A New Mouse Allele of Glutamate Receptor Delta 2 with Cerebellar Atrophy and Progressive Ataxia

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Abstract

Spinocerebellar degenerations (SCDs) are a large class of sporadic or hereditary neurodegenerative disorders characterized by progressive motor defects and degenerative changes in the cerebellum and other parts of the CNS. Here we report the identification and establishment from a C57Bl/6J mouse colony of a novel mouse line developing spontaneous progressive ataxia, which we refer to as ts3. Frequency of the phenotypic expression was consistent with an autosomal recessive Mendelian trait of inheritance, suggesting that a single gene mutation is responsible for the ataxic phenotype of this line. The onset of ataxia was observed at about three weeks of age, which slowly progressed until the hind limbs became entirely paralyzed in many cases. Micro-MRI study revealed significant cerebellar atrophy in all the ataxic mice, although individual variations were observed. Detailed histological analyses demonstrated significant atrophy of the anterior folia with reduced granule cells (GC) and abnormal morphology of cerebellar Purkinje cells (PC). Study by ultra-high voltage electron microscopy (UHVEM) further indicated aberrant morphology of PC dendrites and their spines, suggesting both morphological and functional abnormalities of the PC in the mutants. Immunohistochemical studies also revealed defects in parallel fiber (PF)–PC synapse formation and abnormal distal extension of climbing fibers (CF). Based on the phenotypic similarities of the ts3 mutant with other known ataxic mutants, we performed immunohistological analyses and found that expression levels of two genes and their products, glutamate receptor delta2 (grid2) and its ligand, cerebellin1 (Cbln1), are significantly reduced or undetectable. Finally, we sequenced the candidate genes and detected a large deletion in the coding region of the grid2 gene. Our present study suggests that ts3 is a new allele of the grid2 gene, which causes similar but different phenotypes as compared to other grid2 mutants.

Introduction

Ataxia is defined as a neurological dysfunction that causes loss of motor coordination such as gait imbalance associated with appendicular ataxia and defects in gait or speech [1–3]. Inherited spinocerebellar degenerations (SCDs) are among the main causes of ataxia. SCDs comprise the two most relevant forms of ataxia: the autosomal recessive ataxias and the autosomal dominant spinocerebellar ataxias (SCAs) [1]. The majority of recessive ataxias are caused by loss-of-function (missense) mutations, while SCAs are mostly caused by an insertion of multiple CAG-repeats in the coding region of a particular gene, which is thought to result in a toxic gain-of-function of the protein with poly-glutamine (poly-Q) expansion [1]. Another group of inherited SCDs is the hereditary spastic paraplegias (HSPs, numbered as SPG1-39) characterized by progressive lower limb spasticity and weakness due to distal axonopathy of the corticospinal tract axons [4,5]. Over the last two decades, genetic studies have identified many genes responsible for the inherited SCDs, including 19 genes out of 27 known SCAs and 20 out of 36 established HSPs [3,5].

Increasing numbers of the animal SCD models representing both sporadic mutant mice and genetically-engineered mice have been reported [6–8], including hotfoot and Larcher mutants [9,10]. Their causative mutations were identified in the same gene, grid2, encoding the ionotropic glutamate receptor delta-2 (GluD2)
[11,12], which is selectively expressed in Purkinje cells (PC). At least twenty alleles of the same gene, including ho4jo, ho5jo, ho7jo, ho8jo, ho11jo, ho12jo, ho13jo, ho15jo, ipe (iapalancer), 153Gso and 154Gso have been identified from different mutant mice [11,13–16], and a targeted null mutation in the grid2 gene (grid2 KO mice) has also been reported [17]. These grid2 mutants are commonly characterized by cerebellar atrophy and motor incoordination.

Lurcher (Lc) is a spontaneous semi-dominant mutation in which homozygotes (Lc/Lc) die at birth, and heterozygotes exhibit cerebellar deficiencies and ataxia [9,10]. It is the result of a single missense mutation in the third transmembrane domain in the grid2 gene [11]. Lc is an autosomal dominant and constitutive active mutation which eventually leads to the death of PCs, resulting in their near complete absence as well as the accompanying loss of most GCs and 60–75% of olivary neurons [12,16].

