding within 3′-untranslated region of the claudin-5 gene. The rs10314 variant shows reduced promoter activity compared to the normal variant in the control condition (*P* < 0.05; **P** < 0.01). This difference is absent following the addition of either miRNA. (i) Levels of expression of claudin-5 transcript in polysome fractions post transfection of normal (blue) or rs10314 (red)-expressing cDNA vectors. cDNA, complementary DNA; EV, empty vector; miRNA, micro RNA; UNT, untransfected.
chromosome 22 leaving individuals essentially haploinsufficient for the genes within that region, and the critical BBB associated gene, claudin-5, is located here (Figure 1b). In 2004, it was reported that a single-nucleotide polymorphism in the 3′-UTR of the claudin-5 locus (rs10314) was weakly associated with schizophrenia in a Han Chinese population.28 This weak nominal association was also observed in other studies.29–31 Following sequencing across the remaining claudin-5 allele from a cohort of 67 22q11DS patients, a weak nominal association (**P = 0.0388, two-sided χ²-test) between the claudin-5 variant (rs10314) and those 22q11DS patients who went on to develop schizophrenia was observed (Figure 1c).

Highest frequencies for the derived allele at rs10314 in modern populations (1000 Genomes Project Consortium) are observed to occur in Eastern Eurasia and Siberia (~31%), with notable peaks occurring in Northeast Siberian populations such as the Even, Evenki, Koryak and Yakut (42–67%) (Supplementary Figures 1 and 2 and Supplementary Table 1), with lowest frequencies observed in Afro-Asiatic speaking groups of Western Asia (3.23%).

Generation of cDNA-expressing vectors of normal and rs10314 variant claudin-5 genes showed the rs10314 variant gene expresses 50% less claudin-5 protein in cells (Figure 1d), suggesting that individuals with 22q11DS carrying the rs10314 variant may express up to 75% less claudin-5 at their BBB than the general population (50% for 22q11DS with the wild-type variant due to haploinsufficiency and down by 75% for 22q11DS with the rs10314 variant on top of haploinsufficiency). Interestingly, the variant claudin-5 was less evident at the plasma membrane of transfected cells, with positive immunoreactivity observed in perinuclear bodies, possibly lysosomes (Figure 1e). There was...
however no difference in the levels of claudin-5 transcript over 96 h (Figure 1f), suggesting this variant confers a change in the post-translational processing or stability of the protein product. Transfection of the normal or variant constructs in cells with well-established TJs (Caco-2 cells) showed the same decrease in claudin-5 expression (Supplementary Figure 3).

The rs10314 SNP is a G to a C base change in the 3′-UTR of the claudin-5 gene. In this regard, we sought to examine if micro RNA (miRNA)-binding sites were impacted in any way. In the absence of miR-3934 or miR-125a-3p, two miRNAs in which that variant confers novel binding, the normal variant expresses significantly more promoter activity compared to the rs10314 variant (*P < 0.05 and **P < 0.01 for the respective miRNAs). However, in the absence of either miRNA, there were no significant differences in promoter binding between the normal and rs10314 variants (Figures 1g and h). Isolation of polysome fractions however showed a distinct shift in the levels of claudin-5 transcript in the rs10314 variant to the sub-polysome fractions, suggesting efficient translation is being impacted upon (Figure 1i).

Site-specific and chronic suppression of claudin-5 induces distinct behavioural changes in mice

Such is the impact of the BBB on neural integrity, it can be suggested that each neuron is essentially perfused by its own capillary. We generated inducible AAV vectors expressing doxycycline-inducible claudin-5 shRNA to allow for targeted suppression of claudin-5 in the brains of mice (Figure 2a). We used AAV-2/9 that allows for transduction of endothelial cells within a region of tissue of ~1 mm³ (Supplementary Figure 4; for further details on claudin-5 suppression in brain tissue using this method, see ref. 32). Mice were injected bilaterally in the
hippocampus (Figure 2b) or mPFC (Figure 2c) with AAV-expressing claudin-5 shRNA or a NT AAV as outlined. Claudin-5 levels were significantly suppressed following supplementation of doxycycline in the water of mice both in the hippocampus (*\(P < 0.05\), Figures 2d and e) and mPFC (***\(P < 0.01\), Figures 2f and g). Claudin-5 suppression did not trigger vascular remodelling events as measured by the total stained length of isolecint-IB4 vessels in NT or claudin-5 AAV-injected groups (Supplementary Figure 4c). Suppression of claudin-5 in the hippocampus or mPFC had no impact on the expression levels of occludin or ZO-1 (Supplementary Figure 5). There was also a significant increase in extravasation of a biotinylated agent (600 Da) and fibrinogen (340 kDa) in both the hippocampus (***\(P < 0.01\), Figures 2h and i) and mPFC (***\(P < 0.01\), Figures 2j and k).

