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ORIGINAL PAPER

Zebrin II Is Ectopically Expressed in Microglia in the Cerebellum of *Neurogenin 2* Null Mice

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Abstract

Zebrin II/aldolase C expression in the normal cerebellum is restricted to a Purkinje cell subset and is the canonical marker for stripes and zones. This spatial restriction has been confirmed in over 30 species of mammals, birds, fish, etc. In a transgenic mouse model in which the *Neurogenin 2* gene has been disrupted (*Neurog2*$^{-/-}$), the cerebellum is smaller than normal and Purkinje cell dendrites are disordered, but the basic zone and stripe architecture is preserved. Here, we show that in the *Neurog2*$^{-/-}$ mouse, in addition to the normal Purkinje cell expression, zebrin II is also expressed in a population of cells with a morphology characteristic of microglia. This identity was confirmed by double immunohistochemistry for zebrin II and the microglial marker, Iba1. The expression of zebrin II in cerebellar microglia is not restricted by zone or stripe or lamina. A second zone and stripe marker, PLCβ4, does not show the same ectopic expression. When microglia are compared in control vs. *Neurog2*$^{-/-}$ mice, no difference is seen in apparent number or distribution, suggesting that the ectopic zebrin II immunoreactivity in *Neurog2*$^{-/-}$ cerebellum reflects an ectopic expression rather than the invasion of a new population of microglia from the periphery. This ectopic expression of zebrin II in microglia is unique as it is not seen in numerous other models of cerebellar disruption, such as in *Acp2*$^{-/-}$ mice and in human pontocerebellar hypoplasia. The upregulation of zebrin II in microglia is thus specific to the disruption of *Neurog2* downstream pathways, rather than a generic response to a cerebellar disruption.

Keywords Cerebellum · Microglia · *Neurogenin 2* mutant · Zebrin II · *Acp2* mutant

Introduction

Zebrin II/aldolase C expression in the normal cerebellum is restricted to a Purkinje cell subset and is the canonical marker for stripes, which are arrays of parasagittal bands that extend rostrocaudally throughout the cerebellar cortex [1, 2]. This distribution of zebrin II has been confirmed in over 30 species of mammals, birds, fish, bats, etc. [1, 3–7]. No other cell types in the cerebellum express zebrin II except in the Macaca cerebellum, in which zebrin II is expressed by both Purkinje cells and astrocytes [8, 9]. Lower levels of zebrin II/aldolase C mRNA have also been reported in subpopulations of neurons of the cerebral cortex, striatum, hippocampus, hypothalamic nuclei, primary olfactory cortex, and retina [10, 11].

Neurogenin 2 (*Neurog2*) encodes a basic-helix-loop-helix transcription factor that is required to specify neural identities throughout the neural tube [12–14]. As an example, in the embryonic dorsal telencephalon of *Neurog2*$^{-/-}$ mice, neurons are misspecified, acquiring a GABAergic rather than glutamatergic identity due to the upregulated expression of *Ascl1*, encoding a bHLH transcription factor that specifies distinct neural cell identities [12–14]. In the cerebellar primordium, *Neurog2* is expressed in ventricular zone progenitors in territories that overlap with the expression of Ptf1a, and in prospective cerebellar nuclei, but not in the Math1/Atoh1-positive rhombic lip [15–17]. In a transgenic mouse model in which *Neurog2* has been disrupted (*Neurog2*$^{-/-}$), the cerebellum is smaller than normal and Purkinje cell dendrites are arranged in a disorderly fashion, but the basic zone and stripe architecture is preserved [18]. There is also a reduction in the total number of Purkinje cells in the *Neurog2*$^{-/-}$ cerebellum [18].

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Microglia are the immune cells of the central nervous system, and they are distributed in a non-overlapping territorial fashion and are highly dynamic in both normal and pathological conditions [19, 20]. Microglia play important roles in pathological conditions in the CNS, such as infection, traumatic injury, and neurodegenerative disease [21–23]. There is growing evidence to suggest that microglia are important for the development of neurons and their synaptic connections [24]. In the developing cerebellum, one study demonstrated that microglia promote the death of Purkinje cell neurons [25]. In contrast, in reciprocal neuron–microglial interactions, microglia were able to protect cerebellar granule neurons from apoptosis [26].

In the present study, we found that in the *Neurog2*$^{-/-}$ mouse cerebellum, zebrin II is ectopically expressed in microglia, in addition to its normal expression domain in Purkinje cells. This is a striking observation since the expression of zebrin II in microglia has not been reported in numerous other models of cerebellar disruption: Purkinje cell ectopias, cell death defects, and deafferentation, and shown here, in *acid phosphatase 2 lysosomal* mutants (*Acp2*$^{-/-}$), and human pontocerebellar hypoplasia (PCH). We thus suggest that the ectopic expression of zebrin II in *Neurog2*$^{-/-}$ microglia is a result of the specific disruption of *Neurog2* downstream transcriptional cascades, rather than a generic response to a cerebellar disruption.

Materials and Methods

Animal Maintenance and Tissue Processing

All animal procedures conformed to institutional regulations and the *Guide to the Care and Use of Experimental Animals from the Canadian Council for Animal Care* (CCAC), University of Manitoba Animal Care Committee (ACC), and University of Calgary Animal Care Committee (ACC). All efforts were made to minimize the number of animals used, and the animals were treated in a humane manner. The day of the vaginal plug was considered as embryonic day 0.5 (E0.5), and postnatal day 0 (P0) was deemed as the date of birth. To generate *Neurog2*$^{-/-}$ mice, we set up *Neurog2*$^{+/-}$ heterozygous intercrosses. Animals were collected at postnatal day (P) 15 and *Neurog2*$^{-/-}$ mice were readily apparent as they were runts ($N = 6$). Genotyping was performed according to the methods described by Mattar et al. (2004) [27–30]. *Acp2*$^{-/-}$ mutant mouse embryos were provided by the Institute of Human Genetics in the University Medical Center, Georg-August University, Goettingen, Germany. An *Acp2*$^{-/-}$ colony was established in the Genetic Modeling Center at the Faculty of Health Sciences, University of Manitoba, by breeding mice (C57BL/6) heterozygous for the *Acp2*$^{-/-}$ mutation [31, 32]. Genotyping was performed according to the methods described by Bailey et al. 2014 [31]. Wild-type littermates were used as controls in all studies. Animals were maintained at room temperature and in controlled humidity (50–60%) with a 12-h light-dark cycle. *Acp2*$^{-/-}$ mice cerebella were collected at postnatal day (P) 15 ($N = 4$). The animals were perfused and post-fixed with 4% paraformaldehyde and then cryosectioned at 20 μm and processed for immunohistochemistry according to methods described in Bailey et al. [31].

