Experimental Neurology 348 (2022) 113950

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research paper

Sox-positive cell population in the adult cerebellum increases upon tissue degeneration

Shelanah Salih [a,d,1], Zubair Ahmed Nizamudeen [a,1], Nigel De Melo [a], Lisa Chakrabarti [b], Virginie Sottile [a,c,*]

[a] Wolfson STEM Centre, School of Medicine, The University of Nottingham, Nottingham NG7 2RD, UK
[b] School of Veterinary Medicine and Science, The University of Nottingham, UK
[c] Department of Molecular Medicine, The University of Pavia, Italy
[d] College of Medical and Applied Sciences, Department of Medical Laboratory Sciences, Charmo University, Chamchamal 46023, Iraq

ARTICLE INFO

Keywords:
Cerebellum
Sox1
Bergmann glia
Stem cell marker
Purkinje cell degeneration

ABSTRACT

Adult neurogenesis is well-described in the subventricular and subgranular zones of the mammalian brain. Recent observations that resident glia express stem cell markers in some areas of the brain not traditionally associated with neurogenesis hint to a possible role in tissue repair. The Bergmann glia (BG) population in the cerebellum displays markers and *in vitro* features associated with neural stem cells (NSC), however the physiological relevance of this phenotypic overlap remains unclear in the absence of established *in vivo* evidence of tissue regeneration in the adult cerebellum.

Here, this BG population was analysed in the adult cerebellum of different species and showed conservation of NSC-associated marker expression including Sox1, Sox2 and Sox9, in chick, primate and mouse cerebellum tissue. NSC-like cells isolated from adult mouse cerebellum showed slower growth when compared to lateral ventricle NSC, as well as differences upon differentiation. In a mouse model of cerebellar degeneration, progressive Purkinje cell loss was linked to cerebellar cortex disorganisation and a significant increase in Sox-positive cells compared to matching controls. These results show that this Sox-positive population responds to cerebellar tissue disruption, suggesting it may represent a mobilisable cellular resource for targeted strategies to promote tissue repair.

1. Introduction

While active neurogenesis in the adult brain has been reported in the sub-ventricular zone (SVZ) and the sub-granular zone (SGZ) (Ming and Song, 2011), the adult cerebellum has long been thought to be devoid of neurogenesis activity (Altman and Das, 1966). More recently however, cells displaying properties of self-renewal and multipotency *in vitro* were isolated from postnatal and adult mouse cerebellum (Klein et al., 2005; Lee et al., 2005), suggesting the existence of cerebellar neural stem cells (NSC). A subsequent study showed Bergmann glia (BG), intercalated between Purkinje cells (PC) in the adult Purkinje cell layer (PCL), display features characteristic of NSC such as the expression of Sox1, Sox2 and Sox9, and *in vitro* differentiation capacity (Sottile et al., 2006; Alcock and Sottile, 2009). BG support the migration of Granule cells (GC) during postnatal development and the maturation of neurons including PC and GC (Yamada and Watanabe, 2002). While PC are closely associated with BG throughout life, the loss of PC through trauma or degeneration causes motor coordination dysfunction such as ataxia (Lim et al., 2006).

Although BG express some NSC markers, there is no compelling *in vivo* evidence of their intrinsic ability to replace lost cerebellar neurons, including PC. A previous study suggested that NSC-like cells in the adult rodent CB are quiescent and do not generate neurons or astrocytes *in vivo* under standard physiological conditions (Su et al., 2014), while the presence of a neurogenic subset of Sox2-expressing cells intermingled within the BG was recently reported in mouse but without evidence of PC differentiation (Ahlfeld et al., 2017).

To evaluate the relevance of this Sox-positive population to cerebellar homeostasis, its *in vivo* distribution and properties were analysed in adult tissue, to evaluate its potential in case of tissue damage. The

* Corresponding author at: Department of Molecular Medicine, The University of Pavia, Italy.
E-mail address: virginie.sottile@unipv.it (V. Sottile).
[1] These authors contributed equally to the paper.

https://doi.org/10.1016/j.expneurol.2021.113950
Received 8 October 2021; Received in revised form 1 December 2021; Accepted 7 December 2021
Available online 10 December 2021

response of these Sox-positive BG cells to Purkinje cell degeneration was analysed using the *pcd*^{5J} mouse model (pcd), which carries an autosomal recessive mutation of the Nna1 gene causing the death of virtually all PC by the third to fourth postnatal (P) week (Chakrabarti et al., 2006). *pcd* mice have been observed to lose 25% to 50% of total PCs in the culmen and pyramis vermis of the cerebellum by P25 (Mullen et al., 1976), causing ataxic gait, and slow degeneration of mitral cells and photoreceptor retinal cells (Baltanás et al., 2011; Cendelin, 2014). The present study assessed the features of the Sox-positive BG cells and their response to cerebellar damage occurring during PC degeneration, by investigating *in vivo* changes in the *pcd* mouse model compared to age-matched controls.

2. Materials & methods

Materials were purchased from ThermoFisher Scientific (UK) unless otherwise stated.

2.1. Tissue preparation and immunohistochemistry

Animal cerebellum samples from adult *pcd*^{5J} mouse (Chakrabarti et al., 2009), chick and chimpanzee fixed in 4% PFA were wax-embedded and cut to produce 10 μm serial sagittal sections using a Leica microtome.

Sections were dewaxed, rehydrated (Carson et al., 2015), washed twice for 5 min in PBS+ 0.1% tween 20, before heat-induced antigen retrieval using sodium citrate buffer at pH 6.0 (Namimatsu et al., 2005; Shi et al., 1993). Endogenous peroxidase activity was blocked using 3% H_2O_2 for 10 min, followed by 3 PBS washes for 5 min each and incubation for 1 h using 0.1% fetal calf serum (FCS) blocking solution prepared in PBS. Slides were incubated overnight at 4 °C with the primary antibody for Sox1 (R&D systems, UK), Sox2 (Millipore, UK), Sox9 (Millipore), Calbindin (Sigma-Aldrich, UK), GFAP (Dako, UK), BLBP (Millipore), Nestin (DSHB, USA), βIII-Tubulin (Cell Signalling, UK) diluted in blocking solution. Sections were then washed extensively with PBS, incubated for 1 h at room temperature with FITC-, Texas Red-, and Horseradish Peroxidase (HRP)- conjugated secondary antibodies (Vector Laboratories, UK), and then washed 3 times with PBS for 15 min each. For single immunostaining, slides were treated with DAB (3,3'-Diaminobenzidine) peroxidase substrate kit (Vector Laboratories) according to the manufacturer's instructions, and then mounted with DAPI (4′,6-Diamidino-2-Phenylindole)-containing Vectashield (Vector Laboratories). For double immunostaining, slides were first incubated with DAB then washed 3 times with PBT, before incubation with the Alkaline phosphatase substrate kit (Vector Laboratories) according to the manufacturer's instructions. Finally, slides were washed with PBT 3 times for 5 min each, and mounted with DAPI mounting medium. Images taken with a Nikon Eclipse 90i fluorescence microscope were processed using ImageJ software (https://imagej.nih.gov/ij/). For Sox-positive cell counting, data was normalised as number of cells per 0.7 mm section of PCL (mean ± SEM, $n = 3$). The molecular layer (ML) dimensions were measured perpendicularly from the PCL to the pial layer of the ML (25 line segments per image, 3 images per samples, mean ± SEM).

2.2. Immunocytochemistry

For cell staining, coverslips were washed once with (PBS + 0.1% tween 20) for 10 min at room temperature (RT). Cells were then blocked in 0.1% FCS blocking solution prepared in PBS for 30 min at RT. The cells were then incubated with the primary antibody prepared from the blocking solution for overnight at 4 °C. After 3 washes with PBS, each for 5 min, the cells were then incubated with the secondary antibody prepared in blocking solution and incubated in the dark for 1 h at RT. Coverslips were then rinsed 3 times in PBS for 5 min each, stained with DAPI for 30 min, washed 3 times in PBS for 5 min each and mounted using Fluorogel mounting medium (GeneTex, UK) before being imaged using a Nikon Eclipse 90i fluorescence microscope.

