Novel *CACNA1A* Variant p.Cys256Phe Disrupts Disulfide Bonds and Causes Spinocerebellar Ataxia

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ABSTRACT: Background: Spinocerebellar ataxia (SCA) is a progressive, autosomal dominant neurodegenerative disorder typically associated with CAG repeat expansions.

Objective: We assessed the pathogenicity of the novel, heterozygous missense variant p.Cys256Phe (C256F) in the pore-forming α1-subunit of the Cav2.1 Ca^{2+} channel found in a 63-year-old woman with SCA with no CAG repeat expansion.

Methods: We examined the effect of the C256F variant on channel function using whole-cell patch-clamp recordings in transfected tsA-201 cells.

Results: The maximum Ca^{2+} current density was significantly reduced in the mutant compared to wild-type, which could not be explained by lower expression levels of mutant Cav2.1 α1- protein. Together with a significant increase in current inactivation, this is consistent with a loss of channel function. Molecular modeling predicted disruption of a conserved disulfide bond through the C256F variant.

Conclusions: Our results support the pathogenicity of the C256F variant for the SCA phenotype and provide further insight into Cav2.1 structure and function. © 2021 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society

Key Words: ataxia; autosomal dominant; missense mutation; calcium channels

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Spinocerebellar ataxia (SCA, MIM: 183086) is a progressive, neurodegenerative disease with an autosomal dominant mode of inheritance. Trinucleotide repeat expansions in *CACNA1A*, encoding the pore-forming α1-subunit of voltage-gated Cav2.1 Ca^{2+} channels, belong to the most common causes of SCA (SCA6).[1,2] In addition, single-nucleotide variations in the same gene cause progressive ataxia, episodic ataxia type 2 (EA-2, MIM: 108500), familiar hemiplegic migraine (FHM1, MIM: 141500), and early infantile epileptic encephalopathy 42 (MIM: 617106).[3,4] An association between *CACNA1A* missense mutations and an SCA phenotype is, however, uncommon.

We describe here the novel heterozygous missense mutation p.Cys256Phe (C256F) in a German SCA patient, proving its pathogenicity by demonstrating *loss-of-function* changes in transfected tsA-201 cells. A C256F homology model strongly suggests that the affected cysteine forms a disulfide bond that stabilizes a functionally relevant extracellular domain.

Patients and Methods

The proband was examined at the Department of Neurology, Medical Faculty of the RWTH Aachen University Hospital. Informed consent was obtained for molecular genetic analyses and further scientific use. A next-generation sequencing-based multigene panel was

analyzed at the Center for Genomics and Transcriptomics in Tübingen, Germany. For functional analyses, we introduced the C256F mutation into the human Cav2.1 α1-subunit and expressed it together with accessory β4e- and α2δ2-subunits in tsA-201 HEK cells. Ca^{2+} currents were measured using the whole-cell patch-clamp technique.[5] We generated a Cav2.1 α1 homology model in complex with the α2δ-subunit, using the advanced homology modeling tool in Maestro Release 2019-4. For details, see Supplementary Information.

Results

Patient Phenotype

A 63-year-old woman had been suffering from progressive gait unsteadiness for more than 10 years. In the family history (Fig. 1A), the patient's father was reported with dementia and parkinsonism. Her three siblings were reportedly healthy, but the patient's 32-year-old daughter was affected by an unspecific motor disability reported as infantile cerebral palsy. In our clinical examination, the proband had a pronounced omni-directional, gaze-evoked nystagmus, a reduced suppression of the vestibulo-ocular reflex, ataxic finger-to-nose and heel-to-shin tests on both sides, and a scanning speech pattern. There were no signs of spasticity or muscle weakness, migraine, or episodic neurological symptoms. Except for mild vibratory loss (4/8 at the ankles), we did not observe any sensory involvement. The Scale for the Assessment and Rating of Ataxia score was 6/40 points. A cerebral MRI (magnetic resonance imaging) revealed a profound cerebellar atrophy with preponderance of the vermis area (Fig. 1B). Extensive laboratory tests did not provide any known acquired cause of ataxia in this patient.

