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Article

Role of Transcription Factor Zbtb20 in Developing Cerebellum of Rodents: Regulation of Purkinje Cells Generation and Distribution

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Abstract

Development of cerebellum is a compound process, taking place pre- and postnatally. It is governed by multiple intra- and extracellular factors. Many studies have revealed the molecular mechanisms regulating different aspects of cerebellar development and related pathological entities. However, our understanding about certain factors modulating cerebellar development is still insufficient. Natural mutants and genetically engineered animal models allow to target particular gene products and to understand their role. Therefore, we performed experiments on homozygous knockout mice (Zbtb20^{-/-}) and wild-type animals (controls) to examine the effect of transcription factor Zbtb20 on Purkinje cells (PCs) development during the early postnatal (P) stages – days 4, 8, and 12. Parasagittal sections were treated immunocytochemically with antibody against the PCs' marker Calbindin. Zbtb20 showed time- and space-differential effect: on P4, the lack of Zbtb20 resulted in total upregulation of PCs number, and selectively in folia 3, 8, 9 and 10; on P8 only folium 3 and 10 in the mutants had higher PCs number, while on P12 the opposite was observed – lower PCs' number in the mutants. Additionally, a difference in the PCs' dendritic arborization was detected in Zbtb20^{-/-}. To the best of our knowledge, this is the first study on the role of Zbtb20 on early postnatal PCs development. These findings provide new insights to the cerebellar development, and could have a significant clinical relevance.

Keywords: cerebellum; Zbtb20 knockout mice; transcription factor Zbtb20; development; cerebellar folia; Purkinje cells; Calbindin

1. Introduction

Cerebellum is part of the central nervous system (CNS) playing a pivotal role in motor control. However, it also has cognitive functions such as attention and language, as well as emotional control regarding fear and pleasure responses [1,2]. Cerebellar development has been extensively studied for more than a century. Fundamental studies of Ramon y Cajal provided the first evidence for the neuron theory and created a complete scheme of the cerebellum circuitry [3,4]. His concept that the Purkinje cells (PCs) are continuously varying population is still considered accurate [5,6].

Development of cerebellum is a compound process related to extensive rearrangement of a limited array of specific cell types. The cerebellar primordium derives mainly from the dorsal (alar) neural epithelium lining the first rhombomere, but part of the mesencephalic vesicle also contributes, forming the cerebellar vermis [7,8]. In mice cerebellar development starts at about embryonic (E) day 9 when the neural tube bends and forms the rhombic flexure, followed by migration of the dorsal part of the neural tube close to the midline, which gets displaced laterally. Thus, the fourth ventricle is formed and the primordium of the cerebellum appeared as bilaterally symmetric bulges in the roof of the ventricle at E12.5 [9]. By E15.5 the cerebellar anlage transforms into a smooth cylindrical structure, wrapped around the dorsal part of the brain [7]. The first two postnatal weeks are the time

when folds of cerebellum develop, and three regions become discernible – centrally located vermis, two hemispheres laterally, and the lateral-most paraflocculi. Within each of the regions specific fissures appear along the anterior-posterior axis.

The cytoarchitecture of the cerebellar folia is highly conserved in all mammals. It is relatively simple: only a few cell types (Purkinje, stellate, basket, Golgi, granule cells, Lugaro cells, and unipolar brush cells) are arranged into three layers - from superficially to deep they are known as molecular layer, consisting of interneurons and glial cells, a monolayer of Purkinje cells (PCs) in the middle, and internal granule cell layer [10] (Figure 1). Granule cells are the only excitatory neurons, while all the other are inhibitory [11]. PCs constitute the principal neurons in cerebellar cortex and they provide the only output of it. Their loss is related not only to several neurological disorders such as cerebellar ataxia [12], autism spectrum disorders [13] and progressive myoclonic epilepsies [14] but also to specific peripheral immune alterations [15]. However, our knowledge about the events regulating their development is still limited.