2.3. Cell culture

Brains extracted from 3-month old sacrificed mice were prepared as previously described (Alcock and Sottile, 2009). Briefly, cerebellum and lateral ventricle tissue was isolated, washed in PBS and digested with Accumax (Sigma-Aldrich) for 30 min at 37 °C. Samples were then centrifuged for 5 min, the cells were dissociated and washed with PBS, and seeded onto 6-well tissue culture plates using NSC medium made of DMEM/F12 and Neurobasal (1:1) supplemented with B27, N2, 0.5% Penicillin/Streptomycin, 0.01% Heparin, and the addition of EGF and FGF2 (20 ng/ml each). Cultures were maintained in an incubator at 37 °C under 5% CO_2, and fresh medium was added every 2 days. Cells were passaged using Accutase (Sigma-Aldrich) for 3–5 min at 37 °C and used within 10 passages. For doubling time analysis, cells were seeded in 6-well plates at 1×10^6 cells per well ($n = 3$), cultured for 48 h, dis-aggregated using Accutase and then counted using Countess II Automated Cell Counter according to manufacturer's instructions.

For differentiation, cells were seeded at 50,000 cells/well on Geltrex-coated 13 mm coverslips according to manufacturer's instructions. After attachment, cells were treated for 7 days with NSC medium without growth factors (no EGF or FGF) supplemented with added 0.05% FCS, 1 μM EC23 (Tocris, UK) and 10 ng/μl BDNF, with medium refreshed every 2 days. Cells were fixed using 4% PFA and used within one week for immunostaining. Neuronal perikaryon size was measured by drawing a line across the cell soma (duplicate repeats per condition) using ImageJ.

2.4. Statistical analysis

All analyses were conducted in triplicates unless mentioned otherwise, and graphs were prepared using Prism software (GraphPad version 7). Normality tests were performed using Shapiro-Wilk test. Unpaired parametric two-tailed students *t*-test was performed for statistical analysis unless otherwise stated. Datasets were represented as mean ± SEM with statistical significance level set for *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3. Results

3.1. A conserved population of sox-positive radial glia in adult vertebrate cerebellum

Previous studies have shown that the BG population in the PCL of adult mice cerebellum are positive for the NSC markers Sox1, Sox2 and Sox9 (Alcock and Sottile, 2009). In order to investigate the evolutionary conservation of these NSC characteristics, adult cerebellar tissue from primate, chick and mouse was compared using immunohistochemistry for Sox1, Sox2 and Sox9 and for two radial glia/astrocyte markers, BLBP and GFAP (Feng et al., 1994; Mamber et al., 2012).

The primate, chick and the mouse adult cerebellum showed expression of GFAP and BLBP in the BG population (Fig. 1). GFAP fibers were intercalated with Calbindin-positive PC bodies (blue) in the PCL and dendrites in the upper ML. Similarly, BLBP detection highlighted BG cells in the PCL, with BLBP-stained processes intermingled between the PC dendrites in the ML for all three species. In both primate and chick cerebellum, BG adjacent to PC in the PCL were positive for Sox1, Sox2 and Sox9, in a pattern between Calbindin-positive PC bodies similar to that observed in the adult mouse cerebellum (Fig. 1). This was consistently observed on sagittal sections taken throughout the tissue and confirmed the conserved Sox expression pattern of BG present in the adult cerebellum.

3.2. Morphology and in vitro properties of CB neurosphere cells

The presence of BG cells sharing some stem cells markers in the adult

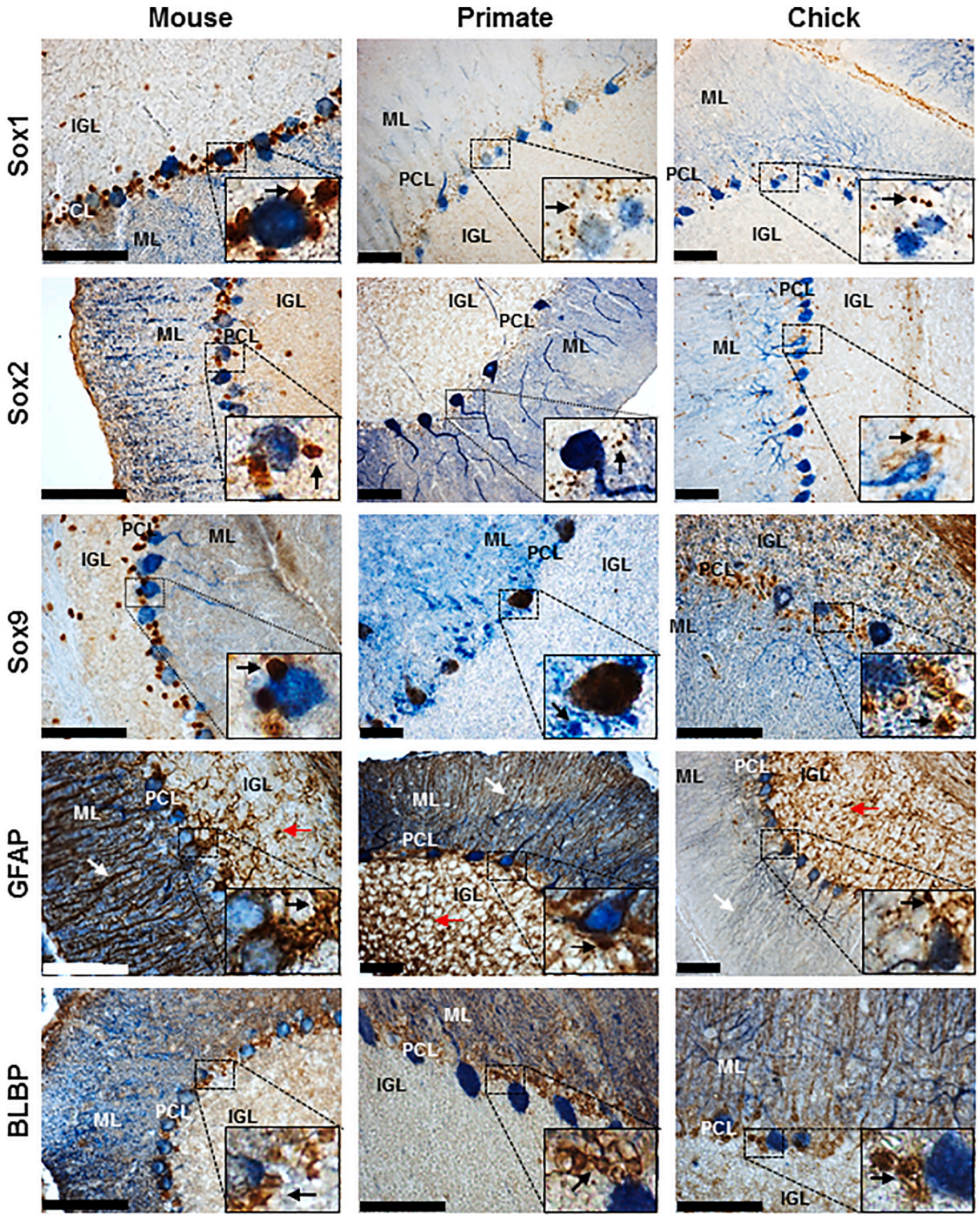

Fig. 1. Expression of NSC associated and radial glial markers in the BG of adult mice, primate and chick. Double immunostaining of Sox1, Sox2, Sox9, GFAP and BLBP (brown) with Calbindin (blue) (except for the primate Sox9-staining, where Calbindin is shown in brown and Sox9 in blue), showing the BG population (arrows) situated in PCL adjacent to Purkinje neurons. Black arrows point to examples of stained cells, white arrows mark the region for GFAP fibers in the ML and red arrows mark GFAP detection in the IGL. Scale bars: 100 μm. IGL: Internal granular layer, ML: Molecular layer, PCL: Purkinje cell layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cerebellum posed the question of a hypothetical capacity to generate new CB cells. To explore the properties of the BG population and a possible ability to form new Purkinje cells, cells isolated from adult mouse cerebellar tissue (CB) were analysed under neural stem cell (NSC) culture conditions alongside cells the lateral ventricles (LV). Within 3 weeks, CB neurospheres were observed which later attached and grew with a radial-like morphology similar to LV neurospheres (Fig. 2A). The growth rate in CB-NSC cultures was slower than in LV-NSC cultures, as illustrated by their doubling time (Fig. 2B). Both cell types in culture stained positive for Nestin (Fig. 2C), although immunohistochemistry

carried out in adult cerebellar tissue showed no detectable Nestin expression in the BG (Suppl. Fig. 1A) in line with existing gene expression data (Supplementary Fig. S1 B). When exposed to differentiating conditions, both cell types showed changes in morphology, and brightfield images at day 7 showed that differentiated CB-NSC produced neuronal-like bodies with larger perikaryon size compared to those observed in differentiated LV-NSC cultures (Fig. 3A-B).

