Recent genetic and molecular biological analyses have revealed many forms of inherited channelopathies. Homozygous ataxic mice, tottering (tg) and leaner (tg\textsuperscript{lah}) mice, have mutations in the P/Q-type Ca\textsuperscript{2+} channel \( \alpha_{1A} \) subunit gene. Although their clinical phenotypes, histological changes, and locations of gene mutations are known, it remains unclear what phenotypes the mutant Ca\textsuperscript{2+} channels manifest, or whether the altered channel properties are the primary consequence of the mutations. To address these questions, we have characterized the electrophysiological properties of Ca\textsuperscript{2+} channels in cerebellar Purkinje cells, where the P-type is the dominant Ca\textsuperscript{2+} channel, dissociated from the normal, \( t_g \), and \( t_g^{\text{lah}} \) mice, and compared them with the properties of the wild-type and mutant \( \alpha_{1A} \) channels recombinantly expressed with the \( \alpha_2 \) and \( \beta \) subunits in baby hamster kidney cells. The most striking feature of Ca\textsuperscript{2+} channel currents of mutant Purkinje cells was a marked reduction in current density, being reduced to \(-60\) and \(-40\%\) of control in \( t_g \) and \( t_g^{\text{lah}} \) mice, respectively, without changes of cell size. The Ca\textsuperscript{2+} channel currents in the \( t_g \) Purkinje cells showed a relative increase in non-inactivating component in voltage-dependent inactivation. Besides the same change, those of the \( t_g^{\text{lah}} \) mice showed a more distinct change in voltage dependence of activation and inactivation, being shifted in the depolarizing direction by \(-10\) mV, with a broader voltage dependence of inactivation. In the recombinant expression system, the \( t_g \) channel with a missense mutation (P601L) and one form of the two possible \( t_g^{\text{lah}} \) aberrant splicing products, \( t_g^{\text{lah}} \) (short) channel, showed a significant reduction in current density, while the other form of the \( t_g^{\text{lah}} \) channels, \( t_g^{\text{lah}} \) (long), had a current density comparable to the normal control. On the other hand, the shift in voltage dependence of activation and inactivation was observed only for the \( t_g^{\text{lah}} \) (long) channel. Comparison of properties of the native and recombinant mutant channels suggests that single tottering mutations are directly responsible for the neuropathic phenotypes of reduction in current density and deviations in gating behavior, which lead to neuronal death and cerebellar atrophy.

Ca\textsuperscript{2+} controls diverse cellular processes, which include muscle contraction, neurotransmitter release, and other forms of secretion, gene expression, and cell proliferation (1). To evoke these cellular responses, Ca\textsuperscript{2+} influx across the plasma membrane makes a major contribution to augmenting the cytosolic free Ca\textsuperscript{2+} concentration. In neurons, voltage-gated Ca\textsuperscript{2+} channels are one of the major transmembrane pathways, together with Ca\textsuperscript{2+}-permeable ligand-gated channels. Electrophysiological and pharmacological studies have defined several types of Ca\textsuperscript{2+} channels in neurons. There are at least five types of high-threshold Ca\textsuperscript{2+} channels (L, N, P, Q, and R) and a low-threshold Ca\textsuperscript{2+} channel (T) (2–5). At the molecular level, the voltage-gated Ca\textsuperscript{2+} channels are composed of the main pore-forming \( \alpha_1 \) subunit and the accessory \( \alpha_2 \) and \( \beta \) subunits (6–8), and optional subunits, such as the \( \gamma \) subunit in the skeletal muscle Ca\textsuperscript{2+} channel (9, 10). Molecular biological analyses have found a gene family of \( \alpha_{1A} \) subunits, which are produced from the same gene by alternative splicing (13) and/or have different subunit compositions (14). The \( \alpha_{1A} \) channel was first described in cerebellar Purkinje cells as a Ca\textsuperscript{2+} channel that is not blocked by \( \omega \)-conotoxin GVIA or dihydropyridines (15), but is highly sensitive to \( \omega \)-agatoxin (\( \omega \)-Ag)\textsubscript{I} Va (16). The \( \alpha_{1A} \) subunit is identified in cerebellar granule cells and is similar to the P-type channel, but has a lower sensitivity to \( \omega \)-Ag Va, and exhibits faster inactivation kinetics (17). Several types of these Ca\textsuperscript{2+} channels are co-localized in a single neuron and are believed to contribute to fine tuning of neuronal activity, because each type of Ca\textsuperscript{2+} channel is modulated in a different manner. Although the critical role of Ca\textsuperscript{2+} channels, particularly the P- and N-type, for transmitter release in the synaptic terminals has been well established (18, 19), the roles of Ca\textsuperscript{2+} channels in integration of signals or synaptic plasticity have been poorly understood.

Recent genetic and molecular biological analyses have identified that mutations of the gene encoding the Ca\textsuperscript{2+} channel \( \alpha_{1A} \) subunit cause cerebellar ataxia and other forms of neurological disorders. A missense mutation was found in the tottering (\( t_g \)) mice, which display a delayed-onset, recessive disorder consisting of ataxia, motor seizure, and absence seizure resembling petit mal epilepsy (20). The \( t_g \) mutation causes substitution of leucine for proline at a position close to the conserved pore-lining region (P region) in the extracellular segment in the second repeat. Mice with an allelic tottering mutation \( \omega \)-Agol (\( t_g^{\text{lah}} \)), which causes severer symptoms, were found to have a
single nucleotide substitution at an exon/intron junction, which results in skipping the exon/intron, or results in failure to splice out the succeeding intron. In both cases, the $tg^{th}$ mutation causes truncation of the normal open reading frame and expression of aberrant C-terminal sequences. Furthermore, in the human $\alpha_{1A}$ Ca$^{2+}$ channel gene, missense mutations were found in familial hemiplegic migraine, mutations disrupting the reading frame in episodic ataxia type-2 (21), and CAG repeat expansion in autosomal dominant spinocerebellar ataxia (SCA6) (22). Here, in order to disclose the causative relationship among the tottering mutations, the affected Ca$^{2+}$ channel properties, and the neurological disorders, we made comprehensive comparison of the mutant Ca$^{2+}$ channel properties in native Purkinje cells of tottering $tg$ and $tg^{th}$ mice, where many other factors can affect the channel phenotype, and in the recombinant expression system, where direct effects of mutations can be evaluated precisely.

**EXPERIMENTAL PROCEDURES**

**Animals**—C57BL/6Jtg and C57BL/6J-os/+; $tg^{th}$ strains were introduced from the Jackson Laboratory (Bar Harbor, ME) to the Eisai Taku Laboratories. The purchased animals of the commercial diet (CE-2, Nihon Cleo, Tokyo, Japan) and water ad libitum under conventional conditions with controlled temperature, humidity, and lighting. The oligosynthesis (Os) heterozygous phenotype is a marker for the $tg^{th}$ heterozygote of $tg$.

