The TMEM240 Protein, Mutated in SCA21, Is Expressed in Purkinje Cells and Synaptic Terminals

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Abstract
A variety of missense mutations and a stop mutation in the gene coding for transmembrane protein 240 (TMEM240) have been reported to be the causative mutations of spinocerebellar ataxia 21 (SCA21). We aimed to investigate the expression of TMEM240 protein in mouse brain at the tissue, cellular, and subcellular levels. Immunofluorescence labeling showed TMEM240 to be expressed in various areas of the brain, with the highest levels in the hippocampus, isocortex, and cerebellum. In the cerebellum, TMEM240 was detected in the deep nuclei and the cerebellar cortex. The protein was expressed in all three layers of the cortex and various cerebellar neurons. TMEM240 was localized to climbing, mossy, and parallel fiber afferents projecting to Purkinje cells, as shown by co-immunostaining with VGLUT1 and VGLUT2. Co-immunostaining with synaptophysin, post-synaptic fractionation, and confirmatory electron microscopy showed TMEM240 to be localized to the post-synaptic side of synapses near the Purkinje-cell soma. Similar results were obtained in human cerebellar sections. These data suggest that TMEM240 may be involved in the organization of the cerebellar network, particularly in synaptic inputs converging on Purkinje cells. This study is the first to describe TMEM240 expression in the normal mouse brain.

Keywords TMEM240 · SCA21 · Cerebellum · Purkinje cell · Synapse · Immunohistochemistry

Introduction
Autosomal dominant cerebellar ataxias (SCAs) constitute a clinically and genetically heterogeneous group of diseases characterized by degeneration of the cerebellum, brainstem, and spinal cord [1]. Spinocerebellar ataxia type 21 (SCA21) is defined by mild but slowly progressive cerebellar ataxia that is frequently associated with varying degrees of cognitive impairment and Parkinsonism. We recently reported that SCA21 is caused by mutations in the transmembrane protein 240 (TMEM240) gene, previously called C1orf70 [2, 3]. Six missense mutations and one stop mutation in the TMEM240 gene have been described in patients in France, China, Japan, and, very recently, Germany, Colombia, and the Netherlands [2–6]. The TMEM240 gene encodes a small transmembrane protein of unknown function, which has not yet been characterized. To date, non-targeted analyses in the mouse have detected TMEM240 protein in synaptic membranes of the brain [7]. TMEM240 is transcribed in the adult human brain—particularly in the cerebral cortex, cerebellum, dentate gyrus, putamen, and caudate nucleus [8, 9]. Immunohistochemical staining of the human cerebellum has shown moderate positivity in Purkinje-cell membranes [9]. Although the cerebellum is well known to play a crucial role in movement coordination, balance, and vestibular control, a growing body of evidence has highlighted the structure’s involvement in non-motor functions, such as cognition, language, and emotion [10, 11]. Cerebellar damage (including the damage observed in SCA) can be associated with cognitive impairment, such as that seen in cerebellar cognitive affective syndrome (CCAS)
[12, 13]. In cases of SCA21, brain MRI has revealed atrophy of the vermis and the cerebellar hemispheres.

The anatomic structure of the cerebellum has been highly conserved throughout evolution. In mammals, the cerebellum is divided into 10 lobes separated by fissures of various depths [14]. The cerebellar cortex has a well-defined architecture comprising three different layers, each of which is composed of distinct but interconnected cell types. The external molecular layer contains not only inhibitory basket and stellate neurons but also climbing and parallel excitatory fibers that connect to Purkinje-cell dendrites. The Purkinje-cell layer consists of a single sheet of Purkinje-cell bodies, ascending Golgi-cell dendrites, descending basket cell dendrites, climbing fibers, and basket cell bodies. The internal granular layer contains a large number of granule cells. A significant proportion of the granular layer is occupied by glomeruli, which consist of dendrodendritic and axodendritic synapses mainly composed of mossy fibers and inhibitory Golgi-cell dendrites that converge on granule-cell dendrites [15]. The granular layer also contains Purkinje-cell axons leading to a broad network of fiber tracts that connect to deep nuclei (the fastigial, interposed, and dentate nuclei). These nuclei receive inhibitory inputs from Purkinje cells, as well as excitatory inputs from mossy and climbing fibers. The deep nuclei project numerous efferents to other parts of the brain and spinal cord [16]. Purkinje cells are the main targets in the cerebellar network; they receive and integrate excitatory and inhibitory inputs and transfer inhibitory signals to deep nuclei. There are two sources of afferents in the cerebellum: climbing and mossy fibers. Climbing fibers arise from the inferior olivary nucleus and pass through the granular layer to connect to Purkinje-cell bodies and dendrites. Mossy fibers arise in various areas of the brain and spinal cord, cross the white matter to reach the glomeruli, and target granule-cell dendrites. The axons of granule cells reach the molecular layer, where they form T-shaped branches, resulting in two branches of parallel fibers [17]. Here, we investigated TMEM240 protein expression in the adult mouse brain and observed expression in various areas, especially the isocortex, hippocampus, and cerebellum. We detected TMEM240 in most cerebellar neurons (especially Purkinje cells) and mossy, parallel, and climbing fibers. Finally, we observed a similar pattern of expression in human cerebellar sections.