To investigate whether the larger neuronal body in differentiated CB-NSC may be indicative of a predisposition to generate PC, immunodetection was carried out for Calbindin, a known PC marker detected throughout the large soma of PC and along their dendrites and axons *in vivo* (Barski et al., 2003; Garcia-Segura et al., 1984). Calbindin-positive signal was detected along the neural extensions of CB-NSC in both day 0 and day 7 differentiated samples (Fig. 3C), albeit with a stronger signal at day 7. In contrast, differentiated LV-NSC showed weaker but detectable signal for Calbindin in their neural extensions (Fig. 3D). Nevertheless, Calbindin detection was rare and typical PC morphology was not observed in either cell types.

3.3. Analysis of Sox+ BG cells in a cerebellar mutant mouse model

To evaluate the changes occurring in the adult CB when the steady state was disrupted, cerebellar tissue from *pcd*^{5J} mutant degenerative model (homozygous) was isolated for immunohistochemical analysis (Chakrabarti et al., 2006). Changes in the BG population were analysed in the mutant model from early (P17) to late (P100) degeneration stages and compared with age-matched WT controls using anti-Calbindin immunodetection to selectively label PC (Fig. 4). At P17, there was no visible loss of PC between mutant and control mice, however PC arborisation appeared reduced in the mutant mice, and in the molecular layer (ML) GFAP-positive BG processes appeared more disorganised with some misplaced cell bodies visible (Fig. 4A).

At P21, the onset of PC death was noticeable in the mutant samples, rapidly evolving to drastic PC loss by P26 (Fig. 4B). By P100, no Calbindin-positive PC were found in the *pcd* cerebellar cortex, which showed a pronounced upregulation of GFAP expression in BG fibers (Fig. 4B) with a few visible vacuolar structures (Fig. 4C) and marked shrinkage of the ML compared to controls (Fig. 4D).

Double immunostaining for Calbindin with Sox1, Sox2, and Sox9 compared at P17 and P100 (Fig. 5A-C) indicated that although PC were lost at P100 in the mutant mice, Sox-positive BG were still numerous (Fig. 5A). Quantification of Sox-positive cells in mutant cerebellum showed a significant increase after P100 compared to WT controls, although this was not the case at P17 (Fig. 5C), indicating an increase in the Sox-positive BG population in response to late degeneration stages.

3.4. Isolation of CB-NSCs from pcd mutant CB

To evaluate the changes in CB-NSC due to *pcd* mutation, CB tissue was isolated from P21 *pcd* mutants and matching WT controls along with cells from lateral ventricle tissue, using a previously established method (Alcock and Sottile, 2009). CB-NSC established from *pcd* and WT mice were able to form neurospheres when maintained in standard NSC culture medium, however mutant-derived cultures contained significantly fewer neurospheres compared to both CB- and LV-derived cultures established from WT controls (Fig. 6A-B). Cell morphology and expression of stem cell markers Nestin and Sox2 remained comparable in both groups (Fig. 6C), suggesting that the CB-NSC population isolated from early stage PC degeneration maintained NSC marker expression *in vitro* at P21.

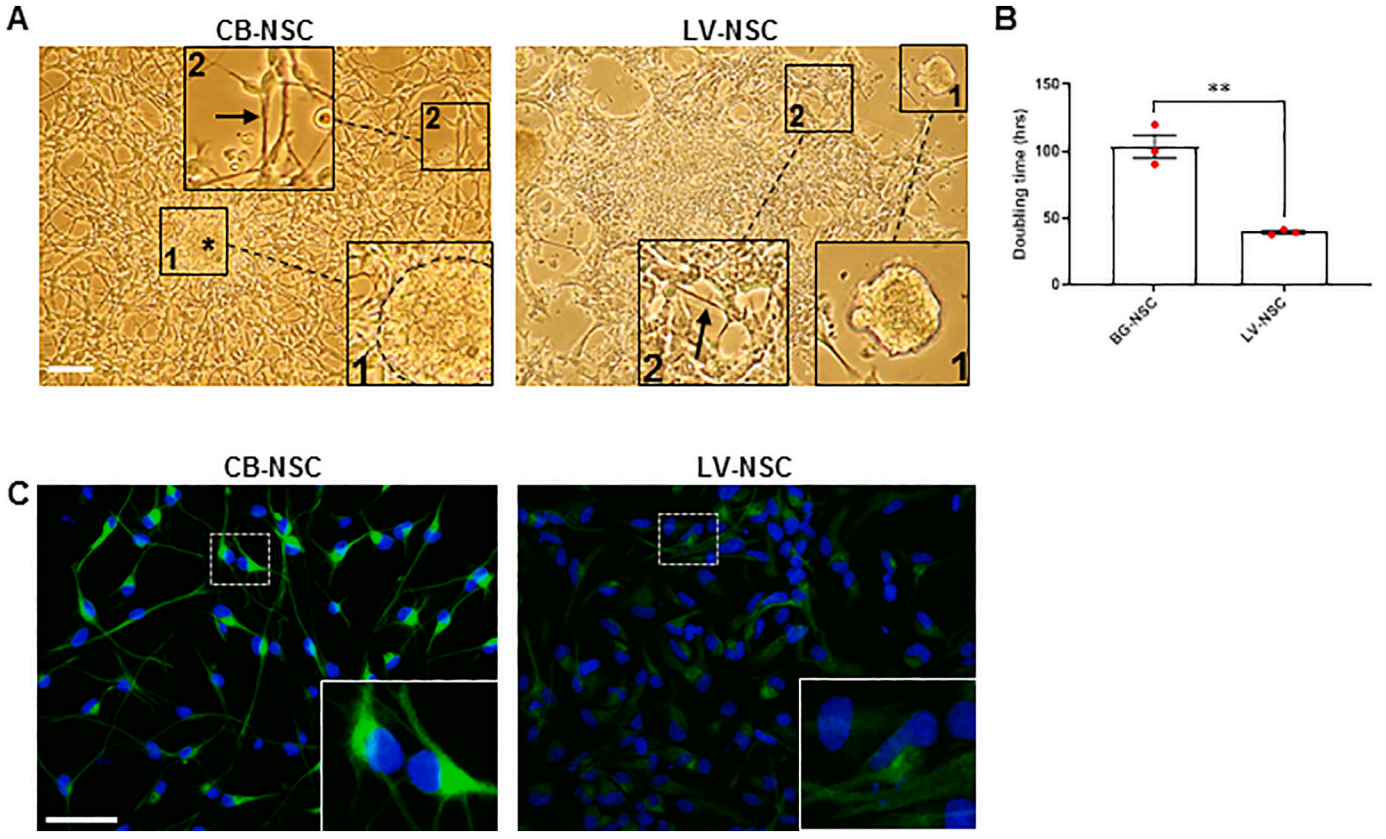

Fig. 2. Comparison of adult CB- and LV- NSC *in vitro*. (A) Brightfield image of CB-NSC (left) and LV-NSC (right) in culture. Box1 show neurosphere structures marked by asterisk, Box 2 shows radial morphology (Black arrow). Scale bar: 125 μm. (B) Doubling time of CB-NSC and LV-NSC ($n = 3$; individual data points shown in red). (C) Immunostaining for Nestin (green) in CB-NSC (left) and LV-NSC (right) cultures. DAPI (blue) used as nuclear counterstain. Scale bar 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