Molecular Genetics

We first ruled out pathogenic repeat expansions in *SCA1*, *SCA2*, *SCA3*, *SCA6* (13 CAG repeats on both alleles, normal <18 repeats, pathogenic >20 repeats in *CACNA1A*), *SCA7*, and *SCA17*. A diagnostic gene panel revealed the heterozygous variant c.767G>T: p.Cys256Phe/C256F in *CACNA1A* (NM_001127221) that has neither been described before nor has been identified in healthy control populations (GnomAD). It affects a highly conserved amino acid position (Figure S1) within a DNA repeat region in exon 5. Two

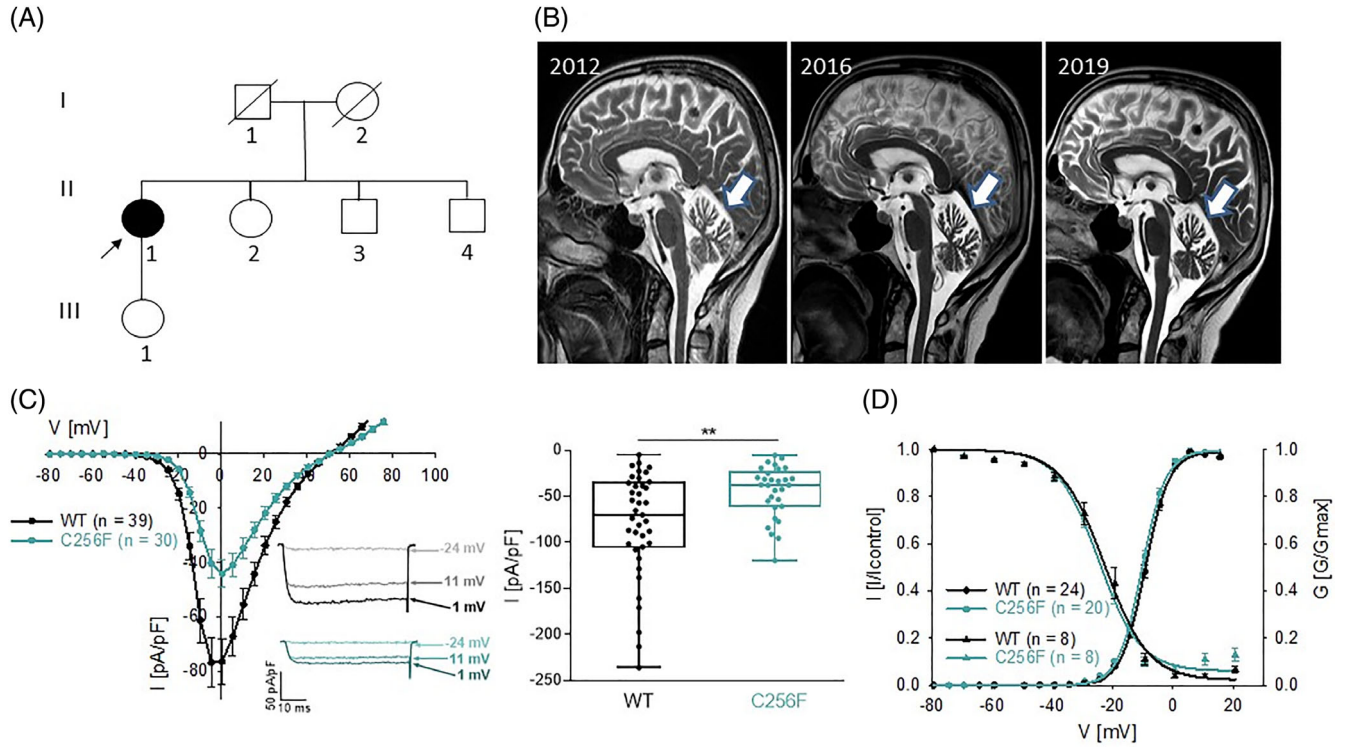

FIG. 1. Pedigree, MRI (magnetic resonance imaging) studies, and effects of C256F on Cav2.1 current density and channel gating. (**A**) Pedigree showing a sporadic case of cerebellar ataxia, with three unaffected siblings. (**B**) Cerebellar atrophy, visible at sagittal T2-weighted MRI sections in the course of 7 years. For quantification of cerebellar volumes, see Figure S4. (**C**) Current–voltage relationship (ICa; mean ± SEM [standard error of the mean]) of WT (wild-type) vs. C256F from parallel (N > 3) transfections. The variant C256F significantly decreased current densities, which is also shown by the boxplots (maximum current density; Student's unpaired *t* test, $^{**}P < 0.01$) and representative current traces obtained from depolarization to −24 mV (activation threshold), 1 mV (the maximal voltage, V_{max}), and 11 mV. Note that the tail current in WT was cut for better visualization. For parameters and statistics, see Table S1. (**D**) Normalized steady-state activation (●) and inactivation (▲) curves of WT versus C256F (mean ± SEM). The mutation C256F had no effect on the voltage dependence of activation and inactivation. For parameters and statistics see Table S1. [Color figure can be viewed at wileyonlinelibrary.com]

