


RESEARCH ARTICLE

Cerebellar developmental deficits underlie neurodegenerative disorder spinocerebellar ataxia type 23Cleo J.L.M. Smeets¹ ; KaiYu Ma² ; Simon E. Fisher^{1,3}; Dineke S. Verbeek^{2,*} ¹ Department of Language and Genetics, Max Planck Institute for Psycholinguistics, Nijmegen, the Netherlands.² Department of Genetics, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands.³ Donders Institute for Brain, Cognition and Behaviour, Radboud University, Nijmegen, the Netherlands.**Keywords**

ataxia, climbing fibers, development, GABAergic transmission, prodynorphin.

Abbreviations

AMPA, α -amino-hydroxy-5-methylisoxazole-4-propionate; BC, basket cell; CF, climbing fiber; Dyn, Dynorphin; GAD67, glutamate decarboxylase 67; NMDA, *N*-methyl-D-aspartate; PC, Purkinje cell; PDYN, prodynorphin; PF, parallel fiber; SCA, spinocerebellar ataxia; vGAT, vesicular GABA transporter; VGCC, voltage-gated calcium channel; vGlut, vesicular glutamate transporter.

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Received 28 May 2020

Accepted 7 October 2020

Published Online Article Accepted 00 Month 2020

doi:10.1111/bpa.12905

Abstract

Spinocerebellar ataxia type 23 (SCA23) is a late-onset neurodegenerative disorder characterized by slowly progressive gait and limb ataxia, for which there is no therapy available. It is caused by pathogenic variants in *PDYN*, which encodes prodynorphin (PDYN). PDYN is processed into the opioid peptides α -neoendorphin and dynorphins (Dyn) A and B; inhibitory neurotransmitters that function in pain signaling, stress-induced responses and addiction. Variants causing SCA23 mostly affect Dyn A, leading to loss of secondary structure and increased peptide stability. *PDYN*^{R212W} mice express human PDYN containing the SCA23 variant p.R212W. These mice show progressive motor deficits from 3 months of age, climbing fiber (CF) deficits from 3 months of age, and Purkinje cell (PC) loss from 12 months of age. A mouse model for SCA1 showed similar CF deficits, and a recent study found additional developmental abnormalities, namely increased GABAergic interneuron connectivity and non-cell autonomous disruption of PC function. As SCA23 mice show a similar pathology to SCA1 mice in adulthood, we hypothesized that SCA23 may also follow SCA1 pathology during development. Examining *PDYN*^{R212W} cerebella during development, we uncovered developmental deficits from 2 weeks of age, namely a reduced number of GABAergic synapses on PC soma, possibly leading to the observed delay in early phase CF elimination between 2 and 3 weeks of age. Furthermore, CFs did not reach terminal height, leaving proximal PC dendrites open to be occupied by parallel fibers (PFs). The observed increase in vGlut1 protein—a marker for PF-PC synapses—indicates that PFs indeed take over CF territory and have increased connectivity with PCs. Additionally, we detected altered expression of several critical Ca²⁺ channel subunits, potentially contributing to altered Ca²⁺ transients in *PDYN*^{R212W} cerebella. These findings indicate that developmental abnormalities contribute to the SCA23 pathology and uncover a developmental role for PDYN in the cerebellum.

INTRODUCTION

Spinocerebellar ataxia type 23 (SCA23) is a late-onset, slowly progressive neurodegenerative disorder for which there is no available therapy. It is caused by pathogenic variants in *PDYN* and characterized by loss of neurons in the Purkinje cell (PC) layer, dentate nuclei and inferior olivary nuclei (2, 52). Patients suffer from gait ataxia, dysarthria, slowed saccades, ocular dysmetria, Babinski's sign and hyperreflexia. *PDYN* encodes the opioid precursor protein prodynorphin (PDYN), which is processed into the opioid peptides α -neoendorphin and dynorphins (Dyn) A and B. These peptides normally function as inhibitory neurotransmitters in pain processing, stress-induced responses, and addiction (1, 9, 19, 43). Dyn A is also known to elicit non-opioid-mediated neurotoxic effects including allodynia, neuronal loss and

paralysis (19, 22, 53). It can cause cell death via the NMDA (*N*-methyl-D-aspartate) receptor (51), and elicit neurotoxic effects via the AMPA (α -amino-hydroxy-5-methylisoxazole-4-propionate) receptor and acid-sensing ion 1a channels (44, 46). Additionally, we have shown that variants causing SCA23 affect the secondary structure of Dyn A, reducing its affinity with its natural κ -opioid receptor as well as peptide stability, leading to peptide aggregation (49).

We previously generated a transgenic mouse, the *PDYN*^{R212W} mouse, overexpressing one copy of human *PDYN* containing the p.R212W variant, and showed that they recapitulate features of SCA23 pathology, showing progressive gait deficits from 3 months of age and loss of motor coordination and balance at 12 months of age (47). Examination of the cerebella of these mice revealed a loss in climbing fiber (CF) height from 3 months of

age, PC loss at 12 months of age and pathologically elevated levels of Dyn A peptide (47). Wild-type and *PDYN*^{WT} mice, overexpressing one copy of wild-type human *PDYN*, did not show consistent changes in these parameters (47).

CFs form a crucial component in synaptic plasticity, a process of great importance to the functioning of the cerebellum; it has long been thought to be the molecular mechanism underlying motor functioning and learning (18, 23, 24, 55). CFs and parallel fibers (PFs) are the excitatory inputs of the cerebellum, and synapse upon the singular output of the cerebellar cortex, the PC (14, 25). CFs form a crucial part of the cerebellar machinery, as they exert enormous control over the synaptic plasticity of the PFs (18, 24, 30, 54). In order to maintain the delicate balance of synaptic plasticity, both CFs and PFs have their own PC dendrite territories. CFs populate the proximal PC dendritic tree, whereas the PFs synapse upon the distal PC dendritic tree (14, 25). CFs and PFs compete for PC territory throughout life, and loss of one of these types of fibers leads to an increase in the other (33, 34). Alterations in components of this process lead to synaptic deficits in several mouse models suffering from ataxia and absence seizures (28, 39). The observed CF deficits in SCA23 are particularly interesting, as similar effects have been observed in other SCA types, including SCA1 (12, 13, 17, 40).

A recent publication demonstrated that in SCA1, stem cells hyperproliferate and preferably differentiate into GABAergic interneurons leading to increased inhibitory connections with PCs and non-cell autonomous PC dysfunction (15). As the CF deficits of SCA23 match those of SCA1 and preliminary data suggested early changes in the SCA23 cerebellum, we hypothesized that SCA23 may also follow this newly identified SCA1 pathology. Therefore, we studied *PDYN*^{R212W} cerebella around the time of cerebellar circuit maturation, from 2 to 8 weeks of age. Interestingly, we found that in SCA23, GABAergic innervation shows reduced synapse connectivity. Here, we report the molecular changes observed in the developing *PDYN*^{R212W} cerebellum, and propose an alternative disease model for SCA23.

MATERIALS AND METHODS

Animals

All animal experiments were performed according to the ethical guidelines of the Animal Welfare Committee of the University of Groningen, the Netherlands. The experimental protocols were approved by the Animal Welfare Committee of the University of Groningen. All efforts were made to reduce the number of animals and minimize their suffering. Transgenic mice were bred and genotyped as previously described (47), and housed with same-sex litter mates under standard conditions including environmental enrichment, with ad libitum access to food and water. Twelve animals were assigned to each group based on genotype, aged to 2, 3,

4 or 8 weeks of age and sacrificed humanely. Cerebellar vermes were dissected and snap-frozen in liquid nitrogen. In preparation for immunohistochemical stainings, mice were perfused with 4% PFA, post-fixed for up to 24 hours in PFA, cryopreserved in 20% and 30% of sucrose solutions until saturated, and then frozen on dry ice. Researchers were blind to genotypes during collection of tissue and data collection.

