Characterizing the molecular etiology of arthrogryposis multiplex congenita in patients with LGI4 mutations

Daniel G. Booth | Nina Kozar | Stephen Bradley | Dies Meijer

Centre for Discovery Brain Sciences and MS Society Edinburgh Centre for MS Research, University of Edinburgh, Edinburgh, United Kingdom

Correspondence
Dies Meijer and Daniel G. Booth, Centre for Discovery Brain Sciences and MS Society Edinburgh Centre for MS Research, University of Edinburgh, Edinburgh, EH16 4SB United Kingdom.
Email: dies.meijer@ed.ac.uk (D. M.) or Email: daniel.booth@nottingham.ac.uk (D. G. B.)

Present address
Daniel G. Booth, Biodiscovery Institute, Queen’s Medical Centre, University of Nottingham, Nottingham, United Kingdom

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Abstract
Disruption of axon-glia interactions in the peripheral nervous system has emerged as a major cause of arthrogryposis multiplex congenita (AMC), a condition characterized by multiple congenital postural abnormalities involving the major joints. Several genes crucially important to the biology of Schwann cells have now been implicated with AMC. One such gene is LGI4 which encodes a secreted glycoprotein. LGI4 is expressed and secreted by Schwann cells and binds its receptor ADAM22 on the axonal membrane to drive myelination. Homozygous mutations in LGI4 or ADAM22 results in severe congenital hypomyelination and joint contractures in mice. Recently bi-allelic LGI4 loss of function mutations has been described in three unrelated families with severe AMC. Two individuals in a fourth, non-consanguineous family were found to be compound heterozygous for two LGI4 missense mutations. It is not known how these missense mutations affect the biology of LGI4. Here we investigated whether these missense mutations affected the secretion of the protein, its ADAM22 binding capacity, or its myelination-promoting function. We demonstrate that the mutations largely affect the progression of the mutant protein through the endomembrane system resulting in severely reduced expression. Importantly, binding to ADAM22 and myelination-promoting activity appear largely unaffected, suggesting that treatment with chemical chaperones to improve secretion of the mutant proteins might prove beneficial.

KEYWORDS
ADAM22, arthrogryposis multiplex congenita, axo-glia interactions, LGI4, myelination, peripheral nerve, Schwann cell

1 | INTRODUCTION

Environmental or genetic factors that affect in utero movement of the fetus will result in congenital contractures, a condition referred to as arthrogryposis multiplex congenita (AMC) (Drachman, 1971; Hageman & Willemsen, 1983; Hall, 2014). Not surprisingly, therefore, AMC, with a prevalence of 1 in 3000 live births, is usually syndromic and has been associated with over 400 medical conditions, including: myasthenia gravis, pulmonary hyperplasia, and diaphragmatic defects (Hall & Kiefer, 2016; Lowry et al., 2010). Consequently, a wide range of environmental and genetic factors have been identified that contribute to AMC. Indeed over 320 different genetic candidates have been identified (Hall & Kiefer, 2016). These genes are implicated in a broad range of processes ranging from embryonic tissue morphogenesis, skeletal muscle development, synaptic transmission, and development of the central nervous system (CNS) and peripheral nervous...
system (PNS). This includes genes involved in myelination and longitudinal polarization of myelinated axons, such as Gpr126 (ADGRG6), Gliomedin (GLDN), Caspr (CNTNAP1), and, most recently, LGI4 (Laquerriere et al., 2014; Maluenda et al., 2016; Mishra et al., 2020; Ravenscroft et al., 2015; Wambach et al., 2017; Xue et al., 2017).

Leucine-rich Glioma-Inactivated 4 (LGI4) belongs to a small family of secreted glyco-proteins (LGI1-4) that have been implicated in a range of neurological conditions including epilepsy and limbic encephalitis (Dalmau et al., 2017; Fukata et al., 2018; Irani & Vincent, 2016; Kegel et al., 2013). LGI function involves binding to the extracellular domain of the transmembrane proteins ADAM22 or ADAM23, which belong to the larger family of “a disintegrin and metalloproteinase” (ADAM) proteins, but lack metalloproteinase activity (Alfandari et al., 2009).

Within the PNS, LGI4 is secreted from Schwann cells and binds to ADAM22 expressed on the axonal membrane (Birmingham et al., 2006; Ozkaynak et al., 2010). Mice carrying homozygous Lgi4 mutant alleles (the “claw paw” allele Lgi4–/– or homozygous Adam22 (Adam22ΔΔ) mutant alleles have a severely reduced lifespan, exhibit pronounced joint contractures (Birmingham et al., 2006; Darbas et al., 2004; Henry et al., 1991; Nishino et al., 2010; Ozkaynak et al., 2010; Sagane et al., 2005) and congenital hypomyelination of the PNS. Functional studies identified a patch of amino acids within the Epitemp domain of Lgi4 that is crucially involved in promoting myelination but distinct from its Adam22 binding interface (Kegel et al., 2014) (Figure 1a).

Recently, the first human AMC patients from five different families have been reported to carry mutations in the LGI4 gene (Mishra et al., 2020; Xue et al., 2017). The most recently described family carries a missense mutation in the LGI4 Met start codon (c.2 T > C) (Mishra et al., 2020). In three of four families described by Xue and colleagues (Xue et al., 2017), mutations generated a premature stop codon or frameshift, resulting in the production of a truncated non-functional protein and ultimately, death of the patients. The fourth family had two children. The first child died 2 h after birth, but the second child survived and was aged 6 at the time of examination in 2016. This patient suffers from a variety of conditions including contractures, apnoea, dysmorphic features, seizures, and verbal development delay but with normal brain MRI (Xue et al., 2017). Interestingly, both children in family 4 were found to be compound heterozygous for the mutations c.773G > C (paternal allele) and c.1301 T > A (maternal allele) encoding full-length LGI4 proteins that contain either the R258P or V434D amino acid substitution. How these missense mutations affect LGI4 function is unknown and the subject of this investigation. In addition, we evaluate how the LGI4 (c.2 T > C) mutation affects the expression of the mutant protein.