**Polymerase Chain Reaction-restriction Enzyme Fragment Length Polymorphism (PCR-RFLP)—A PCR-RFLP method was developed to distinguish between genotypes of $tg$ and $tg^{th}$ mice. In the case of $tg^{th}$, its genotypes can be inferred by the Os phenotype which is tightly linked with $tg^{th}$. However, since recombination between $tg^{th}$ and Os, which leads to an error in $tg^{th}$ genotyping, is possible, we developed a PCR-RFLP method also for $tg^{th}$ genotyping. We first obtained the 5'- and 3'-flanking regions of the $tg$ and $tg^{th}$ mutants using GenomeWalker™ kit for Mouse (CLONTECH, Palo Alto, CA) according to the manufacturer instructions. First PCR was done with an adaptor primer 1 (5'-GTAATACGACTCACTATAGGG-3') and a gene-specific primer, which has the nucleotide sequence of 7 cycles of 25 s at 94 °C and 4 min at 72 °C, 32 cycles of 25 s at 94 °C and 4 min at 67 °C with a final extension for 4 min at 67 °C in MiniCycler™ (MJ Research, Watertown, MA). The resulting PCR products were amplified using a nested adapter primer 2 (5'-ACTATAGGGCACGCGTGGT-3') and a nested gene-specific primer. The conditions of nested PCR consisted of 5 cycles of 25 s at 94 °C and 4 min at 72 °C, 22 cycles of 25 s at 94 °C and 4 min at 67 °C with an additional 4 min at 67 °C. The oligonucleotides used for the cloning are summarized in Fig. 1. The nested PCR products were TA-subcloned to pTBlueR/T-vector (Novagen, Madison, WI). Nucleotide sequences were determined by a Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Norwalk, CA) using the above primers with an ABI Prism™ 377 DNA Sequencer (Perkin-Elmer). PCR conditions for sequencing were 25 cycles of 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C in GeneAmp PCR System 9600 (Perkin-Elmer). The nucleotide sequences of 1633- and 2099-bp genomic fragments including the tet and $tg^{th}$ mutation were determined (GenBank/EMBL accession numbers, AB011375 and AB011276, respectively). PCR primers for amplifying genomic fragments necessary for $tg$ and $tg^{th}$ genotyping were designed based on the above mentioned sequence results. Primers designed were: 5'-GGAAACACGAGCATGAACTCCG-3' (sense) and 5'-GAATGAGGAATTCAGGG-3' (antisense) for $tg$, and 5'-ACGAAGGGCAGTGAAGGG-3' (TGAL1- sense) and 5'-TTCGATGAGGGCATGTTT-3' (TGAL5R, antisense) for $tg^{th}$. Genomic DNA was extracted from the tail as follows: a mouse tail tip about 2 mm in length was cut and put into a 0.5-mL tube with a safety lock. Forty μL of distilled water and a drop of mineral oil (Sigma) were added. The sample was heated at 96 °C for 10 min, and then treated with proteinase K at 55 °C for 90 min. One μL of the DNA-extracted solution was used as a template for PCR. The PCR parameters consisted of 45 cycles of 0.5 min at 94 °C, 1 min at 50 °C, and 1.5 min at 72 °C with a final extension of 10 min at 72 °C. The resulting PCR products were digested with EcoRI (New England Biolabs, Beverly, MA) and BsoNI (New England Biolabs) for $tg$ and $tg^{th}$ genotyping, respectively. The digested products were subjected to electrophoresis on a 2.0% agarose/Et-Br gel and typed. Digestion with the enzymes yielded the following fragments: 296 bp in $tg^{th}$, 127 and 169 bp in $tg$, and 127, 168, and 285 bp in $tg$/+$tg^{th}$, and 91 and 138 bp in $tg$ in Fig. 1C.**

**Construction of Expression Plasmids Encoding Ca$^{2+}$ Channel $\alpha_{1A}$ Subunit with $tg$ or $tg^{th}$ Mutations—**For construction of expression cDNA encoding the $tg$-$\alpha_{1A}$ (BI-2) subunit mutant, a PCR fragment amplified using pSPCBI-2 (23) as a template, a primer BIEL1 (+) (5'-TATGAGAAGGATCTGATGGG-3') (sense) and 5'-GGTGGTTGGCAGATGTTCTCTTCGTCGAAATAAACCGT-3' (antisense), and a PCR fragment amplified using pSPCBI-2, and a primer 2PL (+) (5'-CGCGAAGAAATCTCCGCGCCTG-3') (antisense) and a primer BIIHD1 (+) (5'-CGGCGCAAGTTGTTGTGACG-3') (antisense), were combined with the primers BIEL1 (+) and BIIHD1 (+) in the subsequent PCR. The PCR product was digested with EcoRI and NcoI, and the yielded mouse fragment was substituted for the corresponding EcoRI (5892)/NcoI (6273) sequence of the rabbit $\alpha_{1A}$ (BI-2) cDNA in the recombinant plasmid pK4KBI-2 (24) to obtain pK4KBI-$tg$. For construction of expression cDNA encoding the $tg^{th}$ (long)-$\alpha_{1A}$ (BI-2) subunit mutant with intron, a partial genomic $\alpha_{1A}$ DNA was first isolated from B6.AKR-tg$tg$ mouse liver, by PCR amplification using KlenTag Polymerase (CLONTECH), the primers TGLA-1 (5'-CGGAAGGAGCAGCTCTTAAGCGCCAG-3') (sense) and TGLA-5R (5'-TTCATGGGGAATGATGTTT-3') (antisense), and a PCR fragment amplified using the mouse $\alpha_{1A}$ cDNA, a primer mBI-d1 (+) (5'-GTGGCCAGATCTGTCGGCGCAT-3') (antisense), and a PCR fragment amplified using the mouse $\alpha_{1A}$ cDNA, and a primer TGLA-3R (5'-GCAGATTTCTGAGGACAGCT-3') (antisense), and a PCR fragment amplified using the mouse $\alpha_{1A}$ cDNA, and a primer TGLA-2R (5'-GAGATTGTCAGAAGGAGCTCGCC-3') (antisense), and TGLA-5R, were combined with primers mBI-d1 (+) and TGLA-5R for further PCR. The PCR product was digested with $BgII$ and NcoI, and the yielded mouse fragment was substituted for the corresponding $BgII$/NcoI (6273) sequence of the rabbit $\alpha_{1A}$ (BI-2) cDNA in the recombinant plasmid pK4KBI-2 (24) to obtain pK4KBI-$tg^{th}$ (short).

**Preparation of Dissociated Purkinje Cells—**Purkinje cells were freshly dissociated from 18- to 30-day-old mice under ether anesthesia. The procedure for obtaining dissociated cells from mice is similar to that described elsewhere (25). Coronal slices (400-μm thick) of cerebellum were prepared using a microslicer (DTK-1000, Dosaka, Kyoto, Japan). Coronal slices (400-μm thick) of cerebellum were prepared using a microslicer (DTK-1000, Dosaka, Kyoto, Japan). Purkinje cells were isolated from C57BL/6J (B6) mouse brain poly(A)$^+$ RNA, by PCR amplification using a Marathon cDNA amplification kit (CLONTECH), the primers mBII(5'-CTCAAGACCTACGTTGGTATGC-3') (sense) and mBI-d1(+) (5'-GAATGTTCCAGGACTCTAC-3') (antisense) were used to amplify the 1.3-kb fragment corresponding to the gene encoding the $\alpha_{1A}$ subunit. In the subsequent PCR. The PCR product was digested with $BgII$ and NcoI, and the yielded mouse fragment was substituted for the corresponding $BgII$/NcoI (6273) sequence of the rabbit $\alpha_{1A}$ (BI-2) cDNA in the recombinant plasmid pK4KBI-2 (24) to obtain pK4KBI-$tg^{th}$ (short).
subunits. Electrophysiological measurements and Northern blot analysis were employed to identify functional expression of the α1A channel in BHK-BI-tg. To transiently express tgα1A mutant α1A channels, BHK6 cells were transfected with the recombinant plasmid pK4KI-tgα1A (long) or pK4KI-tgα1A (short) plus pH3-CD8 containing the cDNA of the T-cell antigen receptor (28). Transfection was carried out using SuperFect Transfection Reagent (QIAGEN, Hilden, Germany). Cells were trypsinized, diluted with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 30 units/ml penicillin, and 30 μg/ml streptomycin, and plated onto Celldisk (Sumitomo Bakelite, Tokyo, Japan) 18 h after transfection. Then cells were subjected to measurements 48–66 h after plating on the coverslips. Cells expressing α1A channels were selected through detection of CD8 expression using polystyrene microspheres precoated with antibody to CD8 (Dynabeads M-450 CD8; DYNAOL, Oslo, Norway).

