Postnatal Loss of P/Q-Type Channels Confined to Rhombic-Lip-Derived Neurons Alters Synaptic Transmission at the Parallel Fiber to Purkinje Cell Synapse and Replicates Genomic *Cacna1a* Mutation Phenotype of Ataxia and Seizures in Mice

Takashi Maejima,¹ Patric Wollenweber,¹ Lena U.C. Teusner,¹ Jeffrey L. Noebels,² Stefan Herlitze,¹ and Melanie D. Mark¹

¹Department of Zoology and Neurobiology, Ruhr University Bochum, D-44780 Bochum, Germany, and ²Department of Neurology, Baylor College of Medicine, Houston, Texas 77030

Ataxia, episodic dyskinesia, and thalamocortical seizures are associated with an inherited loss of P/Q-type voltage-gated Ca^{2+} channel function. P/Q-type channels are widely expressed throughout the neuraxis, obscuring identification of the critical networks underlying these complex neurological disorders. We showed recently that the conditional postnatal loss of P/Q-type channels in cerebellar Purkinje cells (PCs) in mice (*purky*) leads to these aberrant phenotypes, suggesting that intrinsic alteration in PC output is a sufficient pathogenic factor for disease initiation. The question arises whether P/Q-type channel deletion confined to a single upstream cerebellar synapse might induce the pathophysiological abnormality of genomically inherited P/Q-type channel disorders. PCs integrate two excitatory inputs, climbing fibers from inferior olive and parallel fibers (PFs) from granule cells (GCs) that receive mossy fiber (MF) input derived from precerebellar nuclei. In this study, we introduce a new mouse model with a selective knock-out of P/Q-type channels in rhombic-lip-derived neurons including the PF and MF pathways (*quirky*). We found that in *quirky* mice, PF-PC synaptic transmission is reduced during low-frequency stimulation. Using focal light stimulation of GCs that express optogenetic light-sensitive channels, channelrhodopsin-2, we found that modulation of PC firing via GC input is reduced in *quirky* mice. Phenotypic analysis revealed that *quirky* mice display ataxia, dyskinesia, and absence epilepsy. These results suggest that developmental alteration of patterned input confined to only one of the main afferent cerebellar excitatory synaptic pathways has a significant role in generating the neurological phenotype associated with the global genomic loss of P/Q-type channel function.

Introduction

P/Q-type voltage-gated Ca^{2+} channels (P/Q-type channels) regulate neurotransmitter release and action potential firing in central neurons. Reduction/loss-of-function mutations in the pore-forming $\alpha 1$ $\text{Ca}_v2.1$ subunit (*Cacna1a*) cause several disorders including ataxia, paroxysmal dyskinesia, and epileptic sei-

zures, as seen in episodic ataxia type 2 spectrum (Jen et al., 2007; Pietrobon, 2010; Rajakulendran et al., 2012). Because P/Q-type channels are abundant throughout the brain, identification of the critical networks underlying these distinct complex neurological disorders would help in the development of new clinical treatments. Although analysis of spontaneous mutants and conventional $\text{Ca}_v2.1$ knock-out mice has provided valuable etiologic considerations, further approaches are required to understand the consequence of P/Q-type channel dysfunction in specific neuronal circuits. To address this question, we and others developed genetically engineered mice containing a floxed *Cacna1a* gene that can be deleted cell type specifically by Cre-dependent recombination (Todorov et al., 2006, 2011; Hashimoto et al., 2011; Mark et al., 2011). First, we used a PCP2 Cre driver line to investigate the Purkinje cell (PC)-specific $\text{Ca}_v2.1$ deletion on neuronal functions and behavior (Mark et al., 2011). We found that the conditional knock-out mice (*purky*) exhibit the full spectrum of behavioral aberrations that is seen in other mutants, suggesting that changes in PC output are sufficient for neurological expression of the disorders. However, we could not exclude the possibility that leakage of Cre recombination outside of the

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Correspondence should be addressed to Melanie D. Mark; Department of Zoology and Neurobiology, ND 7/31, Ruhr-University Bochum, Universitätsstr. 150, D-44780 Bochum, Germany. E-mail: Melanie.Mark@rub.de.

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PCs, as detected in a small number of scattered cells in the cerebral cortex and thalamus, might be involved in the epileptic phenotype. Therefore, we tested another Cre driver line, *Tg^{Gabra6-cre}* mice (Fünfschilling and Reichardt, 2002), in this study. This mouse induces Cre expression under the control of a GABA_A receptor $\alpha 6$ subunit (*Gabra6*) promoter, which has been reported to be unique to cerebellar granule cells (GCs) and a subset of precerebellar nuclei.

GCs are excitatory neurons that are densely packed in the cerebellar granular layer. GCs send parallel fibers (PFs) that make glutamatergic synapses onto PCs, stellate, basket cells, and Golgi cells in the molecular layer. At the glomerulus, GCs receive excitatory input from mossy fibers (MFs) that originate from precerebellar nuclei in the brainstem and spinal cord. MFs also terminate onto deep cerebellar nuclei (DCN) neurons, which can alter the final cerebellar output. To determine whether the loss of P/Q-type channels in GCs could in some way contribute to the disease phenotypes associated with genomic P/Q-type channel mutations, we generated a new conditional knock-out mouse by crossing floxed *Cacna1a* mice with *Tg^{Gabra6-cre}* (*quirky*) mice to induce PF- and MF-pathway-specific deletion of P/Q-type channels. We found that *quirky* mice showed a reduction of PF-PC synaptic transmission in the low-frequency range and a diminution of the excitatory drive of GC transmitter release on PC firing. Phenotypic analysis revealed that *quirky* mice display ataxia, stress- and drug-induced dyskinesia, and absence seizures. In this paper, we discuss the emerging evidence that impaired synaptic transmission confined to one of the main cerebellar excitatory pathways has important implications for the manifestation of P/Q-type channel-associated disease.

Materials and Methods

Mouse strains

Tg(Gabra6-cre)B1Lfr mice [stock #000196-UCD; B6;D2-Tg(*Gabra6-cre*)B1Lfr/Mmucd; Fünfschilling and Reichardt, 2002], *CAG-tdTomato* mice [stock #007905; B6;129S6-Gt(ROSA)^{26Sor}^{tm9(CAG-tdTomato)}Hze/J; Madisen et al., 2010], and C57BL/6J mice (stock #000664) were purchased from the Mutant Mouse Regional Resource Center (MMRRC), the Allen Brain Institute, and the Jackson Laboratory, respectively. *Cacna1a^{Citrine}* mice were generated as described previously (Mark et al., 2011). The animals were cared for according to the guidelines of the animal welfare committee of Nordrhein-Westfalen (LANUV).

Genotyping and real-time genomic PCR

The genetic background of the mice was determined by PCR of genomic DNA from tail biopsy. The following primer pairs to *Cacna1a*, *Cacna1a^{Citrine}* and Cre recombinase were used: *Cacna1a* forward 5' GG GGTCTGACTTCTGATGGA 3', reverse 5' AAGTTGCACACAGGGCTT CT 3'; *Cacna1a^{Citrine}* forward 5' TATATCATGGCCGACAAGCA 3', reverse 5' TTCGGTCTTCAAGGAACC 3'; *Tg^{Gabra6-cre}* forward 5' ATTCTCCACCAACCGTCAGTACG 3', reverse 5' AAAATTTGCCTG CATTACCG 3'. Determination of the zygosity of the Cre recombinase gene in *Cacna1a^{quirk(-/-)}* mice by real-time (RT)-PCR according to the methods described previously in detail (Sakurai et al., 2008). Briefly, genomic DNA (gDNA) from mouse tail biopsies was diluted 1:32, 1:64, 1:128, and 1:256 from *Tg^{Gabra6-cre}* mice as a positive control and from *Cacna1a^{quirk(-/-)}* mice. Reactions were prepared with SYBR Green according to instruction manual (Invitrogen) with 6.25 pmol of each primer and 2 μ l of gDNA, subjected to a 3-step cycling condition of 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min on a Mastercycler realplex 2 (Eppendorf) and the slopes of Ct, dCt, and R2 values of each sample were calculated. Relative quantification of zygosity was performed with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Ct values were used if R2 values of >90% were obtained. Primers used for Cre recombinase were 5'-GAAGATCTTCCAAT TTACTGACCGTACAC-3' and 5'-CCATGAGTGAACGAACCTGGTC

GA-3' and for internal control of gDNA GPDH 5'-TGTGTCCGTCGT GGATCTGA-3' and 5'-CCTGCTTCACCACCTTCTCGA-3'. All RT-PCRs were inspected on an electrophoresis gel for the product size: Cre recombinase and GPDH at 450 and 83 bp, respectively.

