J Toxicol Pathol 2015; 28: 73–78

Original Article

Morphometric analysis of progressive changes in hereditary cerebellar cortical degenerative disease (abiotrophy) in rabbits caused by abnormal synaptogenesis

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Abstract: We previously investigated rabbit hereditary cerebellar cortical degenerative disease, called cerebellar cortical abiotrophy in the veterinary field, and determined that the pathogenesis of this disease is the result of failed synaptogenesis between parallel fibers and Purkinje cells. In this study, longitudinal changes in the development and atrophy of the cerebellum of rabbits with hereditary abiotrophy after birth were morphometrically examined (postnatal day [PD] 15 and 42) using image analysis. Although development of the cerebellum in rabbits with abiotrophy was observed from PD 15 to PD 42, the growth rate of the cerebellum was less than that in normal rabbits. In rabbits with abiotrophy, the number of granular cells undergoing apoptosis was significantly higher at PD 15 and dramatically decreased at PD 42. The number of granular cells did not increase from PD 15 to 42. The synaptogenesis peak at PD 15 occurred when the largest number of apoptotic granular cells in rabbits with abiotrophy was observed. Although 26% to 36% of parallel fiber terminals formed synaptic junctions with Purkinje cell spines, the remainder did not at PD 15 and 42. The rate of failure of synaptogenesis in the present study might be specific to this case of abiotrophy. Morphometric analysis revealed detailed changes in development and atrophy in animals with postnatal cerebellar disease occurring soon after birth. (DOI: 10.1293/tox.2014-0057; J Toxicol Pathol 2015; 28: 73–78)

Key words: abiotrophy, cerebellum, rabbits, morphometry

Introduction

We previously reported that pathogenesis of hereditary cerebellar cortical degenerative disease (cerebellar cortical abiotrophy) in the rabbit is the result of failed synaptogenesis between parallel fibers and Purkinje cells[1, 2]. Typically, a decrease in the cerebellum size is not observed until around postnatal day (PD) 30[1]. However, despite the mild nature of the changes in the cerebellum observed via light microscopy, failed synaptogenesis tends to cause severe clinical signs of ataxia, and affected rabbits were unable to live longer[1]. The failure of synaptogenesis during postnatal development of the brain can cause irregular orientation of neurons or degeneration of neurocytes or both[2-7]. We previously reported irregular orientation of the Purkinje dendritic arbor and apoptotic granular cells during and after migration, in addition to degeneration of Purkinje cells and axons in the advanced stage[1]. While cerebellum degeneration in the brain of adult animals commonly leads to atrophy, how the atrophy interacts with the development of rapidly growing brains of postnatal animals with synaptogenesis failure remains unknown.

Cerebellar cortical abiotrophy is a hereditary and progressive disease with an early onset[8]. Therefore, the presence of longitudinal changes in the postnatal cerebellum is of interest. Here, we examined longitudinal changes during the development and subsequent atrophy of the cerebellum in rabbits with hereditary abiotrophy after birth (PD 15 and 42) by morphometric examination using image analysis.

Materials and Methods

N2 rabbits with and without abiotrophy were produced by backcrossing between F1 female rabbit and male parent rabbit (Wbl:JW SPF) that produce F1 rabbits with ataxia by an autosomal recessive inheritance as has been previously described[1, 2]. Rabbits were cared for in accordance with the

Received: 15 November 2014, Accepted: 8 January 2015
Published online in J-STAGE: 2 February 2015
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principles outlined in the Guide for the Care and Use of Laboratory Animals of the Japanese Association for Laboratory Animal Science and those of our institution.

One animal in each of the affected and control rabbits was euthanized at the same time under intraperitoneal pentobarbital anesthesia at PD 42; this was done because the affected rabbit showed severe ataxia and could not survive any longer. The affected and control rabbits at PD 15 were the same ones we described in a previous report[2]. Central nerve tissues were fixed for approximately 30 min by intracardiac perfusion with approximately 500 ml of 2.5% glutaraldehyde. After perfusion, tissues from the affected and normal rabbits were fixed in 2.5% glutaraldehyde, and central nerve tissues (cerebrum, cerebellum and medulla oblongata) were subjected to histological examination. Central nerve tissues were sagittally sectioned, embedded in paraffin, and serially sectioned into 4-μm specimens for light microscopic examination. Representative sections were stained with hematoxylin and eosin (HE). Small pieces of the middle and posterior lobe of the cerebellar cortex obtained from the affected and normal rabbits were processed for electron microscopy. Pieces were postfixed in 1% osmium tetroxide (OsO_4), dehydrated, and embedded in Epon-Araldite such that the sagittal plane was the cut surface. Semi-thin sections were stained with toluidine blue. Thin sections (approximately 90 nm thick) were stained with uranyl acetate and lead citrate and examined at 80 kV using a transmission electron microscope (H-7600, Hitachi, Tokyo, Japan).