2 | MATERIALS AND METHODS

2.1 | DNA constructs

Point mutations were introduced using overlap PCR. All Lgi expression constructs were cloned in the pcDNA3.1 vector, extending the Lgi open reading frame with a V5 and 6xHis tag, and the retroviral vector pBMN-I-RES-GFP (pBMN-I-GFP was a gift from Garry Nolan (Addgene plasmid # 1736; http://n2t.net/addgene:1736; RRID: Addgene_1736). In these retroviral constructs the Lgi open reading frames start with a signal peptide derived from the mouse kappa light chain immunoglobulin gene, replacing the Lgi protein’s own signal peptide. Adam22-Fc constructs have been previously described (Ozkaynak et al., 2010). The pmScarlet_Giantin_C1 plasmid (a gift from Dorus Gadella (Addgene plasmid # 85048; http://n2t.net/ addgene:85048; RRID:Addgene_85048) used in co-localization studies (Figure 1) and to visualize the Golgi apparatus has been described (Bindels et al., 2017).

2.2 | Virus production

HEK293T Phoenix-ECO (ATCC® CRL-3214™) cells were transfected with pBMN-LGI-RES-GFP constructs, using the polyethyleneimine (PEI) method (Boussif et al., 1995; Reed et al., 2006). Virus was collected in DMEM/F10, 10% heat-inactivated fetal calf serum (FCS), and Penicillin + Streptomycin (Invitrogen) (PS), following a 48-hour expression period. When virus was not used directly, it was snap frozen in liquid nitrogen and stored at −80°C. The titer of viral supernatants was determined using primary mouse 3 T3 cells (Figure S2).

2.3 | Tissue culture methods

Rat Schwann cell cultures were established from sciatic nerves dissected from postnatal day 2–4 Sprague–Dawley rat pups using published methods (Kleitman et al., 2002; Meng et al., 2019). Schwann cells were seeded onto Laminin/collagen (Cultrex®/BME, R&D Systems) and PEI coated 18 mm coverslips and transfected using Lipofectamine 3000 reagent (Invitrogen) as per manufacturers protocol.

Dorsal root ganglia (DRG) were dissected from mouse embryos at embryonic day 13 or postnatal day 4–6 pups (sex not determined) according to established protocols (Kegel et al., 2014; Kleitman et al., 2002; Sleigh et al., 2016). Cells were seeded as explants onto Matrigel (BD Biosciences)/poly-D-lysine (Sigma)-coated 16 mm coverslips (Thermo Scientific) and maintained in MEM (Gibco) supplemented with 3% Fetal Bovine serum (FBS), 50 ng/ml NGF and Penicillin and Streptomycin (M1 medium) in a 5%CO2 incubator at 37°C. The following day, cultures were infected with retrovirus in low serum M1 medium supplemented with 3.75 μg/ml Polyeylene (PFA).

RT4-D6P2T rat Schwannoma cells (obtained through ATCC CRL-2768: here referred to as RT4 cells) and HEK293T cells were
FIGURE 1  The disease-associated LGI4 mutant proteins LGI4(R258P) and LGI4(V434D) are secretion impaired. (a) The Phyre2 web application was used to build a structural model of the LGI4 protein based on the LGI1 crystal structure (Kelley et al., 2015; Yamagata et al., 2018). The LGI4 structure was subsequently visualized using PyMol and the position of the missense mutations V434D and R258P in the seven-bladed propeller structure of the epitempin domain (EPTP domain) is shown in blue. The V434D mutation is in the β sheet of blade 5, close to the amino acid residues that are crucial for LGI4s myelin promoting activity (in red). The R258P mutation is in the solvent exposed β sheet of blade 1. Amino acid residues involved in direct interaction with ADAM22 are indicated in yellow and are identical in LGI1 and LGI4. The stippled outlined amino acids in the EPTP domain and the LRR domain are involved in LGI-LGI interactions, and these amino acids are identical in LGI1 and LGI4. (b) Schematic showing expression constructs. LGI4, LGI4(R258P), and LGI4(V434D), in addition to LGI1 and LGI1(E383A) (not shown) cDNA with extended open reading frames to include V5 (red oval) and 6xHis-histidine (blue oval) genetic tags. (c) Quantitative imaging. Plasmids expressing WT and mutant versions of LGI4 were co-transfected with pmScarlet-Giantin (Scarlet-Golgi) into primary Schwann cells and expressed for 48 h. WT and mutant versions of LGI1 were transfected in parallel as a positive control. Cells were fixed as routine and probed with antibodies targeting V5 (red) and KDEL (ER: green). Representative images showing successfully transfected cells (Scarlet-Golgi positive cells) and intracellular levels of WT or mutant version of LGI1 and LGI4 (V5 immune-reactivity). Bar = 15 μm. Quantification of the images using ImageJ. A KDEL mask was used to measure underlying V5 (individual cells). To account for differences in expression levels between cells, V5 signal was normalized to Scarlet signal and plotted as a column scatter plot (c-lower panel), and statistically analyzed using an unpaired t test. (d) Affinity purification and Western analysis. Cell extracts and conditioned medium were spiked with an equal amount of LGI2 protein and LGI proteins were purified using Ni-NTA beads (Qiagen UK). Western blots were probed with anti-V5 antibody. Band densitometry was performed using ImageJ. LGI4 mutant protein expression levels (arbitrary units) are normalized to LGI2, plotted in a scatter plot, and statistically analyzed using the unpaired t-test. Statistical significance is expressed by a number of stars (p < .001 = *** and p < .0001 = ****). E) Plasmids expressing LGI4(ATG) and mutant LGI4(ACG) were co-transfected with pmScarlet-Giantin (Scarlet-Golgi) into primary Schwann cells and expressed for 48 h. Cells were fixed as routine and probed with antibodies targeting V5 (red) and KDEL (ER: green). Representative images showing successfully transfected cells (Scarlet-Golgi positive cells) and intracellular levels of WT or mutant version of LGI4 (V5 immune-reactivity). Bar = 15 μm. (f) LGI4(ATG) and LGI4(ACG) expression and secretion were analyzed as described under (d)
grown in DMEM supplemented with 10% FBS and Penicillin and Streptomycin at 5% CO₂, 37°C. Cells were transfected using PEI-Max (Polyscienc 24765) or Lipofectamine 3000 (Invitrogen) following established protocols (Reed et al., 2006).