Immunohistochemistry

Sectioning and staining were performed as described previously (47). The primary antibodies used were vesicular glutamate transporter 2 (vGlut2, rabbit, 1:1000, Synaptic Systems, Göttingen, Germany), vesicular GABA transporter (vGAT, rabbit, 1:1000, Synaptic Systems, Göttingen, Germany), glutamate decarboxylase 67 (GAD67, rabbit, 1:1000, Abcam, Cambridge, UK) and Calbindin (mouse, 1:500, Abcam, Cambridge, UK). The secondary anti-rabbit antibody was conjugated with Alexa Fluor 488, the anti-mouse antibody with Cy3 (both: donkey, 1:250, Jackson ImmunoResearch Laboratories, Suffolk, UK). Sections were imaged using an AxioObserver Z1 fluorescence microscope (Carl Zeiss, Oberkochen, Germany), an AxioScan Z1 scanning microscope (Zeiss, Oberkochen, Germany) and the images were analyzed using Fiji software (National Institutes of Health, <http://fiji.sc/>). The presented images were captured using a TCS SP8 confocal microscope (Leica, Wetzlar, Germany) and an AxioObserver Z1 compound microscope equipped with an ApoTome enabling structured illumination microscopy (Carl Zeiss, Oberkochen, Germany).

vGAT and vGlut2 vesicles on PC soma were quantified using a custom-made pipeline. First, PC soma were identified by creating masks using Calbindin stained images. These masks were subsequently used in the vGAT or vGlut2 stained images to count puncta by determining local maxima in each PC soma. Per lobule, 4–7 images were collected and at least five PC soma were analyzed per image. CF height was analyzed by measuring both Calbindin and vGlut2 staining height from the tip of the PC soma, and calculating the ratio. Per lobule 4–7 images were collected and analyzed. GAD67 stainings were performed without Calbindin co-staining. Here, fluorescence intensity was measured in the PC layer. Per lobule, 4–7 images were collected and analyzed.

Reverse transcription PCR and quantitative real-time PCR

Reverse transcription PCR and quantitative real-time PCR were performed as described previously (47). A full list of primers can be found in Table S1.

Protein extraction and Western blotting

Proteins were isolated from snap-frozen mouse vermis. Organs were homogenized in ice-cold RIPA buffer supplemented with a complete protease inhibitor cocktail (Roche, Basel, Switzerland) and PMSF (Sigma-Aldrich, Saint Louis, MO, USA). Samples were centrifuged for 15 minutes at

10 000 rpm, and protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Equal amounts were loaded onto SDS-PAGE gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Life Technologies, Carlsbad, CA, USA) and blocked with 5% non-fat milk in Tris-buffered saline (TBS)-Tween. Blots were probed with primary antibodies against vGlut1 (rabbit, 1:1000, Synaptic Systems, Göttingen, Germany), vGlut2 (rabbit, 1:1000, Synaptic Systems, Göttingen, Germany) and actin (mouse, 1:5000, MP Biomedicals, Irvine, CA, USA). Secondary antibodies were conjugated with horseradish peroxidase (goat, 1:10 000, Jackson ImmunoResearch Laboratories, Suffolk, UK). Densitometric analysis was performed using ImageLab software (BioRad Laboratories, Hercules, CA, USA).

Statistical analysis

All data were normalized against 3-week-old control data and expressed as means \pm SEM. Two-way ANOVA was used to determine the significance of the observed differences between the genotypes and over time ($P < 0.05$ was considered statistically significant).

RESULTS

***PDYN*^{R212W} cerebella show alterations in GABAergic connectivity**

Mice expressing human PDYN containing the p.R212W SCA23 variant (*PDYN*^{R212W} mice) suffer from loss of motor function and balance, coinciding with loss of CF height compared to control mice, as well as to mice expressing wild-type human PDYN (*PDYN*^{WT} mice) (47). This pattern of findings for SCA23 is reminiscent of that seen for SCA1 (12, 13). Therefore, we hypothesized that SCA23 may also mirror other aspects of SCA1 pathology. Edamakanti *et al* found alterations in GABAergic signaling in SCA1 mice (15), leading us to investigate GABAergic signaling in *PDYN*^{R212W} mice. We examined the inhibitory synapses from basket cells (BCs) on PC soma in the vermal lobules at 2, 3, 4 and 8 weeks of age, using the vesicular inhibitory amino acid transporter (vGAT) as a marker for inhibitory synapses and Calbindin as a marker for PCs (Figure 1A). *PDYN*^{R212W} mice showed a reduced number of somatic inhibitory synapses at 2 weeks of age in lobules II, III, IV/V, IX and X as compared to *PDYN*^{WT} and control mice, as well as in lobule I as compared to *PDYN*^{WT} mice (Figure 1B). At 3 weeks of age, *PDYN*^{R212W} lobule IV/V showed fewer synapses as compared to both *PDYN*^{WT} and control, as well as *PDYN*^{R212W} lobules VI and X as compared to control mice (Figure 1B). Lobules II, IV/V and X showed a decreased number of inhibitory synapses in *PDYN*^{R212W} mice as compared to *PDYN*^{WT} and control mice, as well as lobule I as compared to *PDYN*^{WT} mice at 4 weeks of age (Figure 1C). At 8 weeks of age, *PDYN*^{R212W} mice showed fewer somatic synapses in lobules IV/V and VI as compared to both *PDYN*^{WT} and control mice, in lobule II as compared to control mice and in lobules I and IX as

compared to *PDYN*^{WT} mice (Figure 1C). Taken together, these data indicate that SCA23 shows an opposite pathology to SCA1, as inhibitory synapses are lost on *PDYN*^{R212W} PC soma.

CF development is disrupted in *PDYN*^{R212W} mice

As inhibitory connectivity plays a role in the early phase of CF synapse elimination by BC collaterals taking over somatic spines from weak CFs on the PCs, we next investigated CF-PC somatic synapses using vGlut2 as a marker for the CF-PC synapse and Calbindin as a marker for PCs (Figure 2A). Control and *PDYN*^{WT} mice demonstrated normal early phase CF synapse elimination as demonstrated by significant reduction of vGlut2⁺ somatic puncta over time from 2 to 4 weeks of age (Figure 2B,C). *PDYN*^{R212W} mice, however, do not show a significant reduction of these puncta between 2 and 3 weeks of age in lobules II, III, IV/V, VI and IX (Figure 2D). Additionally, in *PDYN*^{R212W} mice, lobule VI does not show significantly fewer vGlut2⁺ synapses from 3 weeks of age on and lobule X does not demonstrate any loss of CF somatic synapses (Figure 2D). While these data suggest that *PDYN*^{R212W} mice do not start out with fewer somatic CF synapses, they point towards a disruption of early phase CF synapse elimination.

As CF development is a finely tuned process, these findings suggested that further CF development could also be affected. Using the same markers, we analyzed the reach of CFs by examining the CF-PC synapses along the PC dendrites (Figure 3A). The PC dendrites of *PDYN*^{R212W} mice showed a significantly reduced CF reach in vermal lobules I, II, III, VI/V, VI and IX at 2 weeks of age in comparison to those of *PDYN*^{WT} and control mice (Figure 3B). The deficit persisted in these lobules and included lobule X at 3, 4 and 8 weeks of age (Figure 3B,C). At 8 weeks of age, significance was lost in lobule IX (Figure 3C). CF reach did not decline between 2 and 8 weeks of age, but does eventually decrease by 12 months of age, as observed previously (47). Altogether, these data indicate disruption of normal CF development in *PDYN*^{R212W} mice, specifically a delay in early phase CF synapse elimination and discontinued CF translocation, leading to a loss of the CF monopoly of the PC proximal dendrites.