Whole Cell Recordings—Electrophysiological measurements were performed on Purkinje cell and BHK cells. BHK cells, stably expressing wild-type or tgα1A channel, were seeded onto plastic coverslips, Celldesk, and incubated in culture medium for 5–8 days. Records were recorded at room temperature (22–25 °C) using whole cell mode of the patch-clamp technique (29) with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA) or an EPC-7 (List Medical, Darmstadt, Germany). Patch pipettes were made from borosilicate glass capillaries (1.5 mm outer diameter, 1.1 mm inner diameter; Narishige, Tokyo, Japan) using a model P-87 Flaming-Brown micropipette puller (Sutter Instrument Co., San Rafael, CA). The patch electrodes were fire-polished. Pipette resistance varied from 1 to 2 MΩ when filled with the pipette solutions described below. The series resistance was electronically compensated to >70% and both the leakage and the remaining capacitance were subtracted by P/6 method. Currents were sampled at 10 kHz after low pass filtering at 1 kHz (−3 db) using the 8-pole Bessel filter (Model 900, Frequency Devices, Haverhill, MA) in the experiments of activation kinetics, otherwise sampled at 10 kHz after low pass filtering at 2 kHz (−3 db). Data were collected and analyzed using the pCLAMP 6.02 software (Axon Instruments). Ba2+ currents were recorded in an external solution that contained (in mM): 3 BaCl2, 155 tetraethylammonium chloride, 10 HEPES, 10 glucose (pH adjusted to 7.4 with tetraethylammonium-OH). The pipette solution contained (in mM): 85 Cs-aspartate, 40 CsCl, 2 MgCl2, 5 EGTA, 2 ATP-Mg, 5 HEPES, 10 creatine phosphate (pH adjusted to 7.4 with CsOH). In the experiments with ω-Aga IVA, the external solution was always supplemented with 0.1 mg/ml cycloheximide. Cytochrome c at 0.1 mg/ml had no effect on Ba2+ currents. Rapid application of drugs were made by a modified “Y-tube” method. Details of this technique have already appeared (30). The external solution surrounding a single cell was completely exchanged within 200 ms. Statistical comparison between normal and mutant mice or mutant channels was performed by Student’s t-test (∗, p < 0.05; ∗∗, p < 0.01; ∗∗∗, p < 0.001).

Single Channel Recordings—The cell-attached patch recording technique was used to measure single-channel currents (29). Patch electrodes were coated with silicone (KE-106, Shinetsu Silicone, Tokyo, Japan) and had resistance of 5–8 MΩ. The bath solution contained (in mM): 85 Cs-aspartate, 40 CsCl, 2 MgCl2, 5 EGTA, 2 ATP-Mg, 5 HEPES, 10 creatine phosphate (pH adjusted to 7.4 with CsOH). In the experiments with ω-Aga IVA, the external solution was always supplemented with 0.1 mg/ml cycloheximide. Cytochrome c at 0.1 mg/ml had no effect on Ba2+ currents. Rapid application of drugs were made by a modified “Y-tube” method. Details of this technique have already appeared (30). The external solution surrounding a single cell was completely exchanged within 200 ms. Statistical comparison between normal and mutant mice or mutant channels was performed by Student’s t-test (∗, p < 0.05; ∗∗, p < 0.01; ∗∗∗, p < 0.001).

RESULTS

Comparison of Ca2+ Channel Currents in Purkinje Cells from Normal, tg, and tgα1A Mice—Mouse α1A genotypes were determined prior to electrophysiological characterization of Ca2+ channel currents in Purkinje neurons. PCR and subsequent digestion of the resulting products by the restriction enzymes AciI and BsaJI, whose sites are only present in normal genomes, distinguished tottering (tg) and leaner (tgα1A), respectively, from normal genotypes (Fig. 1).

Cerebellar Purkinje neurons were freshly dissociated from 18- to 30-day-old normal (C57BL/6J, n = 13), tg (n = 9), and tgα1A mice (n = 7). We first compared maximum amplitudes of currents evoked by step pulses from a holding potential (VH) of −80 mV in 3 mM Ba2+ solution (Fig. 2). In normal mice, maximum amplitudes varied from 1.21 to 12.54 nA, whereas they were reduced in tg mice and tgα1A mice; their maximum amplitudes were at most 5.39 and 4.67 nA, respectively. The mean amplitudes for tg mice (2.95 ± 0.25 nA, n = 27) and tgα1A mice (2.09 ± 0.17 nA, n = 25) were significantly smaller than that for normal mice (5.09 ± 0.29 nA, n = 67). The tgα1A mice grow up slowly presumably due to malnutrition, which might cause a smaller cell size, consequently resulting in the smaller amplitudes. However, the cell capacitance was very similar among the three groups. The mean capacitance was 16.7 ± 0.5 pF (n = 67) for normal mice, 18.0 ± 0.8 pF (n = 27) for tg mice, and 17.4 ± 0.8 pF (n = 25) for tgα1A mice, indicating that cell bodies of cerebellar Purkinje neurons grow up similarly in regard to morphology. The current density, current amplitude divided by cell capacitance, is also an index for comparison of channel activity among the three groups. The current density for both tg mice (184.2 ± 18.1 pA/pF, n = 27) and tgα1A mice (122.8 ± 9.0 pA/pF, n = 25) is significantly lower than that for normal mice (333.4 ± 18.1 pA/pF, n = 67). During preparation of this article, reduction in Ca2+ channel currents in tgα1A Purkinje cells was reported (31).

Do the normal mutant mice exhibit diminished P-type Ca2+ channel activity? We examined this question with a P-type Ca2+ channel blocker, ω-Aga IVA. Application of 100 μM ω-Aga IVA reduced the current density to 25.5 ± 2.5 pA/pF (n = 7) in normal mice, indicating that more than 90% of high threshold current in cerebellar Purkinje cells flow through the P-type Ca2+ channel. This result confirms the previous observation in cerebellar Purkinje neurons (32, 33) showing that ω-Aga IVA
inhibits around 90% of total Ca\(^{2+}\) current. The current density insensitive to \(\omega\)-Aga IVA was 21.7 ± 5.9 pA/pF (n = 4) in \(tg\) mice and 29.8 ± 2.6 pA/pF (n = 12) in \(tg^{la}\) mice. The residual current density in \(tg^{la}\) mice was higher than that in normal mice, but the values were not different substantially among the three groups. It is concluded that current amplitude and current density of P-type Ca\(^{2+}\) channel are reduced in \(tg\) and \(tg^{la}\) mice. Furthermore, \(\omega\)-Aga IVA-insensitive Ca\(^{2+}\) channels, whose amplitude is not affected by the mutations, do not compensate cerebellar Purkinje cells for low channel activity.