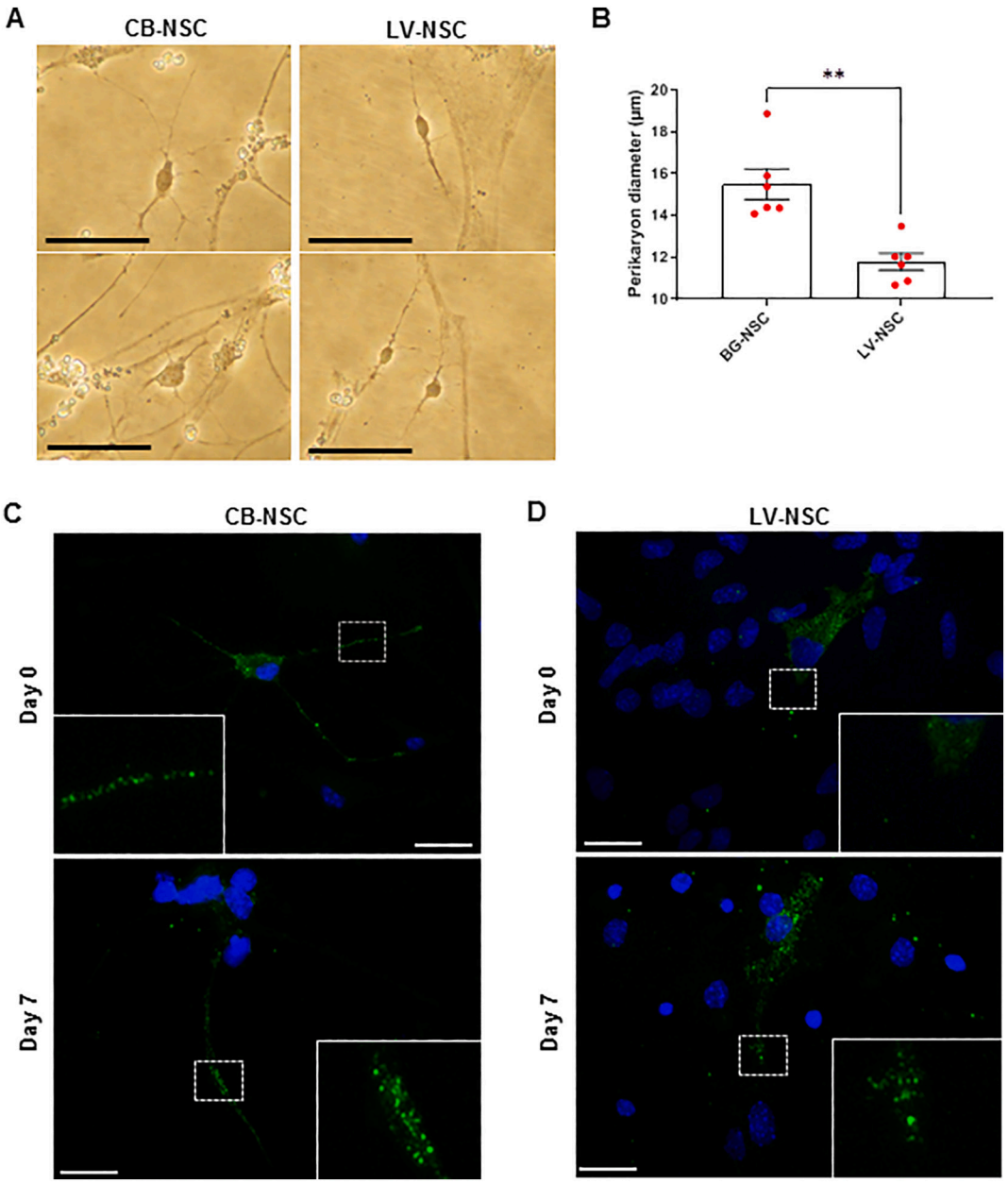

Fig. 3. Differentiation of CB- and LV-NSC *in vitro*. (A) Representative brightfield images showing neuronal bodies (arrows) in differentiated CB-NSC (left) and LV-NSC (right). Scale bar: 125 µm. (B) Perikaryon size measured in CB-NSC and LV-NSC derived neuronal bodies (n = 3; individual data points shown in red). (C—D) Immunostaining for Calbindin (green) in CB-NSC (C) and LV-NSC (D) cells before (top) and after 7 days of differentiation (bottom), with DAPI (blue) used as nuclear counterstain. Scale bar: 25 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In rodents the generation of new neurons has been extensively studied in the classical neurogenic niches of the adult brain: the subventricular zone (SVZ) of the lateral ventricle (LV) and the subgranular zone (SGZ) of the dentate gyrus (Oyarce et al., 2014). In addition to these well-known regions, researchers postulated other potential regions in the adult brain exhibiting neurogenic activity such as the cerebellum, hypothalamic third ventricle, spinal cord, substantia nigra, and amygdala (Oyarce et al., 2014). Although the cerebellum was regarded as a non-neurogenic structure for many years, evidence of some neurogenic activity and the persistence of progenitors in the mature CB has been reported (Alcock and Sottile, 2009; Klein et al., 2005).

4.1. NSC marker expression conserved across mouse, chick, and primate adult BG

Developmental studies have established that the BG population of the cerebellum originates from radial glia, an embryonic progenitor population giving rise to adult NSC in the SVZ and the SGZ (Leto et al., 2016; Yamada and Watanabe, 2002). In addition to a common radial morphology, adult mouse BG also share NSC marker expression such as Sox1, Sox2 and Sox9, and exhibit stem cell properties when isolated *in vitro* (Alcock and Sottile, 2009). The presence of Sox2-positive cells in the adult cerebellum was recently confirmed in mouse, and these were shown to respond to physiological stimuli and increased in number upon local tissue damage (Ahlfeld et al., 2017). These observations portend the hypothesis that the Sox-positive BG population may be mobilisable, responding to damage and thus holding potential as a local source of