Materials and Methods

Animals and Tissue Preparation

Experiments were performed on two-month-old male C57BL/6 mice (Charles River, Lyon, France). Animals were maintained in standard cages and under conventional laboratory conditions (12-h/12-h light/dark cycle, 22 °C), with ad libitum access to food and water. For immunohistochemistry experiments, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) before transcardial perfusion with 0.9% NaCl and then 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) (pH 7.4). For electron microscopy, 0.1% glutaraldehyde [25%] (for the LR White-embedding method) or 1% glutaraldehyde [25%] and picric acid (for the Araldite-embedding method) were added to the perfusion buffer. Brains were removed, post-fixed in 4% paraformaldehyde for 24 h at 4 °C, soaked in 30% sucrose in PBS (for cryoprotection), and then frozen. Sagittal and coronal sections (14 to 40 μm thick) were prepared on a cryostat and mounted on SuperFrost Plus™ Adhesion slides (Thermo Scientific, Brunswick, Germany). Prior to biochemistry analyses, brains were quickly isolated after decapitation and snap-frozen at −80 °C. The mice were maintained in compliance with European standards on the care and use of laboratory animals. Experimental protocols were approved by the local animal ethical committee (approval APAFIS#2264-2015101320441671 from CEEA75, Lille, France).

Post-mortem Brain Samples

Human brains were obtained from the Lille Neurobank (CRB/CIC1403 Biobank, BB-0033-00030, agreement DC-2008-642), which fulfills the criteria of the local laws and regulations on biological resources with donor consent, data protection, and ethical committee review. We used samples from the cerebellum (superior, ventral, and inferior vermis) from four donors with no signs of motor or cognitive decline. The age at death of the four donors was 22, 25, 37, and 78 years of age. All donors died from cardiovascular disease, with no neurological issues. The post-mortem interval was < 48 h. Neuropathological examination excluded any neurodegenerative diseases and the Braak stage was 0. Formalin-fixed and paraffin-embedded tissues were used for immunofluorescence staining of 7-μm-thick sections.

Generation of a New Rabbit Polyclonal Antibody Recognizing TMEM240

Rabbit polyclonal antibodies were raised against a synthetic peptide (PYDGDQSVVDASENY) conjugated to keyhole limpet hemocyanin (KLH) (GeneScript, Piscataway, NJ) and corresponding to amino acids 63 to 77 of human TMEM240. Two rabbits were immunized with the purified immunogenic peptide by intramuscular injection at days 0, 14, 28, and 42 using a commercial service (Agro-Bio, La Ferté Saint-Aubin, France). We screened the sera of the two rabbits at days 28 and 42 by ELISA, followed by western blotting and immunohistochemistry. In each experiment, we used mouse cerebellum lysate as a positive control and incubated it with the sera alone or with either the antigenic peptide or recombinant human
TMEM240 protein (Origene, Herford, Germany). After screening at days 28 and 42, both rabbits were selected and received a final injection at day 64 before collecting the blood. Only the polyclonal antibody from the rabbit giving the best results during screening was used in this study.

**Immunofluorescence**

The specificity of the TMEM240 antibody (C1orf70, G16, sc-245675, Santa Cruz Biotechnology and TMEM240/63-77) was verified in two competition studies: incubation of the primary antibody (5 μg/mL) with (1) human recombinant protein (recombinant protein of human chromosome 1 open reading frame 70 (C1orf70), #TP325178, Origene, Rockville, MD) or (2) blocking peptide (sc-245675P, Santa Cruz Biotechnology) (25 μg/mL) or TMEM240/63-77 peptide (amino acid sequence: RHHHYVIPYDGQ, corresponding to amino acids 55 to 68 of human TMEM240) (25 μg/mL). Another competition study was performed by incubating the TMEM240 antibody with a non-respective blocking peptide (25 μg/mL). Finally, a non-specific blocking peptide control experiment was performed by incubating both TMEM240 antibodies with a non-competitive BDNF peptide (amino acids 166 to 178 #ANT-010, Alomone Labs, Jerusalem, Israel). Sections were blocked in PBS supplemented with 1% serum from the corresponding antibody species (Table S1). For single and double immunohistochemical staining, 40-μm floating sections were incubated with primary antibodies overnight or up to two days at 4 °C in specific blocking serum. The antibodies used for immunostaining are listed in Table S1. After removal of the primary antibody (by washing with 0.1 mol/L PBS, pH 7.4, supplemented with 0.1% Triton™ X100 (Sigma), slices were incubated with the corresponding secondary fluorescent antibody at room temperature for 45 min. Washing was performed using 0.1 mol/L PBS, pH 7.4. An autofluorescence eliminating reagent (Sudan Black) was used on all sections. The cell nuclei were counterstained with DAPI (Life Technologies, dilution 1/5000) for 5 min. Sections were mounted on SuperFrost Plus™ Adhesion slides (Thermo Scientific, Brunswick, Germany) with KPL mounting medium (Seracare, Life Sciences, Millford, MA, USA).