***PDYN*^{R212W} mice display increased PF-PC connectivity**

On the distal PC dendritic tree, CFs and PFs are under intense competition for PC dendritic territory, and loss of CF synapses allows for an increase in PF synapses. This process, known as heterosynaptic competition, is employed during development and synaptic plasticity (33, 34). Given the striking loss of CF-PC synapses, we hypothesized that the number of PF-PC synapses may have increased. However, there is currently no suitable antibody available for a detailed histological quantification of vGlut1, the marker for PF-PC synapses. Therefore, we compared the protein levels of vGlut1 in the vermis of *PDYN*^{R212W} mice to that of *PDYN*^{WT} and

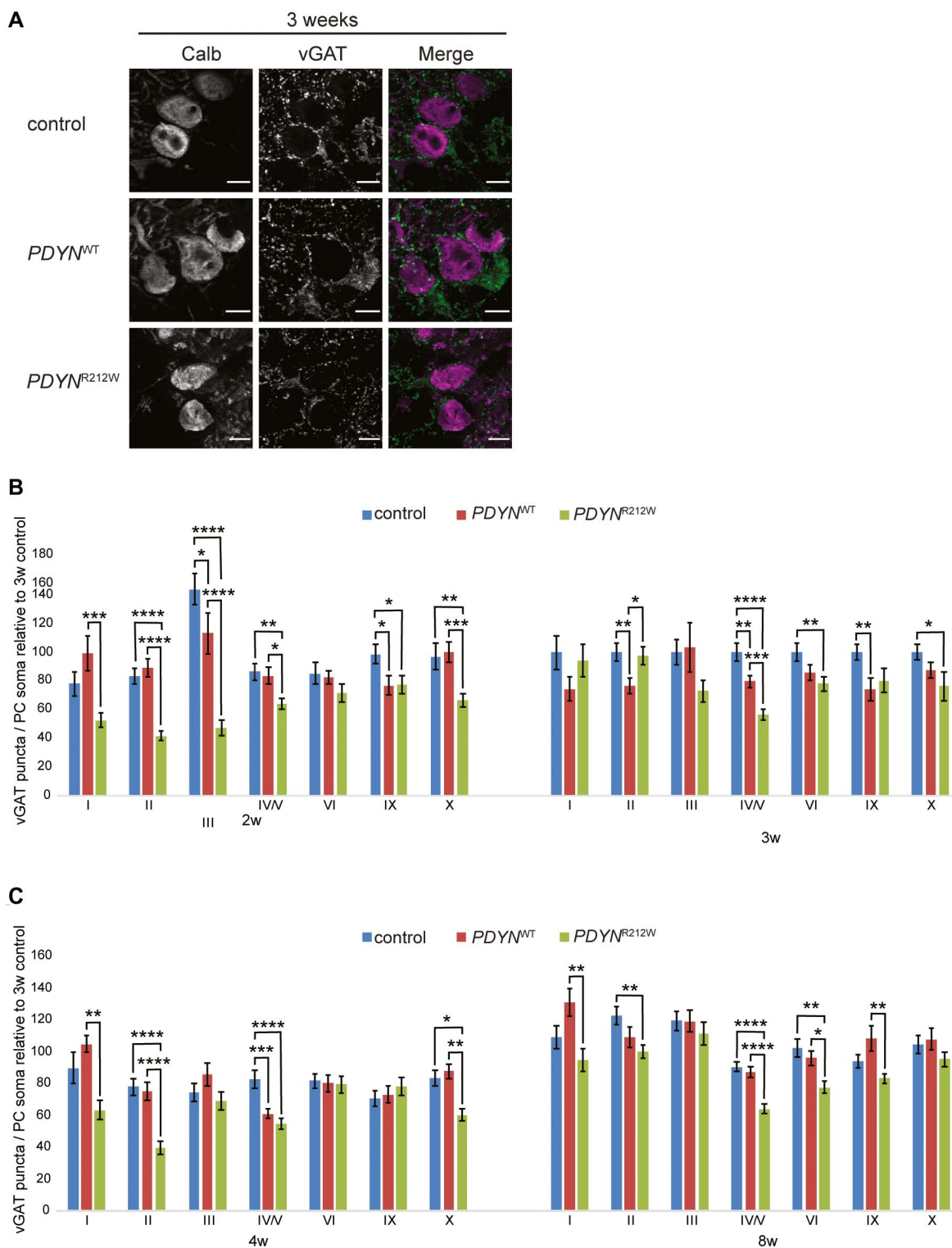


Figure 1. GABAergic innervation of Purkinje cells is reduced in *PDYN*^{R212W} mice. GABAergic presynaptic innervation of Purkinje cell (PC) soma in the vermis was determined by vGAT and Calbindin immunostaining, respectively. (A) Representative fluorescence images of vermal lobule IV/V of control, *PDYN*^{WT} and *PDYN*^{R212W} mice at 3 weeks of age stained with anti-calbindin antibody (magenta) and anti-vGAT (green) antibody. Scale bar = 20 μm. (B-C) Quantification of vGAT

vesicles on PC somas, relative to control mice of 3 weeks of age, in vermal lobules I, II, III, IV/V, VI, IX and X of control, *PDYN*^{WT} and *PDYN*^{R212W} mice of 2, 3, 4 and 8 weeks of age (n = 5 cells per section per lobule [minimum 20 cells] per genotype). *PDYN*^{R212W} mice show reduced amounts of somatic vGAT vesicles early in development. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001. [Colour figure can be viewed at wileyonlinelibrary.com]

