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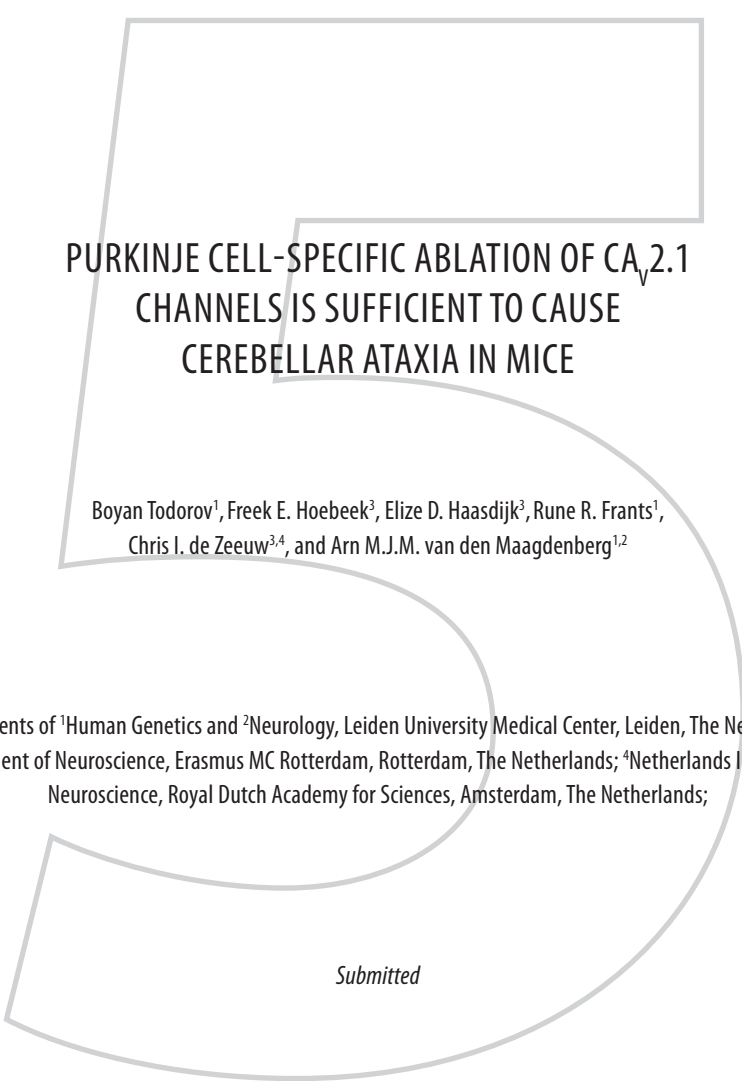
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PURKINJE CELL-SPECIFIC ABLATION OF CA$_V$2.1 CHANNELS IS SUFFICIENT TO CAUSE CEREBELLAR ATAXIA IN MICE

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ABSTRACT

The *Cacna1a* gene encodes the α_{1A} subunit of voltage-gated $Ca_V2.1$ channels that are involved in neurotransmission at many central synapses. $Ca_V2.1$-α_1 knockout (α1KO) mice, which lack $Ca_V2.1$ channels in all neurons, have a very severe phenotype of cerebellar ataxia and dystonia, and usually die around postnatal day 20. As $Ca_V2.1$ channels are expressed throughout the cerebellum, it could not be resolved whether the ataxia was solely due to the lack of $Ca_V2.1$ channel activity in Purkinje cells, or also to the absence of $Ca_V2.1$-dependent synaptic input onto these cells. Here, we crossed conditional *Cacna1a* mice with transgenic mice expressing Cre recombinase, driven by the Purkinje cell-specific *Pcp2* promoter, to specifically ablate $Ca_V2.1$ channels in Purkinje cells. Purkinje cell $Ca_V2.1$-α_1 knockout (PCα1KO) mice aged without the difficulties seen in α1KO mice effectively rescuing the phenotype of early lethality in later. PCα1KO mice exhibited cerebellar ataxia starting around P12, much earlier than the first signs of progressive Purkinje cell loss that occurred around P45. In this mouse model with a cell type-specific ablation of $Ca_V2.1$ channels, we show that ablation of $Ca_V2.1$ channels restricted to Purkinje cells is sufficient to cause cerebellar ataxia. We demonstrate that spatial ablation of $Ca_V2.1$ channels may help unravel cell type-specific mechanisms of human disease.