Hotfoot (ho) is a spontaneous, autosomal recessive mutation, and one of the alleles, ho4jo, was first shown to be caused by a mutation in the grid2 gene [11]. In addition to the ataxic phenotypes common to Lc/+ , both hotfoot mutants and grid2 KO mice are characterized by deficits in parallel fiber (PF)-PCs and climbing fiber (CF) PC synapse formation, as well as impaired induction of long-term depression (LTD) [16,17]. Phenotypic similarities between hotfoot mutants and grid2 KO mice suggest that hotfoot is a loss-of-function mutation. In fact, sensorimotor learning deficits exhibited by hotfoot mutants are greater than those demonstrated by Lc/+ mutants[19,20], although loss of cerebellar cells in hotfoot mutants is less severe [18,21].

Here, we have established a mouse line with an autosomal recessive gene mutation characterized by progressive ataxia and significant cerebellar atrophy. Phenotypic and genetic analyses suggest that the mutation is a new allele of the grid2 gene and another loss-of-function mutation.

Materials and Methods

Mice

Experimental protocols for mice were approved by committees at the National Institute of Biomedical Innovation (DS 23-35), Kinki University (KAME-22-012), Osaka University (FBS 07-001). Mice were maintained under standard conditions of light (8:00 am–8:00 pm) and temperature (23±1°C). The b3 mutant was originally found in the C57BL/6 strain, and additional C57BL/6j mice used for all experiments, including mating, were obtained from SLC Japan. All surgery was performed under 1% isoflurane anesthesia or pentobarbital anesthesia (100 mg/kg body weight, i.p.), and all efforts were made to minimize suffering.

For in vitro fertilization, 4-week-old female C57BL/6j mice were intraperitoneally injected with 5 IU pregnant mare serum gonadotropin (PMSG; Serotropin, ASKA Pharmaceutical), followed by 5 IU human chorionic gonadotropin (hCG; Puberogen, Yell Pharmaceutical) 48 h later. Fifteen hours after hCG injection, oocytes were dissected from the ampulla region of the oviducts and placed in 200 μl droplets of HTF medium (Ark Resource) at 37°C under 5% CO2 in air. Spermatozoa collected from the cauda epididymis of b3 males were incubated for 1–1.5 h in 200 μl droplets of HTF medium to allow capacitation. Oocytes were then inseminated by adding 3–5 μl of the sperm suspension and incubated for 5 h. The fertilized oocytes were washed three times and transferred to fresh drops of KSOR medium (Ark Resource) and cultured overnight. The following day, the 2-cell stage embryos (usually 10 embryos/oviduct) were surgically transferred into the oviducts of pseudopregnant ICR females (0.5 day post coitus) that had been mated with vasectomized males.

When taking footprints, soles of the hind feet were marked with Chinese ink, and the mice were allowed to walk freely on flat paper.

Micro-magnetic resonance imaging (micro-MRI)

An MRI apparatus for small animals at 11.7 T (Bruker, AVANCE 500WB) was used for the experiments. Mice (n = 6) were anesthetized with 1% isoflurane and MRI was performed under different sequences as follows:

1) Sequence for brain imaging: T1 weighted image (Gradient echo: FLASH);

FOV = 2.0 cm, matrix = 256×256, thickness = 0.5 mm, 12 slices,
TR/TE = 100 ms/2.3 ms, NS = 32, scan time = 13 min. MRI was performed 20–24 h after IP injection of Mn solution (0.4 mmol/kg MnCl2/4H2O, 100 mM).

2) Sequence for spinal cord/sciatic nerve imaging:

2-1) T2 weighted image (Spin echo: RARE)

FOV = 2.5 cm, matrix = 256×256, thickness = 0.5 mm, 12 slices,
TR/TE = 4000 ms/25.2 ms, NS = 8, scan time = 8 min.
2-2) Diffusion tensor image (Spin echo)

FOV = 2.5 or 3.0 cm, matrix = 128×128, thickness = 0.5 mm, 8 slices,
TR/TE = 2000 ms/27 ms, b = 1000 s/mm2, 6 directions, NS = 4, scan time = 119 min.

Histology

Mice at the ages of 3 weeks, 4 months and 10 months were used for histological studies. Following the MRI scan, mice were anesthetized with pentobarbital and perfused with 4% paraformaldehyde. The brain was then removed, embedded in paraffin, and used for histological analysis. The sections were stained with hematoxylin-eosin (H&E), toluidine blue, Kluver-Barrera’s (KB) stain, following standard protocols.