We compared sectioned areas of the cerebellum, including the external granular layer, molecular layer, (internal) granular layer, and medulla, in the sagittal plane of the cerebellum in HE-stained sections between the normal rabbits and rabbits with abiotrophy using image analysis software (Image-Pro Plus; Media Cybernetics Inc., Rockville, MD, USA). Granular cells and apoptotic cells in the internal granular layer were counted randomly in 10 areas of HE sections per rabbit under light microscopy at 200-fold magnification using image analysis software at PD 15 and 42 for rabbits with and without abiotrophy. Pyknotic cells scattered in the granular layer were regarded as apoptotic cells, which stained positively by the TUNEL method as described in previous reports[1, 2]. The number of synaptic junctions between parallel fibers and Purkinje cell spines were also counted in 25 areas in the electron microscopic examination using 12,000-fold magnification at PD 15 and 42 for the rabbits with or without abiotrophy. We considered only the synaptic junction that included certain features, such as synaptic vesicles of the parallel fiber terminals and high-density postmembranous thickening of Purkinje cell spines.

Results

Morphometry of the cerebellum

Figure 1 shows the morphometry of a sagittal section of the cerebellum in each animal and each layer in one area of sagittal section of the cerebellum.

The size of the cerebellum of the rabbit with abiotrophy was similar to that of normal one at PD 15. The cerebellum of both developed gradually from PD 15 to 42. However, the growth rate of the cerebellum was lower in rabbits with abiotrophy. The cerebellum size in the normal rabbits increased approximately 2-fold from PD 15 to 42. In contrast, cerebellum development in affected rabbits was stunted, with only a 1.2-fold increase in size. By PD 42, the cerebellum size in affected rabbits was 26% lower than that in the normal rabbits.

The difference in cerebellum size at PD 42 was caused by differences in development of the molecular and granular layers. Compared with the granular layer and medulla, the molecular layer exhibited the largest increase in size during the observation period in the normal rabbits, with a 2.4-fold increase observed in the molecular layer compared with a 1.9-fold increase in the granular layer and 1.7-fold increase in the medulla. In rabbits with abiotrophy, the molecular layer showed a 1.4-fold increase in size, which was markedly less than in the normal rabbits. However, the 1.7-fold increase in the medulla was the same as that observed in the normal rabbits. In contrast, the granular layer showed only a 0.9-fold increase.

The cells of the external granular layer were depleted at PD 42 in both rabbits with or without abiotrophy at the lowest level of quantification, suggesting that the migration rate of granular cells was not a factor in the abiotrophy.

Numbers of granular cells and apoptotic cells

The numerical density of granular and apoptotic cells per unit area and the total number of granular and apoptotic cells in sagittal sections in the granular layer are shown in Figure 2. The number of granular cells was calculated from the densities of both cells and the area of the granular layer.

From PD 15 to 42, the density of granular cells decreased slightly in rabbits with and without abiotrophy due to the simultaneous neuropile development. In contrast, the number of granular cells increased in rabbits without abiotrophy (160%) and decreased in those with abiotrophy (87%).

The density and number of apoptotic cells were very low at both PD 15 and 42 in the normal rabbits, but high at PD 15 and decreased at PD 42 in rabbits with abiotrophy.

Number of parallel fiber-Purkinje cell junctions

Figure 3 shows the density of synaptic junctions between parallel fiber terminals and Purkinje cell spines and the number in sagittal sections in the molecular layer as determined by the electron microscopy examination. The number of synaptic junctions per section was calculated from the density observed upon electron microscopy examination and the area of the molecular layer.