**Image Acquisition**

Fluorescence was observed with Zeiss Axio Imager Z2, confocal Zeiss LSM 710, and Zeiss spinning-disk microscopes. Images were acquired using Zen Lite software and analyzed using Fiji-ImageJ software. The acquisition parameters were selected by the software for each experiment, with corresponding brain sections incubated with the secondary antibody alone. Each image was obtained using the selected acquisition parameters to avoid background staining. For whole brain and cerebellum images, tiles and maximum intensity projection images were obtained on 35-μm-thick sections. Relative grayscale values were obtained by manually drawing regions of interest (ROIs) for various brain structures, such as the cerebellum, deep nuclei, and layers of the cerebellar cortex. Pixel intensities in each ROI were quantified on RGB images converted into grayscale images. The background values were subtracted prior to quantification (the threshold was the same within experiments). The data were normalized by square root transformation to obtain a normal distribution and reduce skewness. Finally, all values were divided by the ROI surface to obtain the relative grayscale values. At least three independent experiments were performed before quantification or co-localization analyses. For statistical analysis, at least 10 images were measured for three different animals for brain structure quantification. Thus, there were 30 independent measurements for each structure. Co-localization of staining was evaluated with Pearson’s correlation coefficient (PCC) using Zen (Zen Black, Carl Zeiss Microscopy, GmbH, Munich, Germany) or Imaris software (Bitplane AG, Zurich, Switzerland).

**Preparation of Pre- and Post-synaptic Density Fractions**

Cerebellar lysates were homogenized in Tris-sucrose buffer (10 mM Tris, 10% sucrose) and the protein concentrations determined using the BCA assay. In total, 100 μg protein lysate was homogenized in 500 μL Tris-sucrose buffer. Homogenates were sonicated and centrifuged at 1000g at 4 °C for 10 min. Supernatants were collected and centrifuged at 12,000g at 4 °C for 20 min. Pellets were washed in 500 μL Tris-EDTA buffer (4 mM Tris, 1 mM EDTA, pH 7.4) and centrifuged two times at 12,000g at 4 °C for 20 min. Pellets were resuspended in Tris-NaCl Triton buffer (20 mM Tris, 100 mM NaCl, 0.5% Triton™X100, pH 7.2) and homogenized at 4 °C for 15 min. After centrifugation at 15,000g at 4 °C for 20 min, the supernatants, corresponding to post-synaptic density (PSD) fractions, were washed three times in Tris-NaCl Triton buffer. The pellets, corresponding to non-PSD fractions, were washed in 100 μL Tris-NaCl-Triton. Samples were mixed with NuPage® LDS 2× sample buffer supplemented with 20% NuPAGE® sample reducing agents (Invitrogen, Carlsbad, CA, USA) and equal volumes were loaded onto gels and analyzed by western blotting. Duplicate samples for PSD and non-PSD fractions were loaded.

**Western Blotting**

Samples were homogenized in an appropriate volume of Tris-sucrose buffer and protein concentrations determined using the BCA assay. One volume of NuPage® LDS 2× sample
buffer, supplemented with 20% NuPAGE® sample reducing agents (Invitrogen, Carlsbad, CA, USA), was added to the homogenates. Samples were then heated 10 min at 100 °C. After loading 30 μg total protein lysate, molecular-weight markers (Novex and Magic Marks, Life Technologies, Carlsbad, CA, USA), and 10 ng recombinant human protein (recombinant protein of human chromosome 1 open reading frame 70 (C1orf70), #TP325178, Origene, Rockville, MD) onto precast 12% Criterion XT Bis-Tris polyacrylamide gels (Bio-Rad, Hercules, CA, USA), electrophoresis was carried out by applying 165 V for 45 min in NuPAGE® MES SDS running buffer (1×). Proteins were transferred to a 0.2-μM nitrocellulose membrane (G&E Healthcare, Chicago, IL, USA) at 100 V for 40 min. The quality of the protein transfer was determined by reversible Ponceau Red coloration (0.2% Xyline Ponceau Red and 3% trichloroacetic acid). Membranes were blocked in 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20 (v/v) (TBS-T) supplemented with 5% (w/v) skimmed milk (TBS-M) or 5% (w/v) bovine serum albumin (TBS-BSA), depending on the antibody, for 1 h (Table S2). Membranes were washed in TBS-T before incubation with primary antibodies overnight at 4 °C. After washing in TBS-T, membranes were incubated with secondary antibodies for 45 min at room temperature. Revelation was performed using ECL™ Western Blotting Detection Reagents (G&E Healthcare, Chicago, IL, USA).