pathogenic variants have been previously described at adjacent positions. At the same position, the exchange from cysteine to arginine was classified as pathogenic (Uniprot) in a patient with EA-2. For C256F, in silico predictions were mostly deleterious (11 pathogenic vs. 2 benign).

Functional Characterization of the C256F Missense Variant

Heterologous expression of wild-type and mutant Cav2.1 channel complexes in tsA-201 cells revealed an approximately 45% and highly significant ($P < 0.01$, Student's unpaired t test) reduction in maximal Ca^{2+} current (ICa) density (ICa normalized to the cell size; pA/pF) for the mutant compared to wild-type (Fig. 1C; Table S1). When analyzed at comparable current amplitudes, no effects on the voltage dependence of activation and inactivation gating were found (Fig. 1D; Table S1). Instead, we found a small but significant increase in current inactivation independent from current amplitude and after prespecified time points of 250 and 500 ms during a 5-second depolarizing pulse to the voltage of maximal inward current (V_{max}) (Figure S2A,B). The reduced current amplitude and faster current inactivation are consistent with a loss of channel function. The observed smaller current densities could not be explained by a lower expression of mutant Cav2.1 α1-protein in Western blots compared to wild-type (Figure S3).

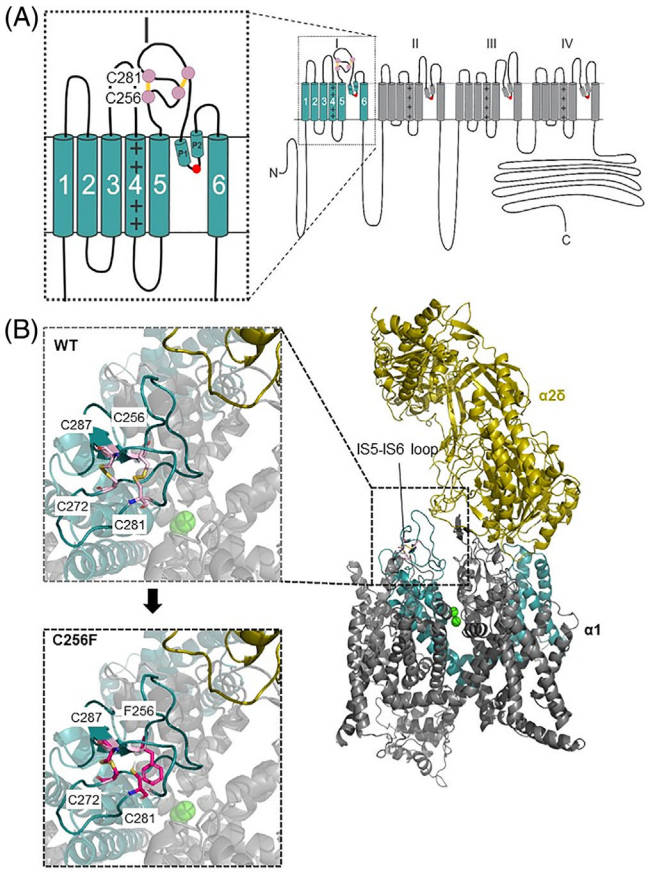

FIG. 2. Structural model of the Cav2.1 α1-subunit in complex with the α2δ-subunit. (**A**) Topology of the Cav2.1 α1-subunit. Inset shows domain I of the α1-subunit (green) with conserved cysteines (pink) forming disulfide bonds (yellow) in the IS5-IS6 loop. P1 and P2 depict pore-forming helices close to the selectivity filter negative charges (red dots). (**B**) Homology model of Cav2.1 WT α1-subunit in complex with the α2δ-subunit (yellow). In the wild-type Cav2.1 α1-protein, C256 is involved in a disulfide bond with C281 and located adjacent to a second disulfide bond formed by C272 and C287 (pink sticks), top inset. In the C256F mutant (blue sticks, bottom inset), this interaction is disrupted. Ca^{2+} ions, bound to the selectivity filter, are shown as light-green spheres, and sulfur, nitrogen, and oxygen molecules are depicted in yellow, dark blue, and red, respectively. [Color figure can be viewed at wileyonlinelibrary.com]