2.4 Immunofluorescence processing, microscopy and image analysis

Transfected primary rat Schwann cells were fixed in 4% PFA/2% sucrose in 0.1 M NaPO₄ pH 7.4 buffer for 15 min at 4°C, washed with PBS, and then permeabilized using 0.1% Triton for 10 min. Next, cells were blocked for 1 h at room temperature in 10% Donkey serum/PBS. Primary antibody incubation (in blocking solution) was carried out at room temperature for 1 h, before washing with PBS (5 × 5 min). Secondary antibody staining (in blocking solution) was carried out at room temperature for 1 h, before washing with PBS (5 × 5 min). Cells were mounted in Mowiol containing DAPI for DNA staining. Samples were examined under a Zeiss Axio Imager widefield fluorescence microscope and images were acquired using a x63 oil lens.

DRG cultures were essentially processed as described above, but with an extended incubation in primary antibody (overnight). Additionally, DRG cultures were also treated with ice-cold methanol for 20 min at −20°C, immediately the following fixation and prior to blocking. DRG samples were imaged with an LSM880 Zeiss Airyscan microscope and using a Plan-Apochromat x20/0.8 M27 air objective. Approximately four 7 × 7 tile scans were acquired per coverslip, collectively covering 4–9 mm².

Images were acquired using an average of 4 line-scans using HeNe633, DPSS 561–10, Argon and Diode 405 lasers. Beam splitters MBS 488/561/633 and MBS 405 were used. Voxel dimensions were 0.123 x 0.123 x 2.5 μm. Z stack consisted of 9–13 sections in total. Tile-scans volumes were “stitched” in XY, projected onto a single slice in Z and saved as an uncompressed tiff file. In Amira (Thermofisher), neurofilament and P0 signals were used to create “auto skeletons,” digital reconstructions of the axonal and myelinated networks. Auto skeleton parameters: Threshold 26–30, smoothing 0.5, iterations 10. “Spatial graph statistics” was used to retrieve parameters such as collective length and diameter and per axonal segment. GFP expressing cells were counted manually.

2.5 Affinity purification and Western blot analysis of LGI proteins

For LGI4 secretion assays, RT4 cells were plated onto 6-well plates and transfected the following day using Lipofectamine 3000 (constructs shown in Figure 1b and Figure S1) in 6-well plates. For experiments presented in Figure 5, 4-Phenylbutyric acid (4PBA) was added to 2.5 mM for 48 h. Conditioned medium and cells were collected 72 h after transfection. Conditioned medium was washed to 100 mM NaPO₄, pH 8, 10 mM Imidazole, 0.1% NP40 and spun at 16,000g to remove death cells and insoluble debris. Cells were washed twice with ice-cold PBS and subsequently collected in 1 ml of 8 M Urea, 0.1 M NaPO₄ pH 8. Cell lysates were briefly sonicated to reduce viscosity. Both cell lysates and conditioned medium were spiked with tissue culture supernatant of HEK293T cells transfected with LGI2 to allow normalization. LGI proteins were purified using Ni-NTA agarose affinity purification and Western blot analysis of LGI proteins.

ice-cold PBS and subsequently collected in 1 ml of 8 M Urea, 0.1 M NaPO₄ pH 8. Cell lysates were briefly sonicated to reduce viscosity. Both cell lysates and conditioned medium were spiked with tissue culture supernatant of HEK293T cells transfected with LGI2 to allow normalization. LGI proteins were purified using Ni-NTA agarose beads for 3 to 4 h at room temperature. Beads were collected by centrifugation and washed twice with 50 mM NaPO₄ pH 8, 300 mM NaCl, 10 mM Imidazole before eluting proteins in 2x Laemmli loading buffer. Samples were reduced by addition of DTT to 50 mM, denatured for 10 min at 70°C, and separated on a 4%–12% or 8% precast Bolt mini protein gel. Proteins were electro-blotted onto Nitrocellulose membranes (Amersham 0.45 μm). Membranes were blocked in milk (Seracare) and incubated with anti-V5 antibody overnight at room temperature. After incubating the membrane with HRP-coupled secondary anti-mouse IgG antibody for 45–60 min, blots were

FIGURE 2 LGI4 mutants bind ADAM22. HEK293 cells were transfected with expression cassettes for ADAM22-Fc together with either wildtype or mutant LGI4 (see Figure 1b). The ADAM22-Fc cassette encodes a fusion protein of the extracellular domain of ADAM22 and the human IgG1 heavy chain (a). Processing of the fusion protein through the secretory pathway results in glycosylation and cleavage of the pro-domain (pro) by the Furin convertase. Calculated molecular mass for the processed and unprocessed forms are indicated. Cell extracts and culture medium were analyzed 48 h after transfection. (b) Immunoblot of ProteinA-agarose affinity purified ADAM22-Fc complexes from cell extract and medium were probed with antibodies against the extracellular domain of ADAM22 and V5 (LGI4). The ADAM22 antibody, which binds the Disintegrin domain of ADAM22, detects a predominant 120kD band in the cell extract, which corresponds to the unprocessed glycosylated fusion protein. The same antibody detects a 90kD band in the medium. This protein corresponds to the fully processed glycosylated form (pro-domain cleaved) of the fusion protein. The LGI4 and LGI4 mutant proteins are detected with the V5 antibody. Lower panel showing anti-LGI4 in a contrast enhanced version of the panel immediately above and illustrates the low level of LGI4 mutant protein secreted in the medium in accordance with Figure 1d.
developed using West Pico Plus chemiluminescent substrate (Invitrogen) and imaged on a Licor Odyssey Fc machine. Images were processed using Fiji. All experiments were performed in triplicate and repeated at least twice. LGI4 signals were normalized to LGI2 signals (both detected by the V5 antibody) and analyzed with the unpaired t test using Graphpad Prism 9.