**Immunogold Electron Microscopy**

Two different fixation procedures were used for the electron microscopy analysis: LR White embedding gives higher sensitivity, whereas Araldite embedding preserves cellular structures. The cerebellum was rapidly dissected, incubated in fixation buffer at 4 °C, and post-fixed in 1% osmic acid, 0.1 M phosphate buffer. The sections were dehydrated using a series of increasing ethanol concentrations, impregnated with 96% ethanol, and stored in pure buffer at 4 °C. After polymerization (4 °C under UV for LR White or 56 °C for Araldite), the sections (85-nm thick) were incubated with the anti-TMEM240 antibodies on sagittal brain sections. We observed no staining of the corpus callosum. TMEM240 immunoreactivity was quantified by immunofluorescence labeling of TMEM240 with Santa Cruz Biotechnology antibody) was lower in the striatum than in the corpus callosum. TMEM240 immunoreactivity (Santa Cruz Biotechnology) was lower in the striatum (5.52 ± 0.1 U, p < 0.0001) and hindbrain (5.54 ± 0.1 U, p < 0.0001) than in the brain as a whole (6.09 ± 0.07 U). TMEM240 immunoreactivity in the midbrain showed mean values similar to those obtained in the brain as a whole (6.16 ± 0.19 U, p = 0.98) and interbrain (defined as the brain region encompassing the thalamus and hypothalamus (6.29 ± 0.15 U, p = 0.13)). We detected higher values in the isocortex (7.95 ± 0.17 U, p < 0.0001), hippocampus (7.39 ± 0.13 U, p = 0.17). We detected higher values in the isocortex (7.95 ± 0.17 U, p < 0.0001), hippocampus (7.39 ± 0.13 U, p = 0.17).

**Results**

**TMEM240 Antibody Specificity**

Competition studies performed in the anterior lobe verified the specificity of the TMEM240 antibodies (Figures S1 and S2 for the TMEM240 sc-245675 (Santa Cruz Biotechnology) and TMEM240/63-77 antibodies, respectively). Incubation of the TMEM240 antibodies with the respective immunogenic blocking peptide (Figures S1.b1-b2 and S2.b1-b2) abolished the TMEM240 immunoreactivity observed when incubating thin sections with the TMEM240 antibodies alone (Figures S1.a1-a2 and S2.a1-a2). Incubation of the TMEM240 antibodies with a non-competitive BDNF peptide (Figures S1.d1-d2 and S2.d1-d2) or a non-respective immunogenic blocking peptide (Data not shown) did not abolish TMEM240 immunoreactivity.

**TMEM240 Protein Expression in the Mouse Brain**

TMEM240 immunoreactivity was quantified by immunofluorescence labeling of TMEM240 with Santa Cruz Biotechnology (Fig. 1a) and TMEM240/63-77 (Fig. 1b) antibodies on sagittal brain sections. We observed no staining of the corpus callosum. TMEM240 immunoreactivity (Santa Cruz Biotechnology antibody) was lower in the striatum (5.52 ± 0.1 U, p < 0.0001) and hindbrain (5.54 ± 0.1 U, p < 0.0001) than in the brain as a whole (6.09 ± 0.07 U). TMEM240 immunoreactivity in the midbrain showed mean values similar to those obtained in the brain as a whole (6.16 ± 0.19 U, p = 0.98) and interbrain (defined as the brain region encompassing the thalamus and hypothalamus (6.29 ± 0.15 U, p = 0.13)). We detected higher values in the isocortex (7.95 ± 0.17 U, p < 0.0001), hippocampus (7.39 ± 0.13 U,