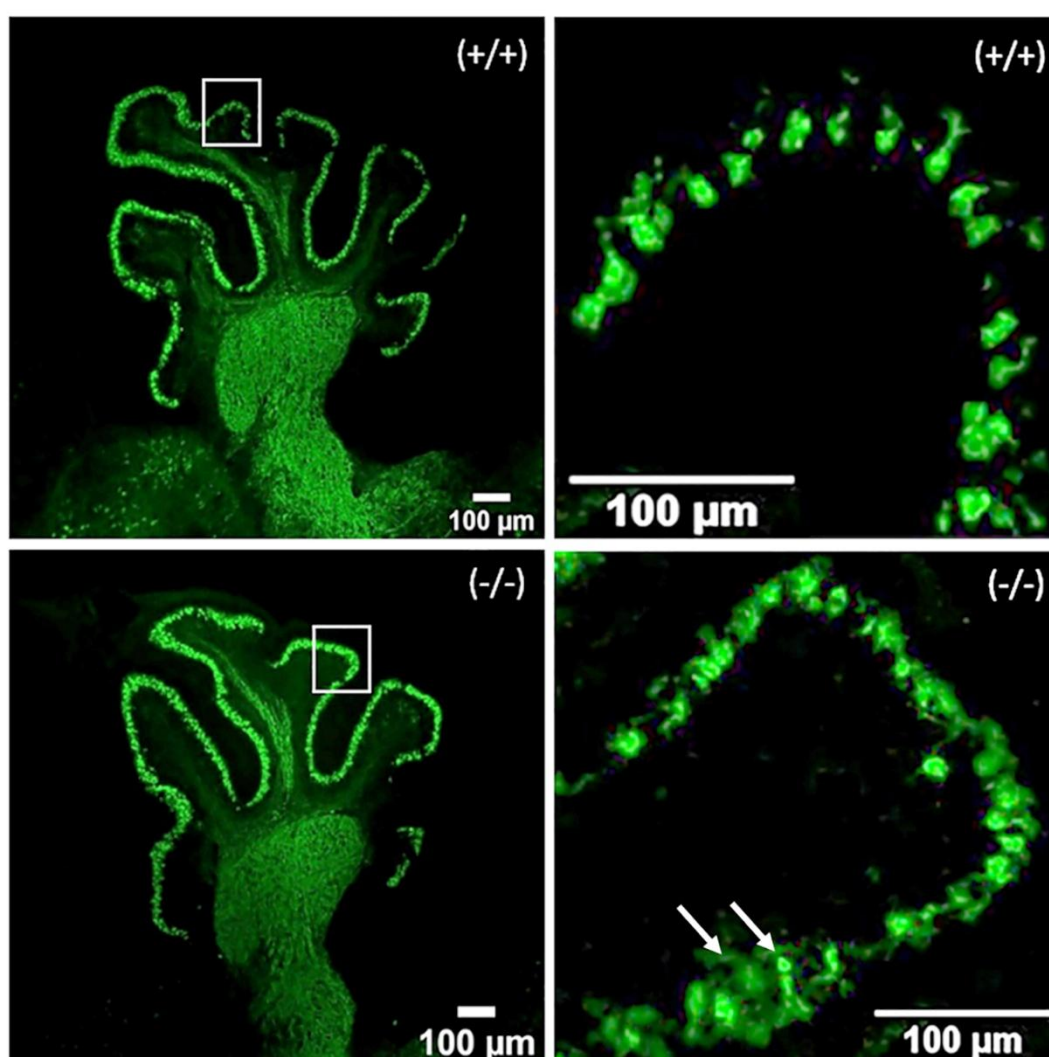


Figure 1. Fluorescent images of parasagittal sections of cerebella from WT ($Zbtb^{+/+}$) and mutant mice ($Zbtb^{-/-}$). Specimens are stained for immunodetection of the PCs marker Calbindin. PCs (green) somas are not yet completely arranged in a thin single-celled layer; dendritic trees are not fully developed, though single or two primary dendritic branches are discernable. Axons (arrows) undergo development as well.

Cerebellar development depends on multiple factors, molecular and cellular, including growth factors, hormones, transcription factors and epigenetic factors, as well as cell-to-cell interactions. Zinc fingers are the largest group of transcription factors with the finger domain, which play a pivotal role

in gene regulation [16]. The zinc finger and BTB domain-containing 20 (Zbtb20) gene encodes for a transcription factor, involved in pre- and postnatal development, as well as in a range of pathological conditions [17,18]. As a transcription factor, Zbtb20 acts as a molecular switch, guaranteeing that genes regulating various cellular processes, including autophagy, apoptosis, differentiation, metabolism, and immune responses, depending on the specific cell type, are expressed at the appropriate time.

Existence of natural mutants hampering cerebellar development and functioning, together with genetically engineered animal models, allowed to target particular gene products in certain cell lineages, thus revealing their developmental and functional implication [19,20]. Zbtb20 gene of the mutants lacks the functionally important BTB domain of the protein, as well as the first zinc finger. Knockout of Zbtb20 results in direct or indirect downregulation of cell division rates in a variety of cell types, including chondrocytes, post-stroke astrocytes, and cortical radial glia progenitors [21–23]. Conditional deletion of Zbtb20 in cortical progenitors, and to lesser extent in differentiating neurons, results in an increase in the number of neurons in layer IV at the expense of layers II and III [24]. Increased Zbtb20 expression in patients with hepatocellular carcinoma is associated with poor clinical prognosis and high relapse rate [25,26]. However, the role of Zbtb20 in cerebellar development, particularly in the PCs genesis and function, has not been studied yet. Understanding the mechanisms behind those processes may pave the road to potential intervention in variety of pathological entities related to PCs insufficient development or degeneration.

2. Results

In order to explore the effect of transcription factor Zbtb20 on the PCs development, we examined specimens from wild-type mice (WT, Zbtb20^{+/+}), used as controls, and knockout mice (Zbtb20^{-/-}), referred to as mutants, at different postnatal stages: postnatal (P) day 4, 8, and 12. The PCs were detected via immunofluorescent method using antibody against Calbindin (CB), a specific marker of these cells. Quantitative analysis was performed to compare the time- and space distribution of PCs in corresponding sections of cerebellum from the two experimental groups. Cell number was counted as average per cerebellar folium, per 1 mm length of the Purkinje layer, and in 10 000 μm^2 from regions of interest (ROI). The latter was particularly important for the comparative analysis of specimens at the early developmental stage (P4), when PCs are not yet arranged in a monolayer.

2.1. Development of PCs at Postnatal Day 4

At postnatal day 4, PCs are undergoing significant changes. At this stage, some of the unique features of the PCs are already noticeable – cells have more or less stellate appearance, somas are not yet arranged in a thin single-celled layer; the dendritic trees and axons undergo rapid development. Overview image of a parasagittal sections of cerebellum from a wild-type (+/+) and knockout mouse (-/-) is shown in Figure 1

Quantitative comparison of PCs distribution among the folia indicated that in most of the folia of the mutant cerebellum the number of PCs per areas of 10 000 μm^2 is higher than in the controls (Figure 2).

Additionally, the number of cells per 1 mm length of randomly chosen areas of the Purkinje layer, without taking into consideration the number of the folium, was counted. Statistical analysis indicated that the average number was significantly higher in the Zbtb20^{-/-} mutants (27 cells/1 mm) compared with the wild-type animals (25 cells/1 mm) (Figure 3). Similar correlation was observed when the total number of PCs per corresponding cerebellar sections were compared: in the mutants - 270 cells per section, vs. 255 cells per section from the controls (Figure 4).