For binding assays, LGI4 (constructs in Figure 1b) was cotransfected with ADAM22-Fc (Figure 2a) and expressed in HEK293T cells for 48 h. Conditioned medium was collected and centrifuged for 10 min at 16,000g at 4°C to remove debris. Cells were washed with PBS before they were scraped from the plate and pelleted. Cell pellets were resuspended in extraction buffer (20 mM Tris pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF and 1x Protease Inhibitor Mix (Sigma P8340) and incubated for 1 h. Insoluble material was removed by centrifugation as above. Conditioned medium and cell lysate were incubated with ProteinA-agarose beads (Repligen) for 2 h at room temperature. Proteins were eluted in 1.5x Laemli loading buffer at 96°C for 4 min and separated on a 4%-12% gradient SDS-PAGE gel (Invitrogen) and analyzed by western blotting as essentially as described above except thatIRDye-coupled secondary antibodies were used.

2.6 Antibodies

Primary antibodies were from the following sources: mouse anti-acetylated tubulin (Sigma T6793) WB 1:1000; mouse anti-neurofilament M (2H3 monoclonal antibody developed by Jessell and colleagues obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of biology, University of Iowa); IF, 1:100), rabbit anti-c-Myc (SC-789, Santa Cruz Biotechnology; WB, 1:1000), mouse anti-V5 (05025C5 and 05021D11, Absea Biotechnology), WB, 1:20, IF, 1:5; mouse anti-Adam22 (Neuromab N57/2), WB 1:100; rat anti Lgi4 (01311H12, Absea Biotechnology), WB 1:100; chicken anti PO (PZO, Aves labs), IF 1000; mouse anti-KDEL (10C3, Abcam) IF 1:500. The following secondary antibodies were used: Alexa594-conjugated donkey anti-chicken, Alexa594-conjugated donkey anti-mouse, Alexa488-conjugated donkey anti-chicken, Alexa647-conjugated donkey anti-mouse, Alexa4594-conjugated donkey anti-chicken, Alexa488-conjugated donkey anti-rabbit (all Jackson Immuno-research). For Western blot anti-mouse IRDye 680CW (LI-COR), anti-rabbit IRDye 800CW (LI-COR), Goat-anti-mouse HRP (Invitrogen A16066) 1:10,000. All Alexa secondary antibodies were used at 1:500 and all LI-COR antibodies used at 1:10,000 dilutions.

2.7 Live imaging microscopy of cocultures

Rat Schwann cells, transduced to express free GFP, were seeded onto DRG-derived neuronal cultures (21 days in vitro) isolated from WT or Adam22Δ1/Δ2 mice. Immediately following seeding, cocultures were mounted into an IncuCyte (Sartorius) for extended live imaging microscopy. Samples were imaged using a 20X objective, with a 10-min frame-rate, for both GFP and phase, over a 12 h period. Using Fiji, uncompressed files (.tiff stacks) underwent Linear Stack Alignment with SIFT prior to analysis using TrackMate. Parameters: Estimated Blob Diameter, 25 μm; Threshold, 0.05; Linking max distance, 30 μm; Gap-closing max distance, 50 μm; Gap-closing max frame gap, 3. Only cells with >10 continuous tracking frames were considered. Schwann cell tracking was delineated into “recruitment” and “migration.” Recruitment phase was considered as the period of non-purposeful, non-directional movement (shown at the track origin of many cells as apparently random “dithering” motion) or/and cell morphology remained round/lacked obvious elongation. Migration phase of tracking was considered when cells moved purposefully and directionally, typically in straight lines (shown in most track footprints as clean lines) (Figure 4cv and cvi).

3 | RESULTS

3.1 In silico analysis

The high sequence homology between LGI4 and LGI1 (48% identities, 20% similarities) and the recent elucidation of the crystal structure of LGI1 (Yamagata et al., 2018) allowed the computational modeling of the LGI4 structure and mapping of the mutated amino acid residues (Kelley et al., 2015) (Figure 1). Polyphen (Adzhubei et al., 2013) and Provean (Choi et al., 2012) algorithms both suggest that the Lgi4 mutations are deleterious. The position of these mutated residues in the second and fifth blade of the LGI4 Epitemin seven-bladed propeller structure (Figure 1) could potentially affect the ADAM22 interaction interface (yellow residues), the myelination promoting interface (red residues), or the general stability of the Epitemin structure. The latter could influence the secretion of the respective mutant LGI4 proteins as it passes through the quality control system of the endomembrane. Like LGI4, the secretion of LGI1 is critical for its function. Both secretion-positive and secretion-impaired LGI1 mutant proteins have been reported to cause autosomal dominant lateral temporal epilepsy (ADLTE) (Dazzo et al., 2016; Yokoi et al., 2015).