**Statistical Analysis**

Data are reported as the mean ± standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism software version 7 (GraphPad Software Inc., La Jolla, CA, USA). The normality of the data distribution was assessed by the Shapiro-Wilk test. Differences between mean values were determined using the paired Student’s t test for data when comparing an independent variable between two groups. For the analysis of one independent variable (mean relative grayscale value in the whole structure of interest) between more than two groups (mean relative grayscale value in different parts of a structure of interest), a one-way analysis of variances (ANOVA) was used with a post hoc Fisher’s least significant difference (LSD) test. The threshold for statistical significance was set to p < 0.05.
p < 0.0001), and cerebellum (7.30 ± 0.35 U, p < 0.0001) (Fig. 1c). We also assessed TMEM240 immunoreactivity in the dentate gyrus, caudate nucleus, putamen, cerebral cortex, and cerebellum, as databases show that human TMEM240 is highly expressed in these regions [8]. We observed higher values in the CA1 and CA2 pyramidal layers (9.25 ± 0.07 U and 8.78 ± 0.12 U, respectively, p < 0.0001) and granular layer of the dentate gyrus (8.40 ± 0.05 U, p < 0.0001) than in the hippocampal formation as a whole (7.39 ± 0.03 U). The lowest value was obtained in the CA3 field (6.68 ± 0.08 U, p < 0.0001) (Figure S3.a). In the area of the striatum, we visualized higher expression in the nucleus accumbens than the caudoputamen (6.48 ± 0.3 U vs 5.36 ± 0.05 U, respectively, p < 0.0001) (Figure S3.b). Compared with the mean intensity in the isocortex as a whole (7.95 ± 0.17 U), we observed the lowest value in the orbital area (6.74 ± 0.14 U, p < 0.0001), values close to that of the isocortex as a whole in the somatosensory-related area (7.7 ± 0.04 U), somatomotor-related area (7.78 ± 0.09 U), visual areas (7.86 ± 0.04 U), and posterior parietal association areas (7.78 ± 0.05 U), and the highest value in the retrosplenial area (8.21 ± 0.03 U, p < 0.0001) (Figure S3.c). We obtained similar TMEM240 quantification values with the TMEM240/63-77 antibody (Figs. 1d, and S3.d-f). We observed a marked background with the commercial TMEM240 antibody (Santa Cruz Biotechnology). However, our home-made antibody (TMEM240/63-77) allowed us to obtain similar staining in the brain and cerebellum with less background. The quantification of TMEM240 expression in the regions, areas, and subparts of the adult mouse brain is presented in Table S3.

**TMEM240 Protein Expression in the Cerebellum**

We analyzed TMEM240 immunoreactivity on sagittal, parasagittal, and coronal cerebellar sections in each cerebellar lobule, cerebellar fiber tracts, and the fastigial, interposed, and dentate cerebellar nuclei. We detected immunoreactivity for TMEM240 in all cerebellar structures analyzed and mild differential expressions between lobules with both TMEM240 antibodies, but with lower values in the cerebellar fiber tracts (Figs. 2 and S4). In the cerebellar cortex (sagittal sections, TMEM240 Santa Cruz Biotechnology antibody, Fig. 2a), we observed a lower value in the lingula (lobule I) (6.65 ± 0.24 U, p < 0.0001) and higher values in the culmen (lobules IV–V)
(7.64 ± 0.27 U, \( p < 0.0001 \)) and folium-tuber vermis (lobule VII) (7.41 ± 0.24 U, \( p < 0.0001 \)) (Fig. 2c) than the mean intensity value for the cerebellum as a whole (7.24 ± 0.60 U). We obtained similar TMEM240 quantification values with the TMEM240/63-77 antibody (Fig. 2b, d). We detected TMEM240 in the fastigial nucleus (5.26 ± 0.32 U) and interposed nucleus (5.71 ± 0.32 U) (Fig. 2c). In the cerebellar cortex (parasagittal sections, TMEM240 Santa Cruz Biotechnology antibody, Figures S4.a-b), we detected lower values in the ansiform Crus 2 lobule (6.70 ± 0.50 U, \( p = 0.0042 \)) and paramedian lobule (6.82 ± 0.46 U; \( p = 0.13 \)) than the mean intensity value for the cerebellum as a whole (7.12 ± 0.42 U). We observed higher values in the simple lobule (7.62 ± 0.42 U, \( p = 0.0002 \)) and ansiform Crus 1 lobule (7.60 ± 0.49 U, \( p = 0.0006 \)). We observed the highest value in the copula pyramidis lobule (7.75 ± 0.49 U, \( p < 0.0001 \)). We also detected TMEM240 in the dentate nucleus (7.03 ± 0.38 U) (Figures S4.a-b). We obtained similar staining in the parasagittal sections with the TMEM240/63-77 antibody (Figure S4.c). In the cerebellar nuclei, TMEM240 was highly expressed in neurons with large cellular bodies and showed a punctuate pattern (Figure S4.d). We detected TMEM240 in the molecular and granular layers and major immunoreactivity in the Purkinje-cell layers with both antibodies, as shown by low magnification (Fig. 3a, d). In the molecular layer, punctuate TMEM240 expression was detected in the Purkinje-cell membrane, the soma, and dendrites (Fig. 3b, c, e, f).