PCH and matched normal control sections were the generous gift of Dr. Mark Del Bigio, Department of Pathology, University of Manitoba ($N = 1$). Consent was obtained from the parents after death for full autopsy. Controls were acquired under University of Manitoba Health Research Ethics Board protocol H2013:217. Samples were fixed in 10% formalin for 10–14 days; tissue samples were dehydrated in graded alcohols and embedded in paraffin. The sections were from brains (2 months old) and the matched control subject. The anterior and posterior vermis of the cerebella were dehydrated and paraffin embedded. Sections of anterior ($N = 5$) and posterior ($N = 5$) cerebellar vermis were cut at 5-μm thickness in the transverse plane used in this study.

Immunohistochemistry

Immunohistochemistry was performed on cerebellar sections, as previously described [31, 33]. We first washed the sections with PBS and continued with 0.3% H_2O_2 to stop endogenous peroxidase activity. The antigen retrieval technique was used for anti-PLCβ4, with sections taken through a series of graded methanol baths prior to the blocking of peroxidase activity. After washing the sections with PBS again, 10% normal goat serum was used to block, and the sections were then incubated with primary antibody overnight at room temperature. After washing with PBS, we incubated the samples with secondary antibody for 2 h. Diaminobenzidine (DAB) and ABC Peroxidase Standard Staining Kit were used as the chromogen to show the reaction signal.

A direct anti-IgG (secondary anti-sera) and high concentrations of Triton X-100 (1%) were applied as a method for labeling myelin, microglial cells, or blood vessels without the use of any specific primary anti-serum [34]. Biotinylated anti-IgGs (α-IgGs; secondary antibodies or anti-sera) were detected with the avidin-biotin-immunoperoxidase (ABC) method and with avidin-bound fluorochromes. To reveal secondary anti-serum binding, the ABC method was used. Biotinylated secondary anti-sera diluted 1:500 in PBS were applied for 2 h. The Vectastain Elite ABC Kit (Vector, Burlingame, CA; #PK-61000) was used at 1:200, being applied for 3 h.

Antibodies

The following antibodies were used in this study: anti-phospholipase C beta 4 (PLCβ4), rabbit polyclonal (Abcam, 1:100); anti-zebrin II (ZII) (a gift from Dr. Richard Hawkes, University of Calgary, Calgary, Alberta, Canada), a mouse monoclonal antibody (1:200); anti-Iba1/AIF1 mouse monoclonal Iba1 (Millipore Sigma, 1:200); and secondary antibodies: goat anti-

mouse IgG, HRP conjugate, (Millipore Sigma, 1:500), and goat anti-rabbit IgG, HRP conjugate (Millipore Sigma, 1:500).

Immunofluorescence

For immunofluorescent staining, sections were processed as above and incubated with the different primary antibodies at 4 °C overnight. After washing, sections were incubated with secondary antibodies: Alexa Fluor 488 goat anti-mouse or anti-rabbit IgG and Alexa Fluor 594 goat anti-rabbit or anti-mouse IgG (Thermo Fisher, 1:1000). For autofluorescence control, we performed no primary antibody staining, incubating with the secondary antibody alone.

Whole-Mount Immunohistochemistry

Whole-mount immunohistochemistry was performed on dissected cerebella according to Sillitoe and Hawkes [35], except that PBS containing 0.2% skim milk (Nestlé Foods Inc., North York, ON, Canada) and 0.1% Triton-X 100 (Sigma, St. Louis, MO, USA) was used as the blocking solution (PBSMT). We added 5% dimethyl sulfoxide (DMSO) to PBSMT for overnight blocking, and after staining, we washed the sample and kept it in PFA. The antigen retrieval technique was required for anti-PLCβ4. Samples were boiled in sodium citrate buffer (95 °C) for 1 h and then cooled down to room temperature.

Imaging

Zeiss Axio Imager M2 microscope, Zeiss Z1 and Z2 Imager, Zeiss LSM 700 confocal microscope, and Zeiss Microscope Software were used to capture and analyze the cerebellar sections. To edit and montage the image, Adobe Photoshop software (CS5.1) was used to finalize the data.

Results

Zone and Stripe Pattern in the Neurog2$^{-/-}$ Cerebellum

Neurog2 is an important determinant of neural cell identity in both the PNS and CNS, with region-specific functions [36]. We set out to test whether *Neurog2* also plays a role in pattern formation within the brain, focusing on the well-characterized zone and stripe patterns in the cerebellum. While the *Neurog2*$^{-/-}$ cerebellum is known to be smaller, it is not known whether it is properly patterned [18].

The most well-studied cerebellar stripe markers are zebrin II and PLCβ4 [5, 37]. We first used anti-PLCβ4 to determine whether zone and stripe expression patterns were altered in *Neurog2*$^{-/-}$ cerebella. In postnatal day (P) 15 *Neurog2*$^{+/+}$ (wild-type) mouse cerebella, the striped expression pattern of Plcβ4 was clearly visible in the anterior zone of the cerebellar cortex, with immunoreactivity mostly localized to a subset of Purkinje cells (Fig. 1a, b).