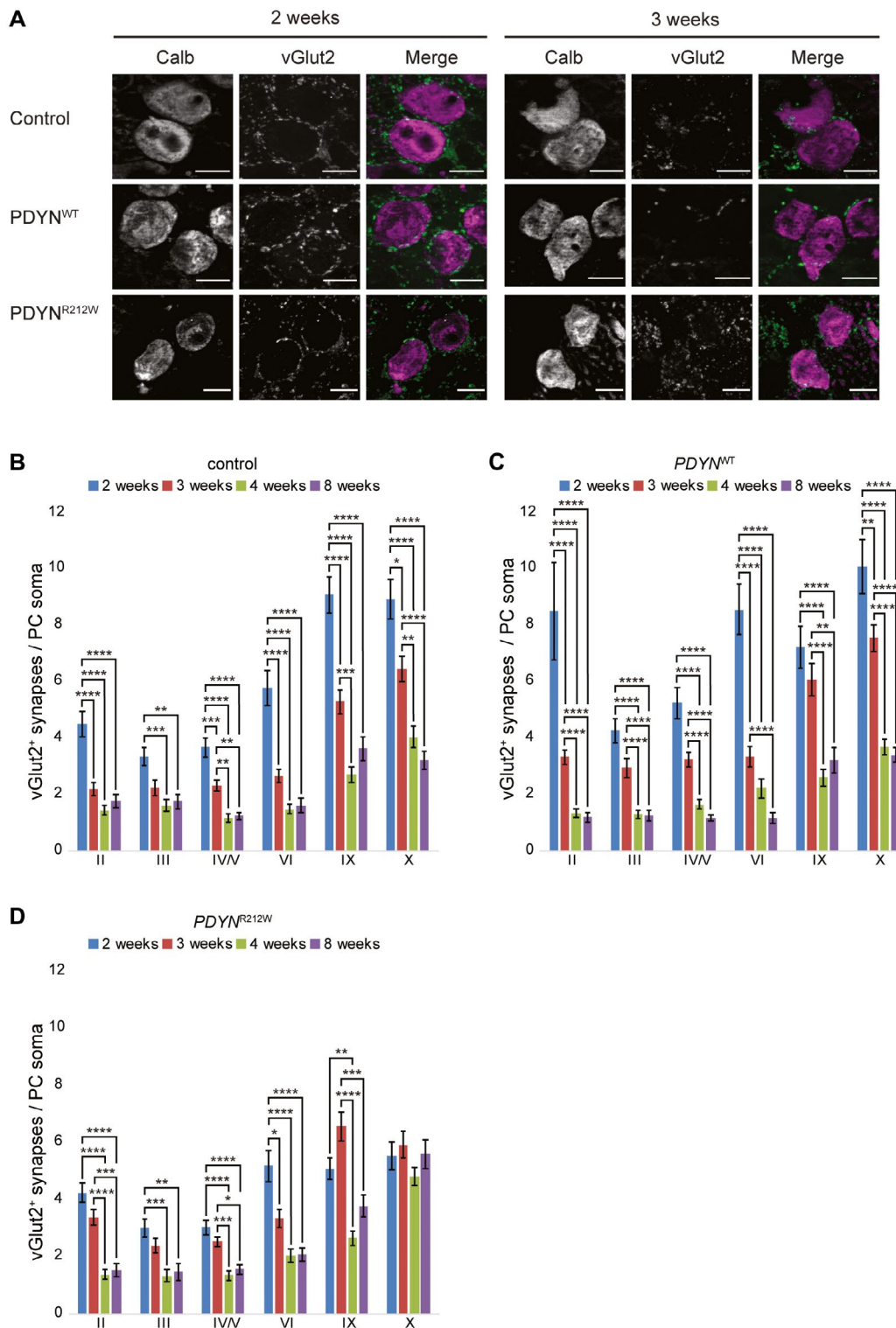


Figure 2. Climbing fiber synapse elimination is impaired in PDYN^{R212W} mice. The presynaptic innervation of Purkinje cells (PC) by climbing fibers (CF) in the vermis during development was assessed by Calbindin and vGlut2 immunostaining, respectively. (A) Representative fluorescence images of vermal lobule X of control, PDYN^{WT} and PDYN^{R212W} mice at 2 and 3 weeks of age stained with anti-Calbindin (magenta) and anti-vGlut2 (green) antibody. Scale bar = 20 μ m (B-D) Quantification of the vGlut2 puncta per PC soma in vermal lobules II, III,

IV/V, VI, IX and X of control (B), PDYN^{WT} (C) and PDYN^{R212W} (D) mice at 2, 3, 4 and 8 weeks of age. Control and PDYN^{WT} mice show a marked reduction of vGlut2 synapses over time in all lobules. In contrast, PDYN^{R212W} mice display a delay in reduction of vGlut2 synapses over time in all lobules except lobule X, which shows no decline of vGlut2 synapses ($n = 5$ cells per section per lobule [minimum 20 cells] per genotype). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. [Colour figure can be viewed at wileyonlinelibrary.com]

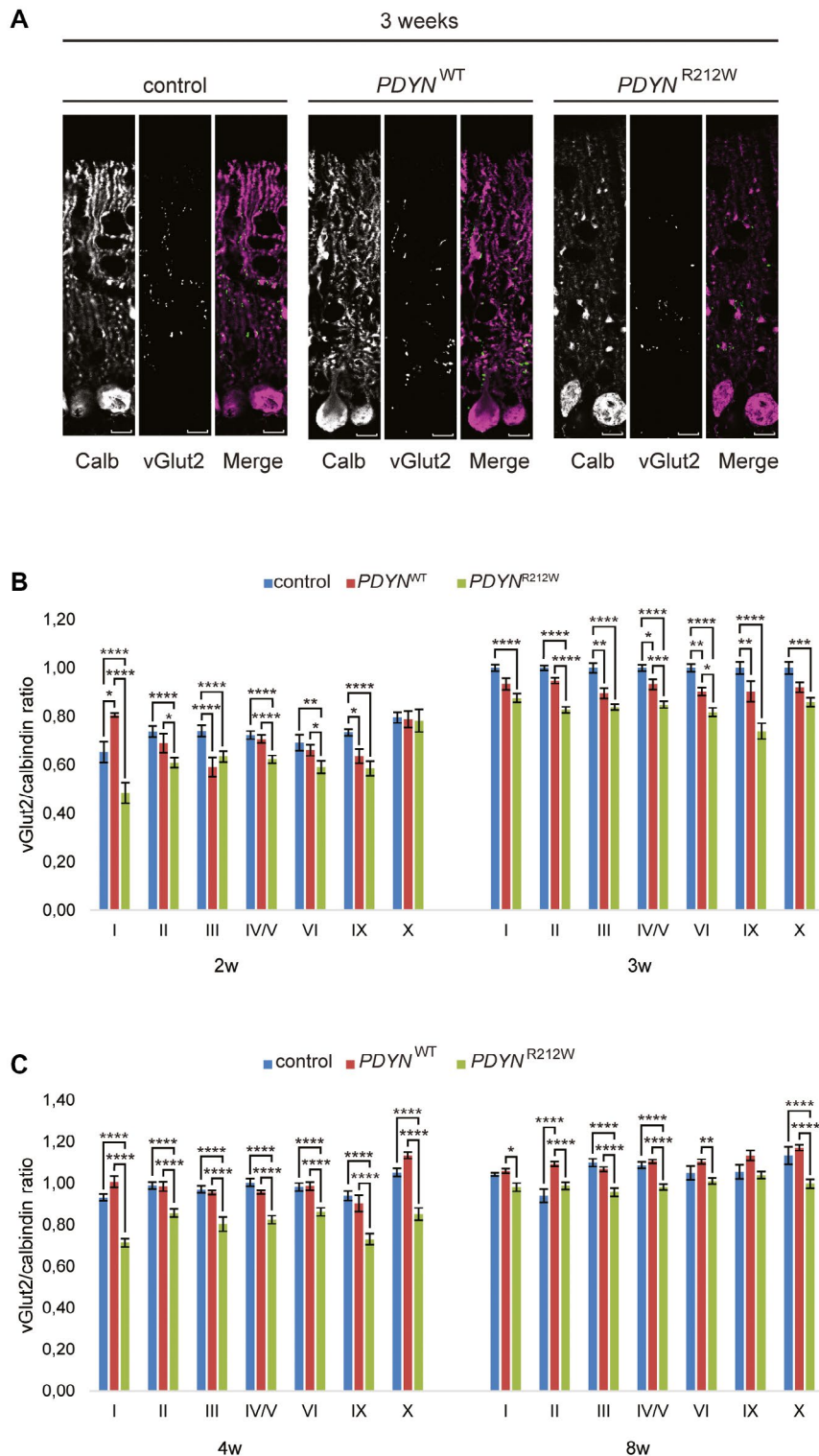


Figure 3. *PDYN*^{R212W} mice show developmental climbing fiber deficits. The distribution of climbing fibers (CF) over the Purkinje cell (PC) dendritic tree in the vermis was assessed by vGlut2 and Calbindin immunostaining, respectively. (A) Representative confocal images of vermal lobule IV/V of control, *PDYN*^{WT} and *PDYN*^{R212W} mice at 3 weeks of age stained with anti-Calbindin (magenta) and anti-vGlut2 (green) antibody. (B–C) Quantification of the relative height of vGlut2 compared to Calbindin staining in vermal lobules I, II, III, IV/V, VI, IX and X of control,

PDYN^{WT} and *PDYN*^{R212W} mice of 2 and 3 weeks of age ($n = 4-7$, per genotype). At 2 weeks of age, *PDYN*^{R212W} mice show significant loss of CF-PC synapses in all lobules except lobule X. At 3 weeks of age, lobule X also displayed reduced CF-PC synapses in *PDYN*^{R212W} mice. All lobules showed reduced CF-PC synapses in *PDYN*^{R212W} mice at 4 weeks of age, while at 8 weeks of age, significance in lobule IX was lost. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. [Colour figure can be viewed at wileyonlinelibrary.com]