Fig. 3 shows Ca\(^{2+}\) channel currents and their current-voltage (I-V) relationships in Purkinje cells from normal and mutant mice. Ba\(^{2+}\) currents were elicited with 30-ms depolarizing pulses from a \(V_m\) of −80 mV to test potentials from −50 to 50 or 60 mV with increments of 10 mV in 3 mM Ba\(^{2+}\) solution. The current density was significantly lower at test potentials between −20 and 20 mV for \(tg\) mice and between −40 and 20 mV for \(tg^{la}\) mice. The Ba\(^{2+}\) currents in normal and \(tg\) mice were similar in the I-V relationship with currents first detectable at voltages near −40 mV, grew to reach the maximal amplitude around −20 mV and then declined toward a zero current asymptote with further depolarization. However, the I-V relationship for \(tg^{la}\) mice was shifted in the depolarizing direction by about 10 mV. To describe activation parameters more accurately, we measured tail currents evoked by clamp-back to the fixed potential of −60 mV after 5-ms step depolarization from −50 to 30 mV for normal and \(tg\) mice or from −45 to 30 mV for \(tg^{la}\) mice with 5 mV increments (Fig. 4A). Peak amplitude of tail currents, which should reflect Ca\(^{2+}\) channel activation, could be fitted by a single Boltzmann function, where the voltages for half-maximal activation and slope factors were −28.0 ± 1.1 and 4.9 ± 0.5 mV (n = 11) for normal mice, −28.3 ± 1.1 and 4.7 ± 0.3 mV (n = 13) for \(tg\) mice, and −19.2 ± 1.3 and 5.4 ± 0.3 mV (n = 13) for \(tg^{la}\) mice, respectively (Table I). The activation curve for \(tg^{la}\) mice was significantly shifted in the depolarizing direction without changing the slope factor (Fig. 4A). When we ignore the negative curvature at membrane potentials over 30 mV, apparent reversal potentials, calculated from the I-V relationships, were 37.6 ± 1.3 mV (n = 52) for normal mice, 35.2 ± 0.7 mV (n = 25) for \(tg\) mice, and 37.9 ± 1.0 mV (n = 20) for \(tg^{la}\) mice, indicating that apparent permeability was not changed in mutant mice.

The voltage dependence of inactivation was measured by the use of 2-s prepulses. Peak current amplitude induced by the test pulse to −20 or −10 mV from various prepulse voltages was normalized to the amplitude induced by the test pulse from a prepulse potential of −100 mV and was plotted against the prepulse potentials (Fig. 4B). Voltage dependence of inactivating components was fitted with the Boltzmann’s equation. The inactivating components induced by the 2-s voltage displacements were significantly smaller for \(tg\) mice (0.53 ± 0.04, n = 9) and \(tg^{la}\) mice (0.53 ± 0.03, n = 9) than for normal mice (0.70 ± 0.04, n = 15) (Table I). In addition, the Boltzmann’s parameters for \(tg^{la}\) mice were different from normal mice. The midpoint of inactivation curve was −23.7 ± 2.0 mV with the slope factor of 10.6 ± 1.6 mV (n = 9) for \(tg^{la}\) mice, −38.9 ± 2.9 mV with the slope factor of 5.3 ± 0.5 mV (n = 15) for normal mice, and −33.9 ± 2.2 mV with the slope factor of 5.4 ± 0.7 mV (n = 9) for \(tg\) mice.

Because mutant mice have different voltage dependence of activation, we next examined the activation kinetics. Ba\(^{2+}\) currents were elicited by 5-ms test pulses to various potentials from −45 to 30 mV with 5 mV increments (Fig. 5A). The time course of the rising phase was well described by a single time constant. The time constant versus test potential relationship was “bell shaped” for normal mice and two mutant mice (Fig. 5B). At a test potential of −30 mV, the time constants for normal mice and \(tg\) mice reached a maximum (1.83 ± 0.23 ms, n = 9 for normal mice, and 2.35 ± 0.37 ms, n = 13 for \(tg\) mice). At test potentials positive to −30 mV, the time constant decreased with increasing test pulse voltage. In accordance with the shift of activation curve for \(tg^{la}\) mice, voltage dependence of the time constant was shifted in the depolarizing direction by 10 mV with a maximum of 2.16 ± 0.20 ms (n = 13 at −20 mV).

The time constants of activation phase were 0.23 ± 0.02 ms (n = 10), 0.28 ± 0.03 ms (n = 13), and 0.46 ± 0.03 ms (n = 13) at a test potential of 10 mV for normal mice, \(tg\) mice, and \(tg^{la}\) mice, respectively. The activation of \(tg^{la}\) mice was statistically slower than that of normal mice and \(tg\) mice at potentials positive to −20 mV.

It is hard to find the difference in the rate of inactivation in Purkinje cells among the three types of mice, because as shown in Fig. 3, Ba\(^{2+}\) currents decayed little within 30-ms test pulses. However, the mutant mice have different inactivation curves from normal mice (Fig. 4B). To further examine the inactivation rate of Ca\(^{2+}\) channel in Purkinje cells from the three types of mice, the decay phase of Ba\(^{2+}\) currents evoked by 2-s test pulses was analyzed. The decay phase was well fitted by two exponential functions with a non-inactivating component (Fig. 6). The two exponential time constants for \(tg\) mice and \(tg^{la}\) mice were similar to those for normal mice (about 50 ms for the fast component and about 1600 ms for the slow component) except the fast exponential time constant for \(tg^{la}\) mice at test potentials of −10 and 0 mV. The mean values of the fast and slow
time constants at a test potential of 0 mV were 51.9 ± 3.3 ms and 1454.6 ± 207.5 ms (n = 15) for normal mice, 47.6 ± 5.2 ms and 1404.3 ± 112.6 ms (n = 9) for tg mice, and 36.2 ± 4.2 ms and 1721.4 ± 201.1 ms (n = 9) for tg<sup>la</sup> mice, respectively. The ratio of the three components, fast, slow, and non-inactivating components at a test potential of 0 mV, were 0.24 ± 0.03, 0.45 ± 0.03, and 0.31 ± 0.03 for normal mice, 0.18 ± 0.02, 0.34 ± 0.04, and 0.48 ± 0.02 for tg mice, and 0.19 ± 0.02, 0.38 ± 0.03, and 0.43 ± 0.02 for tg<sup>la</sup> mice, respectively (Fig. 6C). The ratios of fast and slow inactivating components for tg mice and tg<sup>la</sup> mice were smaller than those for normal mice at all potentials between −20 and 20 mV, although the difference was not statistically significant. The non-inactivating components were significantly larger for tg mice and tg<sup>la</sup> mice at test potentials between −10 and 10 mV, being in agreement with the voltage-dependent inactivation curves in
Alteration of \( \alpha_{\text{IA}} \) Channel Function by Tottering Mutations

**TABLE I**

| Mice     | Activation | Inactivation | Decay component |
|----------|------------|--------------|-----------------|
|          | \( n \)    | \( V_{0.5} \) | \( k \)         | \( n \) | \( V_{0.5} \) | \( k \) |          |
| Normal   | 11         | -28.0 ± 1.1  | 4.92 ± 0.50     | 15     | -36.9 ± 2.9  | 5.32 ± 0.45 | 0.70 ± 0.04 |
| \( \text{tg} \) | 13         | -28.3 ± 1.1  | 4.65 ± 0.33     | 9      | -33.9 ± 2.2  | 5.39 ± 0.67 | 0.53 ± 0.04 |
| \( \text{tg}^{\alpha} \) | 13         | -19.2 ± 1.3a | 5.38 ± 0.30     | 9      | -23.7 ± 2.0a | 10.64 ± 1.64 | 0.53 ± 0.03 |

* Statistically significant (\( p < 0.001 \)) different from normal mice.