Immunohistochemistry

Under pentobarbital anesthesia, mice were fixed transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.2), and cerebellar sections (50 μm in thickness) were prepared using a microslicer (VT1000S, Leica). Immunohistochemical incubations were done at room temperature using phosphate-buffered saline (PBS, pH 7.2) containing 0.1% TritonX-100 (TPBS) for diluent and washing buffers. For immunofluorescence, most sections were successively incubated in a floating state with 10% normal donkey serum for 30 min, a mixture of primary antibodies overnight (1 μg/ml), and a mixture of Alexa Fluor-488-, indocarbocyanine (Cy3)-, and indodicarbocyanine (Cy5)-labeled species-specific secondary antibodies (1:200, Invitrogen; Jackson ImmunoResearch) for 2 h. For immunohistochemical analysis with calbindin antibody, we used fresh frozen sections mounted on silane-coated slide glasses which were fixed with 4% PFA for 10 min and then subjected to immunofluorescence incubation as above. Images were taken with a laser-scanning microscope (FV1000, Olympus) equipped with HeNe/Ar laser, and PlanApo (10x/0.40) and PlanApoN (60x/1.42, oil immersion) objective lens (Olympus). To avoid cross-talk between multiple fluorophores, Alexa 488 (or FITC), Cy3, and Cy5 fluorescent signals were acquired sequentially using the
488 nm, 543 nm, and 633 nm excitation laser lines. Single optical sections were obtained (640×640 pixels, pixel size 110 nm).

For immunohistochemical analysis, we used affinity-purified primary antibodies raised against the following molecules (host species): mouse calbindin (goat, [22]), mouse cerebellin 1 (cbln1, guinea pig, [23]), mouse glial fibrillary acidic protein (GFAP, rabbit, [24]), mouse parvalbumin (guinea pig, [25]), mouse glutamate receptor GluD2 (rabbit, [26]), rat vesicular glutamate transporters VGluT1 and VGluT2, (guinea pig, [27]), and mouse vesicular inhibitory amino acid transporter (VIAAT, guinea pig, [27]).

Electron microscopy

Brains from 10-month-old (30 weeks) mice were perfused with 4% paraformaldehyde/2.5% glutaraldehyde and fixed for 2 hours in the fixative solution. The cerebella were cut in small pieces (about 1 mm³) and washed with phosphate buffer, postfixed in sodium cacodylate-buffered 1.5% osmium tetroxide for 60 min at 4°C, dehydrated using a series of ethanol concentrations, and embedded in Epon resin. The samples were examined under an electron microscope (H-7650, Hitachi or JEM1400, JEOL).

Ultra-high-voltage electron microscopy (UHVEM)

An ultra-high voltage (3MV) electron microscope (H-3000, Hitachi) was used. Mouse brain (4 months old) was fixed with 4%
paraformaldehyde/2.5% glutaraldehyde solution overnight and sagittal brain slices (500 μm thickness) were prepared using Microslicer (Matsunami). Golgi stain was performed on the slices following standard protocol. Following several rinses in 8% sucrose in phosphate buffered saline (PBS), the slices were immersed for 2 h in a mixture of 2.5% paraformaldehyde, 2.5% glutaraldehyde in PBS. After fixation with 1% osmium tetraoxide for 1 h, the thick sections were dehydrated through a graded series of ethanol (60%–100%) and propylene oxide. Finally, they were embedded in Quetol 812 at 45°C for 1 day, and then at 60°C for 2 days. The embedded sections were mounted on epoxy block and cut on an ultramicrotome (Ultracut E; Reichert-Jung) at 10 μm thickness and mounted on 50-mesh grids. Specimens were examined with an ultra-high voltage electron microscope H-3000 (Hitachi) at an accelerating voltage of 2000 kV. Images were recorded with a 4 k×4 k slow scan charge-coupled device (SSCCD) camera F406BK with a pixel size of 15 μm×15 μm (Hitachi) at a nominal magnification of 2,000×.

Figure 2. Cerebellar atrophy in the ts3 mutant mice revealed by Micro-MRI and histological analysis. A) Brain images acquired by micro-MRI (left and middle panels). Horizontal (left) and sagittal (middle) images of cerebella indicate cerebellar atrophy in the ts3 mutant (lower panels) and WT control (upper panels). The right panels show H&E-stained cerebellar sagittal sections of the ts3 mutant (lower) and WT control (upper). Scale bar indicates 1 mm.

B, C) Cerebellar size of ts3 mutants and WT controls. First, the lengths of major and minor axes (mm) of the whole cerebellum were measured by micro-MRI (B, upper panels). They were consequently multiplied and compared between two genotypes (C, upper, n = 6, unit: mm²). To examine significant anterior folial atrophy in ts3 cerebella, we also measured the length of the folia (shown in B, lower) and compared in ts3 and WT mice (C, lower, n = 6, unit: mm). *P<0.05, **P<0.01.