Histology

Animals were anesthetized and perfused intracardially with 4% paraformaldehyde in PBS. Three male brains at 1 and 9 months of age were removed from each genotype, fixed for 1 h at 4°C, and then cryoprotected overnight by incubating in 30% sucrose in PBS. Samples were embedded in optimal cutting temperature medium and immediately frozen in dry ice. Then, 20 μ m cryostat sections were collected, air-dried at room temperature on gelatin-coated slides, and Nissl stained. Images were acquired with a microscope (BX51; Olympus). Relative sizes of cerebellar structures from *Cacna1a^{Citrine}* and *Cacna1a^{quirk(-/-)}* sagittal cerebellar sections were analyzed using Volocity software (Improvision). Immunofluorescent labeling was performed according to standard procedures (Mark et al., 1995). Sagittal cerebellar sections from at least three adult males for each genotype were incubated with the following primary and secondary antibodies: anti-vesicular Glutamate transporter 1 (VGLUT1; Millipore), anti-P/Q-type Ca²⁺ channel (Synaptic Systems), anti-mouse IgG Alexa Fluor 594 (Invitrogen), and anti-rabbit IgG Alexa Fluor 488 (Invitrogen). Sagittal slices were incubated with primary antibodies overnight at 4°C and then washed and incubated with secondary antibodies for 2 h at room temperature. The samples were embedded in Prolong Gold antifade (Invitrogen). Images were acquired with a confocal microscope (TCS SP5II; Leica).

Electrophysiology

Parasagittal cerebellar slices (250 μ m thick) were prepared from at least three mice per genotype for each experiment, as described previously (Edwards et al., 1989; Mark et al., 2011). Mice were killed at postnatal day 21–32 unless noted otherwise. PCs, GCs, and DCN neurons were visually identified under an upright microscope (DMLFSA; Leica) equipped with infrared illumination. During experiments, slices were continuously perfused with an artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, and 20 D-glucose equilibrated with 95% O₂ and 5% CO₂. For recordings of EPSCs in PCs, whole-cell voltage-clamp recordings were made at 30°C with borosilicate glass electrodes (2–4 M Ω) that were filled with an internal solution containing the following (in mM): 60 CsCl, 10 D-gluconic acid, 20 TEA-Cl, 30 HEPES, 20 BAPTA, 4 MgCl₂, 4 ATP, and 0.4 GTP (pH 7.3, adjusted with CsOH). SR95531 (10 μ M; Tocris Bioscience) was added to the external solution to block GABA_A-receptor-mediated inhibitory synaptic transmission. Excitatory synaptic transmission was evoked by a 100 μ s voltage pulse applied through a glass pipette filled with ACSF and placed in the molecular layer for PF-EPSCs or in the vicinity of PC somata for CF-EPSCs. A combination of EPC10/2 amplifier and Patchmaster software (HEKA) was used to control membrane voltage, stimulation timing, and data acquisition. PCs were voltage clamped at -70 mV to search for PF-EPSCs and at -70 mV or -20 mV to search for CF-EPSCs. Series resistance was compensated routinely by 70–80%. Membrane currents were filtered at 3 kHz and digitized at 20 kHz. In GCs and DCN neurons, whole-cell recordings were performed with patch pipettes (8–12 M Ω for GCs and 4–6 M Ω for DCN neurons) filled with an internal solution containing the following (in mM): 126 K-gluconate, 4 NaCl, 10 HEPES, 0.2 EGTA, 2 MgCl₂, 4 ATP, 0.4 GTP, and 10 phosphocreatine (pH 7.3, adjusted with KOH). EPSCs in GCs were recorded by voltage clamping at -70 mV and at $35 \pm 1^\circ$ C in the presence of SR95531 (10 μ M), strychnine (0.5 μ M), and D-(+)-2-amino-5-phosphonopentanoic acid (D-AP5; 30 μ M; Tocris Bioscience) to isolate non-NMDA receptor currents. Recordings of EPSCs in DCN neurons were performed in the presence of SR95531 (10 μ M) and strychnine (0.5 μ M) at -70 mV and at room temperature in the tissue prepared from postnatal 14- to 18-d-old mice. Series resistance was compensated by 70–80%. The EPSCs were evoked by stimulation of the white matter surrounding the deep cerebellar nuclei with a 100 μ s electric pulse delivered through a concentric bipolar electrode. Statistical significance in all experiments was evaluated with *t* test or ANOVA combined with Tukey

test using SigmaStat software (Systat Software) or IGOR Pro software (WaveMetrics). All chemicals were purchased from Sigma unless stated otherwise. ω Agatoxin IVA (Aga-IVA) and ω Conotoxin GVIA (Ctx-GVIA; Peptide Institute) were dissolved in the external solution just before bath application.

Presynaptic calcium imaging

Calcium transients evoked in PF tracts were measured as described previously (Mintz et al., 1995; Regehr and Atluri, 1995). Coronal slices of cerebellum (300 μm thick) were prepared from five mice per genotype at a postnatal age of 20–25 d. A membrane-permeable low-affinity Ca^{2+} indicator, Magnesium Green AM (Invitrogen) was loaded in PFs with a labeling solution for 10 min by a high-pressure stream that is formed through a dye-delivering pipette and a suction pipette situated within the molecular layer. The labeling solution was prepared every day by dissolving 50 μg of the indicator in 20 μl of $\sim 25\%$ pluronic acid/75% DMSO mixture and 400 μl of saline. After 1–2 h of incubation time, fluorometric measurements were performed. Under 488 nm excitation light (Polychrome V; Till Photonics), fluorescent transients were evoked in the molecular layer by a 200 μs electric pulse (10 V) delivered through a concentric bipolar electrode placed near the dye-loading site. The signals from a 30 $\mu\text{m} \times 30 \mu\text{m}$ area $> 300 \mu\text{m}$ away from the loading site were collected through a 60 $\times$ objective lens (LUMPLFLN 60XW; numerical aperture [NA] = 1; Olympus) and a fluorescent filter set (51006 FITC/Texas Red; Chroma Technology) and then converted by a photomultiplier system (PMT-100; Applied Scientific Instrumentation). The output signals were low-pass filtered at 500 Hz (4-pole model 432 Bessel filter; Wavetek) and digitized at 5 kHz using an EPC10/2 amplifier and PatchMaster software, which was also used for regulating the timing of the electrical stimulation and the excitation flash. In this experiment, cerebellar slices were continuously perfused with ACSF including 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX; 10 μM ; Tocris Bioscience) at room temperature. Each fluorescent transient was analyzed offline with IGOR Pro and is presented as $\Delta F/F_0$ showing the change in fluorescence intensity relative to baseline intensity.

Optogenetic focal stimulation of granule cells and extracellular recording of PCs

To stimulate GCs specifically and to monitor the resultant effects on PC activity in slice preparation, we adopted an optogenetic method using a channelrhodopsin-2 (ChR2), a cationic channel that is activated by blue light. We injected an adeno-associated virus (AAV2/9; Vector Core Facility, University of Pennsylvania) containing a double-floxed inverted open reading frame encoding ChR2(H134R) fused to the red fluorescent protein mCherry (ChR2-mCherry) that is under the control of the EF1 α promoter (20297; Addgene) into the cerebellar cortex of *Cacna1a*^{quirk(-/-)} mice and *Tg*^{Gabra6-Cre} mice as a control. Briefly, 2-month-old mice were anesthetized with 1–2% isoflurane in air delivered continuously through a precision vaporizer (WPI) and mounted onto a stereotaxic frame (Narishige). After exposing the cranium, a small hole was drilled in the skull at -6.3 mm from bregma. The virus (4 μl , titer $3.0 \times 10^{13}/\text{ml}$) was pressure injected into the cortex through a micropipette (tip outer diameter, $\sim 30 \mu\text{m}$), lowered 2 mm deep from the top of brain first, and then aliquoted at points 200 μm apart toward the surface. Mice were housed for at least 2 weeks to maximize ChR2 expression levels in GCs before performing electrophysiological experiments. Parasagittal cerebellar slices were perfused continuously with ACSF kept at $35 \pm 1^\circ\text{C}$. To record spontaneous firing of PCs, extracellular recordings were performed as described previously (Dizon and Khodakhah, 2011; Mark et al., 2011). Recording glass pipettes (tip diameter, $< 1 \mu\text{m}$) filled with ACSF were placed close to the axon near the soma of PCs. Spike detection and analysis of instantaneous frequency were performed using a customized analysis protocol written with IGOR Pro software. Focal excitation of ChR2 in GCs was achieved by irradiation of 470 nm light delivered through a quartz glass optical fiber (Thomas Recording) with a blunt end (tip outer diameter, $\sim 35 \mu\text{m}$) and was connected to the LED lamp housing at the other end. An applied light intensity was adjusted manually with a constant-current control unit (Thomas Recording) by reading the output light intensity measured in a

30 $\mu\text{m} \times 30 \mu\text{m}$ area at the outlet of optical fiber with a photomultiplier system. Downward deflection in baseline voltage was induced by a 470 nm light irradiation. The deflection could be observed even with a recording electrode outside of the brain tissue, suggesting that it was due to an artificial effect, most likely a photoelectric effect on a recording electrode.