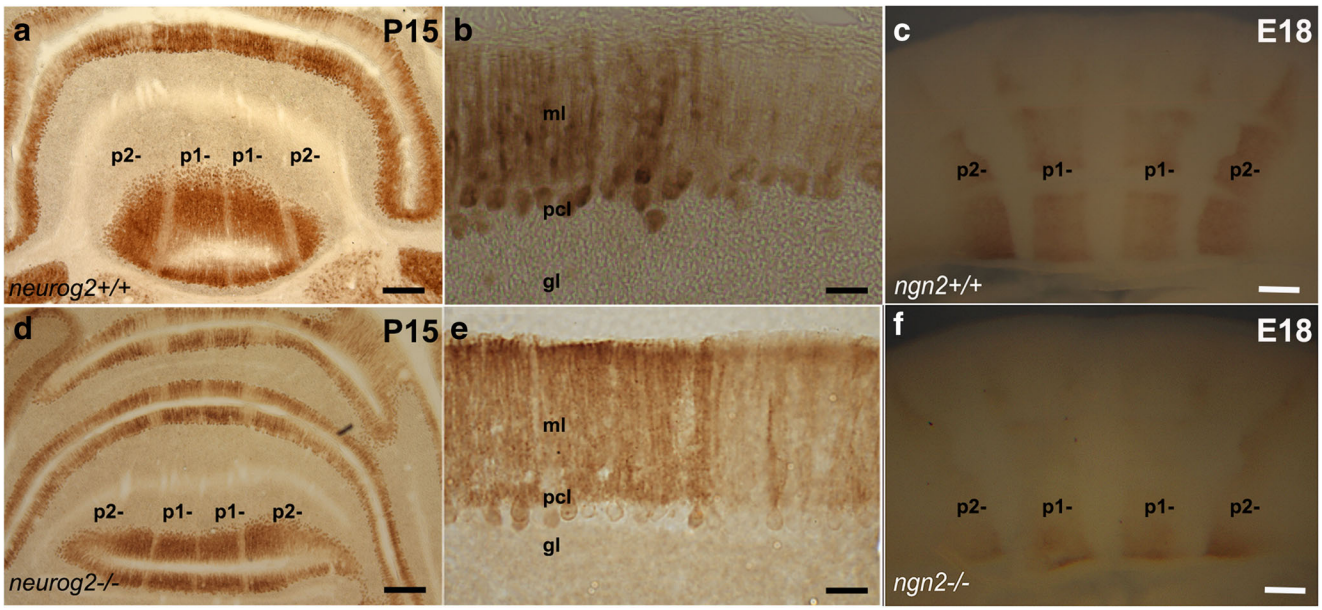

Fig. 1 Plcβ4 expression in P15 and E18 *Neurog2*$^{+/+}$ and *Neurog2*$^{-/-}$ cerebella. **a**–**c** Transverse sections through the cerebellum of P15 *Neurog2*$^{+/+}$ mouse immunostained by anti-Plcβ4 showing the stripe pattern in the cerebellar cortex in the anterior zone (**a**). A higher-magnification image showing that immunoreactivity is mostly localized in a subset of Purkinje cells (**b**). Whole-mount immunostaining of control mouse cerebellum at E18 showing PLCβ4 immunopositive stripes that are separated by narrow immunonegative stripes from the anterior aspect (**c**). **d**–**f** Transverse section through P15 *Neurog2*$^{-/-}$ mouse cerebellum immunostained with anti-Plcβ4 showing normal stripes in the cerebellar cortex (**d**). A higher-magnification image of Plcβ4 immuoreactivity mostly localized in a subset of Purkinje cells (**e**). Whole-mount immunostaining of *Neurog2*$^{-/-}$ mouse cerebellum at E18 showing PLCβ4 immunopositive stripes (**f**). *P* postnatal day, *E* embryonic day, *p* Purkinje cell stripe, *ml* molecular layer, *pcl* Purkinje cell layer, *gl* granule cell layer, Scale bar: **a**, **d** 250 μm, **b**, **e** 50 μm, **c**, **f** 500 μm

Whole-mount immunostaining at embryonic day (E) 18 with PLCβ4 similarly showed wide immunopositive stripes separated by narrow immunonegative stripes (Fig. 1c). In P15 *Neurog2*$^{-/-}$ null mutant cerebella, PLCβ4 was expressed in a normal stripe pattern in the cerebellar cortex, marking a subset of Purkinje cells that were symmetrically distributed around the midline, as observed in control cerebella (Fig. 1d, e). In addition, whole-mount immunostaining of E18.5 *Neurog2*$^{-/-}$ cerebella revealed the normal distribution of PLCβ4 immunopositive stripes (Fig. 1f). The distribution of PLCβ4+ Purkinje cell stripes is thus not perturbed in the absence of *Neurog2*.

In order to further characterize the zone and stripe pattern in the *Neurog2*$^{-/-}$ cerebellum, zebrin II immunoperoxidase staining was performed. Zebrin II was expressed in a subset of Purkinje cells, with zebrin II-positive parasagittal stripes alternating with zebrin II negative stripes in both the anterior and posterior zones in P15 *Neurog2*$^{+/+}$ cerebellar cortices (Fig. 2a, b). Notably, zebrin II was not expressed in other