**Fig. 5. Voltage dependence of activation time constant.** A, families of \( \text{Ba}^{2+} \) currents and the curve fit of their activation phases. \( \text{Ba}^{2+} \) currents were evoked by 5-ms step depolarization from -40 to 20 mV for normal (a) and \( \text{tg} \) mice (b), and from -40 to 10 mV for \( \text{tg}^{\alpha} \) mice (c) in 10-mV increments from a \( V_c \) of -100 mV. Repolarization phases of the currents were blanked. Currents were filtered at 10 kHz and digitized at 100 kHz. The activation phases were well fitted by a single exponential function at all potentials. B, comparison of activation time constant. Activation time constant obtained from currents was plotted as a function of test potential. Data are expressed as mean ± S.E. if they are larger than symbols.

**Fig. 4.**

*Comparison of Wild-type and Mutant \( \alpha_{\text{IA}} \) Channels Expressed in BHK Cells.*—The \( \text{tg} \) mutation is a nucleotide (C to T) substitution (Fig. 1) that replaces Pro-601 with Leu in the extracellular S5-S6 linker region in repeat II (20). The S5-S6 linkers contain the P regions that form a pore structure in \( \text{Ca}^{2+} \) channels. On the other hand, the \( \text{tg}^{\alpha} \) mutation is a nucleotide (G to A) substitution (Fig. 1) at a splice donor site that would cause two aberrant splicing patterns: failure of splicing out an intron and skipping of one exon (20). Inclusion of an intron results in translation of the intron sequence and an out-of-frame read through of subsequent exons, substituting an abnormal 99-amino acid sequence for the C-terminal sequence from Glu-1967 (\( \text{tg}^{\alpha} \) (long)). The exon skip results in an out-of-frame splice, exchanging the C-terminal sequence from Met-1922 with an abnormal 57-amino acid sequence (\( \text{tg}^{\alpha} \) (short)). Because we did not know which is the major product in the \( \text{tg}^{\alpha} \) mice, we constructed both candidates, \( \text{tg}^{\alpha} \) (long) and \( \text{tg}^{\alpha} \) (short).

We attempted to establish the stable cell lines of mutant \( \alpha_{\text{IA}} \) channels in BHK6 cells, which stably express the \( \alpha_{\beta2} \beta_{1A} \) subunits. The stable cell line of the \( \text{tg}^{\alpha} \) channel was successfully established, however, for unknown reasons, we could not obtain cell lines stably expressing \( \text{tg}^{\alpha} \) mutant channels. Therefore, we transiently expressed the \( \text{tg}^{\alpha} \) mutant channels into BHK6 and compared their channel activities with those of the wild-type \( \alpha_{\text{IA}} \) channel transiently expressed in BHK6. Fig. 7A shows typical \( \text{Ca}^{2+} \) channel currents in BHK cells, and Fig. 7B compares current density for the three mutant \( \alpha_{\text{IA}} \) channels with that for the wild-type \( \alpha_{\text{IA}} \) channel. In the stable expression system, current density ranged from 27.8 to 240.3 pA/pF with an average of 109.4 ± 11.3 pA/pF (\( n = 26 \)) for the wild-type \( \alpha_{\text{IA}} \) channel, and from 5.7 to 164.2 pA/pF with an average of 48.1 ± 6.1 pA/pF (\( n = 38 \)) for the \( \text{tg}^{\alpha} \) channel. In the transient expression system, current density was distributed from 27.9 to 130.0 pA/pF with an average of 66.3 ± 7.2 pA/pF (\( n = 20 \)) for the wild-type \( \alpha_{\text{IA}} \) channel, from 17.4 to 205.0 pA/pF with an average of 83.2 ± 12.6 pA/pF (\( n = 18 \)) for the \( \text{tg}^{\alpha} \) channel, and from 9.3 to 117.3 pA/pF with an average of 32.9 ± 10.4 pA/pF (\( n = 10 \)) for the \( \text{tg}^{\alpha} \) (short)-\( \alpha_{\text{IA}} \) channel. The activity of the \( \text{tg}^{\alpha} \) channel was significantly lower than control values. On the other hand, the activity of the \( \text{tg}^{\alpha} \) (long)-\( \alpha_{\text{IA}} \) channel was larger than that of the wild-type \( \alpha_{\text{IA}} \) channels expressed transiently, but the difference was not statistically significant. As shown in the Fig. 7B, current densities of the wild-type \( \alpha_{\text{IA}} \) channel in the transient expression system were smaller than those in the stable cell line, however, kinetics of activation and inactivation and parameters of voltage dependence were similar between the two expression systems (data not shown). Therefore we used the stable expression system for more detailed analysis of the recombinant wild-type \( \alpha_{\text{IA}} \) channels.

Depolarizing pulses for 30 ms from a \( V_c \) of -100 mV evoked inward currents that first appeared at -30 mV and grew with increments of depolarization, reached a peak in the I-V relationship around 0 mV, and then declined with progressively more depolarized voltage steps (Fig. 7C). To draw activation curves, tail currents were recorded at a potential of -50 mV following the termination of 5-ms test pulses to various potentials between -30 and 50 mV with 5-mV increments (Fig. 8A) as in Fig. 4. Tail current amplitude was normalized to the tail current amplitude following a test pulse to 50 mV and plotted against test potentials. The tail current activation shows “S-shaped” curves reaching half-maximal and maximal near -5 and 30 mV, respectively. The curves were symmetric around the half-maximal voltage and thus could be fitted to a single-component Boltzmann equation, in which the voltage for half-maximal activation and slope factors were -7.6 ± 1.0 mV and 5.0 ± 0.4 mV (\( n = 8 \)) for the wild-type \( \alpha_{\text{IA}} \) channel, -8.2 ± 0.5 mV and 5.9 ± 0.3 mV (\( n = 6 \)) for the \( \text{tg}^{\alpha} \) channel, -2.4 ± 0.7 mV and 7.0 ± 0.6 mV (\( n = 9 \)) for the \( \text{tg}^{\alpha} \) (long)-\( \alpha_{\text{IA}} \) channel, and -7.0 ± 1.4 mV and 7.0 ± 0.5 mV (\( n = 6 \)) for the \( \text{tg}^{\alpha} \) channel, respectively (Table II). The activation curve for the \( \text{tg}^{\alpha} \) (long)-\( \alpha_{\text{IA}} \) channel was shifted in the depolarizing direction by about 5 mV. In addition, the voltage dependence of the activation was slightly broader in the \( \text{tg}^{\alpha} \) (long)-\( \alpha_{\text{IA}} \) and the \( \text{tg}^{\alpha} \) (short)-\( \alpha_{\text{IA}} \) channels than in the wild-type \( \alpha_{\text{IA}} \) channel.