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RT-PCR

Reverse transcription PCR (RT-PCR) was performed according to standard protocol. Briefly, cerebella were sampled from ts3 mice and wild-type controls. Total RNAs were extracted using Trizol reagent (Thermo Scientific) and cDNAs were synthesized from the RNAs using Superscript II Reverse Transcriptase (Life Technologies) with either oligo (dT) or random primers (Life Technologies), according to the manufacturers’ protocols. Nested PCR was performed to amplify the grid2 cDNA from ts3 and wild-type cerebella using Ex-Taq (TaKaRa) and primers as follows: 5’-ATGGAAGTTTTCCCCTTGCT-3’, 5’-TCATATGGACGTGCCTCGGTCG -3’. PCR parameters are as follows: 94°C 2 min; 30 cycles of 94°C 1 min, 60°C 1 min; 72°C 3 min, 72°C 7 min.

Western blotting

Western blotting was performed according to standard protocol. Briefly, sampled cerebella were homogenized in RIPA buffer with protein inhibitors and proteins were extracted by sonication and centrifugation. 10 μg of each protein was electrophoresed using SDS-PAGE gel, blotted on PVDF membrane (Bio-Rad), and incubated overnight at 4°C with anti-GluD2 antibody (1: 2000 dilution, rabbit, [26]), anti-Chlb1 antibody (1: 250 dilution, rabbit, [23]), anti-α-tubulin antibody (1: 10,000 dilution, rabbit, MBL) or anti-β-actin antibody (1: 400,000 dilution, mouse, Sigma). Secondary antibodies, anti-rabbit IgG (donkey, GE Healthcare) or anti-mouse IgG (sheep, GE Healthcare) were used for detection of anti-rabbit (GluD2, Chlb1, or α-tubulin) or anti-mouse (β-actin) primary antibody, respectively. ECL Prime Western blotting reagent (GE Healthcare) was used for chemiluminescence detection and images were captured using MYECL Imager (Thermo Scientific).

Statistics

All data are expressed as the mean ± SEM (or SD if specifically described). Statistical significance was evaluated by Student’s t-test and was set at the p<0.05 level, except in a case where chi-square (χ²) analysis was performed to compare frequency distribution of dendritic spine morphology between WT and ts3 mice in the four different categories.
Results

Isolation of a mouse line with behavioral abnormality

While maintaining the C57BL/6J mouse population, we discovered some mice with the ataxic phenotype. The mice could not move smoothly and also fell frequently, most likely due to abnormal control of their hind limbs (Fig. 1A). To clarify hereditary mode of the ataxic phenotype, the mice were mated with normal C57BL/6J mice. However, both male and female failed to produce offspring under natural breeding conditions, presumably due to ataxia. Consequently, we performed in vitro fertilization (IVF) in order to obtain offspring.

As shown in Fig. 1B, one male mouse with ataxia was born via IVF using sperm from a mutant male and oocytes from a mutant female. In contrast, when oocytes from normal females were used for IVF, no offspring displayed abnormality at F1 generation. At F2 generation, however, about 25% of offspring exhibited the same phenotype as the original mice, suggesting that the ataxic phenotype is recessively inherited and that a single gene is responsible for the phenotype. We referred to the mutant mice as ts3.

Progressive changes in abnormal behavior

To characterize the behavioral abnormalities of the ts3 mice more precisely, we first collected footprints of hind soles from mutants and control littermates (Fig. 1C). The mutant mice displayed a short-stepped gait and moved their toes parallel to the direction of movement. Moreover, the ts3 mice did not attach their heels on the ground, suggesting that this behavioral...
abnormality is one of the characteristics of spinocerebellar ataxia. Although the extent of hind limb abnormality was not significant during suckling period (2 weeks old), after weaning (3 weeks old) the mutant mice began to fall frequently. Finally, at 6-weeks, the mice began to walk dragging their hind limbs.

Because mice with spinocerebellar ataxia display frequent limb-clasping, normal and mutant mice were examined by hanging test. When young mice (6-week old) were tested, both normal and ts3 mice were well balanced by opening their limbs (Fig. 1D, upper panel). Some of the older ts3 mice (i.e. after 12 weeks; the figure shows 30-week-old mice), however, exhibited frequent limb-clasping (Fig. 1D). In addition, tail-muscle tonus (not shown) and hind-limb tonus were observed in older ts3 mice after having an object stuck between the thigh and the abdomen, suggesting that the mutant is a potential model for recessive spinocerebellar ataxia.