No significant changes were noted in the numerical density of junctions from PD 15 to 42 in either normal rabbits or rabbits with abiotrophy, because of simultaneous neurofiber and glial fiber development in the molecular layer. However, a distinct difference in the numerical density of junctions was observed between rabbits with and without abiotrophy at each stage. In rabbits with abiotrophy, the densities of the

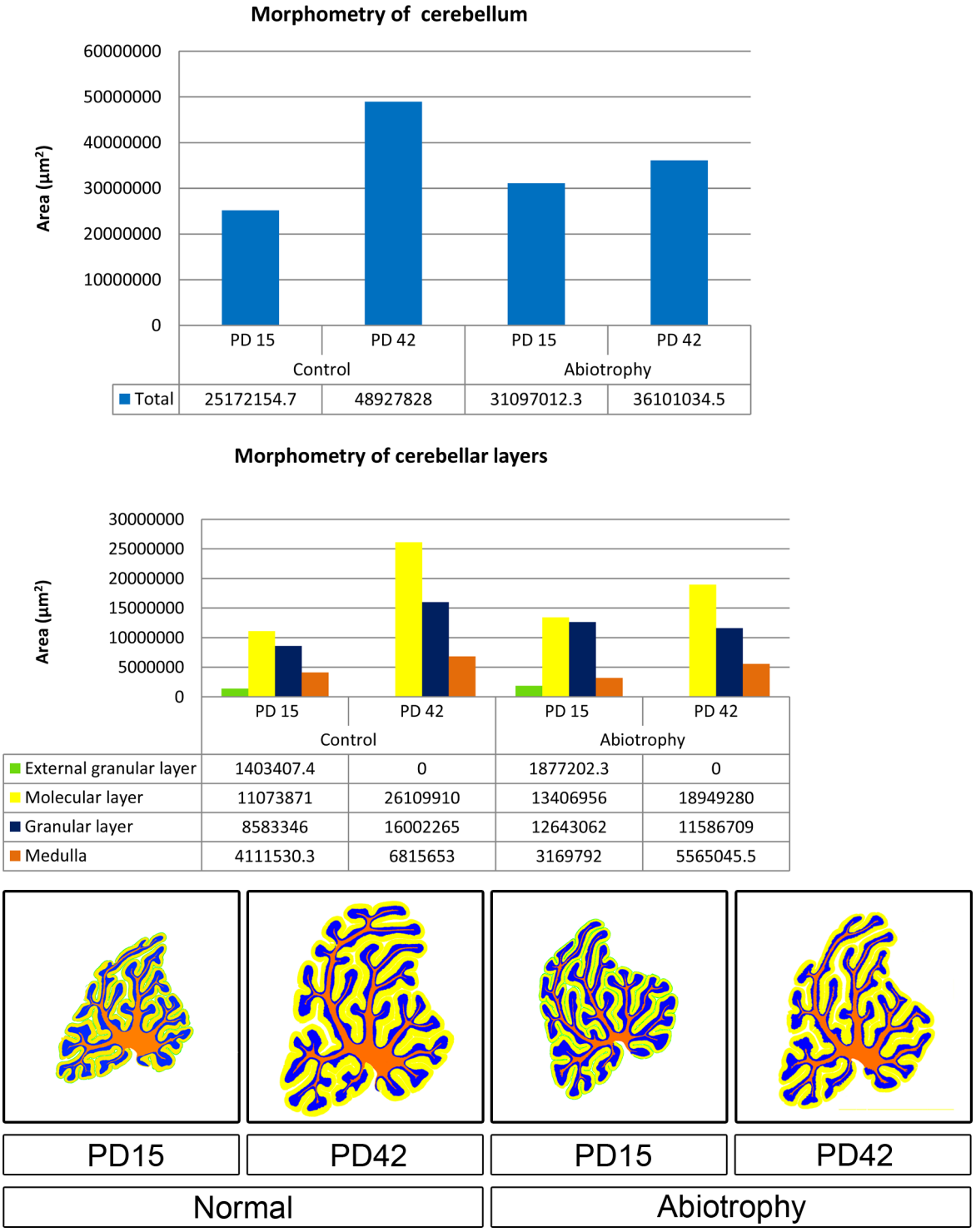

Fig. 1. Morphometry of a sagittal section of the cerebellum and each layer in one area of a sagittal section of the cerebellum. Normal rabbits and rabbits with abiotrophy at PD 15 and 42. Green indicates the external granular layer, yellow indicates the molecular layer, dark blue indicates the granular layer, and orange indicates the medulla.

junctions were reduced by 71% at PD 15 and 60% at PD 42 when compared with those of rabbits without abiotrophy.