Intriguingly, a range of behavioural changes were observed in mice across five domains (learning and memory; depression; anxiety; social behaviour; and locomotor activity). Suppression of claudin-5 in the hippocampus led to a significant impairment in performance in the social novelty task (*\(P < 0.05\)) and a significant decrease in grooming behaviour (*\(P < 0.05\)), suggestive of changes in affect (Figure 2l). Suppression of claudin-5 in the mPFC led to more profound changes, with significant impairments in extravasation of a biotinylated agent (600 Da) and fibrinogen (340 kDa) in both the hippocampus (***\(P < 0.01\), Figures 2h and i) and mPFC (***\(P < 0.01\), Figures 2j and k).

Figure 4. Phenotype of inducible claudin-5 knockdown mice. (a) Reduced spontaneous alternation in the T-maze in claudin-5 knockdown mice (*\(P < 0.05\)). (b) Reduced discrimination index in claudin-5 knockdown mice in the object recognition task (*\(P < 0.05\)). (c) Reduced open arm entries observed in the elevated plus maze in the claudin-5 knockdown mice (*\(P < 0.05\)). (d) Increased side bias in inducible claudin-5 knockdown mice in a spontaneous alternation task in the Y-maze (*\(P < 0.05\)). (e) Decreased acoustic prepulse inhibition (PPI) in inducible claudin-5 knockdown mice with a 77 dB prepulse at 110 dB (***\(P < 0.01\) and 120 dB (*\(P < 0.05\)). (f) Summary of behavioural data following suppression of claudin-5 in the inducible claudin-5 knockdown mouse model. (g) Contrast-enhanced magnetic resonance imaging (MRI) showing significant extravasation of contrast agent in the brain of inducible claudin-5 knockdown mice (right) compared to non-targeting control mice (left; ***\(P < 0.01\)). All assays were performed 2–4 weeks post supplementation of doxycycline (2 mg/ml) to the drinking water. RQ, relative quantity.
Acoustic PPI is a sensorimotor gating phenomenon that is preserved across species and has previously been shown to strongly associate with schizophrenia. Indeed, numerous proposed mouse models of schizophrenia use acoustic PPI as a correlate of a schizophrenia-like phenotype. Here, we show that mice lacking sufficient claudin-5 at the BBB display a reduced acoustic PPI response with a 77 dB pulse at 110 dB (**P < 0.01) and 120 dB (**P < 0.05; Figure 4e). Taken together, the behavioural data presented here suggest a profound link between the gene-dosage effect of claudin-5 and manifestations of many schizophrenia-associated symptoms.

Finally, in vivo measurements of BBB permeability were performed using contrast-enhanced MRI. In claudin-5-suppressed animals, gadolinium extravasation lead to significantly increased contrast in the acquired images (**P < 0.01) compared to NT control mice (Figure 4g). In addition, there was significantly increased extravasation of fibrinogen throughout the brains of claudin-5 suppressed animals (**P < 0.01) compared to littermate controls (Supplementary Figure 16).

Anti-psychotic drugs regulate claudin-5 levels and claudin-5 expression is aberrant in human schizophrenia patients

It is known that systemic biomarkers of BBB dysfunction are increased in individuals with schizophrenia. Evidence for microvascular dysfunction has also previously been linked with the condition, however, a distinct molecular genetic link between endothelial cell dysfunction and schizophrenia has been lacking. We examined the effects of some of the most common anti-psychotic drugs on claudin-5 levels both in vitro in primary brain endothelial cell cultures and in vivo in wild-type C57BL6 mice. We found that lithium, haloperidol and chlorpromazine all significantly increased levels of claudin-5 protein in a dose-dependent manner in vitro (Figure 5a) with no obvious changes in the expression pattern of claudin-5 (Figure 5b). In vivo, we found similar increases in claudin-5 following anti-psychotic administration with significant increases in protein expression for all drugs (**P < 0.05; Figures 5c and d) and in messenger RNA transcription for chlorpromazine alone (**P < 0.05; Figure 5e). We also examined the impact of these drugs on the levels of other TJ components, and found that lithium and haloperidol increased levels of occludin (**P < 0.05) but not ZO-1 in vitro (Supplementary Figures 17a–c), while chlorpromazine alone increased levels of ZO-1 (**P < 0.05) in vivo (Supplementary Figures 17d–f). To explore whether these drugs were affecting claudin-5 expression through Wnt signalling, we analysed downstream components of this signalling and found that levels of Axin-2 and Sox17 expression were unaffected (Supplementary Figure 18).