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PURKINJE CELL-SPECIFIC KNOCKOUT OF CA$_V$2.1 CHANNELS

Keywords: P/Q-type Ca^{2+} channels, conditional, cell-specific knockout, *Cacna1a*

INTRODUCTION

Ca$_V$2.1 channels are plasma membrane multimeric protein complexes that play an important role in neurotransmitter release at most central brain synapses (*Mintz et al., 1992b*). Ca$_V$2.1 channels are characterized by the presence of the pore-forming α$_{1A}$ subunit that is encoded by the *Cacna1a* gene, which is widely expressed throughout the nervous system (*Westenbroek et al., 1995*; *Kulik et al., 2004*). Ca$_V$2.1-α$_1$ knockout (α1KO) mice that are completely devoid of Ca$_V$2.1 channels exhibit a severe phenotype of cerebellar ataxia and dystonia that starts around postnatal day (P) 12, and die, if not given very special care, around P20 (*Jun et al., 1999*; *Fletcher et al., 2001*; *Kaja et al., 2007*; *Katoh et al., 2007*). Histological analysis in survivors indicated that a progressive gradual loss of Purkinje cells started between P45 and P100 (*Fletcher et al., 2001*). Cerebellar ataxia is also part of the phenotype of the naturally occurring *Cacna1a* mouse mutants *leaner*, *tottering*, *rolling Nagoya*, and *rocker* that are characterized by a loss of Ca$_V$2.1 function. All these mutants, except *rocker*, show to different extents granule and/or Purkinje cell death (*Herrup and Wilczynski, 1982*; *Rhyu et al., 1999*; *Sawada et al., 2009*; *Zwingman et al., 2001*). Recently, cerebellar ataxia has been shown to be a likely consequence of the increase in the irregularity of Purkinje cell firing in in *tottering* and *leaner* mice (*Hoebeek et al., 2005; Walter et al., 2006*). However, since both parallel and climbing fibers that innervate Purkinje cells both express Ca$_V$2.1 channels (*Kulik et al., 2004*), the origin of the aberrant Purkinje cell firing in Ca$_V$2.1 mutants can not yet be resolved. Moreover, neurotransmission from these fibers has been shown to be abnormal in various loss-of-function *Cacna1a* mutants (*Matsushita et al., 2002*; *Liu & Friel, 2008*). Thus, it is at present unknown whether the neurobiological consequences in *Cacna1a* mutant mice are the result of a reduced (or absent) Ca$_V$2.1 channel function in Purkinje cells or an abnormal synaptic input onto Purkinje cells.

Therefore, we used the Cre-lox system to ablate Ca$_V$2.1 channels exclusively in Purkinje cells by crossing conditional *Cacna1a* mice that carry a "floxed" allele (*Todorov et al., 2006*) with transgenic mice that express Cre recombinase under the control of the Purkinje cell-specific *Pcp2* promoter (*Barski et al., 2000*). We were not only able to prevent the early lethality seen in α1KO mice, but also could demonstrate that lack of Ca$_V$2.1 channel function in Purkinje cells is sufficient to cause cerebellar ataxia that was associated with severe, progressive Purkinje cell loss starting well after the onset of the ataxia.

EXPERIMENTAL PROCEDURES

Animals

All animal experiments were performed in accordance with guidelines of the respective universities and national legislation. Previously, we generated conditional *Cacna1a* mice using a gene targeting approach (*Todorov et al., 2006*). The *Cacna1a* allele in these mice