The total number of synapses in rabbits with abiotrophy was less than that in those without abiotrophy at PD 15, and did not increase as much as in those without abiotrophy throughout the observation period. While the number of synaptic junctions increased 2.1-fold from PD 15 to 42 in the normal rabbits, the number increased only 1.7-fold from PD 15 to 42 in rabbits with abiotrophy. In rabbits with abiotrophy, the number of junctions was 64% lower at PD

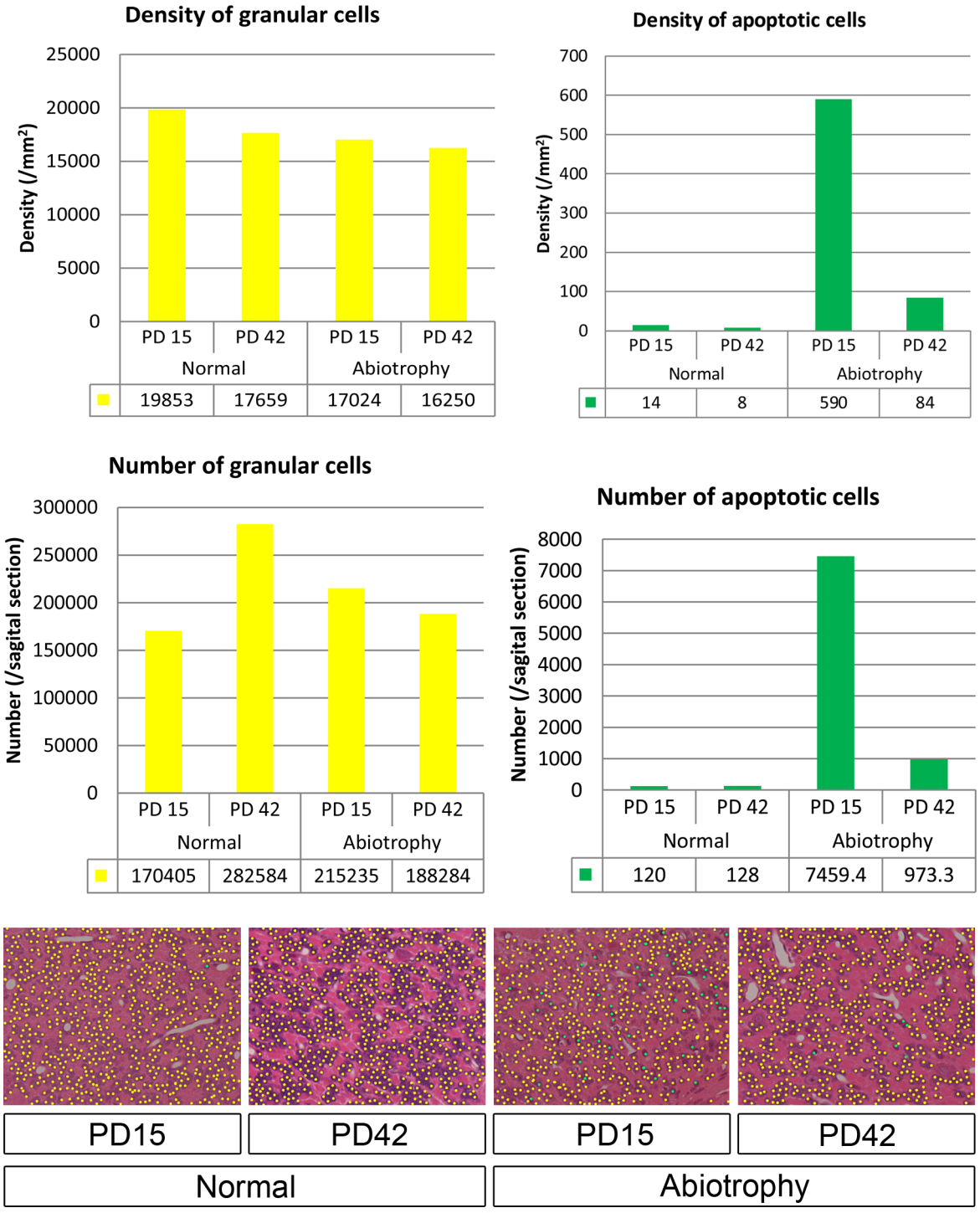

Fig. 2. Density of granular and apoptotic cells per unit area (mm^2) and the numbers of granular and apoptotic cells in sagittal sections in the granular layer. Normal rabbits and rabbits with abiotrophy at PD 15 and 42. Yellow dots indicate granular cells, and green dots indicate apoptotic cells.