3.2 LGI4(R258P) and LGI4(V434D) accumulate in the ER and are secreted at strongly reduced levels

To test whether LGI4(R258P) and LGI4(V434D) mutant proteins are secretion positive or negative we performed a series of expression experiments in cultured cells. Epitope-tagged LGI4 proteins were generated (Figure 1b) and expressed in rat Schwann cells for 48 hours, before processing for light microscopy imaging (Figure 1c). Experiments using wild-type LGI1 (LGI1 WT) and the secretion-impaired mutant LGI1 (LGI1 E383A) were performed in parallel and served as...
control (Kalachikov et al., 2002; Yokoi et al., 2015). Cells were co-transfected with Scarlet Golgi. LGI signal (V5) was normalized to Scarlet Golgi to account for differences in individual cell transfection/expression levels (Figure 1c). V5 signal colocalizing with ER (KDEL) was measured. A significant increase in ER-localized LGI4 fluorescence intensity (red channel) was observed in cells transfected with LGI4(R258P) and LGI4(V434D) mutant proteins (60.6 ± 8.6 and 43.8 ± 8.4 respectively) compared with LGI4 WT (15.7 ± 2.5) transfected cells (Figure 1c–lower panel). As expected, a significant increase in cellular LGI1(E383A) retention was also observed, compared with its WT LGI1 counterpart (27.3 ± 4.0 and 7.5 ± 0.8, respectively). Thus, the intracellular LGI4 and LGI1 mutant proteins co-localize with KDEL immune-reactivity, suggesting that a major portion of the mutant proteins accumulate in the ER compartment.

To establish how much, if any, mutant LGI4 protein was secreted, we next expressed the same expression plasmids in rat Schwannoma (RT4) cells, followed by Ni-NTA affinity purification from cell extract and conditioned medium (Figure 1d). As expected, most of LGI4 was found in the medium (Figure 1d Medium). In contrast, most of the mutant LGI4 was found in the cell extract (Figure 1d Cell). However, low levels of mutant LGI4 protein were still detected in the culture medium (17% and 4% for LGI4(R258P) and LGI4(V434D), respectively) (Figure 1d). Thus, both R258P and V434D mutations severely impair progression of LGI4 through the endo-membrane system, accumulating in the endoplasmic reticulum, resulting in strongly reduced secretion levels of the mutant LGI4 protein.

3.3 The LGI4 c.2 T > C mutation strongly reduces expression of LGI4

We next examined the effect of another type of mutation recently described in an Indian AMC patient (Mishra et al., 2020). This mutation (c.2 T > C) changes the Methionine start codon (AUG) into a Threonine codon (ACG) and could thus severely affect translation efficiency of the LGI4 mRNA (Kearse & Wilusz, 2017). We modified the mouse LGI4 cDNA to reflect the exact context of the human LGI4 start codon (LGI4 ATG) and introduced the c.2 T > C mutation (LGI4 ACC) (see Figure S1). Both plasmids were transfected together with Scarlet-Golgi into rat Schwann cells and their intracellular expression examined. Whereas low levels of LGI4 ACC are observed in the ER of transfected Schwann cells no expression of LGI4 ACC was detected at these levels of sensitivity, suggesting that indeed the LGI4 ACC mRNA is poorly translated (Figure 1e). Western blot analysis of transfected RT4 Schwann cells and conditioned medium confirmed that LGI4 ACC is indeed expressed at much-reduced levels resulting in low levels of secreted LGI4 protein (at ~8% of wildtype levels; Figure 1f).

3.4 Mutant LGI4 proteins bind their receptor ADAM22

We next investigated whether the secretion-impaired LGI4 mutant proteins were also affected in their binding to their receptor ADAM22. We co-expressed in HEK293 cells wild-type or mutant versions of LGI4 together with ADAM22-Fc; a fusion between the ADAM22 extracellular domain and human Fc (IgG1: Figure 2a) and purified the LGI4-ADAM22-Fc complexes from cell extracts and medium using protein A beads (Figure 2b). All versions of LGI4 could be co-purified with ADAM22-Fc from cell extracts, with very little difference observed in the abundance of purified WT or mutant LGI4 (Figure 2b cell lysate). Both WT and mutant versions of LGI4 could also be co-purified from cell medium (Figure 2b medium), however, the amount of co-purified mutant LGI4 was significantly lower than that of LGI4 (84%–88% reduction), in line with our demonstration that mutant LGI4 protein secretion is severely impaired (Figure 1d). Thus, the LGI4 R258P and V434D mutations do not affect binding of the mutant protein to its receptor ADAM22. Additionally, co-expression of the mutant LGI4 protein with its ADAM22 receptor does not improve its secretion nor does mutant LGI4 protein negatively influence ADAM22-Fc processing and secretion.

3.5 Reduced secretion of LGI4 mutant proteins result in strongly reduced levels of myelination

LGI4 is widely expressed, but its function has been best defined within the PNS (Bermingham et al., 2006; Nishino et al., 2010; Ozkaynak et al., 2010). Here, a major function of Schwann cell-derived LGI4 is to promote axonal sorting and myelination through binding of ADAM22 expressed on the axon (Ozkaynak et al., 2010). Structural and functional studies have shown that a small ensemble of amino acids on the side of the doughnut-shaped Epitempin domain (see Figure 1a) are critically required for this myelin-promoting activity of LGI4 (Kegel et al., 2014). Having shown that the LGI4 mutations R258P and V434D affect the level of secretion of the mutant protein but not its ADAM22 binding properties, we now asked whether these mutations affect the myelin-promoting activity of LGI4. To explore the myelin-promoting properties of LGI4 mutants we used genetic complementation in an in vitro myelination assay and advanced microscopy imaging (Figure S2). Briefly, dorsal root ganglia (DRG) were dissected from embryonic claw paw (Lgi4 clp/clp) mice and transduced with retroviral particles (that only infect dividing cells) to express either WT or mutant versions of LGI4 (Figure 1b, see also Figure S3-normalization of viral titers). Free GFP is also expressed from the same construct and is a useful marker to identify successfully infected cells. Claw paw (clp) mice carry a mutation in the Lgi4 gene that severely reduces secretion of the Lgi4(clp) protein and DRG explant cultures derived from these mice do not myelinate (Bermingham et al., 2006; Kegel et al., 2014). Neuronal cultures transduced with viral vectors expressing either LGI4, LGI4(R258P) or LGI4(V434D) were allowed to myelinate, in vitro, for 3 weeks, before processing for super-resolution light microscopic imaging (Figure 3). Myelin was visualized using an antibody against the major myelin protein P-zero (MPZ; green). In agreement with previous studies, the expression of wildtype LGI4 in Lgi4(clp)-DRG cultures results in extensive
myelination (Bermingham et al., 2006; Kegel et al., 2014), whereas the non-transduced control Lgi4(clp)-DRG cultures do not show any myelin (Figure 3a). Crucially, some myelin, albeit markedly reduced, was present in cultures infected with either LGI4(R258P) or LGI4 (V434D) mutants (Figure 3a). This posed an important question; is the reduced level of myelination a direct consequence of the reduced secretion of the mutant protein or is its intrinsic myelin-promoting activity affected?