was "floxed" by introducing *loxP* sites flanking exon 4 (i.e., *Cacna1a*^{flox} mice) (*Fig. 1A*). A genomic deletion of exon 4 was achieved by crossing the conditional mice with *Pcp2*^{Cre} transgenic mice that express Cre recombinase exclusively in Purkinje cells (*Barski et al., 2000*). Homozygous *Cacna1a*^{flox/flox} mice were first crossed with homozygous *Pcp2*^{Cre} mice generating double heterozygous transgenic offspring. Female double transgenic mice were then crossed with *Cacna1a*^{flox/wt} males to generate mice that were *Cacna1a-*
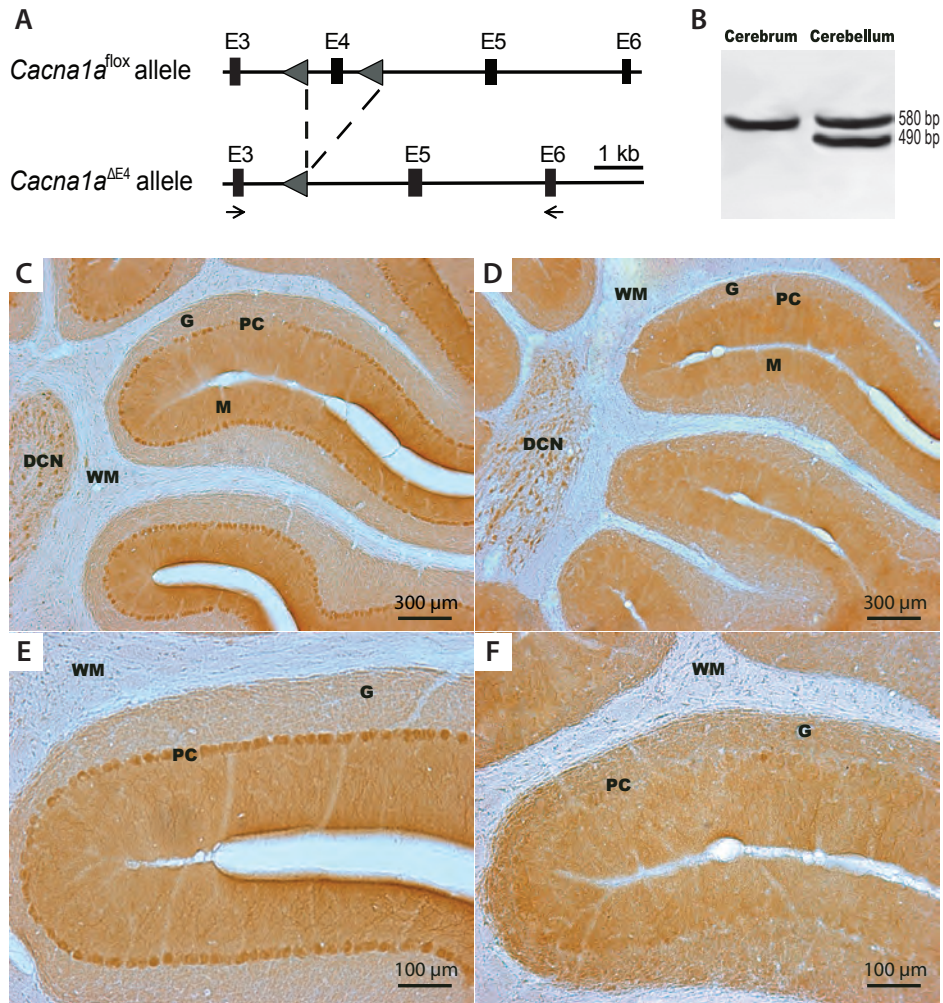
Figure 1. Generation of conditional *Cacna1a* Purkinje cell knockout mice. (**A**) Schematic representation of the genomic structure of the relevant part of the floxed and recombined *Cacna1a* alleles. Black boxes indicate exons (E). Triangles indicate the position of loxP sites. Arrows indicate the position of the primers used for the RT-PCR. (**B**) RT-PCR of cerebellar RNA amplifies a 580-bp product from the non-recombined *Cacna1a* transcript and a 490-bp product from transcripts that lack exon 4. (**C-F**) Anti-Ca$_V$2.1-α_1 immunostaining in cerebellar sections of P30 wild type (**C, E**) and PCα1KO (**D, F**) mice. DCN - deep cerebellar nuclei; G - granule cell layer; M - molecular layer; PC - Purkinje cell layer; WM - white matter.

deficient (and thereby $Ca_V2.1$ channel-deficient) in Purkinje cells (i.e., Purkinje cell $Ca_V2.1$-α_1 KO; PCα1KO). The same cross also produced mice in which *Cacna1a* was not affected (wild-type; WT). Genotyping of the *Cacna1a*^{flox} allele was performed by PCR using primers P1 (5`-acctacagtctgccaggag-3`) and P2 (5`-tgaagcccagacatccttgg-3`) and genomic DNA as a template. Genomic deletion of exon 4 after Cre recombination was confirmed by PCR using primers P3 (5`-agtttctattggacagtgctggt-3`) and P4 (5`-ttgcttagcatgcacagagg-3`). Primers P5 (5'-acttagcctgggggtaactaaact-3') and P6 (5'-ggtatctctgaccagagtcatcct-3') were used to genotype the *Cre* transgene.