Abnormality in the Purkinje and granule cells of the cerebella

Identification of cerebellar atrophy in ts3 mice by Micro-MRI

To investigate the cause of ataxia in the ts3 mice, we first performed micro-MRI on ts3 and WT brains. We found that the cerebella of ts3 mutant mice were significantly smaller than that of WT controls (4 months old, n = 6 each, Fig. 2A, B), measuring the size of different regions of the cerebella for both genotypes and confirming significant cerebellar atrophy in the ts3 mutants (Fig. 2C, D, Fig. S2). We also performed histological analyses on the same animals, with results consistent with those from micro-MRI (Fig. 2A, B). Finally, we examined micro-MRI and histology of the spinal cords and sciatic nerves of the ts3 mice when compared to the WT controls (4 months old, n = 3 each, Fig. 3, Fig. S3). No detectable abnormality (e.g., demyelination or degeneration of upper/lower neurons) was observed in either sciatic nerves (Fig. 3A, B) or spinal cords (Fig. 3C, D, S3) of the ts3 mutants.

Abnormality in the Purkinje and granule cells of the anterior folia in ts3 cerebella

The atrophy observed in the ts3 cerebella was further examined histologically. Fig. 4 shows KB-stained sections of the ts3 and WT cerebella. Reduced numbers of GGs were observed in older ts3 mutants when compared to the WTs (30 weeks old, Fig. 4A, C).
Next, we performed immunohistochemistry using anti-Calbindin antibody to examine PC morphology. As shown in Fig. 4B, dendrite branching of PCs in the molecular layer of ts3 cerebella was abnormal, and each dendrite was significantly thicker in the 30-week-old mutants than in WT control (Fig. 4B, D). Average number of PCs in each lobule was also calculated from serial sections, and significant decreases were observed in lobules VII and VIII in the 30-week-old ts3 mutants (Fig. S4).

Electron microscopic analysis of Purkinje cells in ts3 mutants

To further analyze PC abnormality in the ts3 cerebellum, we performed an electron microscopic study on mutant and normal cerebella. In ts3 cerebella, we observed electron dense accumulations in cell bodies of PC cells (30 weeks old, Fig. 5B, D, E, arrowheads). Higher magnification images demonstrated that the aggregates contain electron dense and electron lucent compartments (Fig. 5E), which are characteristics of lipofuscin. Lipofuscin is composed of highly oxidized cross-linked protein aggregates and lipids. Previous reports suggest that lipofuscin accumulation is a consequence of oxidative stress and is a characteristic feature of senescent postmitotic cells including neurons [28]. These granules were not observed in WT PC cells (Fig. 5A, B).

Abnormal morphology of dendrites and spines in ts3 mutants revealed by ultra-high voltage electron microscopy

We next performed ultra-high voltage electron microscopy to further analyze abnormalities in PCs of the ts3 mutants. As shown in Fig. 6A (indicated by arrowheads), morphology of the dendrites and dendritic spines in the PCs of the ts3 mutants differed from those in the WT cerebella (4 months old). To quantify the difference in dendritic spine morphology, we compared spine density (Fig. 6B) and shapes (Fig. 6C) according to previous report [29]. Dendritic spine density was comparable between hS and WT controls (Fig. 6B), however, frequencies of spine morphology, which was categorized as thin, stubby, mushroom and double-headed, were significantly different between the two genotypes (Fig. 6C, P<0.01). Strikingly, not only spines but also dendrites were malformed; unlike WT, the thickness of dendrites was not...
constant with isthmic portions at intervals observed in the \(t_3\) mutants (Fig. 6A, arrows). These morphological abnormalities have not been described in previous studies.