15 and 71% lower at PD 42 than in those without abiotrophy.

Discussion

In this study, the morphometric analyses of rabbit abiotrophy revealed the longitudinal development of the normal cerebellum, progress of this disease, and pathogenesis. By comparing the size and each layer of the cerebellum, we determined that the development in terms of size was normal during PD 15 and the cerebellum developed in unaffected parts from PD 15 to 42, even in rabbits with abiotrophy. In addition, our data also shows that the diagnosis of postnatal

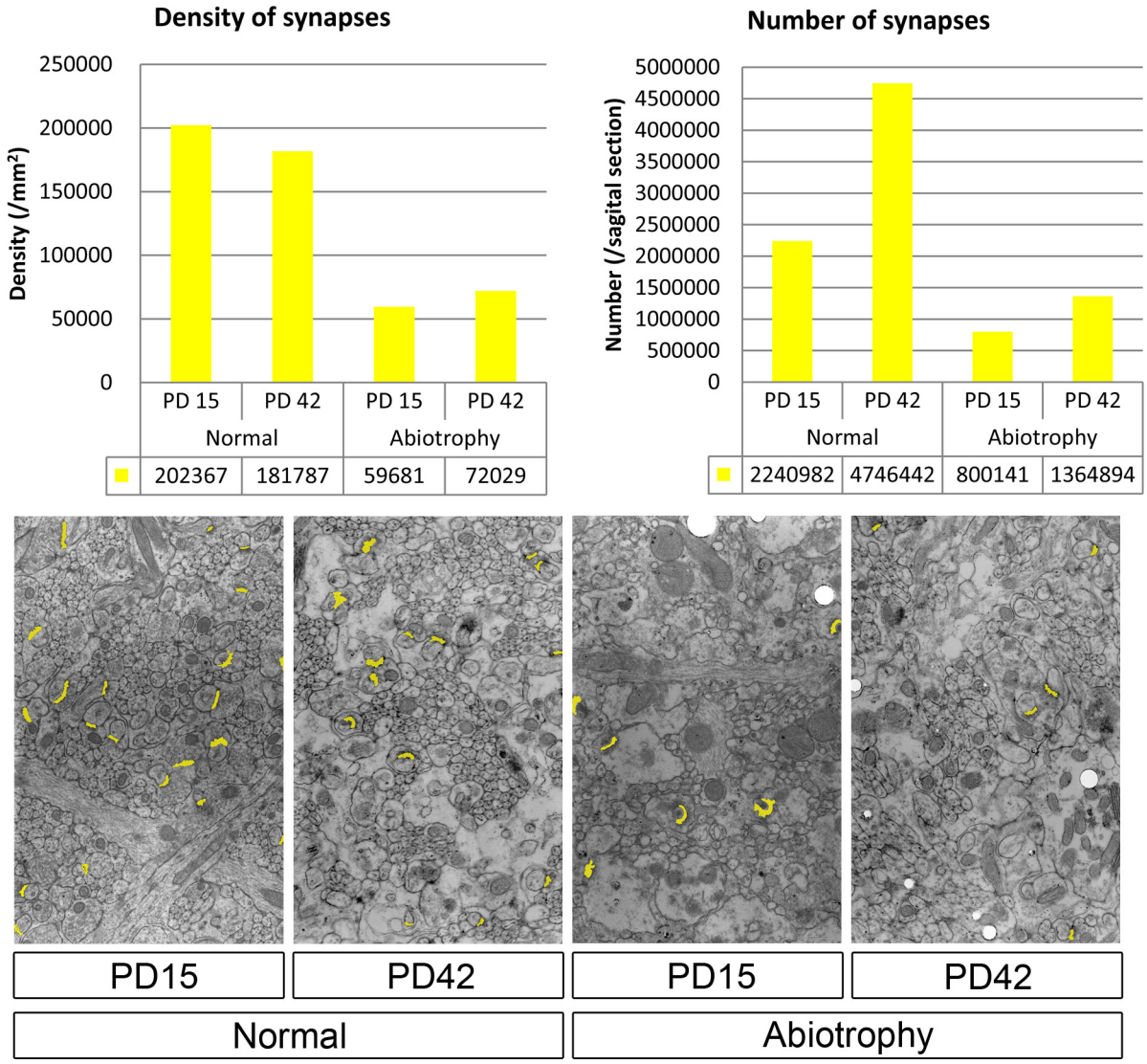

Fig. 3. Density of synaptic junctions between parallel fiber terminals and Purkinje cell spines per unit area (mm^2) and the number in sagittal sections in the molecular layer. Normal rabbits and rabbits with abiotrophy at PD 15 and 42. Yellow patches indicate synaptic junctions.

brain disease in a study with a single time point is difficult. For example, comparison of rabbits with and without abiotrophy at only PD 42 would have led to a diagnosis of cortical atrophy of the cerebellum. However, the size of the cerebellum affected by abiotrophy did increase, albeit only slightly.