Reverse transcription PCR

Total RNA was isolated from freshly dissected cerebellum and cerebrum of mice. For RT-PCR, first-strand cDNA was synthesized using random primers. Subsequently, *Cacna1a*-specific PCRs were performed using primers P7 (5`-gatgacacggaaccatac-3`) and P8 (5`-attgtagaggagatcagtcc-3`) that are located in exons 3 and 6, respectively.

Rotarod test

The accelerating Rotarod (*UGO Basile S.R.L., Commerio VA, Italy*) test was performed on a 4-cm diameter horizontal rotating rod. The test was performed in a semi-dark room with a light source placed at the bottom to discourage mice from jumping off the rod. Following a training period (in which the mice were placed on the rod moving with a low constant speed of 5 rpm for 5 min), the mice were subjected to two trials per day, separated by 30 min, on five consecutive days. Each trial started with the rod moving at a constant speed of 5 rpm; after 10 s the speed was gradually increased to 45 rpm over the following 5 min. The latency to fall (i.e., endurance) was recorded, and the endurance per trial per genotype presented as means ± SEM.

Histology

Mouse brains (16 PCα1KO and 16 wild-type) were obtained at various ages after cardiac perfusion with PBS followed by 4% buffered paraformaldehyde (PFA). Post-fixation was performed for 1h in 4% PFA, followed by overnight incubation in 10% sucrose in 0.1 M phosphate buffer at 4ºC. Subsequently, membranes were removed, tissue was embedded in 10% sucrose in 10% gelatine, and the gelatin was fixed with 30% sucrose in 10% formaline for 2.5 h at room temperature. This was followed by an overnight incubation in 30% sucrose in 0.1 M phosphate buffer at 4ºC. Sagittal sections were cut 40-μm thick on a freezing cryotome (*Leica, Bensheim, Germany*). Some of the sections were processed for silver staining, which selectively stains dying neurons (*Nadler & Evenson, 1983*; *Jaarsma et al., 2000*). The remainder of the sections was processed directly for free-floating immunohistochemistry. In brief, sections were incubated in 10% heat-inactivated normal horse serum (NHS) with 0.5% Triton-X100 in TBS, for 2hrs and then incubated with either primary rabbit polyclonal anti-$Ca_V2.1$-α_1 antibody (ACC-001, 1:100; *Alomone Labs, Jerusalem, Israel*), mouse monoclonal

anti-Cre antibody (AB24607, 1:1,000; *Abcam, Cambridge, UK*), or rabbit polyclonal anti-calbindin antibody (CB38, 1:10,000; *SWANT, Bellinzona, Switzerland*) diluted in 1% NHS with 2% heat-inactivated fetal calf serum and 0.4% Triton-X100 in TBS at 4ºC. Next, sections were washed and incubated for 2hrs at room temperature with a secondary biotin-labeled goat anti-rabbit antibody or goat anti-mouse antibody (1:200; *Vector Laboratories, Burlingame, CA*). For protein detection, sections were incubated with the avidin-biotin-peroxidase complex (ABC kit, 1:100, *Vector Laboratories*) for 2hrs at room temperature, and developed in 0.1 mg/ml diaminobenzidine in 0.005% H_2O_2. Sections were air-dried and coverslipped. Paraffin-embedded cerebellar sections (5 µm) were processed for standard hematoxylin and eosin staining.

RESULTS

Generation of mice that lack Ca$_V$2.1 channels in Purkinje neurons

Homozygous PCα1KO mice were generated by crossing *Cacna1a*flox/flox mice with transgenic mice expressing *Cre* recombinase driven by the Purkinje-specific *Pcp2* gene promoter (i.e., *Pcp2*Cre mice) (*Fig. 1A*). Successful genomic deletion of exon 4 of the *Cacna1a* gene was assessed at the RNA level by RT-PCR on cerebral and cerebellar total RNA (*Fig. 1B*). While only non-recombined PCR product of 580 bps is observed in preparations of the mutants' cerebrum, both non-recombined and recombined (480 bps) products were identified in cerebellar preparations. This result indicated that Cre recombination only occurred in part of the cerebellar neuron population. Immunohistochemistry for α_{1A} protein indicated that Cre recombination was Purkinje cell-specific as no protein signal was present in the soma of virtually any Purkinje neurons of PCα1KO mice (*Fig. 1D, F*). However, α_{1A} protein expression in other cerebellar structures (such as in deep cerebellar nuclei) was not different between PCα1KO and wild-type mice. These findings are in line with the reported Purkinje cell specificity of the *Pcp2*Cre mice (*Barski et al., 2000*).