**FIGURE 3** LGI4 mutant proteins stimulate myelination commensurate with their levels of secretion. LGI4 or LGI4 mutant genes (see Figure 1b) were introduced through retroviral transduction into dorsal root ganglia (DRG) explant cultures derived from clp mice. Following infection, cultures were maintained under myelination conditions for ~3 weeks before fixation with paraformaldehyde and processing for light microscopy. Samples were probed with antibodies directed against GFP (white), Neurofilament (red), and MPZ (green).

(a) Representative microscopic images of untreated (control) cultures and cultures infected with viral particles to express LGI4, LGI4(R258P), and LGI4 (V434D). (b) Multiple tile-scan volumes (7 × 7 to 8 × 8) were acquired and analyzed using Amira.

“Distance Skeleton” shows representative “tracks” of axons (using neurofilament) and myelin (using MPZ). Distance skeleton “heat-map” colors represent fiber thickness (blue = low caliber, red = high caliber). Bar = 200 μm. (c) Summary statistics from the analyses in (b).
We explored this question using automated digital reconstruction software (Amira, Thermofisher). Our volumetric tile-scans, captured for each condition, collectively covered ~4–9 mm² of the center of each coverslip. Using the cultures infected with WT LGI4 as a guide, we were able to confirm that this area corresponded to ~90% of the myelinating axons present on the coverslip (Figure S2B). Amira was used to retrieve a selection of measurements, including total length of axons (as revealed by neurofilament staining) and myelinated axons (as revealed by Myelin Protein Zero staining) for each of the culture conditions (Figure 3b). As expected, no myelinated axons were detected in untreated controls (Figure 3b and c). In contrast we estimated that ~15% of axons were myelinated in cultures expressing WT LGI4 (Figure 3b and c). Strikingly, only an ~0.4%–0.9% of axons were myelinated in cultures expressing LGI4(R258P) or LGI4(V434D) (Figure 3b and c). This level of “hypomyelination” equates to 3%–6% of that observed in the wildtype LGI4 control. This low level of myelin corresponds roughly to the protein abundances measured for direct protein secretion (Figure 1), suggesting that, at least at these low levels of secretion, a relationship exists between the abundance of protein secreted and the amount of myelin produced.

We recently reported that super-resolution light microscopy provides sufficient resolving power to accurately measure axon geometry in myelinating cocultures, including axon diameter (Booth et al., 2019). Therefore, we were able to demonstrate that none of the above findings were a result of differences in axon caliber, as both the myelinated and unmyelinated axons had comparable diameters in each of the samples (Figure 3cii and ciii). This analysis suggests that the R258P and V434D mutations do not severely affect the myelin-promoting activity of the LGI4 protein per se and that myelination is graded to the secretion level of LGI4, at least at these low levels of expression.

3.6 | LGI4/ADAM22 regulates Axon-Schwann cell engagement

Although the importance of LGI4 in axonal sorting and myelination is well established, its precise molecular activity remains ill-defined. The genetic deletion of LGI4, or its axonal receptor ADAM22, results in severe congenital hypomyelination, however it is not yet known whether this is due to a role in local signaling at the Schwann cell/axon interface, and/or whether LGI4 and ADAM22 are also required for Schwann cell engagement with the axonal membrane.

We next considered whether the reduction in myelination in our assays was a direct result of the myelin-promoting ability of the LGI4 (R258P) or LGI4(V434D) expressing Schwann cell per se, or rather, a reduction in the engagement of Schwann cells with axons. We revisited our micrographs to quantify the frequency that GFP expressing Schwann cells could be found aligned to an axon (Figure 4a, Figure S4). Strikingly, a ~50% reduction was found in the number of GFP expressing cells aligned to axons in cultures expressing mutant versions of LGI4 (22 ± 4 and 26 ± 8 Schwann cells per 100 mm of axon for LGI4(R258P) and LGI4(V434D), respectively), compared with the wild type LGI4 control (47 ± 6 Schwann cells per 100 mm of axon) (Figure 4bi). Moreover, the percentage of engaged Schwann cells actively myelinating (as revealed by MPZ staining) is reduced in cultures transduced with Lgi4(R258P) (~8%) or Lgi4(V434D) (~4%) compared with cultures transduced with wildtype Lgi4 (~14%) (Figure 4Bii). It is unlikely that these different levels of engagement and myelination can be attributed to different levels of expression between cultures, as viral titers were normalized (Figure S3). This suggests that LGI4 performs a dual function in Schwann cell engagement and sorting and myelination of axons, via an as of yet undefined pathway.