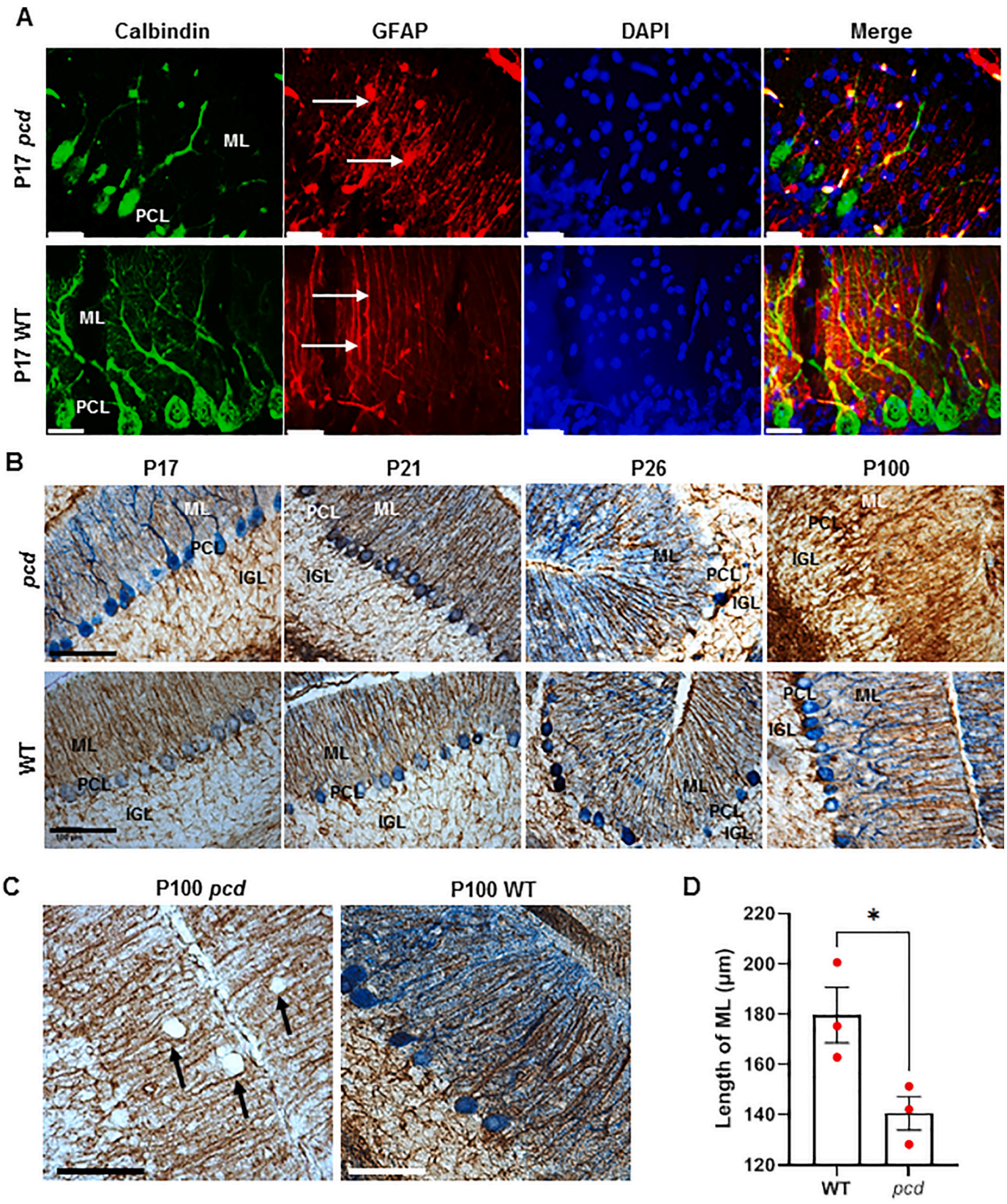

Fig. 4. Postnatal changes in the cerebellum of *pcd* mutant and WT mice. (A) Immunofluorescence staining for GFAP (red) and Calbindin (green) showing a change in arborisation of BG fibers in ML of *pcd* mutant mice at P17 compared to WT control. DAPI was used as a nuclear counterstain. White arrows show continuous radial BG fibers extending to the WT ML, and the thickened bundles in ML of the *pcd* mutant. Scale bar: 30 µm. (B—C) Double immunostaining for GFAP (brown) and Calbindin (blue) showing the BG fibers situated in ML of *pcd* mutant and WT control mice at P17, P21, P26 and P100 (B), with signs of gliosis in the *pcd* Bergman glia at P100. (C) Large vacuoles (black arrows) observed at P100 in the molecular layer. (D) Quantitation of ML length in P100 WT *vs pcd* cerebellar cortex. n = 3 (individual data points shown in red), *$p < 0.05$. Scale bar: (B) 100 µm, (C) 50 µm. P: Postnatal day, WT: Wild type, BG: Bergmann glia, IGL: Internal granular layer, ML: Molecular layer, PCL: Purkinje cell layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

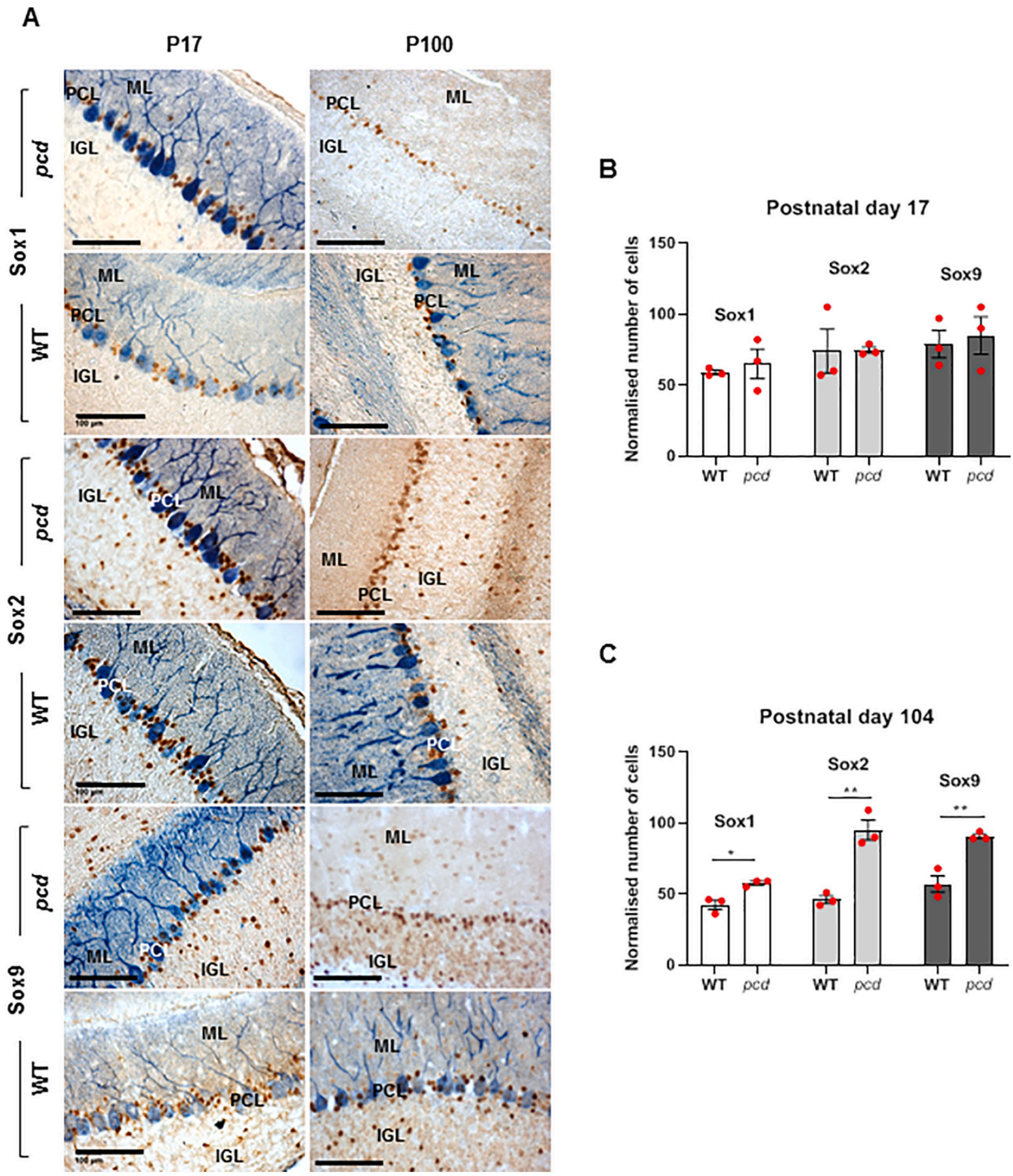

Fig. 5. Changes in Sox-cells in the cerebellum of *pcd* mutant and WT mice. (A) Immunostaining for Sox1, Sox2, Sox9, marking the BG population (brown) in the PCL adjacent to Purkinje neurons marked by Calbindin (blue) in *pcd* mutant and WT control mice, analysed at postnatal day 17 (P17) and 100 (P100). Scale bar: 100 μm. (B—C) Quantification of Sox-positive Bergmann glia in *pcd* mutant and WT mice at P17 (B) and P104 (C). Normalised data shown as number of cells per 0.7 mm section of PCL. (mean ± SEM, n = 3, individual data points shown in red). *p < 0.05. IGL, internal granular layer; ML, molecular layer; PCL, Purkinje cell layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NSC that could play a role in cerebellum repair. Interestingly, a recent ageing study described a medicinal compound able to induce the appearance of a PCNA-positive signal in the cerebellar cortex of adult mice, although the precise nature of these responsive cells was unconfirmed (Ratto et al., 2019).

Here, BG distribution and expression of NSC-associated markers Sox1, Sox2, Sox9, GFAP and BLBP was confirmed in the chick and primate adult cerebellum, indicating this is a conserved feature of adult BG among vertebrates. This Sox-positive population in the adult CB did not show any detectable expression *in vivo* for Nestin and Ki67, which are markers typically associated with proliferative neuroprogenitors *in vitro* (Faiz et al., 2015; Hendrickson et al., 2011) but reportedly absent in quiescent stem cells *in vivo* (Dahlstrand et al., 2014; Gilyarov, 2008; Lendahl et al., 1990; Morshead et al., 1994; Wiese et al., 2004). Additional corroboration for this result was found using the Allen Brain Atlas (https://www.brain-map.org, Lein et al., 2007) indicating the absence