Videoelectroencephalographic recordings

Silver wire electrodes (diameter, 0.005 inches) attached to a microminiature connector were implanted bilaterally into the subdural space over the frontal, temporal, parietal, and occipital cortices of mice under Avertin anesthesia. After surgery, mice were allowed to recover for at least 1 d before recording. Simultaneous cortical EEG activity and behavioral video monitoring were performed using a digital electroencephalograph (Stellate Systems). EEGs were recorded for $\geq 2 \text{ h/d}$ over multiple days. Recordings were made from ≥ 4 male mice per genotype. Mice were evaluated between the ages of 5 and 7 months. EEG signals were filtered through a 3 Hz high-pass filter, a 35 Hz low-pass filter, and a 60 Hz notch filter.

Behavioral analysis

The rotarod, vertical pole, beam walk, and hang wire tests were performed in the same group of 3-month-old mice (6 female, 8 male *Cacna1a*^{Citrine} mice; 4 female, 8 male *Cacna1a*^{quirk(-/-)} mild mice; 3 female, 3 male *Cacna1a*^{quirk(-/-)} severe mice). A gait analysis was done in 9-month-old mice (4 female, 5 male *Cacna1a*^{Citrine} mice; 4 female, 5 male *Cacna1a*^{quirk(-/-)} mild mice; 5 female, 4 male *Cacna1a*^{quirk(-/-)} severe mice). Some of these mice also underwent the other motor tests.

Gait analysis. Footprints were obtained by painting the forepaws with red and hindpaws with blue, nontoxic, water-soluble paint (Pelikan). Mice were placed at one end of a 10 cm wide $\times$ 70 cm long $\times$ 10 cm high tunnel that was connected with their home cage at the other end and was accessible through a hole. Each mouse underwent three pretrials before performing three trials with white paper. Only one footprint pattern per mouse was scanned into the computer with a CanoScan 9000F (Canon), seven steps per footprint were measured using ImageJ software (NIH) as described previously (Klapdor et al., 1997). A total of 42 steps were measured per genotype.

Rotarod. Motor coordination and balance skills were assessed with a rotarod test (Columbus Instruments). Mice were placed on a rod rotating at 4 rpm for 1 min of acclimation. The rod was then accelerated at 0.1 rpm/s up to 40.0 rpm. The test continued until all mice had fallen off the rod. Latency to fall and rotations per minute at the time of fall were recorded for each mouse. Three trials per mouse were run and averaged together.

Vertical pole test. Motor coordination and balance skills were also assessed by the vertical pole test. A 50 cm grooved plastic pole (diameter, 1 cm) was placed vertically and secured to a platform. Mice were placed faced upward at the top of the pole and the latency to turn around and climb down the vertical pole was recorded. Timeout occurred if the mouse did not complete the task after 120 s. If the mouse fell from the top of the pole, a time of 120 s and a “fall” notation was recorded.

Beam walk. To assess fine motor coordination and balance, mice were analyzed for their capability to cross a narrow beam to an enclosed goal box. The horizontally placed, 70-cm-long beam was 10 mm wide and 60 cm above the table surface. One end of the beam was mounted to a small, illuminated platform and the other end was fastened to an enclosed (20 cm^2) goal box. Mice underwent training for 2 d (6 trials/d) before data collection. Briefly, the mouse was placed at an illuminated end of the beam and the time required to traverse the beam to the goal house was recorded. Mice were given a maximum of 120 s to transverse the beam and a fall was also recorded as 120 s. In addition to recording the latency to transverse the beam, time spent immobile (idle), left and right paw slips, and falls were noted (Quinn et al., 2007). Data were averaged over 3 trials per mouse.

Hang wire test. A simple test to assess the balance and grip strength of the mice was performed according to a modified method (Sango et al., 1996). Briefly, mice were hung upside down on a wire screen (12 mm $\times$ 12 mm grid) positioned 50 cm above a cage. The latency to fall into the

Figure 1. Characterization of Cre-recombination pattern with the Gabra6-Cre driver line. Gabra6-Cre driver line was crossed with the td-Tomato reporter line. td-Tomato fluorescence identifies neurons and their axons in which Cre-dependent recombination occurred. **A**, Parasagittal section of whole brain. **B**, Parasagittal section of the cerebellar cortex. **C**, Coronal section of the medulla. External cuneate nucleus, lateral reticular nucleus, and scattered cells in the spinal trigeminal nucleus are positive. **D**, Sagittal section showing pontine nuclei and reticulotegmental nucleus of the pons. **E**, Parasagittal section of the dorsal cochlear nucleus. **F**, Clustered neurons in the parasubiculum. Scattered cells in the dentate granule layer can be seen. Scale bars: **A**, 800 μm ; **B**, 50 μm ; **C**, 400 μm ; **D**, **E**, 200 μm ; **F**, 400 μm .

cage was recorded. Mice that did not fall within the 60 s trial period were removed and given a maximum score of 60 s.

Characterization of dystonic episodes. Adult *Cacna1a*^{Citrine} mice (9 females, 13 males) and *Cacna1a*^{quirk(−/−)} mice (11 females and 11 males for mild; 9 females and 5 or 6 males for severe) between 6 and 9 months of age were monitored in a test cage for spontaneous dystonic episodes. After 2 h of acclimation, spontaneous dystonic episodes were video recorded with Ethovision software (Noldus) and the frequency and duration of attacks over a 24 h period were recorded. Stress- or chemical-induced dystonic episodes were monitored in mice every 5–10 min for 60 min (or until the episode ended) after a change from their home cage, a 10 min cage transport on a laboratory cart, a short 10 min restraint in a 100 ml syringe, a subcutaneous injection of 0.9% NaCl as a vehicle control, a subcutaneous injection of 15 mg/kg caffeine, and an intraperitoneal injection of 2 g/kg ethanol. Behavioral studies were performed according to methods described previously in detail (Fureman et al., 2002). The data are shown as the percentage of mice that had a dystonic episode per mouse group and the average episode duration per group.

Results

Selective knock-down of P/Q-type channel in the mouse cerebellar PF and MF pathways

To establish specific knock-out of P/Q-type channels from GCs in cerebellum and precerebellar nuclei neurons in brainstem, we crossed *Cacna1a*^{Citrine} mice (Mark et al., 2011) with *Tg*^{Gabra6-cre} mice (Fünfschilling and Reichardt, 2002). *Cacna1a*^{Citrine} mice are conditional knock-in mice that carry a Citrine in the first

exon of *Cava2.1* gene, which is flanked by loxP sites to induce Cre-dependent deletion of the P/Q-type channel $\alpha 1$ subunit. *Tg*^{Gabra6-cre} mice induce expression of Cre recombinase under the control of a GABA_A receptor $\alpha 6$ subunit (*Gabra6*) promoter that has been reported to have unique expression in GCs within cerebellum and in a subset of precerebellar nuclei (Fünfschilling and Reichardt, 2002). The Cre recombination occurs between P4.5 and P20, reaching almost complete levels at P15. Therefore, in the newly generated mice, a P/Q-type channel is expected to be deleted postnatally. Initially, we verified the Cre recombination pattern driven by *Tg*^{Gabra6-cre} mice by crossing them with *CAG-tdTomato* reporter mice (Madisen et al., 2010). In the adult brain, td-Tomato expression via Cre recombination was detected clearly in cerebellum (Fig. 1A). Due to the strong td-Tomato fluorescence in the granular and molecular layers where GC somata and their axons are located, td-Tomato-negative cells were visualized easily, including PCs, molecular layer interneurons, and Golgi cells, as described previously (Fig. 1B) (Fünfschilling and Reichardt, 2002). Recombination was also detected in the pontine nuclei, reticulotegmental nucleus of the pons (Fig. 1D), external cuneate nucleus, lateral reticular nucleus, sporadically within the spinal trigeminal nucleus (Fig. 1C), and also in a dorsal cochlear nucleus (Fig. 1E). In contrast, td-Tomato-positive cell bodies were not detected within inferior olive nuclei where CFs originate, in the DCN consist-

ing of output neurons from cerebellum, or the vestibular nuclei. These observations suggest that within the cerebellar excitatory pathways, the MF and PF pathways specifically undergo Cre-mediated recombination by the *Tg*^{Gabra6-cre} driver line, but not CF input or PC/DCN output pathways. However, outside of the hindbrain region, td-Tomato-positive nuclei were detected in hippocampal connected areas such as the pre-/para-/postsubiculum (Fig. 1F), medial amygdaloid nuclei, and the amygdala-hippocampal area (Fig. 1A), sporadically in the colliculus and dentate granule layer. In contrast to *purky* mice, the positive neurons in forebrain were highly confined to these areas. No td-Tomato-positive neurons were detected in the thalamus or basal ganglia, and very few positive neurons were detected in the cerebral cortex.