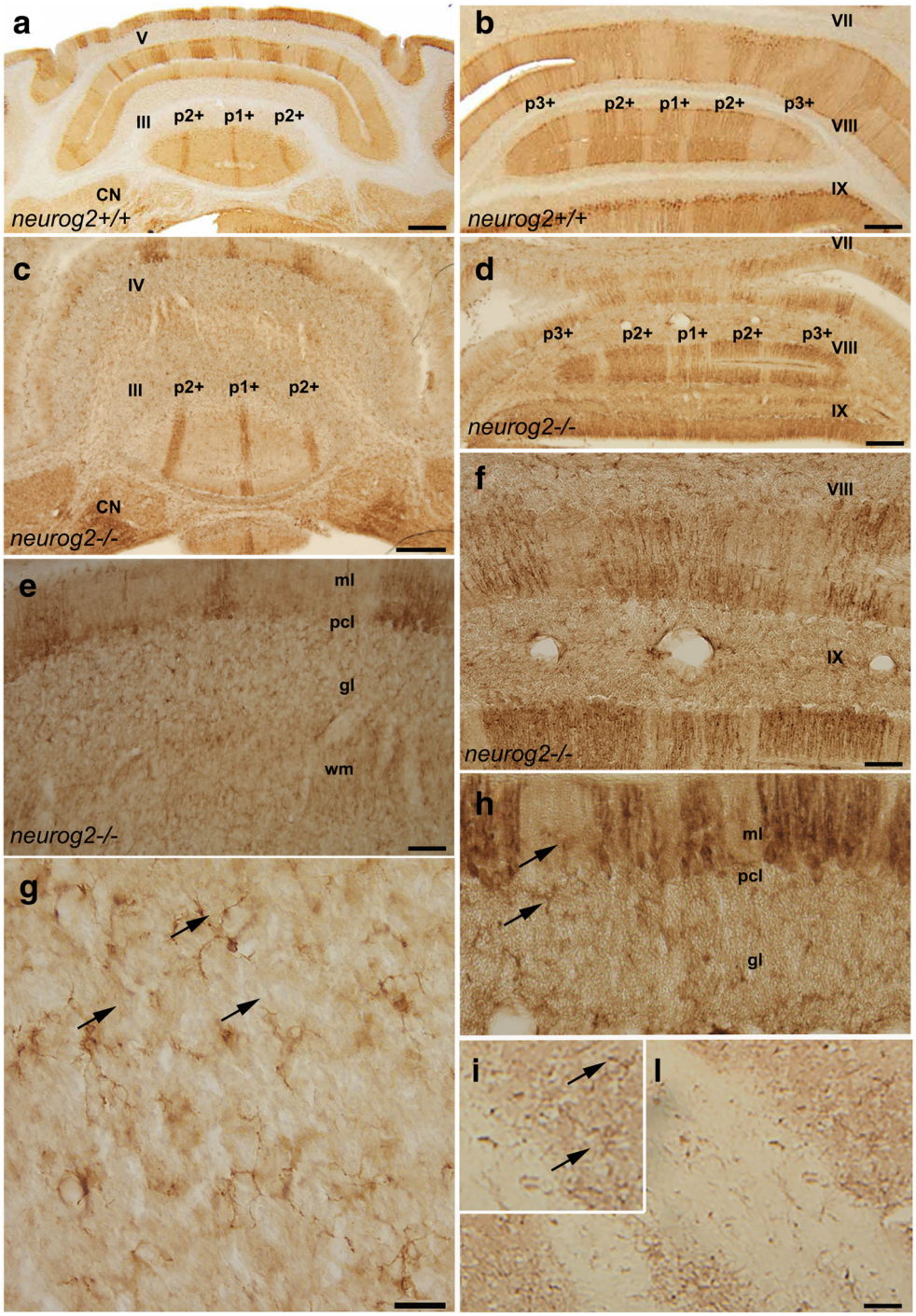

Fig. 2 Zebrin II expression in P15 *Neurog2*$^{-/-}$ and *Neurog2*$^{+/+}$ mouse cerebella. **a**–**h** Transverse sections of P15 *Neurog2*$^{+/+}$ (**a**, **b**) and *Neurog2*$^{-/-}$ (**c**–**h**) cerebellum immunostained with zebrin II. Zebrin II is expressed in a subset of Purkinje cells in the P15 *Neurog2*$^{+/+}$ cerebellar cortex, with patterned parasagittal stripes in anterior (**a**) and posterior (**b**) zones, but there are no other cell immunoreactivities with zebrin II. The P15 *Neurog2*$^{-/-}$ cerebellum showed normal zebrin II+ parasagittal stripes in anterior (**c**) and posterior (**d**) zones and strong immunoreactivities with non-Purkinje cells as well (**e**–**h**). There was no stripe pattern for the ectopic zebrin II+ cells, which were distributed in molecular, Purkinje cell, and granular layers (**e**–**h**). A sagittal section through the brain stem of the *Neurog2* mutant showed Zebrin II expressed in cells with similar morphology that is detected in cerebellum (I (**i**)). Individual lobules in the vermis are indicated in Roman numerals. *p* Purkinje cell stripes, *ml* molecular layer, *pcl* Purkinje cell layer, *gl* granule cell layer, *wm* white matter. Scale bar: **a**–**d** 250 μm, **e**, **f** 100 μm, **g** 20 μm, **i** 50 μm applied to **h**

cerebellar cells. Zebrin II expression in Purkinje cells in the P15 *Neurog2*$^{-/-}$ cerebellar cortex showed the normal pattern of parasagittal stripes (Fig. 2c, d). However, in P15 *Neurog2*$^{-/-}$ cerebella, zebrin II also had strong immunoreactivity in non-Purkinje cells (Fig. 2e–h). These ectopic zebrin II-positive cells did not have a stripe pattern, unlike Purkinje cells, in the P15 *Neurog2*$^{-/-}$ cerebellar cortex (Fig. 2e–h). At higher magnification, the zebrin II-positive, non-Purkinje cells were distributed in the molecular layer, Purkinje cell layer, and granule cell layers (Fig. 2e–h). In addition to the cerebellum, we also observed ectopic zebrin II expression in sagittal sections through the brain stem (Fig. 2I (i)), suggesting that *Neurog2* may also negatively regulate the expression of zebrin II in other CNS domains.

Microglia in the Neurog2$^{+/+}$ and Neurog2$^{-/-}$ Cerebella Have a Similar Distribution

To determine the identity of the ectopic zebrin II-positive cells in *Neurog2*$^{-/-}$ cerebella, we focused on their morphology, and given their highly ramified structure, postulated that they might be microglia (Fig. 3). To examine microglia, we used two different markers: ionizing calcium-binding adaptor molecule 1 (Iba1) [38] and immunoglobulin G receptor (IgGr) [39]. In the P15 *Neurog2*$^{+/+}$ mouse cerebellar cortex, Iba1 staining marked microglia in the molecular layer, Purkinje cell layer, and granule cell layers (Fig. 3a). At higher magnification, the Iba1-positive microglial cells were highly ramified, which is a feature of "resting" microglia (Fig. 3c). Immunostaining with IgGr showed a similar phenotype and distribution of microglia in the P15 *Neurog2*$^{+/+}$ mouse cerebellar cortex (Fig. 3b), and higher-magnification images clearly showed ramified IgGr-positive microglial cells (Fig. 3d). To further assess the distribution of microglia in *Neurog2*$^{-/-}$ cerebella, we did double immunostaining with anti-Iba1 and IgGr in the P15 *Neurog2*$^{-/-}$ mouse cerebellar cortex and showed the co-localization of immunoreactivity in microglia (Fig. 3e–g).

To confirm that the distribution of microglia was normal in *Neurog2*$^{-/-}$ cortices, we counted microglia immunostained with Iba1 in six sections through the vermis of P15 animals, capturing five areas in each zone (each area = 0.6 × 0.64 mm). We calculated microglia number to be 59.69 ± 1.36 per 1 mm^2 in wild type and 55.94 ± 1.28/1 mm^2 in *Neurog2*$^{-/-}$ (mean ± SE). There were thus no significant differences between wild-type and *Neurog2*$^{-/-}$ in microglial distribution or number (Fig. Suppl. 1B). There was also no evidence of microgliosis as we mainly detected ramified, unactivated microglia in the *Neurog2*$^{-/-}$ cerebellum.