The reduced engagement of mutant Schwann cells with axons could be the result of a reduction in the initial attachment of Schwann cells to the axonal membrane and/or a reduction in process elongation and migration along the axon. These cellular processes have been shown to depend on RGD -binding integrins such as the beta3/alphav integrin (Catignas et al., 2021). Moreover, the RGD-binding integrins have been demonstrated to bind ADAM receptors (Cal et al., 2000; D’Abaco et al., 2006), suggesting that LGI4 could modulate Schwann cell attachment and migration through its obligate receptor ADAM22. To begin to distinguish between these possible cellular mechanisms we investigated the initial association and migration of wild-type Schwann cells along ADAM22 mutant axons. We established primary sensory neuron cultures from Adam22Δ/Δ mouse embryos and seeded the axon bed with rat Schwann cells that were transduced with a GFP lentivirus to allow tracing of individual cells. Immediately after seeding cultures were placed in an incubator with live video recording capability (IncuCyte, Sartorius). The behavior of Schwann cells was monitored over a period of 12 h (Videos S1 and S2). We examined the time it took for a Schwann cell to attach to an axon (recruitment time) but also the total distance it migrated along the axon (axonal migration) over the 12-h time frame (Figure 4c and d, di, dii). No significant difference was observed for Schwann cell recruitment. However, a subtle but significant difference revealed increased migration of Schwann cells about the length of Adam22Δ/Δ axons, compared with those of WT controls (53.12 ± 4.63 μm and 36.21 ± 3.76 μm, respectively). This could suggest that Schwann cell migration along the axons relies on LGI4/ADAM22 interaction and is a point of interest for future studies.

3.7 | 4PBA improves secretion of LGI4 mutant proteins

Our data show that the secretion-deficient LGI4(R258P) and LGI4(V434D) proteins are functionally intact, suggesting that increased secretion of these mutant proteins might be of therapeutic benefit. Indeed, it has been demonstrated that in a preclinical seizure mouse model based on the secretion-deficient LGI1 mutant LGI1(E383A), treatment with the compound phenylbutyrate (4PBA) reduces seizure susceptibility in these animals. Phenylbutyrate is marketed under different names and is primarily used in treating urea cycle disorders but has also been described to function as a histone deacetylase inhibitor and chemical chaperone (Vega et al., 2016). It is this chemical chaperone function of 4PBA that is believed to be responsible for
Schwann cell engagement is reduced in cultures transduced with LGI4 mutant genes. (a) Representative examples of GFP expressing Schwann cells (green) aligning with axons (neurofilament - magenta). The upper panels show an overview, whereas the lower panels show two examples of Schwann cells fully engaged with an axon. One myelinating Schwann cell, indicated by P-zero (red) (Zoom/arrow 1) and one non-myelinating Schwann cell (Zoom/arrow 2). Bar = 50 μm. Additional examples shown in Figure S4. (b) Plot showing the total number of Schwann cells engaged with an axon. (bii). The percentage of engaged Schwann cells that were also myelinating. All data were normalized to 100 mm of axon length. Bars = 40 μm. (c) Live cell imaging microscopy. Cocultures of GFP expressing rat Schwann cells, and non-myelinating DRG cultures isolated from WT or Adam22Δ1/Δ1 mice, were imaged for 12 h to monitor Schwann cell recruitment and migration along axons. (ci–civ) Representative stills (T = 0) of GFP expressing Schwann cells (ci and cii) or GFP + Phase (ciii and civ) for WT (ci and ciii) and Adam22Δ1/Δ1 (cii and civ) samples. (c–d) TrackMate (Fiji) was used to map initial Schwann cell recruitment and then migration along the axon. Representative tracking footprints are shown for WT (cv) and Adam22Δ1/Δ1 (cvi) samples. Bars = 50 μm. Quantifications of tacking data are shown for Schwann cell recruitment (dii) and migration along axons (dii). Ncell20 Nrepeat3. Red bars show mean ± SEM. Data statistically analyzed using an unpaired t test (Prism).
We report that both LGI4(R258P) and LGI4(V434D) exhibit severely reduced levels of secretion, but that these residual levels result in some relieve of a “hypomyelination” status in an in vitro Schwann cell-neuron coculture system. Thus, we propose that the clinical phenotype in this compound heterozygous patient largely results from severely reduced secretion of the variant proteins that are otherwise functional and suggest that differences in clinical severity in this LG4-mutant associated AMC patients versus full loss of LGI4 mutant patients is due to differences in the residual secretion of the mutant protein.

Recently, a second report described a fifth family to carry a missense mutation in the start codon (c.2 T > C) of the LGI4 gene (Mishra et al., 2020). One affected child with multiple contractures, hypotonia, and severe respiratory distress, died two and a half months after birth, whereas a second child with milder clinical presentation survived and was examined at two and a half years of age. Both individuals are homozygous for the c.2 T > C mutation. This mutation changes the Methionine start codon into a Threonine codon. No alternative upstream or downstream AUG start codon is present that could provide an alternative translational start site and result in a secreted LGI4 protein. However, translation of mRNAs is not exclusively initiated from an AUG codon as flanking nucleotides play crucial roles in initiation codon selection (Keese & Wilusz, 2017). In particular, the Guanine at position 4 and the Purine (G; see Figure S1) at −3 provide direct interactions with the translation pre-initiation complex and determine the strength of the start codon. Cell transfection assays have shown that the ACG start codon in a proper context results in a more than 10-fold (7%) reduction of reporter protein expression (Ivanov et al., 2010). Indeed, our transfection experiments similarly demonstrate that the LGI4(ACG) mutation results in strongly reduced expression of the protein and consequently reduced secretion to ~8% of wildtype levels. Thus, similar to the two mutations studied here and the mouse Lgi4 claw paw mutation (Bermingham et al., 2006), it is likely that this novel LGI4 mutation represents a strong hypomorphic allele. However, in contrast to these mutant LGI4 proteins, the c.2 T > C allele encodes a fully wildtype LGI4 protein, which might account for the relatively mild clinical phenotype in this patient (Mishra et al., 2020).