The number of apoptotic granular cells was significantly higher at PD 15 and dramatically decreased at PD 42 in rabbits with abiotrophy. The number of granular cells did not increase but instead decreased slightly. Apoptotic cells, although fewer in number, are also found in the normal rabbit in the internal granular layer and tend to increase in number from PD 5 to PD 10[9]. Post-migratory granular cells most likely undergo apoptosis due to failed synaptic contacts during molecular layer formation[9, 10]. This suggests that granular cells without establishment of normal synaptic junctions with Purkinje cell spines undergo apoptosis at PD 15, leading to a reduced number of granular cells at PD 42. As the peak granular cell migration occurred before PD 42, most granular cells that failed to connect with Purkinje cells disappeared by PD 42.

We previously reported decreased numbers and abnormalities of synaptic junctions between parallel fibers and Purkinje cell spines using electron microscopic observation[1, 2]. In the present study, we confirmed this by surveying the number of synaptic junctions in morphometric analysis. In affected rabbits, the total number of junctions was less than half that in the normal rabbits at PD 15, and the number did not notably increase until PD 42. This result suggests that failure of synaptic junctions had already been determined by around PD 15.

In the postnatal development of the cerebellar cortex in rats, granular cells start to migrate around PD 8 and finish migrating around PD 20[11]. Synaptic formation between parallel fibers and Purkinje cell spines occurs around PD 12

to 30 [3, 4, 11, 12]. In mice, cerebellar development proceeds faster than that in rats. Granular cells start to migrate around PD 3 and finish around PD 14. Formation of synaptic junctions between parallel fibers and Purkinje cell spines starts around PD 7 and finishes around PD 20[7]. These observations suggest that synaptic contacts are formed a few days after granular cell migration. In rabbits, previous reports have shown that the thickness of the external granular layer was reduced to only two to three cell layers between PD 15 and 25[10], which is mainly linked to granular cell migration to the internal granular layer. Furthermore, synaptophysin immunoreactivity diffused throughout the entire molecular layer by PD 20 to 30, which indicates that synaptic formation was completed around this period[13]. We therefore speculate that PD 15 was the peak of synaptogenesis post migration and that synaptogenesis was already finished in the cerebellum of rabbits by PD 42. So the peak of synaptogenesis at PD 15 is when the largest number of apoptotic granular cells in rabbits with abiotrophy is observed.

In glutamate receptor δ2 subunit-deficient mutant mice, the percentage of Purkinje cell spines forming synapses has been shown to be 98% to 99% in wild type animals but as low as 55% to 60% in mutants, while the rest of the Purkinje cell spines were not attached to any nerve terminal during the second and third postnatal weeks, which is when synapses and terminals are actively generated[6]. In the present study, the number of synaptic junctions ranged from 64% (PD 15) to 71% (PD 42) less than that of the normal rabbits, suggesting that 36% to 29% of parallel fiber terminals formed synaptic junctions with Purkinje cell spines, while the remainder failed to do so at each stage in rabbits with abiotrophy. The rate of successful synaptic formation in the rabbits with abiotrophy was less than those in glutamate receptor δ2 subunit-deficient mutant mice. We speculate that the rate of failure of synaptogenesis may almost be regular in rabbits with this disease.

Analysis of an early-onset and progressive hereditary cerebellar disease is not easy due to the cerebellum developing in the early postnatal phase and degenerating soon after birth. Determining whether a disease features atrophy or hypoplasia is difficult when investigating a single time point in the postnatal phase. To our knowledge, no reports of morphometric analysis of abiotrophy of the cerebellum have been published. Survey data helped us demonstrate the legitimacy of histological findings and clarify the longitudinal process of the disease, and morphometric analysis revealed detailed changes in development and atrophy in a postnatal cerebellar disease that occurred soon after birth.

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