Distal extension of climbing fiber territory in \(t_3\) mutants

To investigate changes in cellular morphology and neuronal projection in the cerebellum of \(t_3\) mutants, we performed immunohistochemical studies using antibodies to calbindin, VGluT2 (vesicular glutamate transporter 2), VGluT1, VIAAT (vesicular inhibitory amino acid transporter), parvalbumin and GFAP (glial fibrillary acidic protein) (4 months old, Fig. 7). Through calbindin immunofluorescence, cerebella in \(t_3\) mutants was normal in foliation, laminated organization, and monolayer alignment of PCs, although cerebellar size was smaller than control mice as described in Fig. 2 (Fig. 7A-D). However, a striking phenotype was found in the distribution pattern of VGluT2-stained CF terminals. In control mice, CF terminals were distributed along calbindin-stained PC dendrites in the basal four-fifths of the molecular layer (Fig. 7B, E). In contrast, in the \(t_3\) mutants CF terminals were markedly increased in number and distributed throughout the full extent of the molecular layer (Fig. 7D, F). No such genotypic differences were noted for VGluT2-stained PF terminals (Fig. 7G, I), VIAAT-stained interneuron terminals (Fig. 7H, J), parvalbumin-stained inhibitory neurons (Fig. 7K, M) or GFAP-stained Bergmann fibers (Fig. 7L, N).

**Impaired PF–PC synapse formation in \(t_3\) cerebella**

Similar expanded distribution of CF terminals has been reported in mutant mice expressing defective glutamate receptor GluD2, Cbln1, or carbonic anhydrase-related protein 8 (Car8) \([17,30,31]\). In these mutants, PF–PC synapse formation is commonly impaired, as reflected in the emergence of “free spines” lacking synaptic contact with PF terminals and of “mismatched synapses”, in which the postsynaptic density (PSD) in PC spines is longer than the active zone in PF terminals \([17,31–33]\). Therefore, we investigated the formation of PF–PC synapses in \(t_3\) mutants by electron microscopy (4 months old). In control mice, most PC spines contacted PF terminals in a one-to-one fashion, and the PSDs and active zones were well matched at PF–PC synapses. In \(t_3\) mutants, we frequently observed free spines \((f\text{ in Fig. 8C, D)} and mismatched synapses \((m\text{ in Fig. 8C, E)}). Fig. 8F shows frequencies of normal synapses, mismatched synapses and free spines in the two genotypes. Whereas normal synapses were observed in about 99% of the PF–PC synapses in WT mice, the majority of the synapses/spines observed in \(t_3\) mutants were either mismatched \((17.8\pm1.4\%) \) or free \((39.4\pm4.4\%, P<0.001)\).

**Significant reduction of GluD2 and Cbln1 expression in \(t_3\) cerebella**

As the phenotypes in \(t_3\) mutants with abnormalities in PF–PC synapses are similar to those of GluD2, Cbln1 and Car8 mutants, we compared expression of these three gene products by immunohistochemistry (4 months old, Fig. 9). Severe reduction or loss of immunoreactivity was observed in GluD2 (Fig. 9A-D) and Cbln1 (Fig. 9E-H) but not Car8 (Fig. 9I-L), in the cerebella of \(t_3\) mutants. We also performed western blotting analyses and found that, whereas GluD2 expression was not detectable in \(t_3\) cerebella (Fig. 10B), expression level of Cbln1 was comparable in \(t_3\) mutants. We also performed western blotting analyses and found that, whereas GluD2 expression was not detectable in \(t_3\) cerebella (Fig. 10B), expression level of Cbln1 was comparable in \(t_3\) mutants.

**Identification of a large deletion of the grid2 gene and protein in \(t_3\) mutants.** Ultimately, we sequenced the two genes and also performed PCR on genomic DNA or cDNA from \(t_3\) and wild-type cerebella to investigate the possibility that a mutation in one of these genes might be responsible for the defects in \(t_3\) mutant mice. We detected a large deletion of exons in the grid2 gene ranging from exon 3 to 8, resulting in a 1001 bp deletion in the grid2 cDNA (Fig. 10A, C, D, S6A). By genomic PCR analysis, we also discovered the absence of the 3′ portion of the intron 2, suggesting the existence of a mutation within the intron 2, resulting in a frame-shift mutation, causing a GluD2\(^{3\times}\) amino acid substitution from the amino acid 83 and following amino acids (Fig. 10C, D, S6B) and also termination of translation at the amino acid 124 (Fig. 10C, D). The results from Western blotting were consistent in that the GluD2\(^{3\times}\) protein was undetectable using an antibody that recognizes the C-terminus of GluD2\(^{3\times}\) (Fig. 10B).