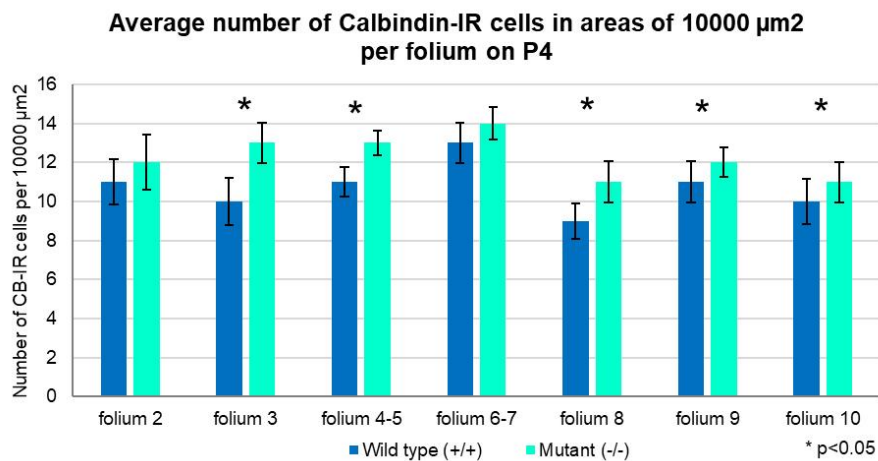


Figure 2. Graphical illustration on average number of Calbindin-IR cells in areas of 10,000 μm^2 per folium on P4. There is a prevalence of the PCs number in the mutants, compared with the wild-type animals. The Independent T-test with a Confidence Interval (CI) of 95% showed statistically significant difference ($p<0.05$) in folia 3, 4-5, 8, 9 and 10. The error bars indicate the Standard Deviation (SD).

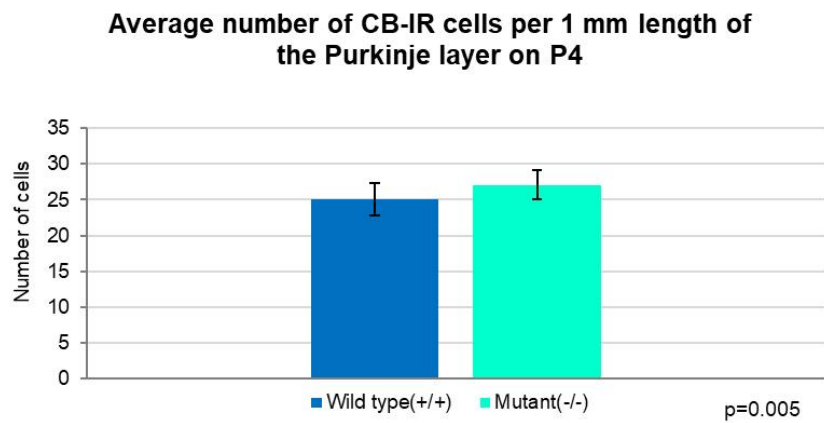


Figure 3. Quantitative analysis on CB-IR cell distribution per 1 mm length of the Purkinje layer in corresponding cerebellar sections at P4. The Independent T-test shows that on average the PCs are significantly more numerous in the mutants than in the wild-type mice ($p=0.005$). Error bars indicate the SD.

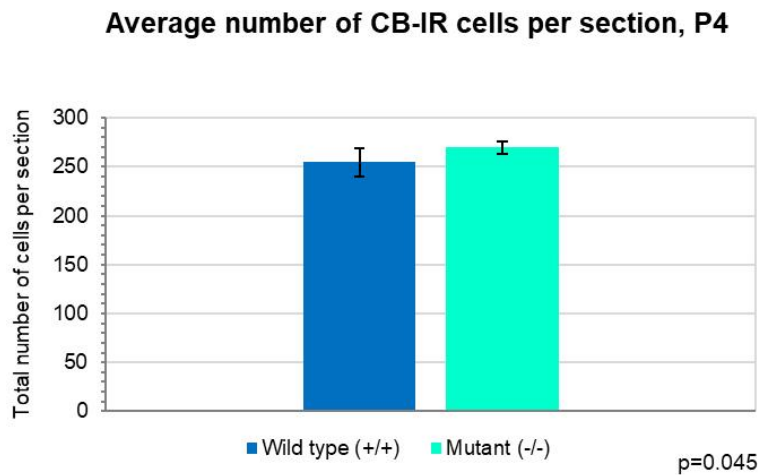


Figure 4. Comparison of the average number of CB-IR cells per section on P4. The total number of Purkinje cells per section was significantly higher in the *Zbtb20*^{-/-} homozygote mutant mice than in the controls (Independent T-test, $p=0.045$). Error bars indicate the SD.

2.2. Development of PCs at Postnatal Day 8

On the P8, PCs showed significant developmental alterations. Their dendritic processes were more complex (Figure 5) and distal spines were observed (not shown here). Additionally, PCs demonstrated branching axonal pattern. The quantitative analysis on average number of PCs per folium showed tremendous difference between the folia in each experimental group, as well as comparing the two types of mice. They were most numerous in folium 4-5 (96 cells in mutants, and 89 in the controls), followed by folium 3 and 6 with (63 vs. 86, and 73 vs. 53 cells, respectively). However, we found more PCs in folia 3 and 8 of the controls, while in the rest of the folia their number prevailed in the homozygous mutants (Figure 6).