**Cellular Distribution of TMEM240 Expression**

TMEM240 immunoreactivity (Santa Cruz Biotechnology) co-localized strongly (PCC > 0.6) with immunostaining for calbindin, a specific Purkinje-cell marker (Fig. 4a1–a3). At a higher magnification, we found immunostaining for...
TMEM240 in the Purkinje-cell membrane, soma, axons, and dendrites, but not in the nuclei (Fig. 4b1–b3). Similar co-staining with calbindin was obtained with the TMEM240/63-77 antibody (Figure S5). Interestingly, immunoreactivity for TMEM240 was found in several calbindin-negative cells in the Purkinje-cell and molecular layers, suggesting that TMEM240 is expressed in stellate and basket cells (Fig. 4b3). Co-staining with parvalbumin (a molecular layer interneuron marker) and TMEM240 (Santa Cruz Biotechnology) showed positive cells for both antibodies. These cells may thus correspond to basket cells in the Purkinje-cell layer and stellate cells in the outer two-thirds of the molecular layer (Figure S6). Moreover, certain cells in the molecular layer were positive for TMEM240 only, which may correspond to excitatory neurons in the molecular layer (Figure S6). The immunoreactivity of TMEM240 in glial cells was negative, as demonstrated by co-staining with glial fibrillary acidic protein (data not shown). We also co-stained for the vesicular glutamate transporters VGLUT1 (Fig. 5a1–a5) and VGLUT2 (Fig. 5b1–b5) to examine the localization of TMEM240 in the cerebellar connections. Co-immunostaining with VGLUT1 in the molecular layer (Fig. 5a4) showed TMEM240 to also be localized to the parallel fibers. In the granular layer, co-staining showed TMEM240 to be localized to the glomeruli, with partial co-staining of TMEM240 and VGLUT1, which could correspond to mossy fibers (Fig. 5a5). Co-immunostaining with VGLUT2 in the molecular layer (Fig. 5b4) showed TMEM240 localization to the climbing fibers. In the glomeruli, co-immunostaining with VGLUT2 showed TMEM240 localization to climbing and mossy fibers (Fig. 5b5).

**Synaptic Distribution of TMEM240 Immunoreactivity in the Murine and Human Brains**

We co-stained for TMEM240 (Santa Cruz Biotechnology) and synaptophysin to examine TMEM240 localization at the cerebellar synapses (Fig. 6a–d). Intense co-immunostaining (PCC > 0.6) showed the presence of TMEM240 in all synapses in all three layers of the cerebellar cortex. At a higher magnification, we observed co-immunostaining with synaptophysin in synapses connecting to the membrane of a Purkinje-cell soma (Fig. 6d). PSD fractionation showed TMEM240 localization in enriched-post-synaptic fractions.
During our study, we focused on male mice. This is a limitation as TMEM240 could have some gender-specific differential expression. However, in our previous clinical
publications [3, 20], we did not observe any gender-based difference within the SCA21 patients, assuming that impact of TMEM240 gene mutations, and presumable TMEM240 protein function, is not gender-related.

Our study characterized TMEM240 expression in the cerebellar neuronal network. Immunofluorescence experiments showed TMEM240 staining in the neurons of the cerebellar cortex and in numerous neuronal projections. TMEM240 was present in afferents from various structures, particularly in neurons that connect to Purkinje cells. We also observed the presence of TMEM240 protein in a high proportion of synapses in the cerebellar cortex; this finding is in accordance with that of a previous report of TMEM240 protein in murine synaptosomes [7].

TMEM240 is widely expressed in Purkinje-cell dendrites, bodies, and axons. Progressive cerebellar cell degeneration and neuronal loss are major, frequent features of SCA. In most cases, neuroimaging and histological analyses have identified Purkinje-cell death as the main pathological feature [21]. Neuropathological data from an autopsied patient with SCA21 revealed a severe loss of Purkinje cells [5]. Our observation of predominant TMEM240 expression in Purkinje cells is consistent with neuropathological evidence showing that the neurodegeneration in SCA21 primarily affects these neurons.

TMEM240 immunostaining of Purkinje cells from normal mice had a punctuate pattern and was distributed equally throughout the soma. This is in contradiction to the report of Seki et al. on cerebellar primary cultures, in which TMEM240 expression was observed in large, cytoplasmic vesicles in the Purkinje-cell soma, dendrites, and axons [20]. The discrepancy between our results and those of their ex vivo cell-based model may be due to overexpression of a flagged version of the TMEM240 protein. The vesicular glutamate transporters of cerebellar afferents mediate glutamate uptake into the synaptic vesicles of excitatory neurons [22]. In the molecular layer, VGLUT2 is exclusively expressed in climbing fibers, whereas parallel fibers express VGLUT1 for vesicular glutamate uptake. In the granular layer, both transporters are detected in mossy fiber terminals [23]. Co-immunostaining with VGLUT1 and VGLUT2 demonstrated the presence of TMEM240 in glutamatergic terminals from climbing, parallel, and mossy fibers. Images were obtained by confocal microscopy.