Because Q-type Ca^{2+} channel currents were originally described in GCs (Randall and Tsien, 1995) and our mutant mice showed odd behaviors, we refer to these mice as *Quirky* or *Cacna1a*^{quirk(−/−)} mice (Fig. 2B). As shown later, we observed that some of the *quirky* mice had clearly lower body weights (Fig. 2C), poor performance on a battery of behavior tests (Fig. 8), and spontaneous and stress-/chemical-induced dyskinesia (Table 1), even though their genetic profile could not be discriminated by PCR or RT-PCR (Fig. 2A, Table 2). Therefore, we categorized *Cacna1a*^{quirk(−/−)} mice into two groups: *Cacna1a*^{quirk(−/−)} mild

mice and *Cacna1a*^{quirk(-/-)} severe mice, and compared these mice with their Cre-negative *Cacna1a*^{Citrine} littermates.

Selective knock-down of P/Q-type channel in the mouse cerebellar GCs causes small changes in cerebellar cortical structure

We first observed that *Cacna1a*^{quirk(-/-)} severe mice showed a 20–30% reduced body weight during the first 3 months of age; however, they recovered in size after 8 months of age, suggesting that *quirky* severe mice are impaired in their nourishment early in life (Fig. 2C). We next compared the gross anatomy of *Cacna1a*^{Citrine} and *Cacna1a*^{quirk(-/-)} mouse brains (Fig. 2D) and found that the forebrains were comparable in size, structure, and weight (data not shown). However, *Cacna1a*^{quirk(-/-)} severe and mild mice showed a 10–20% reduction in overall size of the cerebellum and granular and molecular layers compared with *Cacna1a*^{Citrine} mice at 1 month of age. By 9 months of age only *Cacna1a*^{quirk(-/-)} severe mice continued to display a reduction in their cerebellum and molecular and granular layers (Fig. 2E).

Reduction of P/Q-type channel protein in cerebellar GCs

To confirm the loss of P/Q-type channel protein in the GCs of *Cacna1a*^{quirk(-/-)} mice, cerebellar slices were stained with a specific antibody against the P/Q-type channel and costained with a PF synapse marker, the VGlut1 antibody. In *Cacna1a*^{quirk(-/-)} mice, the fluorescence for P/Q-type channel protein was significantly reduced in the molecular cell layer where PF synapses are located compared with their *Cacna1a*^{Citrine} littermates (Fig. 3). As expected, fluorescent staining for the P/Q-type channel protein remained in PCs.

Selective knock-down of P/Q-type channel in the mouse cerebellar PF and MF pathways causes functional changes at their synapse

The P/Q-type channel is responsible for 40–50% of the total somatic Ca^{2+} current in cultured GCs (Mintz et al., 1992; Pearson et al., 1995; Randall and Tsien, 1995; Doroshenko et al., 1997; Varming et al., 1997) and is the major Ca^{2+} channel at the PF-PC synapse, along with some contribution of N-type and residual-type Ca^{2+} channels (Mintz et al., 1995; Empson and Knopfel, 2010; Myoga and Regehr, 2011). A reduction in P/Q-type channels and/or a change in the contribution of other Ca^{2+} channels at the presynaptic terminal would result in alteration of synaptic transmission and its profile of short-term synaptic plasticity (Catterall and Few, 2008; Neher and Sakaba, 2008). Therefore, we

Figure 2. Conditional knock-down of P/Q-type channels in GCs and precerebellar nucleus neurons reveals changes in cerebellar morphology. **A**, PCR analysis for distinction between wild-type (WT; 1), *Cacna1a*^{Citrine} mice (2), and *Cacna1a*^{quirk(-/-)} mice (3 and 4 from mild and severe mice, respectively) by 3 different primer sets detecting to *Cacna1a* (WT), *Cacna1a*^{Citrine} (Tg), and Cre recombinase (Cre) sequences. **B**, Phenotypic differences among *Cacna1a*^{Citrine} mice, *Cacna1a*^{quirk(-/-)} mild, and *Cacna1a*^{quirk(-/-)} severe mice. **C**, Comparison of body weight gain. Average weights of *Cacna1a*^{Citrine} mice (6 females [F], 9 males [M]), *Cacna1a*^{quirk(-/-)} mild (4 F, 8 M), and *Cacna1a*^{quirk(-/-)} severe mice (11 F, 10 M) were plotted at 1 month of age. * $p < 0.05$, † $p < 0.001$ (ANOVA). **D**, Comparison of brains and the cerebellar structure of *Cacna1a*^{Citrine} and *Cacna1a*^{quirk(-/-)} mild and *Cacna1a*^{quirk(-/-)} severe mice at 6 months of age. Parasagittal sections of cerebellar vermis were stained with cresyl violet. **E**, Relative size of cerebellar structures. Shown are: area of cerebellar slices (whole), granular layers (GL), and molecular layers (ML) determined in *Cacna1a*^{Citrine} (white bar), *Cacna1a*^{quirk(-/-)} mild (gray bar), and *Cacna1a*^{quirk(-/-)} severe (black bar) mice at 1 month (left) and 9 months (right) of age. The area of sagittal cerebellar sections ($n = 6$ slices $\times$ 3 mice) and the thickness of the molecular and granular layers ($n = 6$ areas $\times$ 6 slices $\times$ 3 mice) were measured for each line. * $p < 0.05$, ** $p < 0.01$ (ANOVA).

analyzed the consequences of loss of P/Q-type channel contributions at PF-PC synapses. We first demonstrated using Ca^{2+} imaging that P/Q-type channels contribute up to 50% of the presynaptic Ca^{2+} influx evoked by a single stimulation in control mice, with less contribution of N-type channels (Fig. 3A). The remaining component was blocked by Cd^{2+} , suggesting a high contribution of non-P/Q/N-type channels to the overall Ca^{2+} influx at the PF-PC presynaptic terminals in 3-week-old mice. In PF-PC synapses of *Cacna1a*^{quirk(-/-)} mice, the overall Ca^{2+}

Table 1. Frequency and duration of induced dyskinesias

Treatment	<i>Cacna1a</i> ^{quirk(−/−)} mild			<i>Cacna1a</i> ^{quirk(−/−)} severe		
	Frequency (%)	Duration (min)	<i>n</i>	Frequency (%)	Duration (min)	<i>n</i>
Cage change	0	0	22	57.1	65.2 ± 8.4	14
Cage transport	0	0	22	78.6	67.4 ± 5.6	14
Short restraint	0	0	22	71.4	76.5 ± 6.0	14
Saline	0	0	22	33.3	54.2 ± 13.4	15
Caffeine	0	0	22	93.3	134.4 ± 13.9	15
Ethanol	0	0	22	100	136.0 ± 7.0	15

Behavioral studies were performed as described previously (Fureman et al., 2002). *Cacna1a*^{Citrine} mice (*n* = 22) displayed no dystonic seizures in after any of the treatments. Data represent the percentage of mice that demonstrated a dystonic episode per mouse group and the average duration of the dyskinesia per group. Data represent mean ± SEM.

Table 2. Zygosity determination of Cre recombinase in *Cacna1a*^{quirk(−/−)} mice

Mouse line	<i>n</i>	2 ^{−ΔΔCt}		2 ^{−ΔΔCt}	
		Wild-type	Heterozygosity	Homozygosity	
<i>Cacna1a</i> ^{Citrine}	7	0.00 ± 0.01	—	—	
<i>Cacna1a</i> ^{quirk(−/−)} mild	12	—	0.45 ± 0.16 (12/12)	—	
<i>Cacna1a</i> ^{quirk(−/−)} severe	13	—	0.42 ± 0.04 (10/13)	1.01 ± 0.08 (3/13)	

Zygosity determination by RT-PCR was performed as described previously (Sakurai et al., 2008). *Cacna1a*^{quirk(−/−)} 2^{−ΔΔCt} values were compared with *Tgfab6-cre* 2^{−ΔΔCt} values (homozygosity positive control; 2^{−ΔΔCt} = 1) and are represented as the relative mean ± SD. The number of mice displaying a specific zygosity compared with the total number of mice analyzed per mouse line is shown in parentheses.