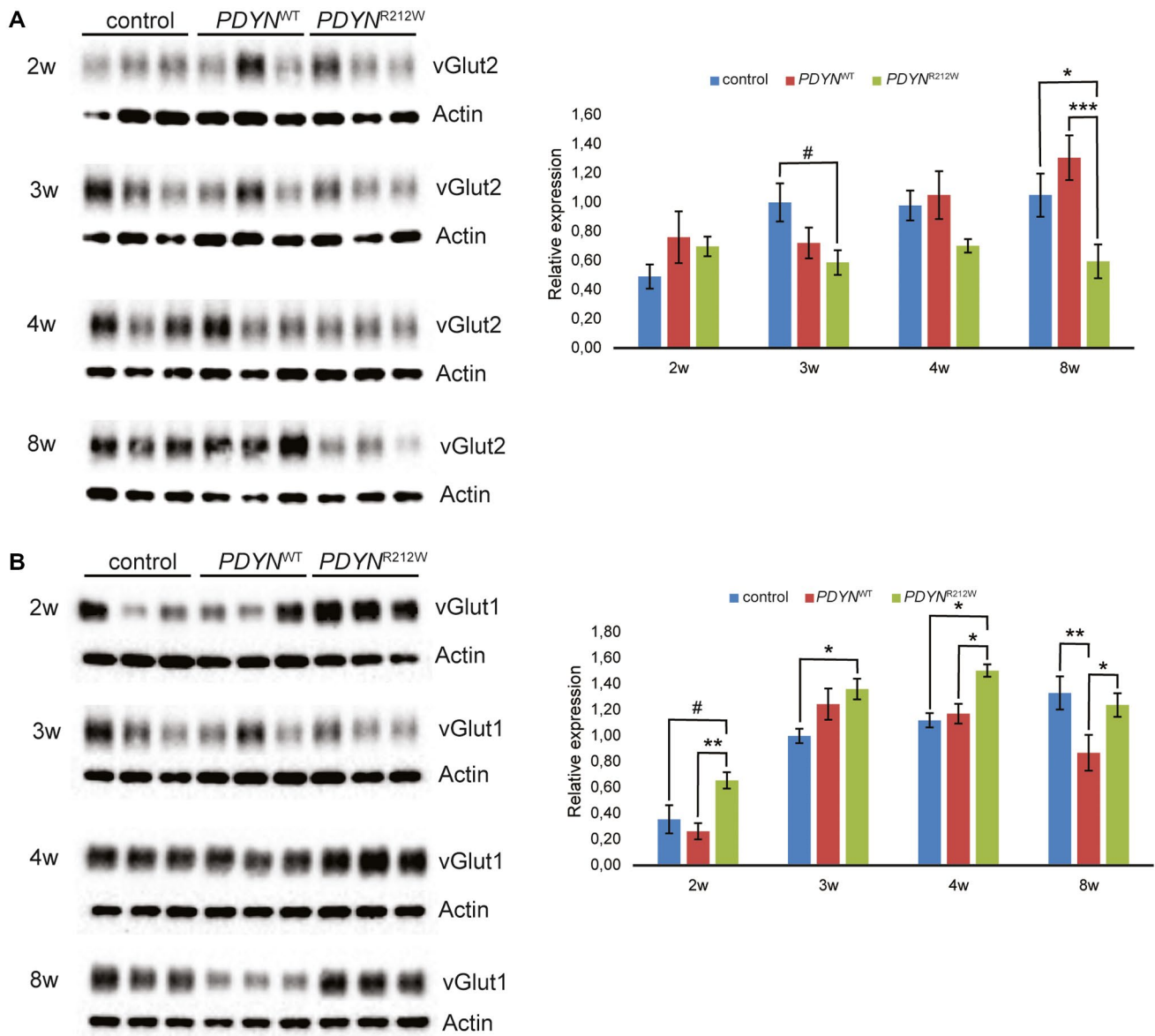


Figure 4. Altered vGlut1 protein levels indicate developmental abnormalities in the vermis of *PDYN*^{R212W} mice. (A,B) The left panel shows representative immunoblots of control, *PDYN*^{WT} and *PDYN*^{R212W} vermal protein lysates, stained for vGlut2 and actin. The right panel shows quantification of these immunoblots using ImageLab software.

control mice. Since vGlut1 and vGlut2 are highly expressed in the cerebellar granule layer, we first ascertained whether we could see a change in vGlut2 protein levels representing the loss of CF-PC synapses by determining vGlut2 protein levels in whole vermis protein lysate. At 8 weeks of age, we observed the expected reduction of overall vGlut2 levels in *PDYN*^{R212W} cerebella (Figure 4A). Although this reduction was only significant at 8 weeks of age, we were able to observe changes in overall vGlut2 protein level, indicating that changes in vGlut1 protein levels point to changes in PF-PC synapse count. The vGlut1 protein levels in *PDYN*^{R212W} vermis were significantly increased at 2, 3 and

vGlut1 protein levels were increased in *PDYN*^{R212W} at 2, 3 and 4 weeks compared with control mice and at 2, 4 and 8 weeks of age compared with *PDYN*^{WT} mice ($n = 3$ per genotype). # $0.10 > p > 0.05$, * $P < 0.05$, ** $P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com]

4 weeks of age compared to control vermis, and at 8 weeks of age compared to *PDYN*^{WT} vermis (Figure 4B). The elevated levels of vGlut1 suggest that PFs have increased their synapse numbers in the vermis of *PDYN*^{R212W} mice, as CFs cannot reach terminal height to populate their natural PC dendritic territory.

Reduced GAD67 expression could indicate internal changes in *PDYN*^{R212W} PCs

As both GABAergic and glutamatergic inputs are altered in *PDYN*^{R212W} mice, we hypothesized that perhaps PCs

adapted internal changes to counteract these alterations. Therefore, we determined the expression of glutamate decarboxylase 67 (GAD67), the main enzyme used by PCs to convert glutamate into GABA. Using GAD67 immunostaining, we determined expression levels by measuring fluorescence intensity in the PC layer of control, *PDYN*^{WT} and *PDYN*^{R212W} mice (Figure 5A). We observed a loss of GAD67 in *PDYN*^{R212W} PCs at 2, 3 and 4 weeks of age as compared to control mice and at 4 weeks as compared to *PDYN*^{WT} mice (Figure 5B). These data suggest that *PDYN*^{R212W} PCs produce less GABA, which could be a reaction to the lost input from basket cells and/or CFs, possibly to normalize their impact on cerebellar nuclei neurons.

Changes in NMDA receptor subunits suggest altered Ca²⁺ signaling

Loss of vGlut2 has been shown to impair glutamatergic transmission (20), and we have previously demonstrated changes in NMDA receptor *Grin2a* subunit expression in *PDYN*^{R212W} mice at 3 months of age (47). Therefore, we determined the mRNA expression levels of the *Grin2* NMDA receptor subunits in the vermis of 2-, 3-, 4- and 8-week-old *PDYN*^{WT}, *PDYN*^{R212W} and control mice. The expression levels of these subunits were relatively low and no significant alterations were detected in *PDYN*^{R212W} mice (Figure S1A). We also determined the mRNA expression levels of the remaining NMDA receptor subunits *Grin1*, *Grin3a* and *-b*. No alterations in *Grin1*, *Grin3a* and *-b* subunit expression were observed in 2-, 3- and 8-week-old *PDYN*^{R212W} mice (Figure 6A). However, the expression of *Grin1* was significantly increased at 4 weeks of age in *PDYN*^{R212W} mice as compared to control and *PDYN*^{WT} mice (Figure 6A), while the inhibitory subunits *Grin3a* and *-b* displayed significantly

decreased expression (Figure 6A). Since *Grin1* is the essential subunit for surface expression of NMDA receptors (31), it is possible that at 4 weeks of age, *PDYN*^{R212W} mice have increased surface expression of NMDA receptors. Taken together, in 4-week-old *PDYN*^{R212W} mice, when NMDA receptors are expressed at PF- and CF-PC synapses in wild-type mice (56), the expression of crucial NMDA receptor subunits is altered, potentially causing altered Ca²⁺ signaling.