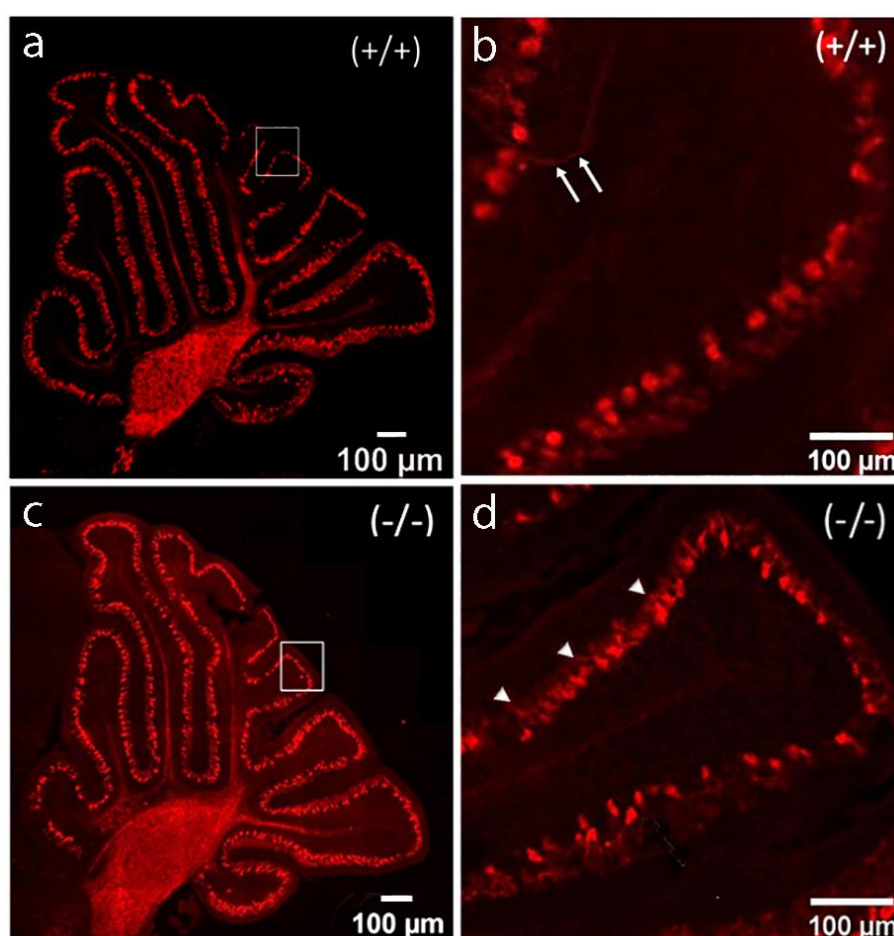


Figure 5. Dark field microphotograph of cerebellar sections from both experimental groups at P8, illustrating the advance in the development of the PCs. (a) Parasagittal cerebellar section from a control mouse and (c) from a mutant at low magnification. (b) Closer view of the framed part at (a). The arrows point at an axon with adult-like branching pattern. (d) Higher magnification of the fragment from (c). More complex dendritic tree arborizations are visible (arrow heads).

When we looked at the density of the PCs in the individual folia we noticed that only in folium 2 there were more CB-IR cells per 10,000 μm^2 in the wild-type mice compared with the mutants, though the difference was insignificant (Figure 7). In the rest of the folia, PCs were more numerous per ROI in the knockout animals, with statistical significance only in folia 3 and 10 ($p<0.05$) (Figure

7). Comparison of the average total number of PCs per section again showed higher density in the mutants ($p=0.02$) (Figure 8).

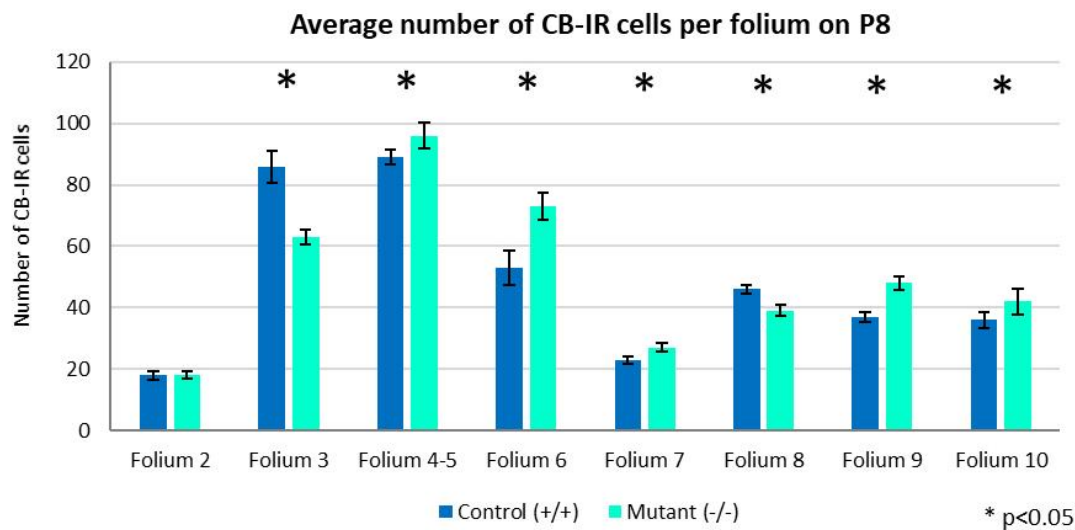


Figure 6. Chart illustrating the distribution of PCs per folia on P8. In most of the folia, the average number of CB-IR cells was higher in the mutants, except for folium 3. There was no difference between folium 2 of the two experimental groups. Independent T-test, $p<0.05$. Error bars indicate the SD.