Figure 3. Loss of P/Q-type channel protein in *Cacna1a*^{quirk(−/−)} mice. Comparison of P/Q-type channel protein expression in parallel fibers between adult *Cacna1a*^{Citrine} and *Cacna1a*^{quirk(−/−)} mice. Cerebellar slices were incubated with primary antibodies against VGlut1 (PF synapse marker in red, top) and P/Q-type channels (green, middle). The bottom panel is a merger between the top and middle panels.

signal amplitude was reduced by 50% compared with control mice and was not sensitive to the P/Q-type channel blocker Aga-IVA, demonstrating the loss of P/Q-type channel at the presynaptic terminal without significant compensation by other Ca²⁺

channels (Fig. 3B). The Ca²⁺ signal from *Cacna1a*^{quirk(−/−)} mice was reduced by ~30% with the N-type channel blocker, Ctx-GVIA, and the 70% remaining current was blocked by Cd²⁺. To further examine contribution of P/Q-type channel to MF synaptic transmission, we recorded MF-derived EPSCs in their two postsynaptic targets, GCs and DCN neurons. In MF-GC synapses from control mice, Aga-IVA reduced the EPSC amplitude and the release success rate by 70%, suggesting that the P/Q-type channel is the major contributor to synaptic transmission at the MF-GC synapse (Fig. 4C). Conversely, Aga-IVA had no effect on the synaptic transmissions in 6 of 8 cells of *Cacna1a*^{quirk(−/−)} mice (Fig. 4D). Further application of Ctx-GVIA reduced the synaptic transmission, suggesting that N-type and other non-P/Q/N-type Ca²⁺ channels determine the synaptic transmission in *Cacna1a*^{quirk(−/−)} mice. The amplitude of recorded EPSCs was not significantly different between the mouse lines (i.e., 46.1 ± 12.9 pA, *n* = 5, in *Cacna1a*^{Citrine} mice and 64.8 ± 12.7 pA, *n* = 8, in *Cacna1a*^{quirk(−/−)} mice at −70 mV). As with MF-GC synapses, P/Q-type channels were a major contributor to MF-DCN synaptic transmission in control mice (Fig. 4E) with a minor contribution from N-type channels. In contrast to *Cacna1a*^{quirk(−/−)} mice, MF-DCN synaptic transmission relied on N-type channels with no contribution of P/Q-type channels (Fig. 4F). Again, the average amplitude of EPSCs at the MF-DCN was not significantly different between the mouse lines (i.e., 313.7 ± 52.7 pA, *n* = 6, in *Cacna1a*^{Citrine} mice and 201.8 ± 62.3 pA, *n* = 5, in *Cacna1a*^{quirk(−/−)} mice at −70 mV). These results suggest that P/Q-type channels are eliminated from MF-GC, MF-DCN, and PF-PC presynaptic terminals. Most likely, N-type and non-P/Q/N-type channels compensate for the loss of P/Q-type channels in mediating synaptic transmission in *Cacna1a*^{quirk(−/−)} mice.

We further investigated the consequences of reducing Ca²⁺ influx at the PF-PC synapses upon synaptic transmitter release. We found that the synaptic transmission evoked by a single electrical stimulation was drastically reduced in *Cacna1a*^{quirk(−/−)} mice compared with control mice (Fig. 5A). The reduction in EPSC amplitude was accompanied by a reduction in paired-pulse facilitation (PPF; Fig. 5B). To explain this reduction in PPF, we compared the presynaptic Ca²⁺ transient for the first and second peak evoked by a 100 Hz (10 ms interval) stimulus train, because the increase of residual presynaptic Ca²⁺ during stimulation is thought to be one of the mechanisms underlying facilitation of synaptic transmitter release. In *Cacna1a*^{quirk(−/−)} mice, the increased Ca²⁺ transient at the second test pulse was smaller than in control mice, although it was comparable in the presence of Aga-IVA, suggesting that the reduced Ca²⁺ transient after the second stimulus was one of the factors causing the reduced PPF in *Cacna1a*^{quirk(−/−)} mice (Fig. 5C–E). The results suggest that the loss of P/Q-type channel at the PF-PC syn-

apse leads to reduced transmitter release due to the reduction in Ca^{2+} influx into the pre-synaptic terminal.

GCs fire at high frequency *in vivo* in response to sensory stimulation. Therefore, we analyzed the fidelity of the synaptic transmission at high-frequency ranges of presynaptic PF activity. Although the synaptic transmission evoked by a single stimulation was highly reduced in *Cacna1a*^{quikr(-/-)} mice (Fig. 5A), high-frequency presynaptic activity ranging from 50–500 Hz revealed robust synaptic transmission (Fig. 6). Even though the increase of EPSC amplitude was initially reduced at lower stimulus frequencies (i.e., 50 and 100 Hz) in *Cacna1a*^{quikr(-/-)} mice (Fig. 6A,B), control level EPSC amplitude was reached after 5 trials at 100 Hz stimulation. Furthermore, the increase in EPSC amplitude during higher-frequency stimulation (200 and 500 Hz) was comparable to that of control mice (Fig. 6C,D). These results indicate that synaptic transmission at the PF-PC synapse in *Cacna1a*^{quikr(-/-)} mice is reduced when GCs spike at low but not high frequencies.

Selective knock-down of the P/Q-type channel in cerebellar GCs reduces the strength of functional connectivity between GCs and PCs

The intrinsic firing rate of PCs is modulated by GC activity, which can be excitatory or inhibitory. The axons of GCs (i.e., PFs) project transversely into the molecular layer and make glutamatergic synapses directly onto PCs. Conversely, GCs inhibit PCs indirectly via the molecular layer interneurons: stellate and basket cells projecting longitudinally. Therefore, we examined the possible changes in functional connectivity between GCs and PCs as a consequence of P/Q-type channel knock-down in GCs and their PF synapses. First, we investigated whether the loss of the P/Q-type channel would affect the properties of GC action potential firing in postnatal 3- to 4-week-old adolescent mice. Although the P/Q-type channel is responsible for 40–50% of the total somatic Ca^{2+} current in cultured GCs (Mintz et al., 1992; Pearson et al., 1995; Randall and Tsien, 1995; Doroshenko et al., 1997; Varming et al., 1997), the loss of P/Q-type channels in GCs did not lead to any change in action potential firing property in response to current injection, suggesting that the P/Q-type channel is not an important contributor to the firing properties of mature GCs (Fig. 7F,G). Our data are consistent with previous studies suggesting that Ca^{2+} -dependent effects on Na^+ -dependent action potential firing are exerted through

PF to PC

MF to GC

MF to DCN

Figure 4. Loss of P/Q-type channel contribution for presynaptic Ca^{2+} influx at parallel fiber to PC synapse and for synaptic transmission at MFs to GCs and DCN neurons in *Cacna1a*^{quikr(-/-)} mice. **A, B**, Time course of peak fluorescent transients ($\Delta F/F_0$) evoked by a single stimulation of the PF tract in the presence of the Ca^{2+} channel blockers 0.2 μM Aga-IVA (Aga), 1 μM Ctx-GVIA (Ctx), and 0.3 mM Cd^{2+} in control mice (**A**) and in *Cacna1a*^{quikr(-/-)} mice (**B**). Inset, Fluorescent transients obtained in the presence of the indicated Ca^{2+} channel blockers. Bars, Summary of the effects of Ca^{2+} channel blockers on the fluorescence transients in control mice ($n = 5$; white) and in *Cacna1a*^{quikr(-/-)} mice ($n = 5$; black). **C, D**, P/Q-type channel contribution for synaptic transmission at MF-GC synapses in control ($n = 5$ cells; **C**) and in *Cacna1a*^{quikr(-/-)} mice ($n = 7$ cells; **D**). Traces, Averaged non-NMDA-receptor-mediated EPSCs obtained in the presence of the Ca^{2+} channel blockers. Summary bars show changes in the relative amplitude of EPSCs (% of control; left bars) and the vesicle-release rate (right bars) by the indicated blockers. **E, F**, Comparison at MF-DCN neuron synapses in control ($n = 6$ cells; **E**) and in *Cacna1a*^{quikr(-/-)} mice ($n = 5$ cells; **F**). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (ANOVA).

N-type Ca^{2+} channels in mature GCs (Rossi et al., 1994; D'Angelo et al., 1997, 1998).