Motor coordination is severely affected in PCα1KO mice

The PCα1KO mice, unlike conventional α1KO mice, have a normal life span. PCα1KO mice appear normal until P10-12 when they start showing an ataxic phenotype with a loss of balance during walking and problems righting themselves, very similar to the ataxia described in conventional α1KO mice (*Supplemental video 1*). Rotarod experiments of PCα1KO mice tested at P30 revealed that these mice were severely ataxic, unable to maintain their balance on the rod for longer than 50 s, in contrast to wild-type control mice, which all stayed on the accelerating rod for more than 200 s (*Fig. 2A*). Both inspection of a video and rotarod testing at P200, did not indicate that the ataxic phenotype had worsened with age in PCα1KO mice (*Supplemental video 2*, *Fig. 2B*).
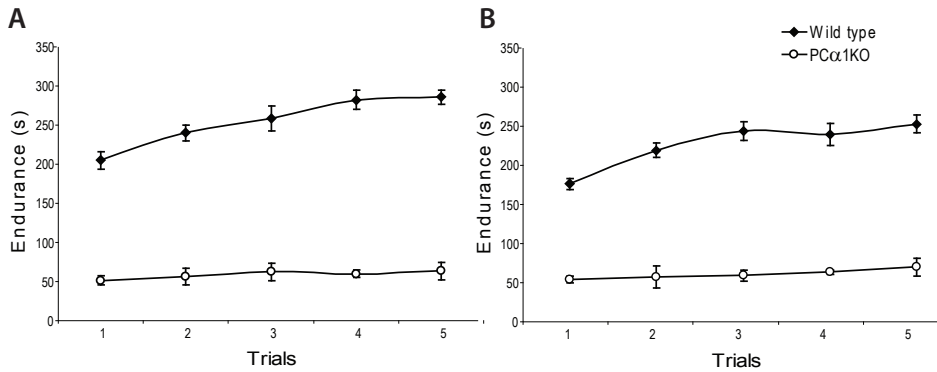
Figure 2. Testing of motor coordination in *Cacna1a* Purkinje knockout mice. Rotarod testing for ataxia in 1-month-old mice (**A**) reveals severe impairment in the performance in PCα1KO mice that was no different also later in their lives (P200) (**B**). Trials were performed on five consecutive days.

Progressive cell death of Purkinje cells in the absence of Ca$_V$2.1 channels

During the first postnatal week, Ca$_V$1 channels are the dominant voltage-gated Ca^{2+} channel type in Purkinje cells (*Liljelund et al., 2000*). In the second week, the role of Ca$_V$2.1 becomes more prominent (*Meacham et al., 2003*), and soon they become by far the dominant Ca$_V$ channel type. This remains the case throughout adulthood (*Mintz et al., 1992a*; *Meacham et al., 2003*). As the *Pcp2* promoter becomes active around P7 (*Barski et al., 2000*), it can be predicted that *Cacna1a* levels in PCα1KO mice will drop before or around that time in development when Ca$_V$2.1 channels normally become important in Purkinje cells.