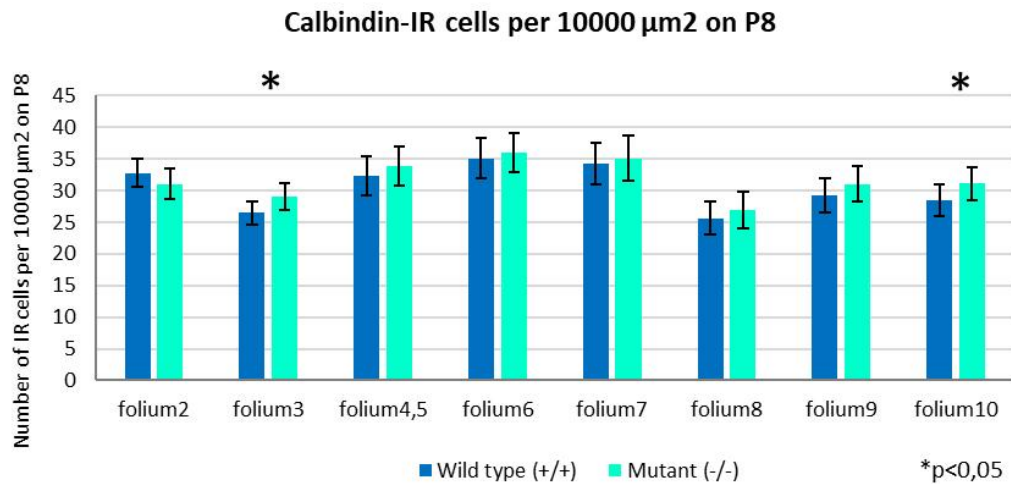


Figure 7. Comparative statistical analysis of the results illustrating the distribution of PCs per 10,000 μm^2 across folia. Except for folium 2, the density of the cells is higher in the *Zbtb20*^{-/-}. Only in folia 3 and 10 was the difference is significant ($p<0.05$).

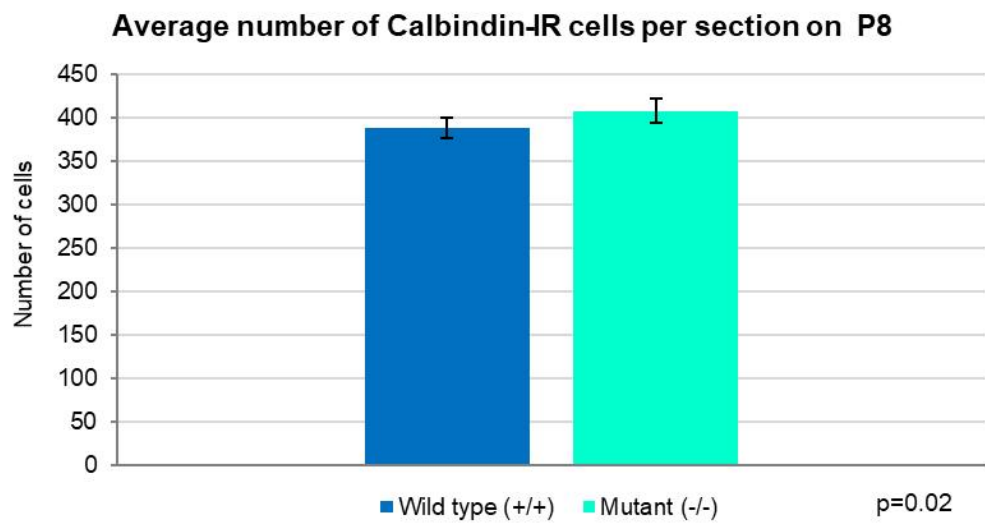


Figure 8. Quantitative illustration of the average number of PCs per section on P8. There is a substantial prevalence in the homozygote mutants compared with the controls (Independent T-test, p=0.02). Error bars indicate the SD.

2.2. Development of PCs at Postnatal Day 8

Changes in the morphology of the PCs at postnatal day 12 are significant, and on the overview photo, the advancement in dendritic tree branching is clearly visible (Figure 9). Highly branched dendrites already exhibit “espalier” morphology, characterized by extend primarily in a plane, which makes them look like thin leaflets. However, at higher magnification, PCs from the *Zbtb20*^{-/-} group (d) showed fewer branch points and less density of the dendritic tree compared with the controls (b).