C256F Homology Model

The recent elucidation of rabbit Cav1.1 heteromeric structures[6] allowed to generate a homology model of Cav2.1 α1 in complex with the α2δ-subunit (Fig. 2). C256 forms a disulfide bond with C281 and is located in close proximity to a second disulfide bond formed by C272 and C287 (Fig. 2A,B, top inset). Notably, all four cysteines are strictly conserved among all 10 voltage-gated Ca^{2+} channel α1-subunits (Figure S1), suggesting they are of high functional importance. These covalent bonds may help to stabilize an otherwise flexible loop region lining the top of the channel pore. For reference, Figure 2 shows the Ca^{2+} ions (light green, Fig. 2B) bound to the selectivity filter (red dots in Fig. 2A). In addition, this loop region forms part of the interaction interface with α2δ-subunits. These subunits are required for proper membrane targeting of the channel complex.[7,8] The C256F missense variant prevents the formation of the disulfide bond with C281, and modeling suggests that its bulky side chain cannot be accommodated in a similar manner as the smaller and polar cysteine side chain (Fig. 2B, bottom inset). This strongly suggests that the C256F mutant disrupts the structural integrity of the loop region via the loss of this disulfide bond, thus increasing its flexibility and/or facilitating altered pairing of cysteine residues.

Discussion

The gene *CACNA1A* has previously been associated with SCA type 6, typically manifesting with late-onset gait disorders.[1,2] Other reports describe a classic phenotype, including directional nystagmus, limb ataxia, and vibratory loss, reminiscent of our patient as well.[2,9] SCA6 diagnosis usually relies on trinucleotide repeat expansions in the *CACNA1A* gene, leading to the expanded polyglutamine tract in the Cav2.1 α1 C-terminus. Not only is the polyglutamine tract expressed with the full-length channel, but also exists in a separate peptide (α1ACT), which serves as a transcription factor and is generated by proteolytic cleavage or independently transcribed from a second cistron.[10]

Heterozygous *loss-of-function* missense or stop variants are typically associated with episodic ataxia, a similar

autosomal dominant syndrome manifesting by attacks of ataxia, nystagmus, vertigo, and sometimes further signs like dystonia. In contrast to progressive ataxia, this condition manifests in childhood and improves or stabilizes over time.

The patient described here represents one of the rare cases with progressive ataxia without (not even borderline) trinucleotide repeat expansions, but with a missense variant in *CACNA1A*. Only few other cases have been published with this extraordinary combination before,[11-13] and functional evidence on these variants is lacking.

This novel variant has not been identified in healthy controls (gnomAD) and affects a conserved position within a functionally relevant region. At the same position, another missense variant, C256R (also not listed in gnomAD), was observed in a family with EA-2.[14] Another missense mutation disrupting the second disulfide bridge in this loop between C272 and C287 (Fig. 2; Figure S1), C287Y, has been identified in association with mild to moderate interictal and progressive ataxia.[15] Interestingly, heterologous expression of C287Y under comparable experimental conditions in COS-7 cells also revealed a strong reduction in current amplitude most likely due to a reduced membrane targeting.[15] This *loss-of-function* phenotype is consistent with other ataxia-associated missense mutations[16] and is very similar to the reduced current amplitudes of the C256F variant reported here. Together, these findings strongly suggest that the disruption of any of the two adjacent disulfide bridges in the extracellular pore loop of homologous repeat I reduces Cav2.1 Ca^{2+} channel activity, explaining a *loss-of-function* mechanism. Taking this clinical, genetic, and functional evidence together, we therefore conclude that *CACNA1A*-encoded Cav2.1 channels do not tolerate disruption of these disulfide bonds and that the novel variant C256F is pathogenic. For the first time, we provide functional evidence on an SCA-related missense variant, showing that episodic ataxia and SCA both share a *loss-of-function* pathomechanism. We recommend the screening of *CACNA1A* for strategically important missense variants in CAG repeat-negative SCA cases. ■

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Data Availability Statement

Data are available on request by contacting the corresponding author. ■

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.