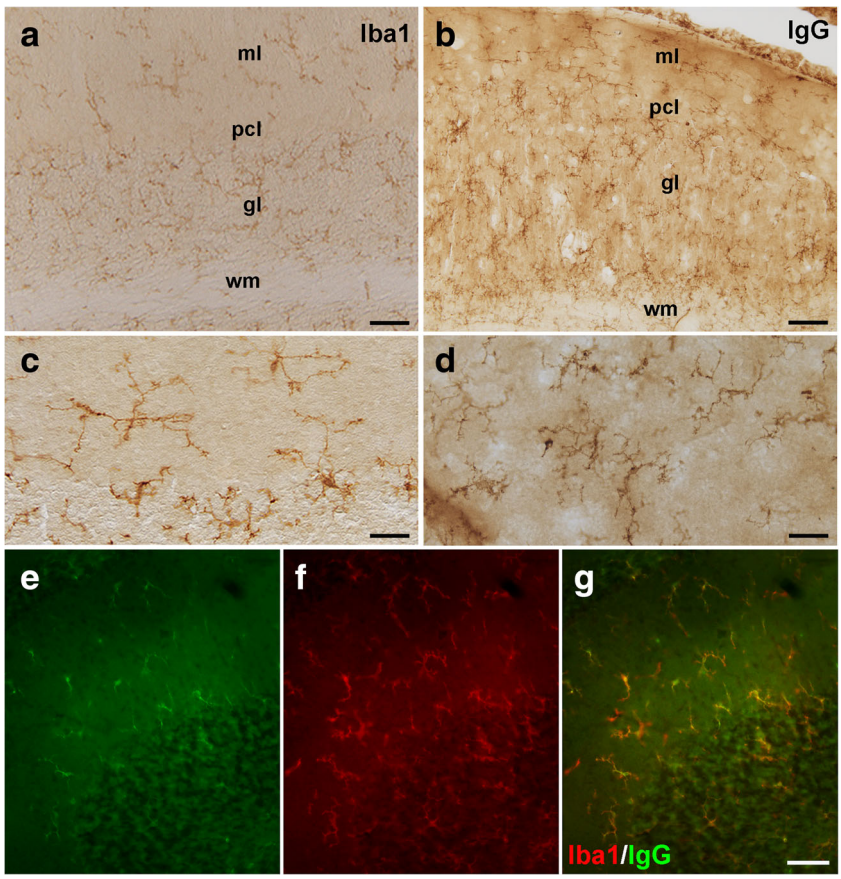

Fig. 3 Iba1 and IgGr are expressed in microglia in P15 *Neurog2*$^{+/+}$ cerebella. **a**–**d** Transverse sections of the P15 *Neurog2*$^{+/+}$ mouse cerebellar cortex immunostained with Iba1, showing microglia distributed in the molecular, Purkinje cell, and granular layers (**a**). Transverse sections of the P15 *Neurog2*$^{+/+}$ mouse cerebellar cortex immunostained with IgGr, which also marks microglia (**b**). Higher-magnification images show ramified Iba1 (**c**) and IgGr (**d**) positive microglia. **e**–**g** Double immunostaining with anti-Iba1 (red) and IgGr (green) of a transverse section through the P15 *Neurog2*$^{-/-}$ mouse cerebellar cortex clearly shows overlapping immunoreactivity in microglia cells. *ml* molecular layer, *pcl* Purkinje cell layer, *gl* granule cell layer, *wm* white matter. Scale bar: **a**, **b** 50 μm, **c**, **d** 20 μm, **g** 50 μm applied to **e**–**g**

Ectopic Zebrin-II Expressing Cells in the Neurog2$^{-/-}$ Cerebellum Are Microglia

To determine whether the cells expressing ectopic zebrin II in P15 *Neurog2*$^{-/-}$ cerebellar cortices were microglia, we turned to dual-label immunofluorescent staining. As shown above, in the P15 *Neurog2*$^{+/+}$ cerebellar cortex, zebrin II immunoreactivity was restricted to a subset of Purkinje cells with a clear pattern of parasagittal stripes, and there were no other cells with zebrin II immunoreactivity (Fig. 4a). In contrast, the *Neurog2*$^{-/-}$ cerebellum showed immunoreactivity in a subset of Purkinje cells with patterned stripes, similar to the normal pattern, but remarkably, there was also strong immunoreactivity in non-Purkinje cells, which were distributed in both zebrin II-positive and zebrin II-negative stripes (Fig. 4b, c).

To determine whether the zebrin II-positive non-Purkinje cells were microglia in the *Neurog2*$^{-/-}$ cerebellar cortex, we performed double immunostaining with anti-zebrin II and anti-Iba1. We found that zebrin II and Iba1 immunostaining overlapped in P15 *Neurog2*$^{-/-}$ cerebella, confirming that the zebrin II-positive non-Purkinje cells in the P15 *Neurog2*$^{-/-}$ cerebellum were microglia (Fig. 4d–f). This overlap was extensive; of the Iba1-positive microglia, 100% co-expressed zebrin-II in *Neurog2*$^{-/-}$ cerebella, whereas there was no co-expression in wild-type controls.

Notably, the zebrin II-positive microglia were not restricted to the zebrin II-positive stripes and were present in zebrin II-negative stripes as well (Fig. 4d–f). Moreover, the zebrin II-positive microglia in the *Neurog2*$^{-/-}$ cerebella were present in all three layers of the cerebellar cortex (Fig. 4d–f). We can thus conclude that zebrin II is ectopically expressed in microglia in the *Neurog2*$^{-/-}$ cerebellar cortex.