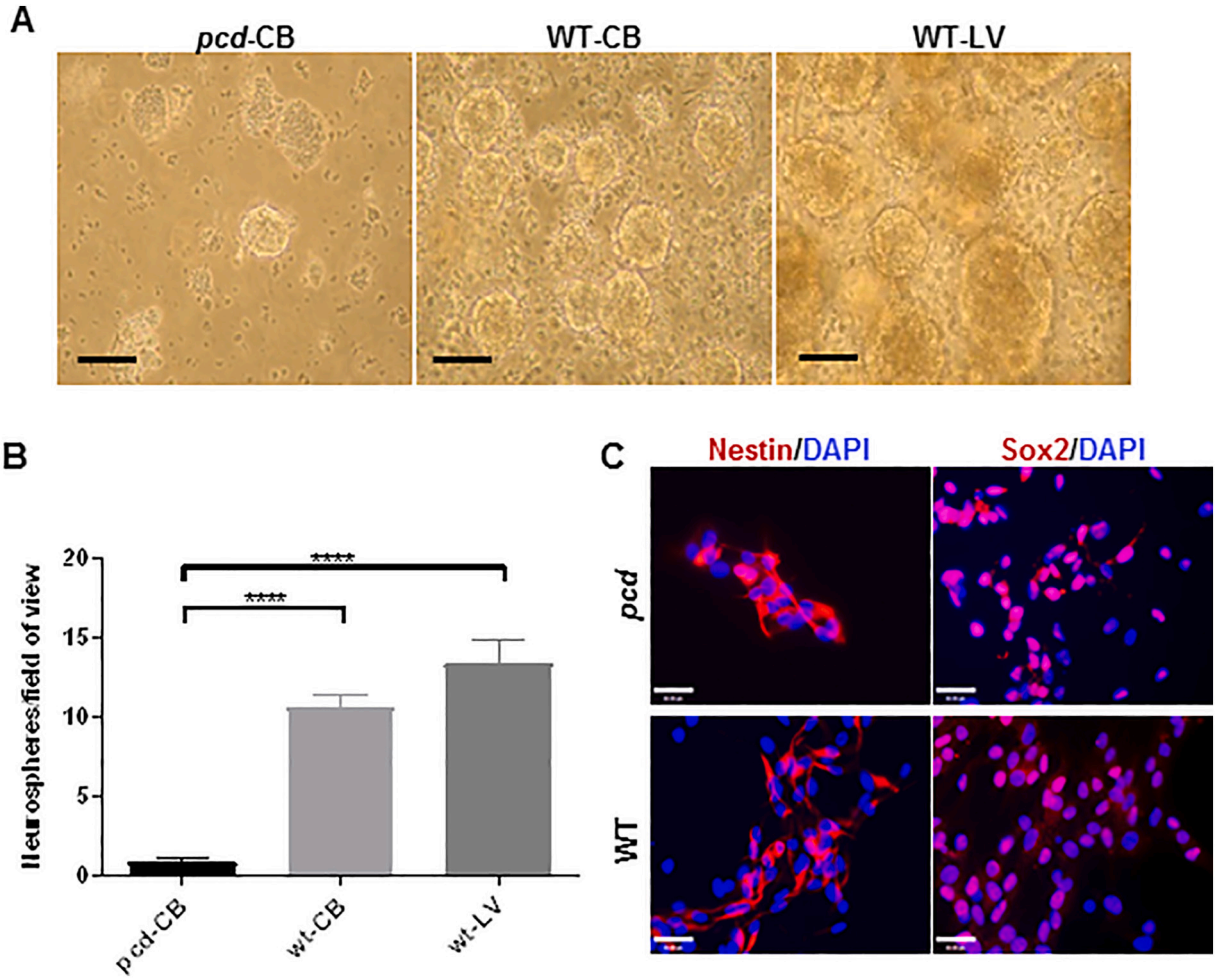

Fig. 6. Cultures isolated from *pcd* and wild type CB. (A) Brightfield images of passage 2 neurospheres from *pcd* and controls, compared to LV controls. Scale bar: 100 μm. (B) The number of neurospheres in *pcd* culture was significantly lower than in cultures from controls. (C) Immunostaining for Nestin and Sox2 expression (red) in CB-derived cultures from *pcd* and WT mice at P21, with DAPI nuclear counterstain (blue). Scale bar: 60 μm. Results are presented as mean ± SEM; $p < 0.05$* calculated by ANOVA with Tukey's multiple comparisons test. $n = 5$. CB: Cerebellum, WT: wild type. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of Nestin transcript in the PCL of the adult mouse cerebellum (Supplementary Fig. S1). This is in contrast with a recent investigation reporting Nestin/Sox2 double positive cells in the adult mouse PCL (Ahlfeld et al., 2017), however no Nestin-positive cells were detected in the PCL of the adult CB used in this study.

When CB-NSC were isolated from P21 *pcd* mutants, cultures grew but produced fewer neurospheres compared to WT controls. Once in culture, these cells still displayed expression of neural stem cell markers Nestin and Sox2, suggesting that adult BG cells are responsive to mitogenic NSC culture conditions. This observation fits with reports that mature BG are able to express Nestin transiently *in situ*, in response to embryonic PC grafts into adult mice (Sotelo et al., 1994), suggesting adult BG can be mobilised *in vitro* and *in vivo*. Studies in zebrafish models have shown that Nestin expression is induced in the cerebellum upon injury, and that Nestin-positive cells can differentiate to replace lost neurons (Kaslin et al., 2013). It has also been shown that Nestin-positive cells in the postnatal PCL, where BG reside, can undergo adaptive reprogramming to replenish irradiated cells in the External Germinal Layer (EGL) of the developing mouse cerebellum (Wojcinski et al., 2017). These reports suggest that Nestin upregulation could prime BG to an activated stem cell-like state for proliferation and neurogenesis *in vivo* and *in vitro*. Future investigations using genetic lineage tracing techniques could help track the origin and fate of Nestin-expressing BG in the cerebellum and explore any fine differences across tissue regions (Dubois et al., 2006; Tsien, 2016).

4.2. BG differentiation features in vitro

Expression of Sox1, Sox2 and Sox9 has been associated with NSC potential, and Sox-positive BG cells isolated *in vitro* have been shown to produce βIII-Tubulin-positive neurons (Alcock and Sottile, 2009), although evidence of their neurogenic potential *in vivo* is lacking. There is limited evidence that PC could be generated *de novo* from adult progenitors. In past studies, adult NSC have failed to generate PC *in vivo* and their efficiency to produce PC *in vitro* was reportedly poor (4.3 ± 0.7%) (Klein et al., 2005), while the *in vitro* maturation of embryonic and adult cerebellum-derived stem cells into Calbindin-positive cells has been described using feeder layers from dissociated E15.5 mouse rhombic lip tissue, which contained GC (Klein et al., 2005; Muguruma et al., 2010, 2015). A subsequent study further suggested the use of fetal cerebellum slices might be required for PC progenitors to become electrophysiologically mature PC (Wang et al., 2015).

Here, CB-NSC differentiated on Geltrex-coated coverslips for up to 7 days *in vitro* produced larger neuronal bodies compared to LV-NSC, a characteristic related to PC *in vivo* (Herndon, 1963). This observation aligns with independent reports that progenitors derived from the cerebellum have distinct intrinsic regional identity compared to SVZ derived NSC (Klein et al., 2005). Although CB-NSC-derived cells showed

Calbindin expression in their neural extensions (in contrast to LV-NSC derived cultures), only very few cells expressed Calbindin, and no cells showed the full PC morphology with dendritic branching. This may not be totally unexpected, as the *in vitro* environment provided by the Geltrex coating could lack many of the signalling and physico-chemical cues required to provide an adequate supporting niche for PC maturation (Klein et al., 2005; Muguruma et al., 2010, 2015; Wang et al., 2015), in addition to the possible requirement of an extended period for differentiation. Along with structural support, acellular tissue components including extracellular vesicles from neighbouring cells may be involved in neuronal differentiation of PC, as described for other processes of neuronal communication and maturation (Frühbeis et al., 2013; Takeda and Xu, 2015), and may still be missing from current culture protocols. Future efforts to optimise and replicate the cellular and acellular requirements of adult BG culture, and systematically survey differentiation markers will be necessary to fully evaluate their differentiation abilities *in vitro*.

4.3. BG changes in pcd mutant

Reactive glia represent proliferating cells, including astrocytes and oligodendrocytes which are found after a lesion or stab wound injury (Pekny and Nilsson, 2005). These cells express NSC markers and have shown potential to generate neurons *in vitro* (Götz et al., 2015), however their *in vivo* potential to produce neurons is still unclear. A previous study reported that GFAP-labelled reactive astrocytes from cortical stab wound lesions formed neurospheres and produced βIII-Tubulin positive neurons, GFAP positive astrocytes and O4 positive oligodendrocytes *in vitro*, but did not produce neurons *in vivo* (Shimada et al., 2012). In the disease model used here, BG showed features of reactive astrocytes *in vivo*, including increased GFAP expression, the appearance of vacuolar structures within the molecular layer (where processes appeared disorganised). The loss of PC was associated with a significant increase in the Sox-positive population at late stages of degeneration, as measured relative to the ML tissue size. It would now be valuable to refine this observation and narrow down the precise stage at which the BG number increases in mutant mice, which could be determined by a systematic time-point analysis between P20 and P100 complemented by an analysis of proliferation markers and BrdU label incorporation. The properties of CB-NSC isolated from *pcd* mutants could also be further analysed at different stages of PC degeneration to evaluate any cellular changes occurring in the Sox-positive population as a result of the neurodegeneration process, while a parallel *in vitro* characterisation of marker expression and differentiation potential could help expose phenotypic changes in the BG population. This may help address the apparent divergence of proliferative levels observed for P21 CB-NSC cells *in vitro* and *in vivo* in mature CB tissue. The question of their neurogenic potential *in vivo* remains unclear, and could be tested in the future using *in vivo* labelling approaches and marker analysis in the mutant model. Even though reactive glia are not considered to readily produce neurons *in vivo* (Aravantinou-Fatorou et al., 2015; Faiz et al., 2015; Shimada et al., 2012; Torper and Götz, 2017), new genetic targeting strategies have opened the prospect of a direct conversion of astroglia towards neuronal lineages (Heinrich et al., 2010, 2011) suggesting that this population in the adult cerebellum may represent a target of choice to promote cellular replacement in ataxic tissue.