The voltage dependence of inactivation was determined by measuring the amplitude of the peak currents evoked by 20-ms
test pulses to 0 mV following 2-s prepulses to potentials between −110 and 10 mV and 10-ms interval at a $V_h$ of −100 mV (Fig. 8B). Peak current amplitude induced by the test pulse to 0 mV from various prepulse voltages was normalized to the peak current amplitude induced by the test pulse from a prepulse potential of −110 mV and was plotted against the prepulse potentials. Voltage dependence of inactivation was fitted with the Boltzmann’s equation. The estimated half-inactivation potential and the slope factor were −52.9 ± 1.4 and 8.7 ± 0.2 mV ($n$ = 12) for the wild-type $\alpha_{1A}$ channel, −52.4 ± 2.7 and 8.3 ± 0.3 mV ($n$ = 7) for the $tg\alpha_{1A}$ channel, −42.2 ± 1.3 and 7.6 ± 0.5 mV ($n$ = 7) for the $tg^{la}(long)\alpha_{1A}$ channel, and −53.5 ± 2.7 and 7.8 ± 0.4 mV ($n$ = 7) for the $tg^{la}(short)\alpha_{1A}$ channel, respectively (Table II). These results indicate that the $tg^{la}(long)\alpha_{1A}$ channel suffers less voltage-dependent inactivation at holding potentials between −70 and −30 mV.

Because the activation kinetics of Ca$^{2+}$ channel currents in Purkinje cells of $tg^{la}$ mice was statistically slower than those of normal and $tg$ Purkinje cells at potentials positive to −20 mV, we next examined the activation kinetics of the wild-type and the three mutant $\alpha_{1A}$ channels in BHK cells. Ba$^{2+}$ currents were elicited by 5-ms test pulses to various potentials from −25 to 45 mV with 5 mV increments. Current activation was well fitted by a single exponential. The time constant versus test potential relationship showed a “bell shape” for all the channels (Fig. 9). Around the voltages for half-maximal activation, the transient $\alpha_{1A}$, 2.19 ± 0.38 ms, $n$ = 9 for the $tg^{la}(long)\alpha_{1A}$ and 1.46 ± 0.18 ms, $n$ = 7 for the $tg^{la}(short)\alpha_{1A}$ channels at 100 mV. At test potentials positive to −5 mV, the time constant for the wild-type and the three mutant $\alpha_{1A}$ channels decreased asymptotically toward 0.3 ms with increasing test pulse voltage. In our expression system in BHK cells, we could not see the slow activation, which was observed in Purkinje neurons dissociated from $tg^{la}$ mice (Fig. 5).

In contrast to the P-type Ba$^{2+}$ currents recorded in Purkinje neurons (Fig. 3A), substantial inactivation occurred in the $\alpha_{1A}$ channel expressed in BHK cells during 30-ms step pulses to potentials positive to −10 mV (Fig. 7A). To compare inactivation kinetics of the three mutant $\alpha_{1A}$ channels with that of the wild-type $\alpha_{1A}$ channel, test pulses lasting 300 ms were given to
Single Channel Properties of Wild-type and Mutant α1A Channels—Because the tg mutation is close to the P region, it is possible that the tg mutation affects the single-channel properties. Therefore we compared the single-channel properties of the wild-type and the tg mutant α1A channels expressed in BHK cells. The α1A channel exhibited several conductance levels, which made it difficult to detect subtle changes in single-channel conductance. As shown in current traces in Fig. 11A, the unitary current amplitudes of well resolved long openings were the same in the wild-type and the tg mutant channels. Fig. 11B shows the amplitude histograms constructed from the currents at a test potential of 10 mV. The single channel conductance was 12.7 ± 0.7 pS (n = 4) for the wild-type α1A channel and 13.8 ± 0.3 pS (n = 3) for the tg-α1A channel. Together with the unaltered reversal potential of the macroscopic I-V relationship, we conclude that the tg mutation does not affect the channel pore properties.

DISCUSSION

In this study we evaluated the properties of the Ca2+ channels in acutely dissociated cerebellar Purkinje cells of the normal and the tottering (tg) and leaner (tgA10) ataxic mutant mice, and compared them with the properties of the wild-type and mutated Ca2+ channels recombinantly expressed in BHK cells, in order to determine which aspects of the Ca2+ channel functions are affected as the primary consequences of the mutations. We used the technique of acute dissociation of the Purkinje cells, because we can obtain quantitative properties of the voltage-gated Ca2+ channels most precisely with the voltage-clamp method. Comparison of the native and recombinant systems was particularly useful for mutations affecting RNA splicing, like tgA10, because these mutations can cause multiple gene products, resulting in complicated phenotypes in native Purkinje cells.

Functional Alterations of Mutant Ca2+ Channels—Comparison of the peak current amplitudes in the Purkinje cells of the normal, tg, and tgA10 mice showed a significant reduction in the tg cells (~60% of control) and a severer reduction in the tgA10 cells (~40% of control). Reduction in the current amplitude was not likely due to the result of nonspecific developmental or nutritional effects, because the size of Purkinje cells of mutant mice was not different from the normal control, at least in the range of age used for measurements. Comparison of the current density thus revealed 45 and 63% reduction in the tg and tgA10 cells, respectively. It is interesting to note that the omega-Aga IVA-insensitive component at a test potential of ~10 mV was not increased in the tg and tgA10 mutant mice, indicating that no compensatory mechanism operates to restore the reduced Ca2+ influx through the P/Q channel.

Reduction of current density was also observed in mutant channels expressed in the BHK cells. Although the level of expression of stably or transiently transformed BHK cells was variable, current density of the cells expressing the Ca2+ channel α1A subunit with the tg mutation or with the tgA10 (short) mutation was significantly smaller than the normal control. Because the tg mutation is a single nucleotide substitution, it is unlikely that the mutation affected the efficiency of transcription and translation. The cDNA constructs encoding the tg (long) and tgA10 (short) mutant channels contains sequences derived from the mouse Ca2+ channel α1A subunit gene. The observation that the expression level of the tgA10 (long) construct, which has a longer mouse genomic insert, slightly increased indicates that the insertion by itself did not affect functional expression strongly. Thus the reduced current density in BHK cells expressing the tg and tgA10 (short) mutants was not caused by altered efficiency of gene expression, but likely resulted from the same post-translational mechanism that im-