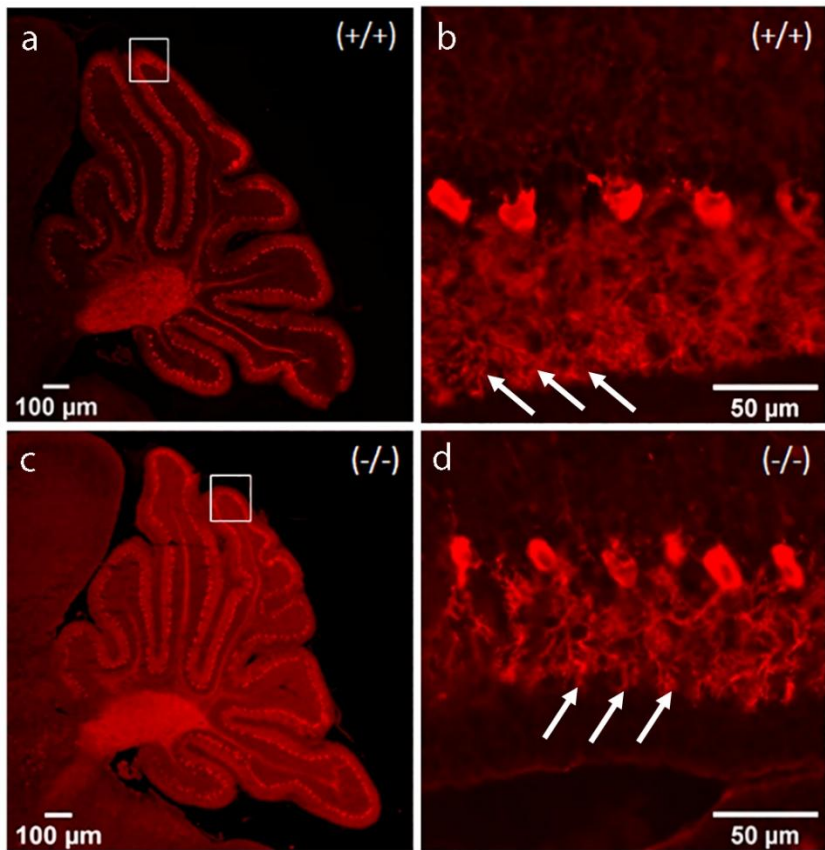


Figure 9. Overview of the developing cerebella on P12, stained for detection of CB; **(a, c)** Parasagittal sections at low magnification. **(b, d)** Higher magnification of the fragments on (a, c). Arrows point at the intricately branched dendrites of the PCs. Of notice is the less dense branching in the mutants.

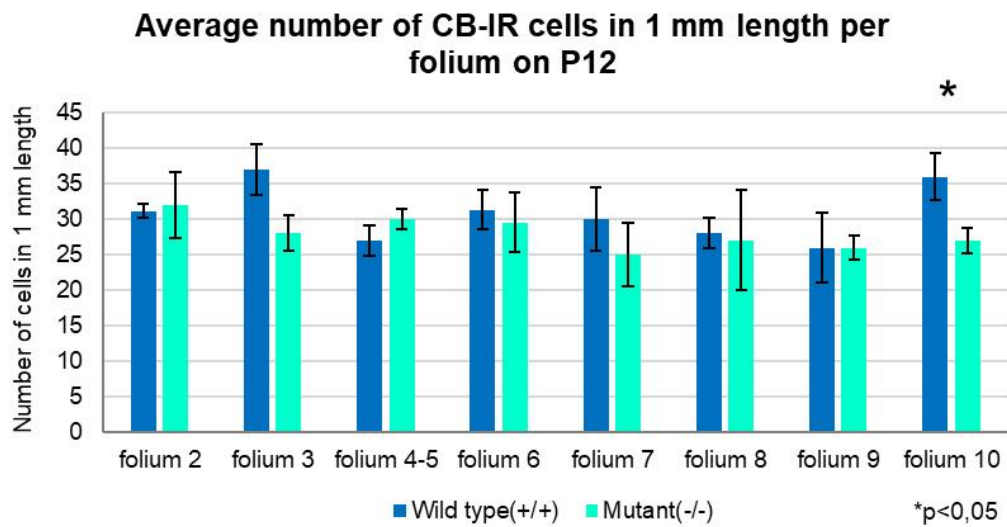


Figure 10. Graph representing the distribution of PCs within 1 mm of the Purkinje layer in the individual folia. At P12 most of the folia of the wild-type (*Zbtb20*^{+/+}) mice express higher number of cells, a significant difference found only in folium 10 (Independent T-test, $p<0.05$). Error bars indicate the SD.

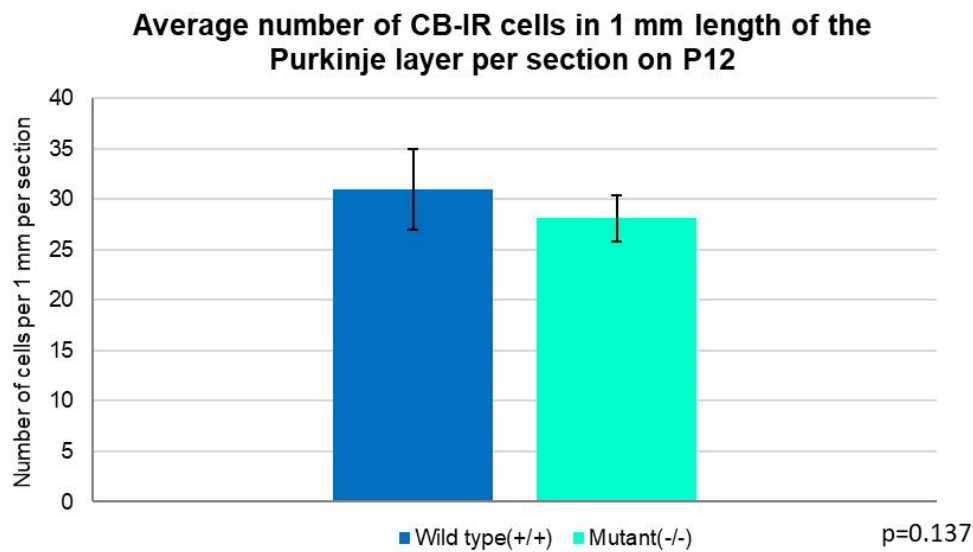


Figure 11. Graph summarizing the number of Purkinje cells per 1 mm length of the layer in the entire section. On postnatal day 12, the average number of PCs was slightly higher in the wild-type (31) vs. 28 in the *Zbtb20*^{-/-} group (Independent T-test, $p=0.137$). Error bars indicate the SD.