Although PCα1KO become ataxic around P10-12, with clumsy motor behavior, a histological analysis at P30 did not reveal any signs of cytoarchitectural cerebellar
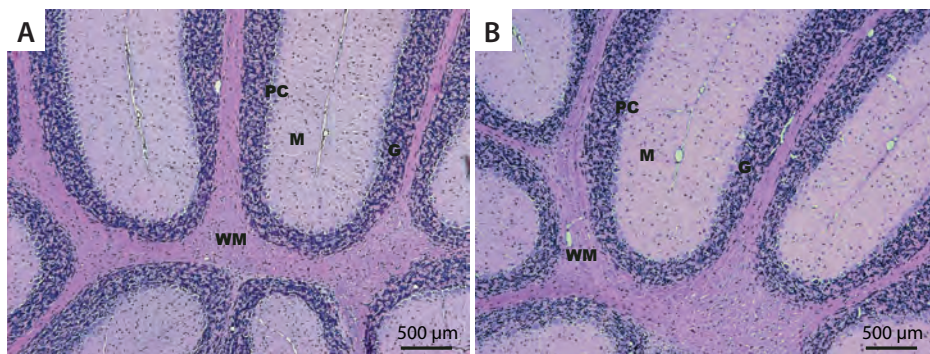
Figure 3. Cerebellar morphology in young *Cacna1a* Purkinje knockout mice. Hematoxilin and eosin staining reveals similar gross cerebellar morphology in PCα1KO (**A**) and wild-type (**B**) mice at P30. G - granule cell layer; PC - Purkinje cell layer; M - molecular layer; WM- white matter.
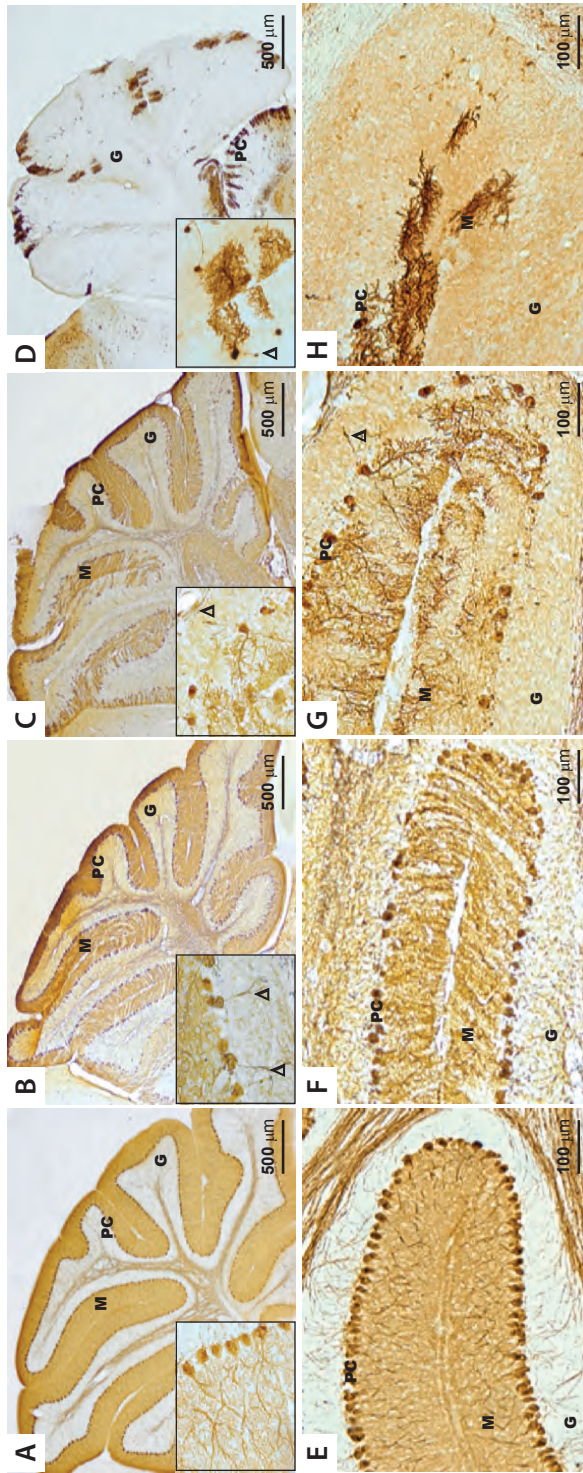
Figure 4. Calbindin D 28K staining of sagittal cerebellar sections of PCα1KO mice at different ages. (**A, E**) Calbindin staining reveals normal foliation of the cerebellum at P30 in PCα1KO mice. Purkinje cells are organized in regular rows with no evidence of abnormal morphology. (**B, F**) Purkinje cell loss, particularly in the anterior lobes of PCα1KO mice at P60. Abnormalities in Purkinje cell morphology, such as axonal swellings (arrowheads in inset), can be seen. (**C, G**) Progressive Purkinje cell loss in PCα1KO mice at P100 is now also more prominent in posterior lobes. Axonal swellings are indicated by arrowheads. (**D, H**) Hardly any Purkinje cells remain in PCα1KO mice at P200. Rare Purkinje cells often show axonal swelling (arrowhead). G - granule cell layer; PC - Purkinje cell layer; M - molecular layer; WM - white matter.