different voltages from a Vh of ~100 mV. The decay phase was well fitted by a two-exponential function with a non-inactivating component (Fig. 10). The two exponential time constants and their fractions of the tg-α1A and the tgA10 (short)-α1A channels were not significantly different from the corresponding values of the wild-type α1A channel at all test potentials. In the tgA10 (long)-α1A channel, these values did not deviate from the control values at test potentials positive to ~5 mV, whereas deviation was observed at some test potentials negative to ~10 mV. This may be due to small voltage-dependent inactivation below ~10 mV. The mean values of the fast and slow time constants at a test potential of 10 mV are 21.0 ± 1.4 and 94.7 ± 5.6 ms (n = 11) for the wild-type α1A channel, 23.1 ± 5.8 and 152.5 ± 42.6 ms (n = 6) for the tg-α1A channel, and 25.2 ± 3.0 and 123.5 ± 13.9 ms (n = 13) for the tgA10 (long)-α1A channel, and 20.3 ± 3.0 and 65.9 ± 7.3 ms (n = 7) for the tgA10 (short)-α1A channel, respectively. The ratio of the time components, fast, slow, and non-inactivating components, were 0.28 ± 0.03, 0.69 ± 0.04, and 0.03 ± 0.01 for the wild-type α1A channel, 0.28 ± 0.09, 0.65 ± 0.06, and 0.07 ± 0.03 for the tg-α1A channel, 0.24 ± 0.02, 0.71 ± 0.02, and 0.05 ± 0.01 for the tgA10 (long)-α1A channel, and 0.33 ± 0.06, 0.63 ± 0.05, and 0.04 ± 0.01 for the tgA10 (short)-α1A channel at a test potential of 10 mV, respectively (Fig. 10).
Due to decreased ion conductance caused through modification and association with the channels. Furthermore, it is possible that altered interaction C-terminal sequence would alter the density of functional capability or dihydropyridine binding (23, 37–39), the changes in the conformational changes to affect the availability of the functional test potential. Data are expressed as mean ± S.E.

| Channel | Activation | Inactivation |
|---------|------------|-------------|
|         | n          | $V_{0.5}$   | mV | k    | $V_{0.5}$ | mV | h |
| Wild-type | 8 | −7.6 ± 1.0 | 5.02 ± 0.38 | 12 | −52.9 ± 1.4 | 8.72 ± 0.23 |
| tg      | 6 | −8.2 ± 0.5 | 5.90 ± 0.29 | 7 | −52.4 ± 2.7 | 8.34 ± 0.30 |
| $tg^{la}$ (long) | 9 | −2.4 ± 0.7* | 7.01 ± 0.61b | 7 | −42.2 ± 1.3a | 7.61 ± 0.56 |
| $tg^{la}$ (short) | 6 | −7.0 ± 1.4 | 6.95 ± 0.49b | 7 | −53.5 ± 2.7 | 7.80 ± 0.38 |

*Statistically significant (p < 0.001) different from wild-type channel.

Statistically significant (p < 0.05 different from wild-type channel.

FIG. 9. Voltage dependence of activation time constant. Inset represents families of Ba2+ currents and the curve fit of their activation phases. Wild-type $\alpha_{1A}$ channel currents were evoked by 5 ms step depolarization from −20 to 30 mV in 10-mV increments from a $V_h$ of −100 mV. Repolarization phases of the currents were blanked. Currents were filtered at 10 kHz and digitized at 100 kHz. The activation phases were well fitted by a single exponential function at all potentials. Activation time constant obtained from currents was plotted as a function of test potential. Data are expressed as mean ± S.E. of 8, 7, 9, and 7 BHK cells expressing wild-type (○), $tg^{la}$ (△), $tg^{la}$ (long)- (□), and $tg^{la}$ (short)-$\alpha_{1A}$ channels (■), respectively. Vertical bars show mean ± S.E. if they are larger than symbols.

Pairs the channel activity in the mutant Purkinje cells.

The $tg$ mutation is located in the extracellular region close to the pore-forming P region of the second repeat (20). Replacement of proline for leucine might conceivably cause a substantial conformational change and alter the ion-conducting pathway, because a proline residue has its side chain fixed to the main chain and stabilizes the protein structure. However, neither the apparent reversal potential nor the single-channel conductance changed in the $tg$ mutant Ca2+ channel (Figs. 3 and 11), suggesting that the reduction in current density is not due to decreased ion conductance caused through modification of the pore structure by the $tg$ mutation.

The $tg^{la}$ mutations result in truncation of the normal open reading frame expression of aberrant C-terminal sequences (20). The C-terminal portion of the Ca2+ channels (34, 35), together with the linker portion connecting the repeats I and II (26), is involved in interaction with the $\beta$ subunit. Because association with the $\beta$ subunits affects availability of the functional Ca2+ channels, judged from enhanced opening probability or dihydropyridine binding (23, 37–39), the changes in the C-terminal sequence would alter the density of functional channels. Furthermore, it is possible that altered interaction with the $\beta$ subunit affects the gating properties of the mutant channel. Alternatively, alteration in the C-terminal region may cause impaired incorporation into the membrane or accelerated degradation of the channel protein. The $tg$ mutation may cause conformational changes to affect the availability of the functional Ca2+ channel or the gating mechanism, or alternatively to modify metabolism of the channel protein.

The $tg^{la}$ and $tg^{la}$ mutations also affect macroscopic gating. The Ca2+ channel in Purkinje cells from $tg^{la}$ mice showed a shift in current-voltage relationship in the depolarizing direction by ~10 mV. This change can be attributable to the shifts in voltage dependence of activation and inactivation in the depolarizing direction. Of the two forms of $tg^{la}$ mutant channels in expressed BHK cells, only the $tg^{la}$ (long) form showed the similar depolarizing shifts of voltage dependence, while the voltage dependence of the $tg^{la}$ (short) form, whose current density was markedly reduced, remained normal. The larger slope factor of voltage dependence of inactivation in the $tg^{la}$ Purkinje cells (Fig. 4B) could be explained by a mixture of the $tg^{la}$ (long) type and the $tg^{la}$ (short) type channels. Taken together with the reduced current density of the $tg^{la}$ (short) form, these results indicate that both $tg^{la}$ (long) and $tg^{la}$ (short) forms are expressed in the $tg^{la}$ Purkinje cells. The Ca2+ channel current in the $tg$ Purkinje cells showed no changes in the voltage dependence in activation or inactivation, and the voltage dependence of the $tg$ mutant channel in BHK cells was also normal.

The $tg^{la}$ mutation affected gating kinetics. Activation of the $tg^{la}$ channel in Purkinje cells was slower than the normal control. This change was not reproduced in the BHK cells, activation of the $tg^{la}$ (long) form was normal and that of the $tg^{la}$ (short) was slightly faster. The slower activation in the $tg^{la}$ Purkinje cells may be mainly due to the $\omega$-Aga IVA-insensitive component, which shows slower activation kinetics. Other kinetic changes in the $tg$ and $tg^{la}$ Purkinje cells were the increased proportion of the non-inactivating component. This altered property must be the consequence of mutations, because this result cannot be explained by contamination of the $\omega$-Aga IVA-insensitive component which inactivates faster. This mutant phenotype was not reproduced in the BHK cells, primarily because of the nature of the $\beta_1$ subunit we used in this study, which confers the fast inactivating character on the $\alpha_{1A}$ channel. We used the $\beta_1$ subunit because we used this subunit in our previous studies. On the other hand, cerebellar Purkinje cells express multiple isoforms of the $\beta$ subunits (40). Such a subtle difference may have caused functional differences between the Purkinje cells from ataxic mice and the BHK transfects with mutant $\alpha_{1A}$ subunits, although involvement of other proteins is also possible.