We also evaluated the strength of the functional connectivity between GCs and PCs by adopting an optogenetic approach. We expressed ChR2 in GCs to stimulate GCs specifically in the

Figure 5. Loss of P/Q-type channel in cerebellar GC reduces synaptic input and changes PPF at parallel fiber to PC synapse. **A**, Averaged input (stimulus intensity) to output (EPSC amplitude) relationship of PF-PC synaptic transmission in control (white circles; $n = 22$ cells) and in *Cacna1a^{quirk(-/-)}* mice (black circles; $n = 28$ cells). Inset, Representative traces of PF-EPSCs evoked at each stimulus intensity. **B**, Averaged paired-pulse ratio (second to first EPSC amplitude) are plotted as a function of interstimulus interval in control (white circles; $n = 18$ cells) and in *Cacna1a^{quirk(-/-)}* mice (black circles; $n = 15$ cells). Inset, Representative traces of PF-EPSCs evoked by a paired stimuli (50 ms interval). * $p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$ (ANOVA). **C**, Representative fluorescence transients ($\Delta F/F_0$; average of 8 consecutive traces) elicited by a train of five stimuli of PF fiber tract (100 Hz) in control (top) and *Cacna1a^{quirk(-/-)}* (bottom) mice. The fluorescence transient acquired during 0.2 μM Aga-IVA application is superimposed in control mice. **D**, Normalized fluorescence traces to the first peak of each trace. The three traces shown in C are normalized and superimposed. Thick line is a control trace in control mice; dashed line, trace obtained during Aga-IVA application in control mice; thin line, control trace in *Cacna1a^{quirk(-/-)}* mice. **E**, Ratio of second peak to first peak of transients summarized in each condition (all, $n = 5$). # $p < 0.05$ (paired t test); * $p < 0.05$ (t test).

cerebellar cortex by a short light pulse and monitored the functional consequences of GC transmitter release on PC activity in slice preparations. We injected AAV2 virus containing a double-floxed inverted open reading frame encoding ChR2-mCherry, which is under the control of the EF1 α promoter, into the cerebellar cortex of *Cacna1a^{quirk(-/-)}* mice and *Tg^{Gabra6-Cre}* mice as controls for specific expression of ChR2 in GCs. To allow for sufficient ChR2 expression in GCs (Fig. 7A), we performed electrophysiological experiments in parasagittal cerebellar slices 2 weeks after virus injection. We monitored spontaneous firing of PCs with extracellular recording and focally irradiated ChR2-expressing GCs through a quartz glass optical fiber (tip outer diameter, ~35 μm; Fig. 7B) connected to a 470 nm LED lamp. PCs fired AP with a frequency of 39.1 ± 2.4 Hz ($n = 21$) in

Figure 6. High-frequency activity reveals robust synaptic transmission at the PF-PC synapse in *Cacna1a^{quirk(-/-)}* mice. Analysis of 5 PF-EPSC trains evoked by different frequencies: 50 Hz (**A**), 100 Hz (**B**), 200 Hz (**C**), and 500 Hz (**D**). Each peak after stimulus artifacts is detected and the amplitude from baseline is simply plotted against the pulse number. The averages ± SEM are shown for control mice (white circles; $n = 9$ cells) and *Cacna1a^{quirk(-/-)}* mice (black circles; $n = 8$ cells). Inset, Representative traces of PF-EPSCs obtained in a same cell from each genotype. **A**, top, and **B-D**, left, Control mice. **A**, bottom, and **B-D**, right, *Cacna1a^{quirk(-/-)}* mice. Scale bars, 100 pA and 20 ms. * $p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$ (ANOVA).

Cacna1a^{quirk(-/-)} mice, which was comparable to that in *Tg^{Gabra6-Cre}* control mice (34.5 ± 1.5 Hz, $n = 25$), suggesting that intrinsic PC firing is not altered in *Cacna1a^{quirk(-/-)}* mice (Fig. 7D). The optical fiber was placed in the GC layer right beneath a recorded PC soma (50 μm apart). In close proximity to the PC soma, GC activity has been reported to increase PC firing dominantly (Dizon and Khodakhah, 2011). As shown in Figure 7C, a short (300 ms) light pulse increased PC firing immediately, which then declined to a certain plateau level during the light pulse. The increase in PC firing frequency was completely blocked by CNQX (10 μM, $n = 6$, data not shown), suggesting that non-NMDA-receptor-mediated but not GABAergic synaptic transmission modulates PC firing. The modulation/increase in PC firing frequency was dependent on the light intensity, because incrementing the light intensity increased the firing frequency (Fig. 7D, E). However, the relationship of light intensity and the resultant increment of the firing rate was impaired in *Cacna1a^{quirk(-/-)}* mice compared with *Tg^{Gabra6-Cre}* control mice (Fig. 7E). These results suggest that the selective knock-down of the P/Q-type channel in cerebellar GCs leads to a reduction of GC excitatory input onto PCs.

Because PCs take on a single CF innervation through a set of developmental processes of synapse formation and elimination, which has been reported to be influenced by PF-PC synapse activity (Hashimoto et al., 2009) and is impaired in many P/Q-type channel mutants (Bosman and Konnerth, 2009; Hashimoto et al., 2009; Pietrobon, 2010; Kano and Hashimoto, 2011), we investigated CF-PC synapse formation in *Cacna1a^{quirk(-/-)}* mice. Only 2 of 31 PCs tested were found to be multiply innervated in 3-week-old mice, and the amplitude of evoked CF-EPSCs in *Cacna1a^{quirk(-/-)}* mice was 3.31 ± 0.59 nA (at -20 mV, $n = 21$), which was comparable to that of control mice (Hashimoto et al.,

2011). Therefore, the results suggest that PF-PC synaptic transmission is impaired while sparing the normal development of CF excitatory input in *Cacna1a*^{quirk(-/-)} mice.

Cacna1a^{quirk(-/-)} mice have ataxia, episodic dyskinesia, and absence epilepsy

Loss-of-function and reduction-of-function mutations of the P/Q-type channel are associated with a complex neurological phenotype including ataxia, dyskinesia, and absence epilepsy. We have shown recently that the loss of the P/Q-type channel in cerebellar PCs accompanied with a sparse loss of the channel throughout forebrain (*purky*) is associated with all three abnormal phenotypes (Mark et al., 2011). Because GCs modulate PC activity, and therefore the output of cerebellar cortex, we studied *quirky* mice to determine whether postnatal loss of P/Q-type channels in GCs, precerebellar nuclei, and other confined forebrain structures leads to behavioral phenotypes similar to those observed in *purky* mice.

We first observed that <25% of *Cacna1a*^{quirk(-/-)} mice developed spontaneous episodes of paroxysmal dyskinesia 3 weeks after birth, as has also been described in *tottering* and other alleles (Noebels and Sidman, 1979). These episodes lasted 59.6 ± 4.4 min and occurred 2.9 ± 0.3 times per day ($n = 15$). Furthermore, in these mice, the dystonic seizure could be rapidly induced by stress (i.e., cage change, cage transport, short restraint, or saline injection) or chemicals such as caffeine or ethanol (Table 1). However, other *Cacna1a*^{quirk(-/-)} mice did not develop seizures. Therefore, we divided the *Cacna1a*^{quirk(-/-)} mice into two groups: mild and severe. The *Cacna1a*^{quirk(-/-)} severe mice showed ataxia in all tests analyzed, including the footprint, rotarod, pole, hang wire, and beam walk tests (Fig. 8A–E). In contrast, the *Cacna1a*^{quirk(-/-)} mild mutants showed slightly wider stride lengths, as seen in the footprint analysis (Fig. 8A), and motor impairment only in the beam walk test (Fig. 8E), but not on the other tests performed. These *Cacna1a*^{quirk(-/-)} mild mice took 4 times as long as their *Cacna1a*^{Citrine} controls to cross the beam, but were twice as fast as the *Cacna1a*^{quirk(-/-)} severe mice. Both *Cacna1a*^{quirk(-/-)} mild and *Cacna1a*^{quirk(-/-)} severe mice showed an increased number of slips when they walked over the beam. Differences in motor coordination also became obvious in the footprint analysis. Both *Cacna1a*^{quirk(-/-)} mild and *Cacna1a*^{quirk(-/-)} severe mice had an increased relative stride width compared with control mice, whereas stride length was decreased by 10–15% only in the *Cacna1a*^{quirk(-/-)} severe mice compared with the other mouse lines tested. It has to be noted that, except for the footprint analysis, all other tests performed (i.e., pole, incline screen, hang wire, and beam walk tests) would often trigger a dystonic episode in

Figure 7. Optical fiber-guided focal stimulation of GCs with Chr2 and the effects on PC spiking. **A**, Sagittal section showing Chr2-mCherry expression in cerebellar GCs after injection of Cre-dependent AAV2 into cerebellum of *Tg*^{Gabra6-cre} mice. **B**, Image of the quartz glass optical fiber with a blunt end tip (tip outer diameter, $\sim 35 \mu\text{m}$) that was used for focal light application. **C**, Placement of an optical fiber and a recording pipette within a cerebellar slice. The optical fiber was placed in GC layer right beneath a recorded PC soma ($50 \mu\text{m}$ apart). Light intensity was monitored at the outlet of the optical fiber ($30 \times 30 \mu\text{m}$) by a photomultiplier. **D**, Example trace (upper) and time course of corresponding instantaneous frequency (bottom plot) of PCs spontaneous firing obtained by extracellular recordings in cerebellar slices of control *Tg*^{Gabra6-cre} mice (left column, white plots) and *Cacna1a*^{quirk(-/-)} mice (right column, black plots) are shown in each panel. A lower-intensity (0.2 V read by a photomultiplier; upper panels) or a higher-intensity (0.5 V; lower panels) light pulse was flashed at the time indicated by black bars (300 ms long, 470 nm). **E**, Plots showing the averaged input (light intensity) to output (change in PC spike frequency) relationship in control *Tg*^{Gabra6-cre} mice (white circles; $n = 25$ cells) and in *Cacna1a*^{quirk(-/-)} mice (black circles; $n = 21$ cells). The change in frequency was determined by subtracting the baseline frequency from the averaged frequency obtained in light-illumination. **F**, Example traces of voltage responses to injected current (22 pA, 400 ms) in GCs obtained from 3-week-old control (left) and *Cacna1a*^{quirk(-/-)} (right) mice. **G**, Averaged input (injected current) to output (spike frequency) relationship of GC membrane responses in control (white circles; $n = 16$ cells) and in *Cacna1a*^{quirk(-/-)} mice (black circles; $n = 21$ cells). * $p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$ (ANOVA). Scale bars: **A**, $50 \mu\text{m}$, inset $300 \mu\text{m}$; **C**, $20 \mu\text{m}$; **D**, $200 \mu\text{V}$, 200 ms ; **F**, 20 mV , 100 ms .