**Discussion**

The present study demonstrated that the aforementioned novel mutant mice exhibit progressive ataxia, with onset at approximately 3 weeks of age. The major symptoms of ataxia are paraplegia and gait imbalance, possibly due to hind limb incoordination. Cerebellar atrophy, morphological abnormality of PCs, reduced number of GGs and evidence for aberrant PF–PC...
Figure 10. Identification of the ts3 mutation in the grid2 gene. A) RT-PCR was performed on mRNA extracted from either ts3 or WT cerebella. A band that was roughly 1 kb shorter was detected from the mutant cDNA, suggesting a large deletion in the grid2 DNA of ts3 mice. B) Proteins were extracted from the ts3 and WT cerebella and Western blotting was performed using anti-GluD2 and β-actin antibodies. The GluD2 protein was not detected in ts3 mutant cerebella. C) Schematic representation of the deletion in ts3 genomic DNA (a) and protein (b). a) The 6 exons (exon 3 to 8) are missing, and only portions of intron 2 and 9 were detected by genomic PCR (DNA sequences between the two Xs in the WT are missing in the ts3 mutants). b) The deletion in the grid2 DNA results in protein truncation (from amino acids 83 to 145) and also a frame-shift mutation. The resulting gene product in ts3 mutants is 124 amino acids in length. D) Sequencing analyses confirmed that whereas exon 4 (enclosed sequences in the upper line) follows exon 3 sequences in the WT cDNA, exons 9 (shaded sequences in light gray) and 10 (shaded in dark gray) follow exon 3 in grid2ts3. The deletion in the grid2ts3 gene causes a frame-shift mutation from amino acid 83 (C to W) and following amino acids in the GluD2ts3 protein and terminates the protein in a truncated, 124 amino acid form due to a termination codon (tga, *).

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synapse formation in ts3 mutants strongly suggest that ataxia is caused by progressive dysfunction in the cerebellar network. Morphological analyses in the present study revealed that PC dendritic spines were abnormal. Electron microscopic studies further demonstrated that PF–PC synapses were also abnormal in ts3 mutants, with a large number of “mismatched synapses” and “free spines” being observed [34]. In normal PF-PC synapses, length of the postsynaptic density (PSD) of the PC spine is well-matched with that of the active zone of PF. In contrast, PSD in ts3 mutants was significantly longer than the active zone of the PF terminal, which is similar to previous observations in Car8, grid2, and Cbln1 mutant mice [30,31,33].

In previous studies, all three mutants demonstrated cerebellar dysfunctions, resulting in abnormalities such as motor dyscoordination, defects in PF–PC synapse formation, and expansion of CF territory. There are, however, differences in the phenotypes of these mutants when compared to that of ts3. For instance, Car8 KO mice demonstrated expansion of CF territory and free spines, but mismatched synapses were not observed [30]. Phenotypes of Cbln1- and grid2-disrupted (KO) mice were similar according to previous reports, demonstrating motor dyscoordination, increase in the number and distribution of CF terminals, and significant rates of free/mismatched synapses [17,31,35]. However, our study indicated that there are also discrepancies between ts3 mutants and Car8- and grid2 KO mice, such as lower body weight, failure to produce offspring, and significant individual differences observed in ts3 but not in the two KO mice.

Our immunohistochemical data indicated that expression levels of both GluD2 and Cbln1 in ts3 mutant cerebella were significantly reduced or undetectable, respectively, whereas the protein level of Car8 was comparable to that of WT controls (Fig. 9). However, the results were inconsistent with our western blotting data, showing that Cbln1 expression levels were comparable in ts3 and control cerebella (Fig. S5). It had previously been reported that Cbln1 immunoreactivity was not detectable in the grid2-disrupted mice [36]. In contrast, expression levels of GluD2 protein are normal in Cbln1 mutant mice (our unpublished data). Taken together, our data raised the possibility that either grid2 or a gene affecting grid2 expression is mutated in ts3 mutant mice, although the possibility that Cbln1 is mutated in ts3 mice could not be excluded. We subsequently conducted further examination by sequencing and PCR, resulting in identification of the grid2 deletion, ranging from exon 3 to 8. Discrepancy of phenotypes between ts3 and other grid2 mutants suggests that the ts3 mutation, which disrupts the majority of the coding region but retains N-terminal portion of the protein, causes phenotypic differences when compared with other mutants. It has been reported that the N-terminal domain of GluD2 (Arg321-Try339) [37], which is deleted in ts3 mutants, is crucial for Cbln1 binding and induction of presynaptic differentiation, suggesting at least that GluD2<sup>Arg321-Try339</sup> does not function through binding to Cbln1. Alternatively, there is a possibility that another gene is also mutated in ts3 mutants. Although our observations indicated that only homozygous ts3 mice, but not heterozygotes or WTs, showed phenotypic abnormalities, this does not entirely exclude the possibility that mutations occur on multiple genes in a linkage group on the same chromosome. Regarding any inconsistency between our immunohistochemical and western blotting data on Cbln1 expression, one possible explanation is that Cbln1 protein was washed away during the process of immunostaining due to lack of normal PF-PC synapse formation, as suggested in a previous report [36].