VGCC dysregulation may be evidence of a compensatory mechanism for *PDYN*-R212W expression

As *PDYN*^{R212W} mice display alterations in the development of crucial PC inputs and NMDA receptor subunit expression is affected, we hypothesized that the expression of voltage-gated Ca²⁺ channels (VGCCs) may also be affected. *Cav2.1*, a VGCC encoded by *Cacna1a*, the SCA6 disease gene (60), is crucial for proper CF maturation and regulates the expression of several genes involved in PC development (11, 26, 33, 38). Based on these prior findings, dysregulation of *Cacna1a* could potentially underlie the observed CF and PC deficits (28, 39), and ultimately ataxia (3, 7, 16, 28, 29). To investigate whether *PDYN*^{R212W} mice exhibit altered Ca²⁺ signaling via VGCCs, we assessed the mRNA expression levels of the cerebellar VGCC subunits *Cacna1a* and *-c*, *Cacna2d2* and *-3*, *Cacnb2* and *-4* and *Cacng2* and *-7* in the vermis of *PDYN*^{WT}, *PDYN*^{R212W} and control mice. The mRNA level of *Cacna1a* was significantly increased at 3, 4 and 8 weeks of age in *PDYN*^{R212W} mice (Figure 6B). A similar effect was observed for *Cacna1c*, its expression was increased at 2, 4 and 8 weeks of age in *PDYN*^{R212W} mice (Figure 6B). At 8 weeks of age, the expression of

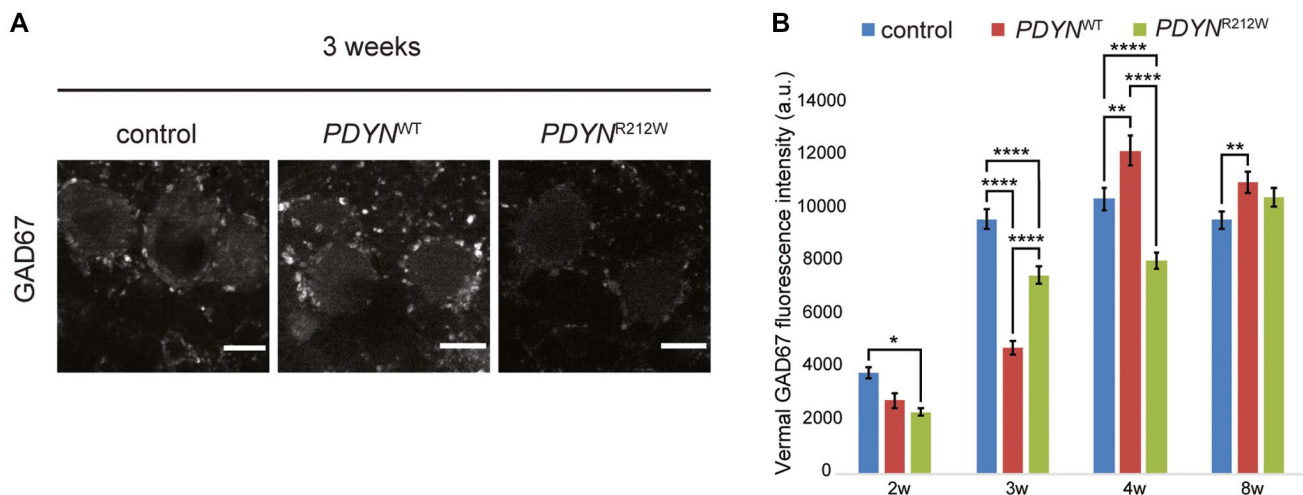


Figure 5. *PDYN*^{R212W} mice show reduced GABAergic transmission. The GABAergic inhibitory transmission by interneurons in the vermis was assessed with GAD67 immunostaining. (A) Representative fluorescence images of vermal lobule IV/V of control, *PDYN*^{WT} and *PDYN*^{R212W} mice at 3 weeks of age stained with anti-GAD67 antibody. Scale bar = 20 μ m (B) Quantification of the relative fluorescence

intensity of GAD67 in the total vermis of control, *PDYN*^{WT} and *PDYN*^{R212W} mice of 2, 3, 4 and 8 weeks of age (n = 20–30 images per genotype, minimum 24 measurements). *PDYN*^{R212W} vermis shows reduced GAD67 fluorescence intensity as compared to control mice at 2, 3 and 4 weeks of age. **P* < 0.05, ***P* < 0.01 and *****P* < 0.0001. [Colour figure can be viewed at wileyonlinelibrary.com]

both *Cacna2d2* and -3 was upregulated in *PDYN*^{R212W} vermis (Figure 6B), which was also observed for *Cacnb2* and -4 (Figure 6C). Additionally, *Cacnb2* expression was also increased at 2 weeks of age, and *Cacnb4* at 4 weeks of age (Figure 6C). As these last four subunits are auxiliary

subunits, regulating the function of *Cacna1a* and *Cacna1c*, the observed increases could be a response to the increased *Cacna1a* and *Cacna1c* mRNA levels. *Cacng2* and *Cacng7* mRNA expression was increased at 8 and 4 weeks of age, respectively (Figure 6C). Since *Cacng2* and *Cacng7* primarily