**Fig. 5** Localization of TMEM240 in mossy, parallel, and climbing fibers.
(a1–a5) Double immunofluorescence labeling of TMEM240 (Santa Cruz Biotechnology) (in red) and VGLUT1 (in green) in sagittal cerebellar sections. Scale bar, 50 μm. (a4) Magnification of an image showing co-staining in the molecular layer. The white arrows indicate TMEM240 localization in parallel fibers. (a5) Magnification of an image showing co-staining in the granular layer. The white arrow indicates TMEM240 and VGLUT1 co-localization in glomeruli. (b1–b5) Double immunofluorescence labeling of TMEM240 (Santa Cruz Biotechnology) (in red) and VGLUT2 (in green) in sagittal cerebellar sections. Scale bar, 50 μm. (b4) Magnification of an image showing co-staining in the molecular layer. The white arrow indicates TMEM240 localization in climbing fibers. (b5) Magnification of an image showing co-staining in the granular layer. The white arrows indicate TMEM240 in climbing fibers and mossy fibers. Images were obtained by confocal microscopy. GL granular layer, ML molecular layer, PL Purkinje-cell layer.
and mossy fibers. Mutations in glutamate transporters and/or receptors lead to Purkinje-cell degeneration and ataxia in murine models and humans [24, 25]. TMEM240 mutations in SCA21 may interfere with neuronal glutamate homeostasis and thus cerebellar function. Functional studies of wild type and mutated proteins in cell-based and animal models are needed to validate this hypothesis.

**Conclusion**

We characterized TMEM240 expression in the cerebellar neuronal network, including neurons of the cerebellar cortex, particularly Purkinje cells. Our work provides insights into the physiological expression of TMEM240 in the cerebellum. These insights are likely to be of great value in the establishment of murine or zebrafish models of SCA21.

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**Contributors** Mégane HOMA: Had a major role in the acquisition of data, including all immunohistochemical analyses, interpreted the data, and drafted the manuscript.
Anne LOYENS: Had a major role in the acquisition of the transmission electron micrographs.
Sabha EDDARKAOUI: Had a major role in the production of a new polyclonal antibody.
Emilie FAIVRE: Had a major role in animal and tissue preparation. Revised the manuscript for critical intellectual content.
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Vincent HUIN and Bernard SABLONNIÈRE: Interpreted the data and revised the manuscript for critical intellectual content.

**Fig. 6** TMEM240 localization in synapses. (a–d) Double immunofluorescence staining of TMEM240 in red (Santa Cruz Biotechnology) (a) and synaptophysin in green (b) in murine sagittal cerebellar sections. (c) Double immunofluorescence (merge) of both TMEM240 and synaptophysin. Scale bar, 10 μm. (d) Magnification of double immunofluorescence staining, focusing on a Purkinje-cell soma.
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**Fig. 7** Electron microscopy analysis. Transmission electron micrographs of TMEM240 immunogold staining (Santa Cruz Biotechnology antibody) of murine cerebellar sections embedded in Araldite resin (a, b) or LR White resin (c, d). Micrographs showing Purkinje-cell soma (a, c). Scale bar, 10 μm. Magnification of immunogold stained Purkinje-cell synapses (b, d). Scale bar, 2 μm. The white arrows indicate TMEM240 localization on the post-synaptic side of the Purkinje-cell membrane. GL granular layer, PC Purkinje cell, PCm Purkinje-cell membrane.

**Fig. 8** TMEM240 localization in human cerebellar cortex sections. Double immunostaining of TMEM240 (Santa Cruz Biotechnology) (in red) and synaptophysin (in green) in human cerebellar cortex sections (a1, b1-b3). Scale bar: 10 μm. Magnifications of a glomerulus (a2), the molecular layer (a3), Purkinje-cell soma (a4). Gl: glomerulus, GL: granular layer, ML: molecular layer, PL: Purkinje-cell layer.
Compliance with Ethical Standards

Ethical Approval  All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted. Experimental protocols were approved by the local animal ethical committee (approval APAFIS#2264-2015101320441671 from CEEA75, Lille, France). Human brains were obtained from the Lille Neurobank (CRB/CIC1403 Biobank, BB-0033-00030, agreement DC-2008-642), which fulfills the criteria of the local laws and regulations on biological resources with donor consent, data protection, and ethical committee review.

Conflict of Interest  The authors declare that they have no conflicts of interest.

Availability of Data and Material  All data generated or analyzed during this study are included in this published article and its supplementary information files.

References

1. Hersheson J, Haworth A, Houlden H. The inherited ataxias: genetic heterogeneity, mutation databases, and future directions in research and clinical diagnostics. Hum Mutat. 2012;33:1324–32.

2. Delplanque J, Devos D, Vuillaume I, De Becdelievre A, Vangelder E, Maureag CA, et al. Slowly progressive spinocerebellar ataxia with extrapyramidal signs and mild cognitive impairment (SCA21). Cerebellum. 2008;7:179–83.

3. Delplanque J, Devos D, Huin V, Genet A, Sand O, Moreau C, et al. TMEM240 mutations cause spinocerebellar ataxia 21 with mental retardation and severe cognitive impairment. Brain. 2014;137:2657–63.