Cacna1a^{quirk(-/-)} severe mice that could interfere with completion of the test.

After performing a developmental behavior study of both *Cacna1a*^{quirk(-/-)} mild and *Cacna1a*^{quirk(-/-)} severe mice at 1–6 months of age, we did not observe a decline or recovery in motor coordination or dyskinesia (data not shown). Therefore, based on our developmental behavior tests, we can conclude that there were no observed *Cacna1a*^{quirk(-/-)} mild mice that progressed to a *Cacna1a*^{quirk(-/-)} severe phenotype.

After comparing 11 litters, we counted 47 *Cacna1a*^{quirk(-/-)} mild mice (29 males, 18 females; ratio of 1.6) and 38 *Cacna1a*^{quirk(-/-)} severe mice (22 males, 16 females; ratio of 1.4). The ratio of *Cacna1a*^{quirk(-/-)} mild to *Cacna1a*^{quirk(-/-)} severe mice was 1.2 and did not correspond to the expected Mendelian genetics. To examine the possibility that the zygosity of the Cre recombinase was contributing to the differences in phe-

Figure 8. Behavioral analysis and absence epilepsy of $\text{Cacna1a}^{\text{quirk}(-/-)}$ mice. Comparisons are shown for $\text{Cacna1a}^{\text{Citrine}}$ mice (white bar), $\text{Cacna1a}^{\text{quirk}(-/-)}$ mild mice (gray bar), and $\text{Cacna1a}^{\text{quirk}(-/-)}$ severe mice (black bar) for results from the following tests: (A) footprint, (B) rotarod, (C) pole test, (D) hang wire, and (E) beam walk. The details of the analysis and the number of mice tested are described in the Materials and Methods. * $p < 0.05$; ** $p < 0.01$ (ANOVA). F, Spontaneous electrocorticographic spike-wave discharge activity recorded from awake adult $\text{Cacna1a}^{\text{quirk}(-/-)}$ mice. Traces from left and right temporal (LT and RT) and left and right parietal (LP and RP) hemispheres show bilateral, spike-wave synchronous discharge during behavioral arrest. Intermittent interictal EEG spike discharges are also present.

notypes from $\text{Cacna1a}^{\text{quirk}(-/-)}$ mice, we performed quantitative genomic RT-PCR. We found that $\text{Cacna1a}^{\text{quirk}(-/-)}$ severe mice were both heterozygous (10 of 13 mice) and homozygous (3 of 13 mice) for Cre recombinase, whereas $\text{Cacna1a}^{\text{quirk}(-/-)}$ mild mice were only heterozygous for Cre recombinase (12 of 12 mice; Table 2). Because the three mice demonstrating homozygosity for Cre recombinase displayed the *quirky* severe phenotype, this is suggestive that $\text{Cacna1a}^{\text{quirk}(-/-)}$ severe mice homozygous for Cre recombinase are likely to develop a more severe *quirky* phenotype. However, $\text{Cacna1a}^{\text{quirk}(-/-)}$ mice that are heterozygous for Cre recombinase can display either *quirky* mild or *quirky* severe phenotypes, so there must be another explanation for the differences in *quirky* phenotypes.

Cortical spike-wave behavioral arrest seizures

To determine whether $\text{Cacna1a}^{\text{quirk}(-/-)}$ mice exhibited spike-and-wave epilepsy, we performed chronic videoelectroencephalographic monitoring. These studies revealed the spontaneous appearance of bilaterally synchronous and symmetrical 6–8 Hz spike-wave discharges while the mouse was awake. Aberrant synchronous discharges occurred in 6 of 6 mice monitored, with a range of 0–3 episodes per hour and a mean burst duration of 1–1.7 s (Fig. 8F). Behavioral arrest and, on occasion, myoclonic movements of the vibrissae or neck accompanied spike-wave episodes and normal motor behavior resumed immediately on termination of the cortical discharge. Isolated interictal cortical spike discharges were also observed infrequently. These patterns were seen in $\text{Cacna1a}^{\text{quirk}(-/-)}$ mice and were never observed in $\text{Cacna1a}^{\text{Citrine}}$ mice ($n = 4$).

Finally, a comparison of our present data obtained on *quirky* mice and published data on *purky* mice (Mark et al., 2011) is summarized in Table 3. *Quirky* mice showed milder reduction in the size of the cerebellum and cortical layer than *purky* mice, in which degeneration of PCs was evident with age. Due to PC-specific elimination of the P/Q-type channel, *purky* mice showed severe impairment of action potential firing in PCs, whereas normal firing patterns were observed in PCs from *quirky* mice by *in vitro* studies. In addition, disruption of excitatory CF and PF synapse formation onto PCs in another PC-specific P/Q-type channel knock-out mouse model has been reported (Hashimoto et al., 2011; Miyazaki

Table 3. Comparison of *Purky* and *Quirky* mice

	<i>Purky</i>	<i>Quirky</i>
Cerebellar anatomy	Decrease	Decrease (severe*)
Area of cerebellar cortex (% of control)	60 ± 1	86 ± 0
Molecular/granule cell layer (% of control)	39 ± 1/73 ± 1	87 ± 1/89 ± 0
Intrinsic PC firing		
<i>In vivo/in vitro</i>	Decrease/loss	ND/normal
Synaptic transmission		
PF-PC	Increase ^a	Decrease
CF-PC	Multiple innervation ^b	Mono-innervation
PC-DCN	Decrease	ND
Ataxia on gait	Moderate	Milder (mild*), mild (severe*)
Dystonic seizures	Yes	No (mild*), yes (severe*)
Frequency (#/d)/duration (min)	2.3 ± 4.1/41 ± 5	2.9 ± 0.3/60 ± 4
Stress and drug induced	Yes	No (mild*), yes (severe*)
Cre expression in BG/SN	Rare/no ^c	No/no
Absence seizures	Yes	Yes
Cre expression in Th/CC	Sparse/sparse ^c	No/rare

Severe* indicates *quirky* severe mice; mild*, *quirky* mild mice. ND, Not determined; BG, basal ganglia; SN, substantia nigra; Th, thalamus; CC, cerebellar cortex. Data represent mean ± SEM.

^aMiyazaki et al., 2012.

^bHashimoto et al., 2011.

^cMadisen et al., 2010.

et al., 2012). However, *quirky* mice demonstrated impairment of synaptic transmission in only PFs and not in CFs. All three phenotypes—ataxia, dyskinesia, and absence seizures—were displayed in both *quirky* and *purky* mice, although differences in the degree of ataxia and the manifestation of dyskinesia were observed. Both mouse lines showed unexpected sparse leakage of Cre recombination outside of the cerebellar circuitry upon crossing with td-Tomato reporter mice. Therefore, we cannot exclude the possibility that P/Q-type channel deletion outside of the cerebellum is involved in the pathogenesis. However, both mice demonstrated only sparse td-Tomato-stained cells in the basal ganglia and substantia nigra, regions primarily implicated in dystonia in humans, and also sparse td-Tomato-stained cells in the cerebral cortex and thalamus, regions that have been implicated in absence seizures. Our findings in *purky* and *quirky* mice suggest that pathological changes in activity cerebellar circuitry are involved in the development and manifestation of ataxia, dyskinesia, and absence seizures.

Discussion

Ataxia, dyskinesia, and absence epilepsy arise from selective deletion of P/Q-type channels in cerebellar circuits

We demonstrated recently that the postnatal loss of P/Q-type channels in cerebellar PCs and sparsely throughout the forebrain (*purky*) was sufficient to induce the three salient abnormal phenotypes seen in several loss-of-function mutants, including *tottering* and other tg alleles (Pietrobon, 2010; Mark et al., 2011). In *purky* mice, PC firing and synaptic transmission to DCN neurons are impaired, indicating that deficient output from the cerebellar cortex is implicated in the full phenotypic spectrum. To our surprise, we also found similar phenotypes—ataxia, episodic dyskinesia, and absence epilepsy—in *quirky* mice, in which P/Q-type channels are deleted postnatally from cerebellar GCs, precerebellar nuclei of brainstem, and several confined nuclei of forebrain (Fig. 1, Fig. 8, Table 1). As shown in our electrophysiological data, although transmission at MF-DCN and MF-GC synapses is well compensated by N-type channels, it is severely impaired at PF-PC synapses, especially in the low-frequency range (Fig. 6), resulting

in a reduced excitatory modulation of PC firing in *quirky* mice (Fig. 7). Afferent inputs onto PCs through MF/PF pathways in *quirky* are high-pass filtered, resulting in the loss of their inhibitory gain and/or timing from PCs onto DCN neurons. These observations suggest that a postnatal pathological alteration in signal transduction at the MF/PF pathway due to a loss of P/Q-type channels can initiate the aberrant *quirky* phenotypes.