abnormalities (*Fig. 3*). Still, as $Ca_V2.1$ channels gate more than 90% of the high-voltage-gated Ca^{2+}-influx in Purkinje cells, loss of their function may affect these cells' morphology and survival. Therefore, we investigated calbindin-stained cerebellar sections of PCα1KO mice at various ages (i.e., P30, P60, P100, and P200) (*Fig. 4*). As was also observed in α1KO mice (*Fletcher et al., 2001*), no overt cerebellar malformations were visible at P30 in PCα1KO mice (*Fig. 4A, E*), suggesting that the development of Purkinje cells occurs normally despite the absence of $Ca_V2.1$ function. The apparent normal morphology of Purkinje cells at P30 shows that it is not loss, but dysfunction of these neurons that brings about the ataxic phenotype at an early age.

However, over time we observed an increasing loss of Purkinje cells (*Fig. 4*). At P60 and P100, the loss of Purkinje cells is most clearly visible in the anterior cerebellar lobes. At these ages, there are many Purkinje cells that show structural abnormalities, such as somatic sprouting and axonal swellings (*Fig. 4B, C, G*). At P200, PCα1KO mice were almost completely devoid of Purkinje cells (*Fig. 4D, H*).

Neurodegenerative changes in PCα1KO mice

In order to visualize dying neurons, we used silver impregnation on tissue from mice of various ages (*Fig. 5*). At P30, PCα1KO mice show little argyrophylic staining (mainly in the molecular layer) (*Fig. 5A, D*), confirming that there are no overt degenerative changes in the cerebellum when PCα1KO mice become ataxic. At P45, the level of argyrophylic
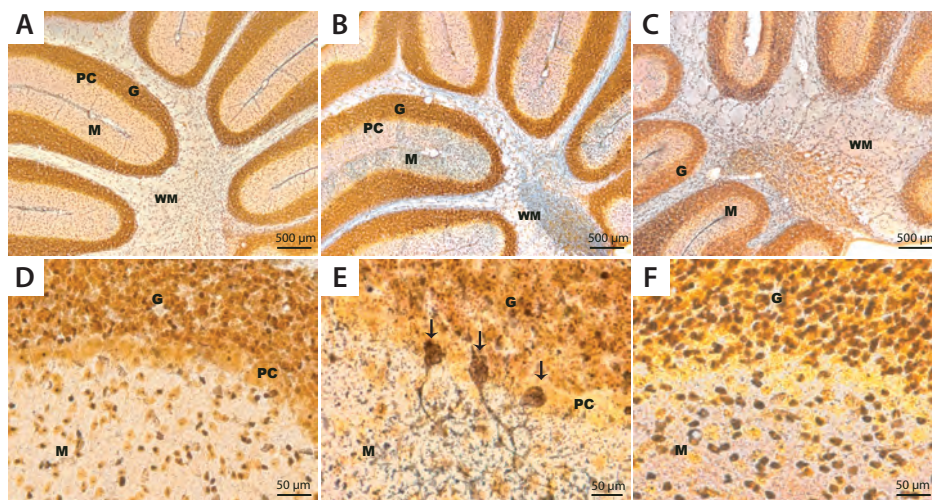
Figure 5. Silver impregnation of cerebellar sections of PCα1KO mice at different ages. (**A, D**) No apparent neurodegeneration is seen in the cerebellum of PCα1KO mice at P30. (**B, E**) Silver-stained Purkinje cell bodies as well as dendrites and axons indicating dying cells in the cerebellum of PCα1KO mice at P45. Arrows indicate dying Purkinje cells in (E). (**C, F**) No Purkinje cells are present. Silver-stained cells can be found in the granular and molecular layers indicating some granule and interneuron loss at P200. G - granule cell layer; PC - Purkinje cell layer; M - molecular layer; WM - white matter.

staining in PCα1KO mice is dramatically increased. Neuronal degeneration seems restricted to the Purkinje cell and molecular layers with many dying Purkinje cells and silver-stained axons and dendrites, randomly dispersed throughout the cerebellar cortex (*Fig. 5B, E*). Consequently, the first degenerative changes in Purkinje cells must appear between P30 and P45. At P200, the molecular and granule cell layers can be seen (*Fig. 5C, F*) despite the absence of Purkinje cells (*see Fig. 4D, H*); both layers contain a considerable number of dying neurons. Apparently, the loss of their target cells, results in some loss of molecular layer interneurons and granule cells.