In this study, we also found individual phenotypic differences between ts3 mutants, although their genetic background is almost identical. First, whereas most of those we observed developed paraplegia, some mice developed mild appendicular ataxia, including fore limbs. Rates of ataxic progression also displayed individual differences. Second, severity of cerebellar atrophy also differed between mice, which does not significantly correlate with difference in behavioral abnormalities. Third, some mice showed anterior folial atrophy in which the anterior part of the cerebellar folia was significantly smaller in size than its posterior counterpart (Fig. 2B, 4A), although this does not seem to directly correlate with severity in ataxia (Fig. 4A). In other ts3 mutants, however, neither the anterior part nor the whole cerebella were smaller than those of WTs.

In summary, the present study demonstrated that the ts3 mutant is a SCD model that exhibits unique features in its behavioral and neuropathological abnormalities when compared with other known SCD model mice. Further studies, including an electrophysiological approach, might address the fundamental question of whether the mutated gene is involved in development, survival, or playing a role in a signaling pathway in the cerebellar neural network.

Supporting Information

**Figure S1 Reduced body weights in ts3 mutant mice.** The average body weight (BW) of ts3 mutants and WT controls at 4, 8, and 16 weeks of age (n = 3, means +/- SD). ***P<0.001, *P<0.05. Note that the average BWs are significantly lower in ts3 mutants than in normal controls at all ages examined. (TIF)

**Figure S2 Relative cerebellar size is significantly smaller in ts3 mutants.** Cerebral and cerebellar sizes were measured from MR images as shown in Fig. 2. A) MR images of the WT and ts3 brains with long and short axes as indicated by red lines to quantify cerebral size. B) Average cerebral size was measured by multiplying length of long and short axes (mm, n = 3). C) To examine whether relative cerebellar size is smaller than that of the cerebrum, ratios of average cerebellar and cerebral sizes (cerebellum/cerebrum, n = 3) in the two different genotypes are shown. Note that relative cerebellar size (cerebellum/cerebrum) is significantly smaller in ts3 mutant mice than that of WT controls, although entire brain size is also smaller. D) Average body weight for mice used in the experiments are shown (n = 3). *P<0.05, ***P<0.001. (TIF)

**Figure S3 Spinal cord morphology at different levels along antero-posterior axis.** Coronal sections of ts3 and WT spinal cord were H&E stained to confirm normal spinal cord morphology for both genotypes, as shown in Fig. 2. (TIF)

**Figure S4 Significant reduction in the average number of Purkinje cells in older ts3 mutants.** Average number of Purkinje cells in each lobule (number/mm²) in ts3 mutant and control mice at 4 weeks (A) and 1 year old (B). In 1-year-old ts3 mutant mice, significant decreases in PC cells were observed in lobules VII and VIII of the mutant cerebella when compared with WT controls. **P<0.01. (TIF)

**Figure S5 Expression level of Cbln1 in ts3 cerebellum is comparable with that of control.** Western blotting on ts3/+ and ts3/ts3 cerebellum demonstrated that Cbln1 is expressed at comparable levels in ts3 mutants and controls, whereas GluD2 is not detectable in ts3 cerebella. This is in contrast to the results of WTs.

A New grid2 Mutant Allele with Cerebellar Ataxia
shown in Fig. 9 by immunohistochemistry, where Cbln1 was not detectable in ts3 cerebella.

**(TH)**

**Figure S6 Detection of a large deletion of the grid2 DNA and GluD2 protein in ts3 mutant mice.** Sequencing analysis revealed a 1001 bp deletion in the ts3 grid2 cDNA corresponding to sequences from exon 3 to 8 (highlighted in A), causing deletion of 333 amino acids (83-415) in the GluD2 protein (highlighted in B). This DNA deletion also results in a frame-shift mutation, and the final gene product is 124 amino acids in length (see Fig. 10B).

**(TH)**

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**Author Contributions**

Conceived and designed the experiments: NH. Performed the experiments: Y. Miyoshi YY TM K. Saigoh NK Y. Monobe NH. Analyzed the data: Y. Miyoshi YY K. Suzuki TM SW NH. Contributed reagents/materials/analysis tools: MK JM. Wrote the paper: YY MW NH.