We distinguished two phenotypes of *quirky* mice based on their neurological severity: severe and mild. Severe *quirky* mice developed dystonic attacks and showed ataxic phenotypes in all behavioral tests. In contrast, mild *quirky* mice did not develop dystonic features, even when challenged by stress or chemicals, and showed deficits only in beam walk and gait analyses (Fig. 8, Table 1). Other differences could be detected in the development of body weight and in the size of the granular and molecular layers (Fig. 2C–E), but the electrophysiological experiments did not show any separable data clusters. We first investigated whether the heterozygosity of Cre recombinase would cause differences in the severity of phenotypes. However, zygosity as determined by RT-PCR revealed no differences (Table 2). Malnutrition may also contribute to these differences, but recovery of dystonic or ataxic phenotypes was not observed. Another possibility is that *quirky* siblings have mixed genetic backgrounds [i.e., C57BL/6J and 129S6/SvEvTac(129)]. The severity of dystonia has been found recently to be modulated by genetic background in DYT1 dystonia mice (Tanabe et al., 2012). In addition, knock-out mice of *Engrailed-1*, which is involved in the development of the mid-/hindbrain region including the cerebellum, revealed differences in mid-/hindbrain development depending on the genetic background (Bilovocky et al., 2003). As seen in episodic ataxia type 2 patients, the symptom varies from intra-/inter-patient's family members. This means that the severity of the symptoms in episodic ataxia 2 patients varies within the same immediate family and extended family. Therefore there must be other environmental or genetic factors that influence the phenotype severity of episodic ataxia 2 patients (Pietrobon, 2010). The severe *quirky* mice displayed more frequent and longer episodes of dyskinesia than *purky* mice (Table 3). In contrast, *quirky* mice were less ataxic under stress-free gait analysis and open-field conditions, suggesting that the site of the channel deletion influences the phenotypic pattern. Nevertheless, in agreement with previous reports in P/Q-type channel mutants and dystonia model mice (Pizoli et al., 2002; Walter et al., 2006; Jen et al., 2007; Neychev et al., 2008; Alviña and Khodakhah, 2010; Calderon et al., 2011), our findings in *purky* and *quirky* mice provide strong evidence for the involvement of the cerebellum in the generation of dystonia and ataxia.

Because both *purky* and *quirky* mice develop absence seizures, it is intriguing to speculate that postnatal developmental changes in the cerebellar output contribute significantly to the thalamocortical dysrhythmia. This is a challenging hypothesis, because absence epilepsy involves aberrant oscillations centered in the thalamocortical loop (Linás et al., 2005; Weiergraber et al., 2010). However, studies in rat models of absence epilepsy have revealed that thalamocortical spike wave discharges are correlated with changes in cerebellar activity and suggested that the cerebellar output may contribute to thalamocortical dysrhythmia (Kandel and Buzsáki, 1993; Hartmann and Bower, 1998). In addition, R-type ($Ca_v2.3$) channels play important roles in PF-PC synaptic plasticity (Myoga and Regehr, 2011), their deletion in mice produces spike-wave absence epilepsy (Zaman et al., 2011), and they are specifically reduced in the cerebellum in a rat model of absence (Lakaye et al., 2002). An involvement of the cerebellum and precerebellar nuclei has also been suggested

for other diseases associated with thalamocortical dysrhythmia, such as Parkinson's disease, tinnitus, and depression (Davis et al., 2000; Llinás et al., 2005; Brozoski et al., 2007; Rolland et al., 2007). The evidence suggests that pathological changes in cerebellar outflow activity are involved in the development and maintenance of abnormal thalamocortical oscillatory behavior. A final possibility inherent to the cre-lox approach that cannot be excluded is that sparse leakage of Cre recombination and P/Q-type channel elimination outside of the cerebellar circuitry could contribute to the generation of absence epilepsy in *quirky* mice. The strong td-Tomato fluorescence detected in the pre-/para-/postsubiculum, medial amygdaloid nuclei, and amygdalohippocampal region, all regions that, while frequently involved in temporal lobe epileptogenesis (Funahashi et al., 1999; Stafstrom, 2005), have not been identified as drivers of seizures with a thalamocortical spike-wave absence pattern.

P/Q-type channels determine short-term synaptic plasticity at the PF-PC synapse

Short-term synaptic plasticity, defined as the presynaptic change in neurotransmitter release, involves presynaptic Ca²⁺ level and Ca²⁺ channel function (Fisher et al., 1997; Zucker and Regehr, 2002; Catterall and Few, 2008). The change occurs in the millisecond to minute time course and is an important mechanism for integration and processing information. It has been shown in several P/Q-type channel mutants that the PF-PC synaptic transmission is reduced to ~30–50% compared with wild-type control mice (Matsushita et al., 2002; Zhou et al., 2003; Kodama et al., 2006; Liu and Friel, 2008). The effects seem to be different among the mutants and can be either presynaptically or postsynaptically derived (Kodama et al., 2006). We found that the postnatal loss of P/Q-type channels specifically in GCs within cerebellum reduced the PF-PC synaptic transmission to ~20% of that of control mice, as indicated in the input-output relationship (Fig. 5A), confirming that the transmission depends strongly on P/Q-type channels in the normal case. The amplitude reduction in *quirky* mice was accompanied by a reduction in PPF (Fig. 5B), which is different from previous results in mutant and total knock-out (*null*) mice, which showed no change (*rocker* and *tottering*) or increased (*leaner*, *rolling*, and *null*) in PPF (Matsushita et al., 2002; Miyazaki et al., 2004; Kodama et al., 2006; Liu and Friel, 2008). Generally, when release probability is reduced, such as after being induced by exposure to lower-Ca²⁺ ACSF or modulators, the PPF is increased along with the amplitude reduction. The discrepancy might be explained by the following observations. We first observed that during presynaptic Ca²⁺-imaging at PF terminals, the ratio of Ca²⁺ influx after the second stimulus relative to the first stimulus was smaller in *quirky* mice compared with control mice (Fig. 5E). This suggests that the relative release probability after the second stimulus would be reduced in *quirky* mice, resulting in a decrease in the PPF. Another possible explanation could be that the extent of functional coupling or physical distance between each type of Ca²⁺ channel (e.g., N-type, P/Q-type, and R-type channels) and exocytosis-triggering Ca²⁺ sensor proteins might be different (Eggermann et al., 2012). In rat PF-PC synapses, the relationship between Ca²⁺ influx and transmitter release (or EPSCs) has been fitted by the polynomial equation, EPSC = $k(\text{Ca}_{\text{influx}}^{2+})^n$ (Mintz et al., 1995; Myoga and Regehr, 2011). The polynomial degree “*n*” has been determined for each type of Ca²⁺ channels and found to be ~4 for P/Q-type channels, ~2 for N-type channels, and 2–3 for R-type channels. Therefore, the PPF profile of PF-PC synaptic transmission in *quirky* mice might follow the weaker relationships made by such looser-coupling Ca²⁺ channels. The other possibility is that P/Q-type channels are the targets of Ca²⁺-dependent modulation for mediating short-term synaptic plasticity (Lee et al., 1999; Mochida et

al., 2008), suggesting that the loss of the target protein in *quirky* mice might change the PPF. Although the EPSCs elicited by a single- or lower-frequency stimulation are smaller in *quirky* mice, repetitive stimulation at higher frequencies (>50 Hz) reveals comparable EPSC amplitude (Fig. 6). A possible explanation for this result is that Ca²⁺ influx through P/Q-type channels is dominant at low-frequency stimulation, whereas during high-frequency stimulation, more N/R-type Ca²⁺ channels are recruited to mediate transmitter release. The recovery of EPSC amplitude during high-frequency stimulation also suggests that the postnatal loss of P/Q-type channels at the PF-PC synapse does not affect the functional machinery of transmitter release. This agrees with the observation that no structural change of PF-PC synapses has been observed in an equivalent mouse model with GC-specific ablation of P/Q-type channels in cerebellum (Miyazaki et al., 2012). Therefore, *quirky* mice provide a new model with which to study the development and manifestation of ataxia, dyskinesia, and absence epilepsy associated with inherited genomic P/Q-type channel mutations.

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