DISCUSSION

In this study we used conditional gene targeting to ablate $Ca_V2.1$ calcium channels selectively in Purkinje cells in mice. This approach allowed us to study, for the first time, the specific contribution of these channels in Purkinje cells to the development of ataxia that is associated with mutations in the *Cacna1a* gene. Our cell type-specific knockout strategy using conditional *Cacna1a* KO mice (*Todorov et al., 2006*) succeeded in circumventing early postnatal lethality, a very prominent feature in conventional *Cacna1a* KO mice that lack $Ca_V2.1$ channels in all neurons (*Jun et al., 1999*; *Fletcher et al., 2001*; *Kaja et al., 2007*; *Katoh et al., 2007*). In line with its well-defined patterns of expression in Purkinje cells in *Pcp2*[Cre] mice (*Barksi et al., 2000*), Cre recombination affected α_{1A} expression in almost all Purkinje neurons, as was evident by the lack of staining in these cells at P30, while $\alpha1$ expression seemed to be preserved in the other neuronal types in the cerebellum and the brain (*data not shown*). We could clearly demonstrate that lack of $Ca_V2.1$ channels in Purkinje cells was sufficient to cause an ataxic phenotype, since the ataxia occurred prior to any cerebellar structural abnormalities. This finding adds to existing evidence that Purkinje cell dysfunction rather than loss of these cells is the primary cause of cerebellar ataxia associated with abnormal $Ca_V2.1$ channel function.

Visual inspection of the mice revealed that the ataxia in PCα1KO mice started at P10-P12, around the same age as in α1KO mice that lack $Ca_V2.1$ channels in all neurons. Loss of $Ca_V2.1$ function in Purkinje cells apparently does not affect normal formation of the cerebellar cortex, similar to what was shown for conventional $Ca_V2.1$ KO mice (*Jun et al., 1999*; *Fletcher et al., 2001*). This is quite remarkable since $Ca_V2.1$ channels play a crucial role in neurotransmission of Purkinje cells (*Mintz et al., 1992a*).

First signs of neurodegenerative changes in PCα1KO mice were observed around P45. Silver impregnation revealed clear argyrophylic staining in the Purkinje cell and molecular layers. At higher magnification, stained Purkinje cell bodies and dendrites were seen, indicating that these cells were dying. From P60, Purkinje cell loss was clearly visible. Many of the remaining Purkinje cells showed abnormalities such as torpedos or axonal swelling. Purkinje cell loss was more prominent in the anterior lobes, similar to what was reported for α1KO mice (*Fletcher et al., 2001*). At older ages

(i.e., P100 and P200), Purkinje cells loss became progressively worse, while neurons in the granule cell and molecular layers were relatively spared.

In *lurcher* mice that carry a mutation in the delta 2 glutamate receptor (GluRδ2), a similar progressive postnatal Purkinje cell degeneration is followed by large scale dying of granule cells after the loss of their target Purkinje cells (*Caddy & Biscoe, 1979*). In PCα1KO mice, the initial neuronal loss was also restricted to Purkinje cells, in concordance with the specificity of the L7 promoter. However, only moderate granule cell death was detected later in life, likely as the consequence of absent critical interaction with Purkinje cells.

This report represents the first account of a cell type-specific ablation of $Ca_V2.1$ channels leading to a disease phenotype. We could clearly demonstrate that specific ablation of these channels in Purkinje cells was sufficient to cause cerebellar ataxia that preceded progressive Purkinje cell loss. Conditional *Cacna1a* mice appear especially useful for dissecting the cell-specific role of $Ca_V2.1$ channels in neuronal circuitries (such as in the cerebellum) and in revealing the mechanisms underlying human disease.

ACKNOWLEDGEMENTS

SUPPLEMENTARY MATERIAL

Video 1. Video recording at P20 indicates an ataxic phenotype in a PCα1KO mouse.

Video 2. Video recording at P200 indicates no obvious progression of the ataxic phenotype in a PCα1KO mouse compared to that seen at P20.

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