5. Conclusion

The results presented demonstrate the conservation of a cell population expressing several markers common to neural progenitor cells in the mouse, primate, and chick adult cerebellum. These Sox-positive cells were detected around the PC bodies, consistent with the Bergmann glia population. Following the postnatal loss of PC in a neurodegenerative mouse model, these Sox-positive cells remained visible in the PCL, showing some process disorganisation with increased GFAP reactivity, and at late time-points these cells were observed to increase in numbers in the disease model compared to matching controls. These results show that the Sox-positive cells present in adult cerebellum are activated in case of PC loss. The physiological basis and possible impact of this cell response in terms of cerebellar regeneration are unclear. Although these cells display some stem cell characteristics, their potential *in vivo* may be dependent on the local tissue's ability to provide the molecular signals needed for neuronal repair. These observations call for investigative strategies seeking to target this responsive cerebellar population *in vivo*, and assess its capacity to produce new PC if exposed to an inductive environment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2021.113950.

Declaration of Competing Interest

The authors declare they have no competing interests.

Acknowledgements

We are grateful to C. Lopez-Margolles and M. Barbadillo-Muñoz for their invaluable help with tissue handling. This work was supported by the Engineering and Physical Sciences Research Council [grant number 1118937] (ND) and a scholarship from the Ministry of Higher Education-Kurdistan Regional Government (SS). A kind donation from R. Jones in the memory of A. Stacey is gratefully acknowledged. VS is supported by a grant from the Italian Ministry of Education, University and Research (MIUR) to the Department of Molecular Medicine of the University of Pavia under the initiative 'Dipartimenti di Eccellenza (2018-2022)'.

References

Ahlfeld, J., Filser, S., Schmidt, F., Wefers, A.K., Merk, D.J., Glaß, R., Herms, J., Schüller, U., 2017. Neurogenesis from Sox2 expressing cells in the adult cerebellar cortex. Sci. Rep. 7, 6137. https://doi.org/10.1038/s41598-017-06150-x.

Alcock, J., Sottile, V., 2009. Dynamic distribution and stem cell characteristics of Sox1-expressing cells in the cerebellar cortex. Cell Res. 19, 1324–1333. https://doi.org/10.1038/cr.2009.119.

Altman, J., Das, G.D., 1966. Autoradiographic and histological studies of postnatal neurogenesis. I. a longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions. J. Comp. Neurol. 126, 337–389. https://doi.org/10.1002/cne.901260302.

Aravantinou-Fatorou, K., Ortega, F., Chroni-Tzartou, D., Antoniou, N., Poulopoulou, C., Politis, P.K., Berninger, B., Matsas, R., Thomaidou, D., 2015. CEND1 and NEUROGENIN2 reprogram mouse astrocytes and embryonic fibroblasts to induced neural precursors and differentiated neurons. Stem Cell Rep. 5, 405–418. https://doi.org/10.1016/j.stemcr.2015.07.012.

Baltanás, F.C., Casafont, I., Lafarga, V., Weruaga, E., Alonso, J.R., Berciano, M.T., Lafarga, M., 2011. Purkinje cell degeneration in pcd mice reveals large scale chromatin reorganization and gene silencing linked to defective DNA repair. J. Biol. Chem. 286, 28287–28302. https://doi.org/10.1074/jbc.M111.246041.

Barski, J.J., Hartmann, J., Rose, C.R., Hoebeek, F., Mörl, K., Noll-Hussong, M., De Zeeuw, C.I., Konnerth, A., Meyer, M., 2003. Calbindin in cerebellar Purkinje cells is a critical determinant of the precision of motor coordination. J. Neurosci. 23, 3469–3477. https://doi.org/10.1523/JNEUROSCI.23-08-03469.2003.

Carson, F.L., Hladik, C., Cappellano, C.H., 2015. Histotechnology: A Self-Instructional Text. American Society for Clinical Pathology, Chicago.

Cendelin, J., 2014. From mice to men: lessons from mutant ataxic mice. Cerebellum Ataxias 1. https://doi.org/10.1186/2053-8871-1-4.

Chakrabarti, L., Neal, J.T., Miles, M., Martinez, R.A., Smith, A.C., Sopher, B.L., La Spada, A.L., 2006. The Purkinje cell degeneration 5J mutation is a single amino acid insertion that destabilizes Nna1 protein. Mamm. Genome 17, 103–110. https://doi.org/10.1007/s00335-005-0096-x.

Chakrabarti, L., Eng, J., Ivanov, N., Garden, G.A., La Spada, A.R., 2009. Autophagy activation and enhanced mitophagy characterize the Purkinje cells of pcd mice prior to neuronal death. Mol. Brain 2, 24. https://doi.org/10.1186/1756-6606-2-24.

Dahlstrand, J., Lardelli, M., Lendahl, U., 2014. Nestin mRNA Expression Correlates with the Central Nervous System Progenitor Cell State in Many, But Not All, Regions of Developing Central Nervous System, vol. 84, pp. 109–129. https://doi.org/10.1016/0165-3806(94)00162-S.

Dubois, N.C., Hofmann, D., Kaloulis, K., Bishop, J.M., Trumpp, A., 2006. Nestin-Cre transgenic mouse line Nes-Cre1 mediates highly efficient Cre/loxP mediated recombination in the nervous system, kidney, and somite-derived tissues. Genesis 44, 355–360. https://doi.org/10.1002/dvg.20226.

Faiz, M., Sachewsky, N., Gascón, S., Bang, K.W.A., Morshead, C.M., Nagy, A., 2015. Adult neural stem cells from the subventricular zone give rise to reactive astrocytes in the cortex after stroke. Cell Stem Cell 17, 624–634. https://doi.org/10.1016/j.stem.2015.08.002.

Feng, L., Hatten, M.E., Heintz, N., 1994. Brain lipid-binding protein (BLBP): a novel signaling system in the developing mammalian CNS. Neuron 12, 895–908. https://doi.org/10.1016/0896-6273(94)90341-7.

Frühbeis, C., Fröhlich, D., Kuo, W.P., Krämer-Albers, E.M., 2013. Extracellular vesicles as mediators of neuron-glia communication. Front. Cell. Neurosci. 7, 182. https://doi.org/10.3389/fncel.2013.00182.

Garcia-Segura, L.M., Baetens, D., Roth, J., Norman, A.W., Orci, L., 1984. Immunohistochemical mapping of calcium-binding protein immunoreactivity in the rat central nervous system. Brain Res. 296, 75–86. https://doi.org/10.1016/0006-8993(84)90512-2.

Gilyarov, A.V., 2008. Nestin in central nervous system cells. Neurosci. Behav. Physiol. 38, 165–169. https://doi.org/10.1007/s11055-008-0025-z.

Götz, M., Sirko, S., Beckers, J., Irmler, M., 2015. Reactive astrocytes as neural stem or progenitor cells: in vivo lineage, in vitro potential, and genome-wide expression analysis. Glia 63, 1452–1468.

Heinrich, C., Blum, R., Gascón, S., Masserdotti, G., Tripathi, P., Sánchez, R., Tiedt, S., Schroeder, T., Götz, M., Berninger, B., 2010. Directing astroglia from the cerebral cortex into subtype specific functional neurons. PLoS Biol. 8, e1000373 https://doi.org/10.1371/journal.pbio.1000373.

Heinrich, C., Gascón, S., Masserdotti, G., Lepier, A., Sanchez, R., Simon-Ebert, T., Schroeder, T., Götz, M., Berninger, B., 2011. Generation of subtype-specific neurons from postnatal astroglia of the mouse cerebral cortex. Nat. Protoc. 6, 214–228. https://doi.org/10.1038/nprot.2010.188.

Hendrickson, M.L., Rao, A.J., Demerdash, O.N.A., Kalil, R.E., 2011. Expression of nestin by neural cells in the adult rat and human brain. PLoS One 6, e18535. https://doi.org/10.1371/journal.pone.0018535.