4. Zeng S, Zeng J, He M, Zeng X, Zhou Y, Liu Z et al. Spinocerebellar ataxia type 21 exists in the Chinese Han population. Scientific Reports [Internet]. 2016 [cited 2019 Dec 17];6. Available from: http://www.nature.com/articles/srep19897.

5. Yahikoza H, Miyatake S, Sakai T, Uehara T, Yamada M, Hanyu N, et al. A Japanese family of spinocerebellar ataxia type 21: clinical and neuropathological studies. Cerebellum. 2018;17:525–30.

6. Traschütz A, van Gaalen J, Roelofs F, Vreeburg M, Ramsden EJ, Deiminger N, et al. The movement disorder spectrum of SCA21 (ATX-TMEM240): 3 novel families and systematic review of the literature. Parkinsonism Relat Disord. 2019;62:215–20.

7. Trinidad JC, Barkan DT, Gulledge BF, Thalhammer A, Sali A, Schoepfer R, et al. Global identification and characterization of both O-GlcNAcylation and phosphorylation at the murine synapse. Mol Cell Proteomics. 2012;11:215–29.

8. Hawrylycz MJ, Lein ES, Guillozet-Bongarčik AL, Shen EH, Ng L, Miller JA, et al. An anatomically comprehensive atlas of the adult human brain transcriptome. Nature. 2012;489:391–9.

9. Uhlen M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome. Science. 2015;347:1260419–9.

10. Koziol LF, Budding D, Andresen N, D’Arrigo S, Bulgheroni S, Imamizu H, et al. Consensus paper: the cerebellum’s role in movement and cognition. Cerebellum. 2014;13:151–77.

11. Mariën P, Ackermann H, Adamszak M, Barwood CHS, Beaton A, Desmond J et al. Consensus paper: language and the cerebellum: an ongoing enigma. The cerebellum [Internet]. 2013 [cited 2019 Dec 17]; Available from: http://link.springer.com/10.1007/s12311-013-0540-5.

12. Schmahmann JD, Sherman JC. Cerebellar cognitive affective syndrome. Int Rev Neurobiol. 1997;41:433–40.

13. Schmahmann JD, Sherman JC. The cerebellar cognitive affective syndrome. Brain. 1998;121(PT4):561–79.

14. Larsell O. The morphogenesis and adult pattern of the lobules and fissures of the cerebellum of the white rat. J Comp Neurol. 1952:97:381–356.

15. Eccles JC, Ito M, Szentágóthai J. Architectural design of the cerebellar cortex. The cerebellum as a neuronal machine [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 1967 [cited 2019 Dec 17]; p. 195–204. Available from: http://link.springer.com/10.1007/978-3-662-13147-3_12.

16. Sillito RV, Joyner AL. Morphology, molecular codes, and circuitry produce the three-dimensional complexity of the cerebellum. Annu Rev Cell Dev Biol. 2007;23:549–77.

17. Beckinghausen J, Sillito RV. Insights into cerebellar development and connectivity. Neurosci Lett. 2019;688:2–13.

18. Seki T, Sato M, Kibe Y, Ohta T, Oshima M, Konno A, et al. Lysosomal dysfunction and early glial activation are involved in the pathogenesis of spinocerebellar ataxia type 21 caused by mutant transmembrane protein 240. Neurobiol Dis. 2018;120:34–50.

19. Schmahmann JD. The cerebellum and cognition. Neurosci Lett. 2019;688:62–75.

20. Devos D, Schraen-Maschke S, Vuillaume I, Dujardin K, Nazé P, Willetteaux C, et al. Clinical features and genetic analysis of a new form of spinocerebellar ataxia. Neurology. 2001;56:234–8.

21. Matilla-Dueñas A, Ashizawa T, Brice A, Magri S, McFarland KN, Pandolfo M, et al. Consensus paper: pathological mechanisms underlying neurodegeneration in spinocerebellar ataxias. Cerebellum. 2014;13:269–302.

22. Gebre SA, Reeber SL, Sillito RV. Parasagittal compartmentation of cerebellar mossy fibers as revealed by the patterned expression of vesicular glutamate transporters VGLUT1 and VGLUT2. Brain Struct Funct. 2012;217:165–80.

23. Hioki H, Fujiyama F, Taki K, Tomioka R, Furuta T, Tamamaki N, et al. Differential distribution of vesicular glutamate transporters in the rat cerebellar cortex. Neuroscience. 2003;117:1–6.

24. Yue Z, Horton A, Bravin M, DeJager PL, Selimi F, Heintz N. A novel protein complex linking the delta 2 glutamate receptor and autophagy: implications for neurodegeneration in lurcher mice. Neuron. 2002;35:921–33.

25. Jen JC, Wan J, Palos TP, Howard BD, Baloh RW. Mutation in the glutamate transporter EAAT1 causes episodic ataxia, hemiplegia, and seizures. Neurology. 2005;65:529–34.

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