Defects in Cerebellar Development Are Not Normally Associated With Ectopic Zebrin II Expression in Microglia

To understand whether ectopic zebrin II expression in microglia in the *Neurog2*$^{-/-}$ cerebellum was a common cerebellar reaction to an intrinsic pathological condition, we also performed zebrin II immunostaining on P15 *Acp2*$^{-/-}$ mutants. These mice have cerebellar defects, including defects in microglia [40, 41], leading us to speculate that microglial changes may be an underlying cause of the ectopic expression of zebrin II. In the P15 *Acp2*$^{-/-}$ cerebellum, Iba1 immunostaining marked both ramified and activated microglia (Fig. 5a, b). The overall number of microglia increased approximately 5-fold in *Acp2*$^{-/-}$ cerebella (wild type 59.03 ± 5.61 and *Acp2*$^{-/-}$ 321.53 ± 24.39; mean ± SEM), and in these mutants, approximately 80% of the microglia were unramified with large

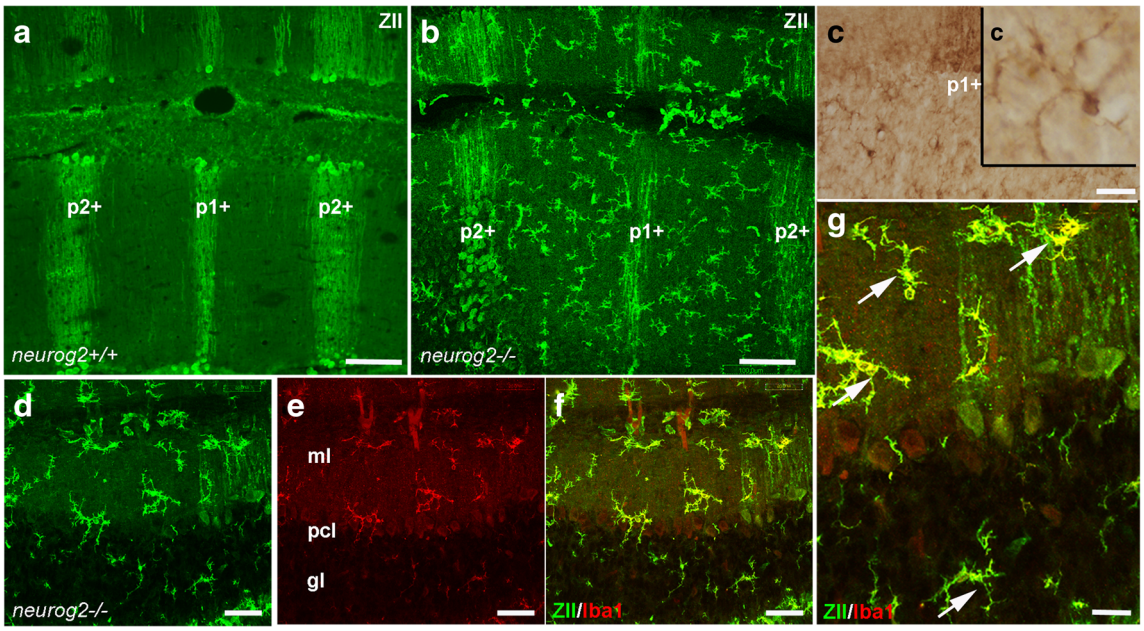

Fig. 4 Zebrin II-positive cells in *Neurog2*$^{-/-}$ cerebella are microglia. **a**–**g** Zebrin II expression in P15 *Neurog2*$^{+/+}$ cerebella shows immunoreactivity in only a subset of Purkinje cells with clear pattern parasagittal stripes, but there are no other cells with zebrin II immunoreactivity (**a**). Zebrin II expression in P15 *Neurog2*$^{-/-}$ cerebella shows immunoreactivity in only a subset of Purkinje cells with a clear pattern of parasagittal stripes, and ectopic zebrin II expression in microglial cells (**b**). (*C*) The *Neurog2*$^{-/-}$ cerebellum shows immunoperoxidase reactivity with zebrin II in Purkinje cells and strong immuonreactivities with microglia (**c**). A single microglia is shown at higher magnification in (*C*). High-magnification images of P15 *Neurog2*$^{-/-}$ cerebella co-immunostained with anti-zebrin II (green) (**d**–**g**) and Iba1 (red) (**e**–**g**), confirming the microglial identity of the ectopic zebrin II-positive cells. The distribution of microglia in the *Neurog2*$^{-/-}$ cerebellum revealed a condensed appearance with small branches that are assumed to be activated and are distributed in both ZII+ and ZII− stripes (white arrows) (**g**). *p* Purkinje cell stripes, *ml* molecular layer, *pcl* Purkinje cell layer, *gl* granule cell layer. Scale bar: **a**, **b** 100 μm, **c** 50 μm 10 μm, **d**–**f** 50 μm, **g** 40 μm

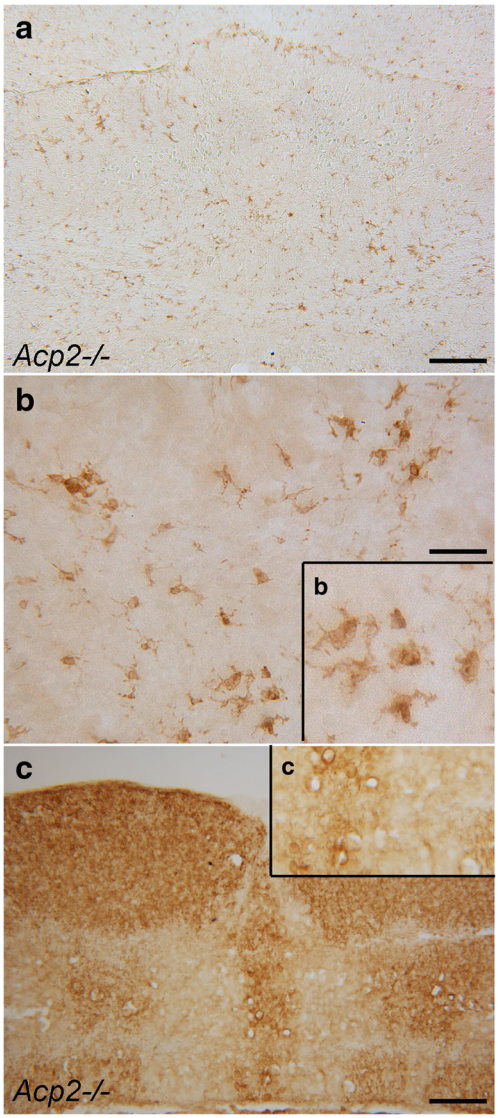

Fig. 5 Iba1-positive microglia are not zebrin II positive in *Acp2*$^{-/-}$ cerebella. **a**, **B**) A transverse section of the *Acp2*$^{-/-}$ mouse cerebellar cortex at P15 immunostained with Iba1 shows microgliosis, and a higher magnification clearly shows ramified and activated microglial cells in (b). **C**) A transverse section of the *Acp2*$^{-/-}$ mouse cerebellar cortex at P15 immunostained with zebrin II shows expression in a subset of Purkinje cell stripes, but not in other cells in cerebellum, (c) A higher magnification of C. Scale bar: **a** 50 μm, **b** 20 μm, **c** 50 μm

somata. However, zebrin II expression in the *Acp2*$^{-/-}$ cerebellum was restricted to a subset of Purkinje cells, and was not seen in microglia (Fig. 5c). The presence of activated microglia is thus not necessarily associated with ectopic zebrin II expression. One reason for the lack of ectopic zebrin II in the *Acp2*$^{-/-}$ cerebellum may be because *Neurog2* is upregulated (instead of lost) in *Acp2*$^{-/-}$ cerebella based on RNA-sequencing data (Fig. Suppl. 1A).