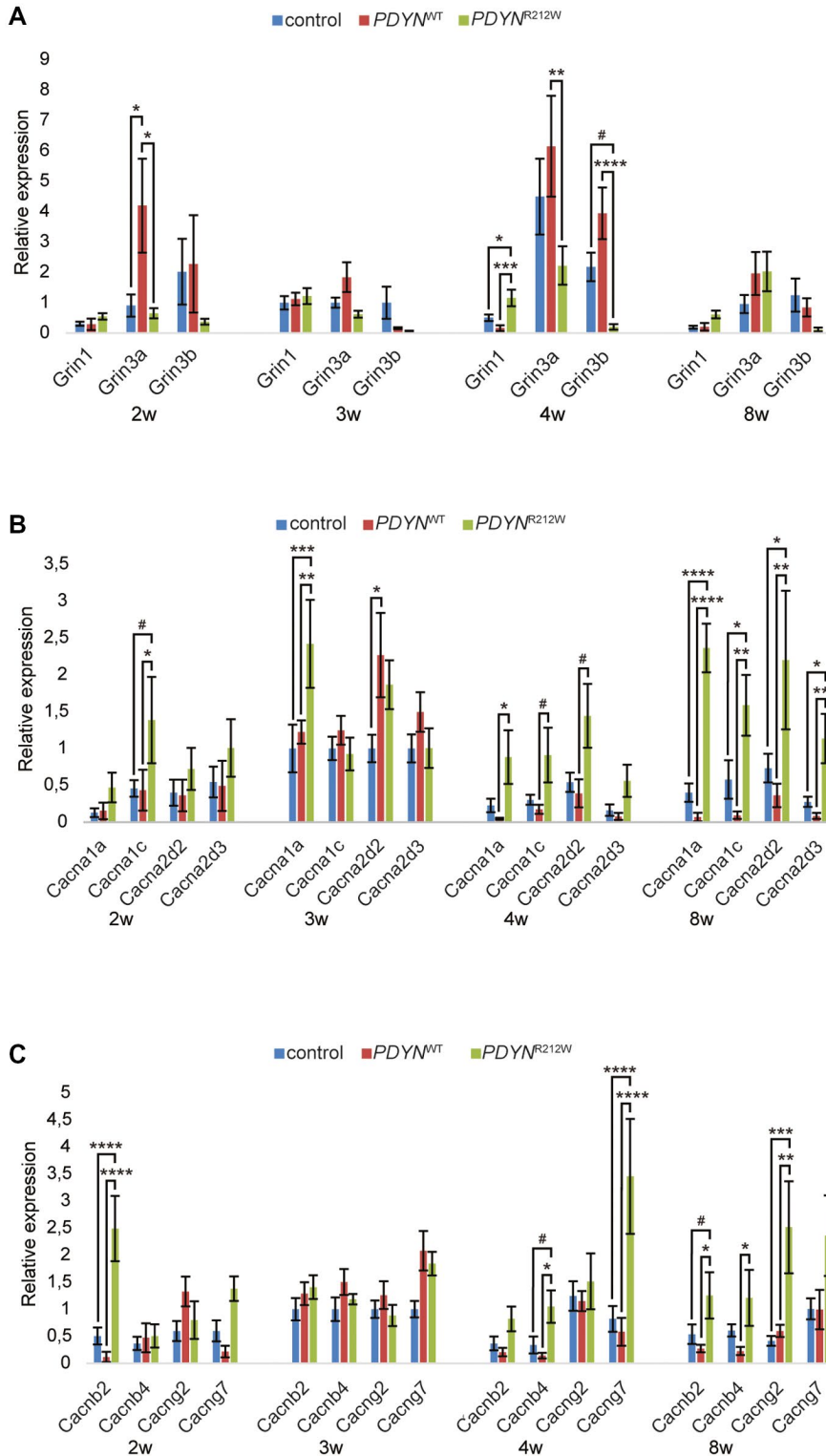


Figure 6. Altered expression of Ca^{2+} channel subunits suggests a compensatory mechanism in $PDYN^{R212W}$ mice. Quantification of RT-qPCR data from 2, 3, 4 and 8-week-old cerebella from control, $PDYN^{MT}$ and $PDYN^{R212W}$ mice ($n = 6$, per genotype). (A) At 4 weeks of age, *Grin1* showed elevated expression in $PDYN^{R212W}$ as compared to $PDYN^{MT}$ and control mice. Simultaneously, *Grin3a* and *-b* expression was significantly reduced in these mice as compared to control and $PDYN^{MT}$ mice, respectively. For *Grin3a*, expression was increased in $PDYN^{MT}$ compared with control and $PDYN^{R212W}$ mice, but this was likely due to expression of $PDYN$ -WT. (B) *Cacna1a* expression was elevated at 3, 4 and 8 weeks of age

in $PDYN^{R212W}$ as compared to control and $PDYN^{MT}$ mice, while *Cacna1c* expression was increased at 2, 4 and 8 weeks of age. In 3, 4 and 8-week-old $PDYN^{R212W}$ mice, *Cacna2d2* was elevated, and at 8 weeks of age, *Cacna2a3* expression was elevated in $PDYN^{R212W}$ mice as well compared with control and $PDYN^{MT}$ mice. (C) *Cacnb2* expression was increased at 2 and 8 weeks of age in $PDYN^{R212W}$ mice compared to both control and $PDYN^{MT}$ mice, while *Cacnb4* expression was enhanced at 4 weeks of age. For *Cacng2* and *Cacng7*, expression was elevated at 8 and 4 weeks of age, respectively. # 0.10 > $p > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. [Colour figure can be viewed at wileyonlinelibrary.com]

regulate trafficking, localization and biophysical properties of AMPA receptors (6,58), we also studied the mRNA levels of *Grial-4*. However, we found no correlation with the expression levels of *Cacng2* and *Cacng7* (Figure S1B). These data demonstrate that Cav2.1, a key player in CF maturation, is markedly upregulated in $PDYN^{R212W}$ vermis around the time of CF maturation. We therefore suggest that dysregulated expression of crucial VGCCs and their auxiliary subunits contributes to the CF maturation deficits and loss of CF-PC connectivity in $PDYN^{R212W}$ cerebella.

DISCUSSION

Our data are the first to demonstrate a neurodevelopmental role for PDYN, as we observed cerebellar developmental deficits in $PDYN^{R212W}$ mice that include loss of GABAergic connectivity, disrupted CF development, increased PF-PC connectivity and dysregulation of key VGCC subunits that are involved in CF maturation between 2 and 8 weeks of age. Moreover, the loss of CF-PC synapses persisted up to 12 months of age, and likely contributes to PC degeneration (47). The alterations in the maturation and number of CF-PC synapses, the number of BC-PC synapses and the expression of vGlut2 and vGlut1 support our hypothesis that developmental deficits in synaptic wiring contribute to motor dysfunction and ataxia. While one study found development of the brain was not affected in Pdyn knockdown mice, the cerebellum was not studied in detail (32). Our evidence leads us to propose that PDYN has different functions in the cerebrum versus the cerebellum, and that, in the cerebellum, it plays key roles in development.

BCs innervate the PC soma and form the pinceau on the PC initial axonal segment, inhibiting PC firing. A recent publication demonstrated that the loss of stellate and basket cell GABAergic transmission does not affect PC dendritic tree development and maintenance (5). However, Brown *et al* also demonstrated that stellate and basket cells cooperate to establish the correct rate and pattern of simple and complex spike firing of PCs *in vivo*, and that loss of BC inhibition increased PC simple spike firing, while it decreased the PC complex firing rate (5). The loss of vGAT⁺ somatic synapses in $PDYN^{R212W}$ vermis suggests that SCA23 PCs suffer from reduced inhibition leading to changes in simple and complex spike firing rates as well as reduced synchronous firing of PC zones, altering cerebellar output and leading to motor

dysfunction and ataxia (21, 27, 41). The observed loss of GAD67 likely contributes to this altered cerebellar output. Notably, reduced levels of GAD67 mRNA in PCs have been observed in the cerebella of people with autism (59). Additionally, BC-PC synapse formation is critical for early phase CF synapse elimination, as BC collaterals take over PC somatic spines from CFs (26). Therefore, the absence of BC-PC synapses may underlie the observed delay in early CF synapse elimination. Nakayama *et al.* have shown that diazepam, a GABA_A receptor sensitizer, can restore impaired CF maturation due to altered GABAergic transmission (37). Hence, we put forward the malformation of GABAergic innervation as a possible therapeutic target for SCA23.

Loss of vGlut2 in the vermis of $PDYN^{R212W}$ mice supports a developmental deficit in the cerebellum, in line with findings showing developmental roles for vGlut2 in the hippocampus (20). A vGlut2 conditional knockout mouse displayed increased open-field exploratory behavior and impaired spatial learning and memory, a phenotype similar to that of NMDA receptor knockdown mice (20, 42). Deficiency of vGlut2 led to reductions of evoked glutamate transmission, neurotransmitter release probability and long-term depression at hippocampal CA3-CA1 synapses during postnatal development. This led to a loss of arborization of the dendritic tree and reductions in the number of dendritic spines in adult mice, suggesting widespread alterations in synaptic connectivity (20). We hypothesize that vGlut2 serves a similar purpose in the cerebellum, with deficiency leading to reduced glutamatergic transmission and, consequently, disturbed Ca^{2+} signaling and malformation of PC dendrites. This hypothesis is strengthened by our previous observation that cultured neurons of $PDYN^{R212W}$ cerebella show reduced neuronal excitability (47), which could be caused by the loss of vGlut2 expression.

$PDYN^{R212W}$ mice displayed elevated vGlut1 levels at 2, 3 and 4 weeks of age, which suggests that PFs in these animals extended their innervation territory on PCs to include more proximal dendrites. The timing of increased vGlut1 expression coincides with late phase CF synapse elimination, a process critically dependent on normal PF-PC synapse formation (26), suggesting that aberrant PF-PC synapse formation supports the malformation of CFs. A cause of aberrant PF-PC synapse formation could be the increased expression levels of *Cacna1a*, which encodes Cav2.1, at 3, 4 and 8 weeks of age. Increased expression of this subunit