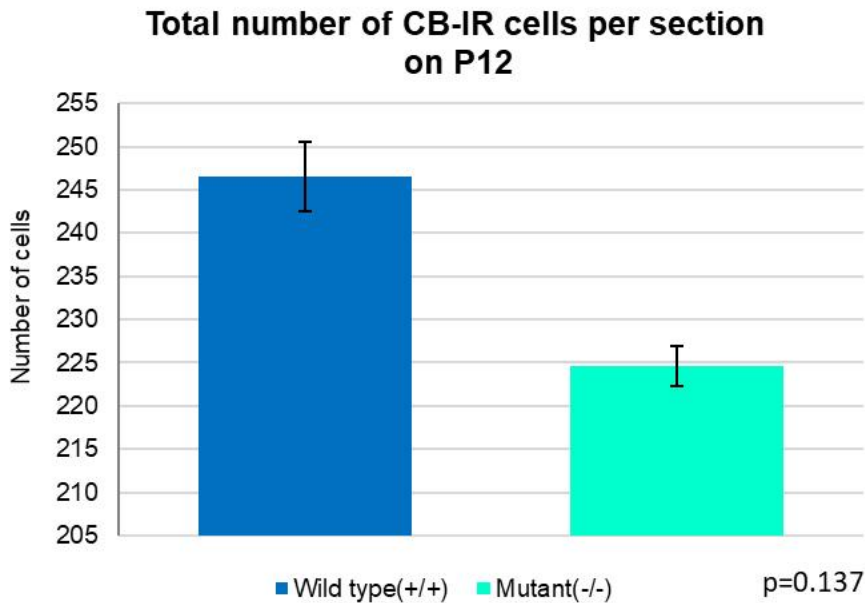


Figure 12. The average total number of PCs in entire cerebellar section of control animal (247 cells) insignificantly exceeded the one of the mutants (225 cells) (Independent T-test, $p=0.137$). Error bars indicate the SD.

3. Discussion

Here we investigated the role of *Zbtb20* in developing cerebellar cortex, particularly its effect on Purkinje cells generation and distribution along the middle cortical layer. Our results showed that the transcription factor has time- and space-differential effect on these processes: on P4, the lack of *Zbtb20* in knockout mice resulted in upregulated number of PCs per 10,000 μm^2 , as well as selectively in folia 3, 8, 9 and 10; on P8 the PCs' number was higher only in folia 3 and 10 of the knockout animals, while on P12 we observed the opposite – slightly lower number of PCs in most of the mutant folia, but in folium 10 this difference was significant. To the best of our knowledge, this is the first such study on the role of *Zbtb20* on early postnatal development of the Purkinje cells.

Undoubtedly, PCs are the most conspicuous neurons in the cerebellum, and the processes of formation of their space-filling and non-overlapping dendritic tree pattern has been studied extensively [27,28]. Purkinje cell dendrites grow in two stages: first the thicker primary dendrites are formed and then thinner once covered in spines [29]. Mature PCs have distinctively flat dendrite, almost two-dimensional, providing maximal contact with perpendicular parallel fibers. During the early postnatal development, PCs grow two or more dendritic trees, and then only one stays as a primary tree, while the others retract [30]. The events are regulated via environmental factors, such as hormones, neuronal growth factors, protein kinase C [28], and particularly the parallel fiber of granule cells [31,32], which, via their synaptic inputs, stimulate the elaboration of dendritic trees [33–35]. The mechanisms of these interactions have been studied widely, using in vitro and in vivo methods, including genetically manipulated experimental animal models, for more precise identification of molecules involved.

Validation of the synaptotrophic hypothesis [33] was provided by genetic manipulation of the best-characterized proteins responsible for synaptogenesis between granule cells and PCs – the presynaptic ligand cerebellin-1 (*Cbln1*) and postsynaptic receptor *GluD2*, which also binds neurexin, a presynaptic plasmalemma protein [32], thus forming a tripartite synaptic adhesion complex [36,37]. *GluD2* sparse knockout mice lose nearly half of synapses between the parallel fibers and PCs' dendrites. Additionally, density of the branch points at the PCs' bodies appears lower, compared with the wild-type, as early as P10 [32]. Surprisingly, without specifically investigating the type of dendritic arborizations, we observe a similar phenomenon in the specimens from *Zbtb20*^{-/-} group. Whether this phenomenon is due to more intense retraction or underdevelopment of dendritic trees

is still unclear. Nevertheless, we cannot rule out the possibility that Zbtb20 is involved in PCs' synaptogenesis. Therefore, further research is needed to clarify this probable function of the transcription factor, and to focus also on effect on dendritic spines development.

In this study we observed dynamic spatio-temporal pattern of PCs distribution among the cerebellar folia during the early postnatal period. Initially, at stage P4, the number of PCs was higher in most of the folia of Zbtb20^{-/-} mutants compared with the wild-type controls. However, this changed gradually, and on P12 most of the cerebellar folia showed the opposite proportion; even in folium 10 of the controls the population of CB positive PCs was significantly higher than in the corresponding folium in knockout animals. These results suggest that Zbtb20 probably suppresses precursors' proliferation and generation of PCs immediately after birth, thereafter, the effect gradually weakens, and by the end of the second week it is completely absent in all cerebellar folia of the mutants.

Alterations in the PCs population developments, similar to which we found in our Zbtb20-deficit mice, has been related to mutations in the gene encoding β -III spectrin. Spectrin-actin network at the plasma membrane is required to maintain cellular morphology and function [38]. Experiments with functional β -III spectrin knockout mouse (β -III^{-/-}) provided evidence that the animals develop motor deficits and age-related PCs loss [39]. The phenomenon was related to PCs dysfunction recapitulating behavior and neuropathology of spinocerebellar ataxia 5 in humans [40]. However, despite the similarity in the histological findings in these and our experiments, we still cannot say what the exact role of Zbtb20 is in cerebellar pathology.