Herndon, R.M., 1963. The fine structure of the purkinje cell. J. Cell Biol. 18, 167–180.

Kaslin, J., Kroehne, V., Benato, F., Argenton, F., Brand, M., 2013. Development and specification of cerebellar stem and progenitor cells in zebrafish: from embryo to adult. Neural Dev. 8, 9. https://doi.org/10.1186/1749-8104-8-9.

Klein, C., Butt, S.J.B., Machold, R.P., Johnson, J.E., Fishell, G., 2005. Cerebellum- and forebrain-derived stem cells possess intrinsic regional character. Development 132, 4497–4508. https://doi.org/10.1242/dev.02037.

Lee, A., Kessler, J.D., Read, T.A., Kaiser, C., Corbeil, D., Huttner, W.B., Johnson, J.E., Wechsler-Reya, R.J., 2005. Isolation of neural stem cells from the postnatal cerebellum. Nat. Neurosci. 8, 723–729. https://doi.org/10.1038/nn1473.

Lein, E.S., et al., 2007. Genome-wide atlas of gene expression in the adult mouse brain. Nature 445, 168–176. https://doi.org/10.1038/nature05453.

Lendahl, U., Zimmerman, L.B., McKay, R.D., 1990. CNS stem cells express a new class of intermediate filament protein. Cell 60, 585–595.

Leto, K., Arancillo, M., Becker, E.B., Buffo, A., Chiang, C., Ding, B., Dobyns, W.B., Dusart, I., Haldipur, P., Hatten, M.E., Hoshino, M., Joyner, A.L., Kano, M., Kilpatrick, D.L., Koibuchi, N., Marino, S., Martinez, S., Millen, K.J., Millner, T.O., Miyata, T., Parmigiani, E., Schilling, K., Sekerková, G., Sillitoe, R.V., Sotelo, C., Uesaka, N., Wefers, A., Wingate, R.J., Hawkes, R., 2016. Consensus paper: cerebellar development. Cerebellum 15, 789–828. https://doi.org/10.1007/s12311-015-0724-2.

Lim, J., Hao, T., Shaw, C., Patel, A.J., Szabo, G., Rual, J.F., Fisk, C.J., Li, N., Smolyar, A., Hill, D.E., Barabási, A.L., Vidal, M., Zoghbi, H.Y., 2006. A protein-protein interaction network for human inherited ataxias and disorders of Purkinje cell degeneration. Cell 125, 801–814. https://doi.org/10.1016/j.cell.2006.03.032.

Mamber, C., Kamphuis, W., Haring, N.L., Peprah, N., Middeldorp, J., Hol, E.M., 2012. GFAPδ expression in glia of the developmental and adolescent mouse brain. PLoS One 7, e52659. https://doi.org/10.1371/journal.pone.0052659.

Ming, G., Song, H., 2011. Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron 70, 687–702. https://doi.org/10.1016/j.neuron.2011.05.001.

Morshead, C.M., Reynolds, B.A., Craig, C.G., McBurney, M.W., Staines, W.A., Morassutti, D., Weiss, S., van der Kooy, D., 1994. Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron 13, 1071–1082.

Muguruma, K., Nishiyama, A., Ono, Y., Miyawaki, H., Mizuhara, E., Hori, S., Kakizuka, A., Obata, K., Yanagawa, Y., Hirano, T., Sasai, Y., 2010. Ontogeny-recapitulating generation and tissue integration of ES cell-derived Purkinje cells. Nat. Neurosci. 13, 1171–1180. https://doi.org/10.1038/nn.2638.

Muguruma, K., Nishiyama, A., Kawakami, H., Hashimoto, K., Sasai, Y., 2015. Self-organization of polarized cerebellar tissue in 3D culture of human pluripotent stem cells. Cell Rep. 10, 537–550. https://doi.org/10.1016/j.celrep.2014.12.051.

Mullen, R.J., Eicher, E.M., Sidman, R.L., 1976. Purkinje cell degeneration, a new neurological mutation in the mouse. Proc. Natl. Acad. Sci. U. S. A. 73, 208–212.

Namimatsu, S., Ghazizadeh, M., Sugisaki, Y., 2005. Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. J. Histochem. Cytochem. 53, 3–11. https://doi.org/10.1177/002215540505300102.

Oyarce, K., Bongarzone, E.R., Nualart, F., 2014. Unconventional neurogenic niches and neurogenesis modulation by vitamins. J. Stem Cell Res. Ther. 4, 184. https://doi.org/10.4172/2157-7633.1000184.

Pekny, M., Nilsson, M., 2005. Astrocyte activation and reactive gliosis. Glia 50, 427–434. https://doi.org/10.1002/glia.20207.

Ratto, D., Corana, F., Mannucci, B., Priori, E.C., Cobelli, F., Roda, E., Ferrari, B., Occhinegro, A., Di Iorio, C., De Luca, F., Cesaroni, V., Girometta, C., Bottone, M.G., Savino, E., Kawagishi, H., Rossi, P., 2019. Hericium erinaceus improves recognition memory and induces hippocampal and cerebellar neurogenesis in frail mice during aging. Nutrients 11 (4), 715. https://doi.org/10.3390/nu11040715.

Shi, S.R., Chaiwun, B., Young, L., Cote, R.J., Taylor, C.R., 1993. Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. J. Histochem. Cytochem. 41, 1599–1604. https://doi.org/10.1177/41.11.7691930.

Shimada, I.S., LeComte, M.D., Granger, J.C., Quinlan, N.J., Spees, J.L., 2012. Self-renewal and differentiation of reactive astrocyte-derived neural stem/progenitor cells isolated from the cortical peri-infarct area after stroke. J. Neurosci. 32, 7926–7940. https://doi.org/10.1523/JNEUROSCI.4303-11.2012.

Sotelo, C., Alvarado-Mallart, R.M., Frain, M., Vernet, M., 1994. Molecular plasticity of adult Bergmann fibers is associated with radial migration of grafted Purkinje cells. J. Neurosci. 14, 124–133.

Sottile, V., Li, M., Scotting, P.J., 2006. Stem cell marker expression in the Bergmann glia population of the adult mouse brain. Brain Res. 1099, 8–17.

Su, X., Guan, W., Yu, Y.-C., Fu, Y., 2014. Cerebellar stem cells do not produce neurons and astrocytes in adult mouse. Biochem. Biophys. Res. Commun. 450, 378–383. https://doi.org/10.1016/j.bbrc.2014.05.131.

Takeda, Y.S., Xu, Q., 2015. Neuronal differentiation of human Mesenchymal stem cells using Exosomes derived from differentiating neuronal cells. PLoS One 10, e0135111. https://doi.org/10.1371/journal.pone.0135111.

Torper, O., Götz, M., 2017. Brain repair from intrinsic cell sources: turning reactive glia into neurons. Prog. Brain Res. 230, 69–97. https://doi.org/10.1016/bs.pbr.2016.12.010.

Tsien, J.Z., 2016. Cre-lox Neurogenetics: 20 years of versatile applications in brain research and counting. Front. Genet. 7 https://doi.org/10.3389/fgene.2016.00019.

Wang, S., Wang, B., Pan, N., Fu, L., Wang, C., Song, G., An, J., Liu, Z., Zhu, W., Guan, Y., Xu, Z.Q.D., Chan, P., Chen, Z., Zhang, Y.A., 2015. Differentiation of human induced pluripotent stem cells to mature functional Purkinje neurons. Sci. Rep. 5, 9232. https://doi.org/10.1038/srep09232.

Wiese, C., Rolletschek, A., Kania, G., Blyszczuk, P., Tarasov, K.V., Tarasova, Y., Wersto, R.P., Boheler, K.R., Wobus, A.M., 2004. Nestin expression–a property of multi-lineage progenitor cells? Cell. Mol. Life Sci. 61, 2510–2522. https://doi.org/10.1007/s00018-004-4144-6.

Wojcinski, A., Lawton, A.K., Bayin, N.S., Lao, Z., Stephen, D.N., Joyner, A.L., 2017. Cerebellar granule cell replenishment postinjury by adaptive reprogramming of nestin+ progenitors. Nat. Neurosci. 20, 1361–1370. https://doi.org/10.1038/nn.4621.

Yamada, K., Watanabe, M., 2002. Cytodifferentiation of Bergmann glia and its relationship with purkinje cells. Anat. Sci. Int. 77, 94–108. https://doi.org/10.1046/j.0022-7722.2002.00021.x.