In addition, we examined brain sections from the parietal lobe of 24 schizophrenia donor brains and 24 age-matched normal control brains (Supplementary Figure 19). We stained for claudin-5 levels in brain sections, and assessed pattern and density of staining in a blinded manner. While overall levels of protein were not changed, an aberrant pattern of claudin-5 immunoreactivity was observed in 62% of the schizophrenia cases—usually claudin-5 is continuously expressed across the length of a blood vessel, but was discontinuous in many of the samples taken from individuals with schizophrenia (Figure 5f). Quantification of claudin-5 expression in blood vessels revealed that, while there were no significant differences between the diagnosis groups in the absence of the rs10314 variant, claudin-5 levels were significantly lower in individuals with a diagnosis of schizophrenia who had the rs10314 variant compared to control subjects who harboured the rs10314 variant (**P < 0.05, Figure 5g).
DISCUSSION

Current treatment options for schizophrenia patients largely and almost exclusively include the use of anti-psychotic therapies and adjunct psychosocial therapy, including psychotherapy and cognitive behavioural therapy. However, anti-psychotic drugs are often discontinued by patients due to inefficacy or intolerable side effects. In addition, the average life expectancy of people with schizophrenia can be between 10–25 years less than normal, with a recent meta-analysis suggesting up to 16 000 people die annually as a result of having to live with the condition.  

There is now a clear and urgent need to better understand the underlying molecular aetiology of schizophrenia and to develop new forms of therapy for this debilitating condition. 

To our knowledge, this is the first molecular-based evidence of the involvement of the BBB to be described in schizophrenia, and brings together an environmental- and genetic-based model for the molecular aetiology of schizophrenia. The protein product of the claudin-5 gene is a four pass transmembrane protein consisting of two extracellular loops that reside at the apical periphery of contacting endothelial cells of the BBB. These extracellular loops can respond to the microenvironment and can allow for rapid remodelling of the TJ dependent on a range of environmental stimuli.

The underlying molecular complexity of the BBB and how it relates to health and disease is only beginning to be unravelled. Indeed, the BBB is not a static microenvironment, it is highly dynamic in both homoeostatic physiology and indeed in pathology. At the BBB, claudin-5 is by far the dominant TJ component, but claudin-3 and claudin-12 are also present. Our understanding of the claudins has been considerably improved through genetic knockout models. In particular, the role of claudin-5 in forming the BBB was confirmed in mice genetically engineered to lack claudin-5. Nitta et al. 33 showed that claudin-5 knockout mice have an impaired BBB that was compromised in a size-selective manner to molecules up to 800 Da in size. However, complete ablation of claudin-5 is lethal, with mice dying within hours of birth. Intriguingly, however, in claudin-5 knockout mice, a barrier can still form and remains intact to molecules 4–1 kDa molecular weight. Our data obtained from inducible ‘knockdown’ mice now suggests a link between the gene-dosage effect of claudin-5 and the onset of schizophrenia-like characteristics in this mouse model. This does not by any means suggest that variations in claudin-5 is a direct cause of schizophrenia, but that it may be a contributing factor in the development of schizophrenia. It is known that claudin-5 levels are impacted in other neurological disorders such as ischaemia and traumatic brain injury 17 and neurodegenerative disorders. 37–39

While the original studies describing the association of the rs10314 variant in the 3′-UTR of claudin-5 with schizophrenia in the general population were weak, our studies here showing a nominal association in a cohort of individuals with 22q11DS with a single remaining claudin-5 allele are important. The central role of claudin-5 at the BBB cannot be underestimated and it has been
identified as an ohnologue, or a gene duplicate originating from whole-genome duplication. Pertinently, ohnologues represent critical dosage-sensitive elements of the genome, responsible for some of the deleterious phenotypes observed for pathogenic copy-number variations and as such are readily identifiable candidate genes for schizophrenia.8,10,41

Critically, it appears that some of the most common antipsychotic drugs can potentially regulate claudin-5 protein levels in a dose-dependent manner. These findings are important given the fact that brain endothelial cells are the first membranous interface these drugs encounter in the cerebrum post administration. This is the first example of these drugs being biologically active in brain endothelial cells and may be fundamental to their mode of action. This is highly suggestive that these drugs can regulate the integrity of the BBB and may go some way to explaining why select drugs have efficacy in some patients and not others. In addition, this suggests that claudin-5 may be a therapeutic target for schizophrenia, with drugs that regulate its expression representing a more targeted and ultimately safer approach to treatment.

At the anatomical level, the central pathological findings in the brains of schizophrenia patients are centred on a distinct loss of cortical grey matter, cortical thinning and reduced numbers of synaptic structures on cortical pyramidal neurons.42 There are however no pathognomonic markers of schizophrenia observable at the histological level post-mortem. Here, we were able to identify an aberrant pattern of claudin-5 immunoreactivity in 62% of schizophrenia patient brains based on an analysis of claudin-5 levels.

Recognizing that schizophrenia is a disorder with a cerebral vascular component will impact the way this condition is treated and lead to improved medicines for patients living with the condition. These findings will also lead to a greater understanding of other neuropsychiatric conditions such as bipolar disorder and affective disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

CG: performed in vivo and in vitro experiments. JK: designed and performed behavioural analyses, data analysis and contributed to writing the paper. MM: genotyping of 22q11DS patients and mice. YG and JH: performed miRNA expression analyses. LMC and RM: analysis of rs10314 variant in various populations. VS, SRH and SYG: and scalable system for studying gene function in mice using conditional RNA. 

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