Our findings of changes in the shape and number of CB-expressing PCs in Zbtb20^{-/-} mutants is in accordance with the recently reported induced alterations in PCs of fetuin-A knockout mouse, also a model of Parkinson's disease [41]. In this single study, fetuin-A expression in cerebellum was found reduced proportionally to the severity of Parkinson's disease. However, the exact mechanisms of PCs' impairment are still elusive. Nevertheless, fetuin-A is known as a multifunctional protein involved in a myriad of essential biological processes. It is essential for the mineral homeostasis, particularly calcium metabolism, it has immunomodulatory functions, and plays a role in brain development and protection [42,43]. The neuroprotective function was ascribed to the anti-inflammatory effect of fetuin-A, due to reduction of TNF-alpha synthesis, as well as downregulation of macrophage and microglial activity [44]. Whether Zbtb20 acts through similar mechanisms, we do not yet know.

Overall, the role of Zbtb20 in the CNS under normal and pathological conditions has been extensively studied. Zbtb20 was found essential for cochlear maturation, hearing [45] and nociception [46], olfactory bulb development [23], dendritic arborization and dendritic spine morphology in the cerebral cortex [47], pyramidal hippocampal neurons generation [48], etc. Pathological entities, underlined by mutations in Zbtb20, comprise a broad spectrum of morphological, neurological and behavioral alterations [49–52]. However, there is still a lack of knowledge about the guiding role of Zbtb20 in cerebellar development. Our study is the first to reveal some of the effects of Zbtb20 on PCs population, and to certain extent on their appearance. It suggests that some of the cerebellar disorders could also be etiologically related to this transcription factor. We believe that deeper insights into the topic will be essential for the design of new effective strategies for neuroprotection and the alleviation of some neurodegenerative disorders.

4. Materials and Methods

4.1. Experimental Animals and Tissues Preparation

The samples of Zbtb20 knockout mutant mice and normal wild-type animals were used in this study. The procedure for generation of homozygous Zbtb20^{-/-} mutant mice has been previously described in detail [53]. Permission for the animal experiments was received by the Niedersächsische Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES)/Oldenburg, resolution № 33.9-42502-04-11/0622 of 07.12.2011. The animals have been handled in accordance with the German Animal Protection Law. Briefly, animals were sacrificed at P4, P8, and P12 via transcardiac perfusion

with ice-cold 4% paraformaldehyde. The brains were isolated, and the cerebella were dissected and immersed in paraformaldehyde at 4°C for post-fixation. Prior to sectioning, tissues were cryoprotected with PBS/Sucrose (30%), and embedded in OCT. The sections were cut at 10 µm.

4.2. Immunofluorescent Staining

Immunostaining was performed on 36 slices, equally distributed in two major groups, i.e., WT and mutants, and 3 subgroups of P4, P8, and P12, respectively. The removal of OCT was followed by antigen retrieval for 5 min at 95°C in citrate buffer (pH 6.0). Appropriate washes in PBS (3 × 5 min) followed this and subsequent treatments. Sections were immersed in 1% bovine serum albumin (in PBS containing 0.01% Triton-X) for 20 min. and incubated for 20 h at room temperature in monoclonal rabbit primary antibody against Calbindin (CB), marker for the Purkinje cells (1:500, dilution Sigma). Following several washes, the specimens were incubated in the secondary antibody (IgG conjugated to Alexa fluorochrome) AF546 or AF488 goat anti-rabbit (Invitrogen, dilution 1:200) was for 2 hours, at room temperature. The samples were counterstained with the fluorescent dye 4',6-diamidino-2-phenylindole DAPI (cat. # 10236276001, Merck, Germany, dilution 1:1000) at room temperature for 15 min. After several washes in PBS, specimens were rinsed in distilled water, and embedded in fluorescent mounting medium (Carl Roth, Germany).

4.3. Imaging and Statistical Analysis

Miscoscopic images were captured using a fully motorized wide-field microscope Zeiss AxioImager Z.2 (Carl Zeiss, Germany) equipped with the appropriate filter, and an AxioCam Mrm rev.3 monochrome CCD camera (Carl Zeiss, Germany), and AxioVision v.4.9 software through an EC Plan-Neofluar objectives 5x, 10x, 20x, and 40x. Shading from the irregular illumination field was corrected during the acquisition via the camera built-in shading correction. The images were saved.zvi format (Zeiss) of the AxioVision software, stitched together, and exported to an 8-bit TIFF format for further processing and analysis with the ImageJ2 (Version 2.3.0/1.53f, Fiji). The number of PCs was counted per folium - in 1 mm length of the Purkinje layer or in regions of interest (ROI) along the layer, with size of 100x100 µm. The latter was applied because at the early postnatal stage (P4), the PCs are not yet arranged in a monolayer. However, for consistency, we continued applying it to the specimens from the later stages of development, P8 and P12. Additionally, quantitative analyses were performed per section – calculating the total number of PCs.

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 29.0.0.0 (241) (IBM Corp., Armonk, NY, USA). Independent T-test was applied for the given values with a Confidence Interval (CI) of 95%. Statistical significance between the two experimental groups was considered if $p < 0.05$. Error bars indicate the Standard Deviation (SD).

Author Contributions: Conceptualization, I.V. and I.S.; methodology, I.V. and I.S.; formal analysis, I.V., I.S., M.B. and T.P.; investigation, I.V.; data curation, I.S.; writing—original draft preparation, I.S.; writing—review and editing, I.V., M.B. and T.P; visualization, I.V. and I.S.; supervision, I.S.; project administration, I.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

Zbtb20	Zink finger and BTB domain-containing protein 20
PCs	Purkinje cells
CB	Calbindin
IR	Immunoreactive

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