The present data described above include the most comprehensive description of the properties of Ca2+ channel current in Purkinje cells of $tg$ and $tg^{la}$ mutant mice and the thorough comparison between the native and recombinant systems. The reduction of the current density and most of the changes in gating were reproduced in the recombinant system using BHK cells. These supportive results in BHK cells strongly suggest that the neuropathic phenotype of the $tg$ and $tg^{la}$ Purkinje cells is the primary consequence of the mutations of the $\alpha_{1A}$ Ca2+ channel.
Fig. 10. Voltage dependence of inactivation time constant. A, Ba$^{2+}$ currents evoked by 300-ms test pulse to 10 mV from a $V_{h}$ of $-100$ mV in a BHK cell expressing wild-type $\alpha_{1A}$ channel. Current decay was fitted by a sum of two exponential functions with time constants of 19.9 and 92.4 ms, whose amplitude was $-0.79$ and $-1.70$ nA, respectively. Its sustained component was $-0.11$ nA. B, voltage dependence of the two inactivation time constants, $\tau_{fast}$ (a) and $\tau_{slow}$ (b). The mean inactivation time constant was plotted as a function of test potential from $-20$ to $40$ mV. C, voltage dependence of the fraction of the three components, fast (a), slow inactivation (b), and sustained components (c). The components were plotted against test potentials. Data are expressed as mean $\pm$ S.E. of 7, 6, 13, and 7 BHK cells expressing wild-type (○), $tg$- (△), $tg^{x}$- (long- □), and $tg^{y}$- (short-) $\alpha_{1A}$ channels (■), respectively. Vertical bars show mean $\pm$ S.E. if they are larger than symbols.

Fig. 11. Single-channel recordings from wild-type and $tg$-$\alpha_{1A}$ channels. A, currents were recorded in cell-attached configuration from BHK cells expressing wild-type $\alpha_{1A}$ channel (a and b) and $tg$-$\alpha_{1A}$ channel (c and d) using 110 mM Ba$^{2+}$ as charge carrier. Typical single-channel currents elicited by 150 ms stepping to 0 (a and c) and 10 mV (b and d). Arrows indicate beginning and end of test depolarization. B, amplitude histogram at a test potential of 10 mV was constructed from 160 and 176 traces of wild-type $\alpha_{1A}$ channel (a) and $tg$-$\alpha_{1A}$ channel (b), respectively. Histograms were fitted with three Gaussian functions. The components had means of $-0.08$, $-0.57$, and $-1.12$ pA for wild-type $\alpha_{1A}$ channel, and $-0.04$, $-0.59$, and $-1.25$ pA for $tg$-$\alpha_{1A}$ channel.

Reduced Ca$^{2+}$ Influx as the Cause of Cerebellar Atrophy—Impaired inactivation is a frequently observed mechanism of dysfunction for other ion channels. For example, mutations of the skeletal muscle Na$^{+}$ channel can cause slower inactivation, leading to hyperkalemic periodic paralysis (41). Based on the histological data that shrinkage and apoptosis of Purkinje cells are observed in $tg$ and $tg^{x}$ mutant mice (42, 43), our initial guess guided by the “Ca$^{2+}$-overload hypothesis” (44) was that mutations would affect the inactivation process, leading to prolonged opening of the channel, and that chronic excess entry of Ca$^{2+}$ into neurons and excess activation of Ca$^{2+}$-dependent intracellular signaling pathways would ultimately lead to cell death. Contrary to the prediction, the present results unambiguously demonstrate that the Ca$^{2+}$ conductance of the Purkinje cells of $tg$ and $tg^{x}$ mutant mice is markedly reduced. Thus our observation indicates that insufficient Ca$^{2+}$ influx leads to shrinkage and apoptosis of the Purkinje cells and ultimately to cerebellar atrophy. Consistent with this idea is the recent report that a mutation near the P region of the first repeat of the human Ca$^{2+}$-$\alpha_{1A}$ subunit causes autosomal dominant progressive ataxia (45). The mutation is located very close to the selectivity filter, and substitutes arginine for glycine, presumably reducing the channel conductance because of the electrorepulsive force between Ca$^{2+}$ and the positively charged arginine residue. This idea is borne out by the morphological studies that a decrease in the levels of intracellular free Ca$^{2+}$, induced by organic Ca$^{2+}$-antagonists or by low extracellular K$^+$, triggers the apoptotic process, which is prevented by the application of Bay K8644, L-type Ca$^{2+}$ channel agonist (46, 47). Furthermore, altered P-type channel activity is suggested to cause the $tg$-like symptoms. Lethargic (lh) mice, which have the mutation of the $\beta_{4}$ subunit, suffer from neurological symptoms, including ataxia, absence epilepsy, and spontaneous focal motor seizure (48–50), while histopathological changes are not observed (51). The $\beta_{4}$ subunit is expressed most exclusively in neuronal tissues, with a high level in cerebellum (40, 52, 53). The affinity of $\beta_{4}$ subunit binding to the $\alpha_{4}$ subunit interaction domain is highest among the four $\beta$ subunits (54). Because these four different $\beta$ subunits change both activation and inactivation of $\alpha_{4}$ channel differently (14), and because omission of the $\beta$ subunit significantly reduces the functional activity of the P/Q type channel (23), it is most likely that the Ca$^{2+}$-channel activity in the Purkinje cells of lh mice is decreased. Together with the Ca$^{2+}$-overload hypothesis, neuronal cell death triggered by low levels of intracellular free Ca$^{2+}$ demonstrates that strict Ca$^{2+}$ homeostasis is essential for normal functions of neurons.

Molecular Mechanism of Cerebellar Dysfunction—the mechanism how reduced Ca$^{2+}$ conductance causes cerebellar dysfunction and atrophy is unknown at present, but we can raise several possibilities on the basis of current knowledge of functional roles of Ca$^{2+}$ in neurons. Cerebellar Purkinje cells have unique properties. Branches of the dendritic tree can generate spikes independently, contributing to local processing and integration of information. Because the Ca$^{2+}$ channel plays a critical role in generating action potentials in dendrites (55), impaired Ca$^{2+}$ conductance would cause failures in spike generation, and consequently in signal processing. Alternatively, the Purkinje cells in the mutant mice may not receive proper transsynaptic inputs strong enough to maintain their activity. Because the P/Q type is probably the main presynaptic Ca$^{2+}$
channel involved in neurotransmitter release from the synaptic terminals of parallel and climbing fibers (56, 57), the tg and tgα-mutations would impair the Ca\(^{2+}\) influx, resulting in reduction in quantal contents.

Reduced Ca\(^{2+}\) conductance in the Purkinje cells would exert not only immediate effects mentioned above but also long-term effects related to synaptic plasticity. Parallel fiber synapses onto Purkinje cells undergo long-term depression, when parallel fiber inputs coincide with depolarization caused by climbing fiber inputs. Because injecting a Ca\(^{2+}\) chelator into Purkinje cells abolishes long-term depression, elevation of Ca\(^{2+}\) is considered to be a prerequisite for induction of long-term depression (58). Reduced Ca\(^{2+}\) conductance caused by the mutations would certainly impair long-term depression, and consequently prevent coordinated movements.

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Single Tottering Mutations Responsible for the Neuropathic Phenotype of the P-type Calcium Channel

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