To extend our observation, we used human cerebella sections with PCH, which have a gross disruption of cerebellar development and neuronal death with abundant activated microglia. Similarly, microgliosis was observed in the PCH cerebellum, with a 3-fold increase in microglial number (control = 154.51 ± 1.71 and PCH = 394.50 ± 20.16), with 95% of the microglia in the PCH sample appearing as unramified with large somata. However, while we detected activated microglia in the PCH cerebellum immunostained with Iba1 (Fig. 6b, i), zebrin II expression was restricted to a subset of Purkinje cells and was not seen in microglia (Fig. 6d, h). The ectopic expression of zebrin II in microglia is thus not a general consequence of abnormal cerebellar development and microglial activation, but it is associated with the loss of *Neurog2* expression.

Discussion

In this study, we found that the patterned parasagittal stripes of zebrin II and PLCβ4 are intact in the *Neurog2*$^{-/-}$ cerebellum, with normal zone and stripe patterns. However, we unexpectedly found that zebrin II is ectopically expressed in microglia in the *Neurog2*$^{-/-}$ cerebellum, with no restriction by lamina, stripe, or zone. The strong ectopic zebrin II expression in microglia in the *Neurog2*$^{-/-}$ mouse cerebellum is extremely unusual, and has never been seen in normal mice—either in the adult or during development—nor in over 30 species of mammals, birds, fish, bat, etc. [1, 3–7]. One important caveat to this statement is that there may be differences in tissue processing, immunostaining procedures, or a focus on a narrow part of the cerebellum that prevented us and others from observing zebrin II expression in microglia. However, we are confident that our finding of ectopic zebrin II expression in *Neurog2*$^{-/-}$ cerebella is not artifactual, and is truly novel, as the ectopic staining is very robust and at high levels. Moreover, we also noted ectopic zebrin II expression in the *Neurog2*$^{-/-}$ brain stem, suggesting that *Neurog2* may regulate gene expression in microglia throughout the CNS, a topic of future research.

Our finding that *Neurog2* has an essential function in microglia is of particular significance given that *Neurog2* is considered to be a neural-specific gene, and microglia arise from a hematopoietic lineage, infiltrating the embryonic brain early in development, upon which they expand to become the resident immune cells of the CNS [42]. This finding thus provides support for a new non-neural function for *Neurog2*.

To understand why zebrin II is ectopically expressed in *Neurog2*$^{-/-}$ microglia, we first asked whether it is because of a reaction to a pathological condition or due to cerebellar abnormalities in the *Neurog2*$^{-/-}$ mutant cerebellum. Importantly, zebrin II is not ectopically expressed in microglia in other mutants in which cerebellar architecture is disrupted (e.g., weaver [43], lurcher [44], staggerer [45], disabled [46, 47], reeler [45, 48, 49]), although there are often signs of "background" astroglial staining in some of these models [9]. Microglial zebrin II expression is thus not a necessary consequence of the disruption of normal cerebellar development. Also, ectopic zebrin II