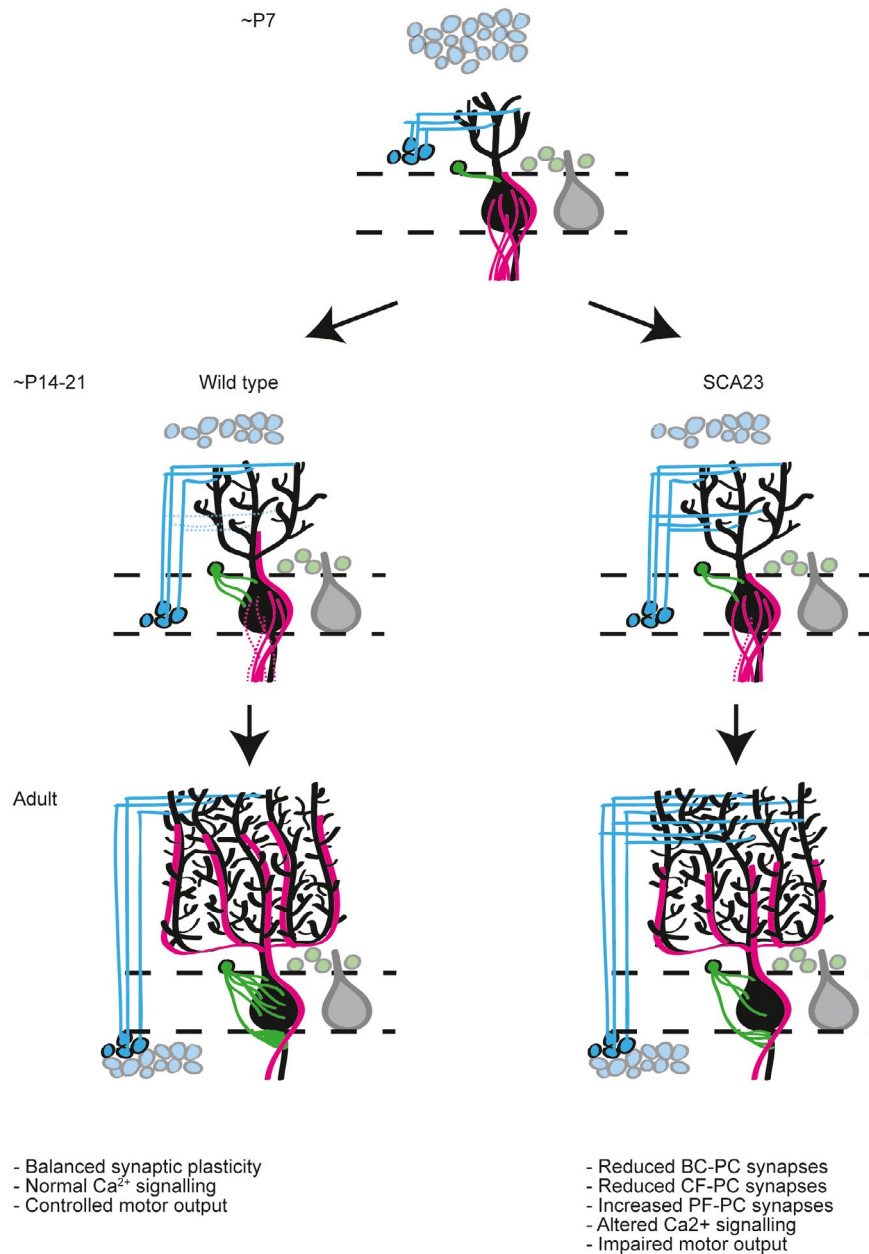


Figure 7. Cerebellar developmental abnormalities underlie SCA23. Under normal conditions, cerebellar development progresses in highly regulated, clear phases (left panels). In SCA23 cerebella, we have observed several disruptions of cerebellar development. First, basket cells (in green) form fewer somatic synapses in SCA23 cerebella. Second, climbing fibers (CFs, in pink) display a delay in CF synapse elimination, as well as a failure to reach terminal height to innervate the

proximal Purkinje cell (PC) dendrites. These deficits likely lead to the third disruption, an increase in parallel fiber-PC synapses (in blue). These developmental abnormalities, combined with our other findings, very likely lead to altered Ca²⁺ signaling and, consequently, impaired motor output and ataxia. [Colour figure can be viewed at wileyonlinelibrary.com]

may affect heterosynaptic competition between PFs and CFs and distal extension of CFs (33). However, as *Cacna1a* is elevated from 3 weeks of age, the increase in vGlut1 at 2 weeks of age could be caused by the loss of vGlut2 at that time. *Cacna1a* dysregulation may also disrupt CF maturation, distal CF extension and Ca²⁺ signaling, as Cav2.1

plays crucial roles in these processes (8, 33, 56). Moreover, the C-terminal tail of the channel functions as a transcription factor, coordinating the expression of genes involved in PC development (11). PC loss has not been observed until 12 months of age (47). Therefore, altered cerebellar development may lead to PC dysfunction before 12 months

of age. Increased expression of *Cacnalc*, encoding Cav1.2, very likely contributes to the SCA23 pathology, as it plays significant roles in neuronal activity and survival, dendritic development, synaptic plasticity, memory formation and learning (4, 35, 36, 50, 57). Additionally, an intronic variant in *CACNA1C* was recently proposed to be disease-causing in a Chinese family with autosomal dominant cerebellar ataxia (10), suggesting a role for *Cacnalc* in the pathology of ataxias. Furthermore, as a loss of vGlut2 could lead to reduced glutamatergic transmission, and consequently, disturbed Ca²⁺ signaling, the increases in expression of these VGCCs and their auxiliary subunits could be a compensatory mechanism for these changes. Altered expression of other Ca²⁺ channel subunits including *Cacng2*, *Cacng7*, *Grin1*, *Grin3a* and *Grin3b* further hint towards dysregulation of Ca²⁺ signaling, however, due to the absence of consistent changes, we cannot draw strong conclusions from these data.

It has long been established that the loss of CF input causes PCs to malfunction, as CFs play a dual role in cerebellar functioning; 1) control of synaptic plasticity at dendritic PC synapses, and 2) generating the distinct complex spike output in the PC axon, and motor learning and performance crucially depend on them (48). Additionally, CF deficits have been observed in other SCA types as well, including SCA1 (12, 13, 17, 45). The arrested development of CFs in *PDYN*^{R212W} mice, therefore, likely underlies the ataxic phenotype (47). Interestingly, while the SCA23 pathology may mirror that of SCA1 in adulthood, the underlying developmental deficits oppose each other. In SCA1, Edamakanti *et al.* found hyperproliferation of stem cells that preferentially differentiated into GABAergic interneurons, leading to increased GABAergic interneuron connectivity and non-cell autonomous disruption of PC function (15). As discussed above, there could be several explanations as to why CFs develop abnormally in SCA23. We believe that the most likely culprit is the loss of GABAergic connectivity that should be provided to PCs by the BCs, as this is the earliest disruption in normal cerebellar development that we have detected (Figure 7). How expression of *PDYN*-R212W leads to dysfunction of BCs should be studied further.

In conclusion, the early loss of BC-PC and, consequently, CF-PC synapses plays a crucial role in the neuropathology of SCA23. We previously hypothesized that an increase in intracellular Ca²⁺ underlies the SCA23 pathology (47). With the evidence presented here, it now appears more likely that the disturbance in Ca²⁺ signaling lies on the other end of the scale, with decreased intracellular Ca²⁺ disrupting normal cellular functioning. This fits the SCA23 pathology more closely, as increased intracellular Ca²⁺ would lead to PC loss more quickly than previously observed (47). We demonstrate a developmental role for *PDYN* in the cerebellum and show that developmental abnormalities in neuronal wiring and disturbance in the PC simple/complex spike balance underlie SCA23. In addition, we propose that diazepam should be explored as a potential therapy, as it could sensitize remaining GABA_A receptors and thereby alleviate SCA23 symptoms.

ACKNOWLEDGEMENTS

We would like to thank Kate Mc Intyre for editing this manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

FUNDING INFORMATION

This work was supported by a Rosalind Franklin Fellowship from the University of Groningen, the Jan Kornelis de Cock-Stichting, the U4 PhD program of the Behavioral and Cognitive Neuroscience graduate school of the University of Groningen and the Max Planck Society. Part of this work was performed at the University Medical Centre Groningen Microscopy and Imaging Centre, which is sponsored by the Netherlands Organization for Scientific Research (NWO grants 40-00506-98-9021 and 175-010-2009-023). None of the funding bodies were involved in the collection, analysis and interpretation of data, nor in the writing of the manuscript.

DATA AVAILABILITY STATEMENT

The data from this study will be made available upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table S1. qPCR primers.
Supplementary Material