The data presented here suggest that an increase in the secreted mutant protein, but largely functional, LGI4 could be beneficial and improve outlook for these patients. Therapeutic strategies that aim to increase secretion or cell surface expression of mutant proteins have been developed for a range of diseases. These strategies focus on the use of osmolytes or molecular chaperones that have shown promise in Cystic Fibrosis and in preclinical mouse models of neurodegenerative diseases such as Parkinson Disease (Brown et al., 1996; Chanoux & Rubenstein, 2012; Cortez & Sim, 2014; Vega et al., 2016). Of particular relevance here is a study by Yokoi and colleagues (Yokoi et al., 2015) who demonstrated that in a preclinical epilepsy mouse model associated with secretion-deficient-LGI1 (LGI1E383A) protein expression, treatment with the compound 4PBA reduces seizure susceptibility. Although the mechanisms of action of these drugs are not always fully understood, it has been demonstrated that some of these compounds alleviate the misfolding of mutant proteins sequestered in the ER, allowing a degree of enhanced secretion (Vega et al., 2016).

4 | DISCUSSION

Here we have dissected the molecular pathways of disease-causing LGI4 mutations, the first to be described in a living human patient.
Our microscopy experiments using transfected Schwann cells indicate that both LGI4(R258P) and LGI4(V434D) mutant proteins are similarly retained in the ER (Figure 1). Indeed, treatment of LGI4(R258P) and LGI4(V434D) with 4PBA increases secretion of these mutant proteins, although statistical significance was only obtained for LGI4(V434D).

Importantly, our results further demonstrate that these mutant proteins retain much of their functional properties. These results provide a promising starting point to screen for additional molecular chaperones and osmolytes to reduce ER stress and improve secretion of these mutant proteins.

The third LGI4 mutation analyzed here, LGI4(c.2 T > C), is of a completely different nature. Successful strategies to improve expression from this LGI4 allele will have to target start codon selection mechanisms. A daunting task as any intervention with this mechanism will affect the translation of a multitude of mRNAs. A large screen in yeast yielded two related quinoline-containing compounds that improved translation from non-canonical start codons (Takacs et al., 2011). However, this was achieved at the expense of fidelity of translation from the canonical AUG start codon.

Our observation that the number of LGI4(R258P) or LGI4(V434D) expressing Schwann cells associated with axons is reduced in Lgi4 mutant cultures relative to wild-type Schwann cells, suggests a role for LGI4-ADAM22 in engagement or stabilization of axon-Schwann cell contacts. Whereas the role of Lgi4 in stimulating proliferation of Schwann cell precursors and promoting sorting and myelination at later stages of Schwann cell development, has been well-documented, a role for Lgi4 in axon-glia adhesion has not been reported earlier. Several cell-adhesion molecules such as N-Cadherin and L1 have been suggested to be involved in Schwann cell-axon adhesion (Seilheimer et al., 1989; Wanner et al., 2006). Moreover, an involvement of RGD-binding integrins such as αvβ3 has been inferred from the observation that Schwann cells adhere to RGD-modified scaffolds and RGD peptide mimetics inhibit Schwann cell migration in vitro (Berti et al., 2006; Catignas et al., 2021; Milner et al., 1997; Sedaghati et al., 2014).

ADAM22 has been demonstrated to interact with α9 and β3 containing integrins and its close relative ADAM23 interacts with αβ3 (D’Abaco et al., 2006; Goldsmith et al., 2004). We have speculated before that Lgi4-mediated ADAM22-integrin interactions could contribute to axonal sorting and myelination (Ozkaynak et al., 2010). By extension, it is possible that Lgi4 contributes to Schwann cell-axon contacts through ADAM22-integrin interactions. However, our live-imaging experiments with Adam22Δ1/Δ1 DRG neurons and wild-type rat Schwann cells suggest that the initial association of Schwann cells with axons is not affected by the LGI4-ADAM22 interaction. It is of note in this context that DRG neurons also express ADAM23, an alternative receptor for LG4, and it is possible that in the absence of ADAM22 the early axonal recruitment events are partly mediated by ADAM23. In support of this suggestion is the observation that the hypomyelination phenotype of young (postnatal day12) claw paw (Lgi4ΔβαΔcΔ) and Lgi4Δv1-2/Δ1-Δ3 mice is more severe than in age-matched Adam22Δ1/Δ1 mice. Future research into the role of LGI4-ADAM in Schwann cell axon interactions will address this possible redundant role of ADAM23.

Another mechanism that could contribute to the reduced engagement with axons of LGI4(R258P) and LGI4(V434D) expressing Schwann cells is ER stress caused by the accumulation of mutant LGI4 protein in the ER compartment of the cell. ER stress, and in particular the unfolded protein response (UPR), has been shown to contribute to a number of peripheral neuropathies characterized by accumulation of mutant protein in the ER (see Clayton & Popko, 2016; Scherer & Wrabetz, 2008; Volpi et al., 2017). Whether LGI4(R258P) and LGI4(V434D) proteins indeed cause ER stress and a maladaptive UPR, is the subject of future research.

In conclusion, we have demonstrated how different AMC-associated LGI4 mutations affect the biology of the LGI4 protein in different ways. Whereas the c.2 T > C mutation severely affects translation efficiency of an otherwise intact protein, the LGI4(R258P) and LGI4(V434D) mutations affect progress through the endomembrane system with accumulation of the mutant proteins in the ER. We provide evidence that the mutant proteins retain much of their functions such as ADAM22 binding and stimulation of myelination and propose that the clinical symptoms in these patients are largely a consequence of the strongly reduced secretion of the mutant proteins. We further show that treatment with molecular chaperone 4PBA improves secretion of mutant protein, which is of potential clinical benefit. How much LGI4 protein expression is required to achieve clinical improvement is not known and will require the generation of a series of hypomorphic alleles for LGI4 and careful measurement of LGI4 protein levels. The genetic complementation essay in myelinating DRG cultures used here, provides an excellent system to screen larger numbers of LGI4 mutant proteins and test the effect of candidate molecules on LGI4 protein secretion and myelination.

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CONFLICT OF INTEREST

The authors declare no competing or financial interests.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no data sets were generated or analyzed during the current study.
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