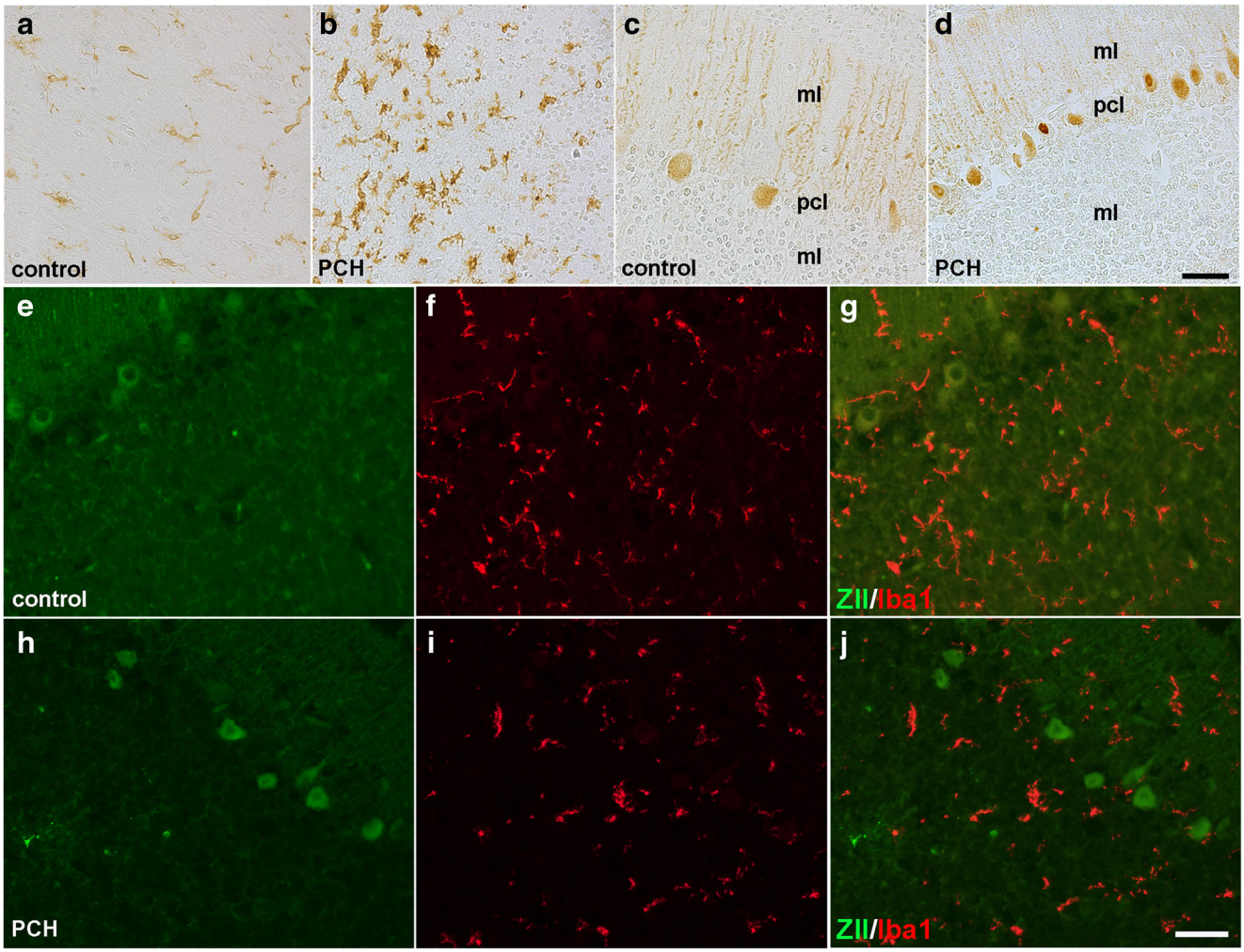

Fig. 6 Iba1-positive microglia are not zebrin II positive in PCH. **a**, **B**) A horizontal section of the control cerebellar cortex immunostained with Iba1 shows ramified microglial cells (**a**). A horizontal section of the PCH cerebellar cortex immunostained with Iba1 shows ramified and activated microglial cells (**b**). **c**, **d** Horizontal sections of control (**c**) and PCH (**d**) cerebellar cortices showing zebrin II expression in Purkinje cells, but not other cells in the cerebellum. **e**–**j** Double immunostaining with zebrin II (green) and Iba1 (red) of a horizontal section of control (**e**–**g**) and PCH (**h**–**j**) cerebellar cortices, showing that none of the Iba1-positive microglia ectopically express zebrin II. Scale bar: **d** 50 μm applied to **a**–**d**, **j** 40 μm applied to **e**–**j**

immunoreactivity was not observed in Purkinje cell death models like Niemann-Pick, in spite of massive Purkinje cell loss which starts first with the death of zebrin II-negative immunoreactive cells, followed progressively by the loss of zebrin II-positive immunoreactive cells (Niemann-Pick A/B [50] and C [51]). Similarly, neonatal methylazoxymethanol-induced lesions result in a massive reduction of the external granular layer, but zebrin II immunoreactivity shows no fundamental abnormalities in the Purkinje cell compartments, and no ectopic zebrin II immunopositive microglia were seen [52]. Moreover, there is no report of microglial zebrin II expression in other cerebellar disruptions such as afferent lesions [53].

We examined whether zebrin II might be ectopically expressed in microglia in another mouse model: the *Acp2*$^{-/-}$ mice, which have severe cerebellar defects, including reduced size, abnormal lobulation, Purkinje cell migratory defects, reduced granule cells, and neuronal degeneration with hypoplastic vermis [31]. Despite these severe anomalies, and the demonstration of clear microgliosis with abundant activated microglia (Iba1 positive) in the *Acp2*$^{-/-}$ cerebellum, none of the microglial cells ectopically expressed zebrin II (Fig. 5). Similarly, our study of PCH did not reveal any ectopic zebrin II expression in microglia (Fig. 5).

Numerous microglia (Iba1 positive) are present in the normal cerebellum [20, 38, 54]. Visual comparison of sections from *Neurog2*$^{+/+}$ and *Neurog2*$^{-/-}$ cerebella immunostained for Iba1 positive showed no difference in number (Fig. Suppl. 1B) or morphology of microglia (Fig. 4). This data suggests that there is an upregulation of zebrin II expression in the *Neurog2*$^{-/-}$ mutant microglia rather than an invasion of the cerebellum by a novel zebrin II-positive microglial population from the periphery.

Like other proneural genes, *Neurog2* participates in the specification of neural cell fates and neuronal identities [36]. To carry out these functions, *Neurog2* both activates and represses the expression of downstream genes [55]. While the activation of neuronal differentiation genes by Neurog2 is carried out in many cases by direct transcriptional activation, Neurog2 represses genes through indirect means [55]. For example, Neurog2 can repress an astrocytic program by sequestering CBP/Stat/Smad co-activators from binding the regulatory regions of GFAP and other astrocytic lineage genes [56]. Conversely, Neurog2 prevents the ectopic expression of *Ebf1* by turning on the expression of *Tbr2*, which acts as a transcriptional repressor [55]. How Neurog2 normally represses zebrin II remains to be investigated. It will also be of interest to see whether Neurog2 is required to repress the expression of other neuronal markers in cerebellar microglia.

An interesting observation is that zebrin II/aldolase C is not generally thought of as an easily inducible gene: indeed, changing the expression of zebrin II in Purkinje cells has proven remarkably refractory to a wide range of genetic and experimental interventions (reviewed in [3, 5]). There are studies describing a potential neuroprotective role for zebrin II/aldolase C. For example, in Purkinje cell death models, such as Niemen-Pick [50, 51] and the tottering and leaner mutants [57], the zebrin II-negative population typically dies earlier. However, this is not always the case, as the homozygous nervous (nr/nr) mutant mouse shows selective death of zebrin II-positive stripes [58]. In the leaner and tottering cerebella, there is ectopic expression of tyrosine hydroxylase adjacent to the zebrin II-positive stripes. However, the ectopic expression of tyrosine hydroxylase does not appear to be neuroprotective [59], and it can therefore be assumed that it may be the same in *Neurog2*$^{-/-}$ mutants.

Conclusion

The ectopic expression of zebrin II that we observed in microglia in the *Neurog2*$^{-/-}$ cerebellar cortex is a unique phenotype that points to a new and novel non-neural role for *Neurog2* as microglia arise from a hematopoietic lineage. Although in this study we did not show the exact mechanism that results in this phenomenon, it is likely to be the result of the specific disruption of Neurog2 transcriptional cascades, rather than a generic response to cerebellar disruption. Indeed, models of cerebellar abnormalities that have activated or ramified Iba1-positive microglia do not show ectopic zebrin II expression, implying that ectopic zebrin II expression in the cerebellar microglia in *Neurog2*$^{-/-}$ mice is specific to the mutation and not a reaction to intrinsic cerebellar pathological condition.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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