CEREBELLUM DEVELOPMENTAL CHALLENGES: FROM MORPHOLOGY TO MOLECULAR ISSUES

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ABSTRACT

Introduction: It is known that, throughout the development of the nervous system, the cellular migratory routes are an important part of its expansion; therefore, the cerebellum is 'sprinkled' with cellular changes during its growth. The aim of this study was to analyse the morphological features of the cerebellum cells in all the layers, during its development. **Material and methods:** We examined 14 cases of human cerebellum, ranging between 1 to 12 months by histopathology and immunohistochemistry. **Results:** Haematoxylin and eosin staining method confirmed the age-linked migration of the cells from the external granular layer into the internal granular layer. Moreover, immunohistochemical evaluation using PROX1 and NFAP showed positivity for the Purkinje cells. However, these cells exposed negativity on NSE stained specimens. On the other hand, the transience of the EGL was analyzed using OCT3/4, which showed the migration of the EGL cells through the molecular layer to the IGL. Also, GFAP and NFAP proved to be a useful tool for the identification of the climbing fibres and the variation of their density connected the age of the patient. **Conclusions:** The human cerebellum undergoes different morphological and molecular changes throughout its evolution during embryogenesis. The markers used in our study have proved to present a differential, stage-dependant reactivity and appeared as useful tools for the identification of different cerebellar structures. Our study is a challenging attempt to understand the basics of cerebellar development at a morphological and molecular level and may bring new perspectives for a better approach of cerebellar associated pathologies.
Key Words: human cerebellum, external granular layer (EGL), internal granular layer (IGL), embryogenesis, developmental changes

INTRODUCTION

The cerebellum is one of the first neurological structures that begins to differentiate during the embryological period. As it has been stated by Herculano-Houzel S. : 'For every neuron added to the cerebral cortex in evolution, four neurons are added to the cerebellum'. [1].

Neural development is one of the earliest systems to begin with and the last to be completed after birth [2]. Cerebellar development occurs during late embryogenesis and early postnatal period [2]. Due to this aspect, the cellular organization of the cerebellum also continues to undergo changes many months after birth [2]. The birthdate of a neuron defines its position within the cortex [2]. Earlier born neurons are the first to migrate and occupy deeper layers compared to later born neurons that occupy more exterior positions [2]. In the normal development of the cerebellum, endogenous Sonic hedgehog (a protein which mediates signalling activities of the notochord and floor plate), stimulates rapid proliferation of cerebellar granule neuron progenitors (CGNPs) in the external granule layer (EGL) [3].

The external granular layer (EGL) is defined by its transience and proliferation [4]. It migrates during the embryological development through the molecular layer (ML) and through the layer of Purkinje cells (PcL) along the Bergman glial fibres, ending its journey into the internal granular layer (IGL), thus leading to the formation of the mature cerebellum [4, 5].

For the first time, Ramón y Cajal was able to present the existence of the EGL on the surface of the cerebellum containing many mitotically active cells (Altman and Bayer, 1997) and its radial migration towards the ultimate destination. (Ramón y Cajal, 1894).

The tempo of transit amplification within the EGL is driven by diffusible sonic hedgehog (Shh), secreted by the Purkinje cells [4]. Due to this reason, as the sonic hedgehog is expressed in Purkinje cells, Shh may not only be involved in the proliferation of the EGL, but also in cerebellum foliation by regulating the position and/or size of lobes during development [4].

The proliferation of the external granular layer has also been shown to be influenced by a number of extracellular matrix (ECM) components, such as β1-integrin, for example, which has an unexpected function in cortical development [3].

Starting from these premises, the aim of this study was to analyse the cellular morphology changes in the cerebellum layers during its development.

MATERIAL AND METHODS

We included in the present study 14 cases of human cerebellum ranging from the age of one month till the age of 12 months, some of them being associated with different pathologies such as Down Syndrome, meningitis, fibrosis, tetraparesis and acute lymphatic leukemia. One slide from each case was stained with haematoxylin and eosin method for histopathologic evaluation. Specimens from each case were immunohistochemically processed using PROX1, OCT ¾, NSE, GFAP, NFAP.

RESULTS

Microscopic evaluation of the haematoxylin and eosin stained specimens revealed the age-related migration of the cells from the EGL. Thus, on specimens containing 1 month old cerebellum, the EGL contained 5 cell layers, at 5 months, 3 cell layer, at 6 months, it contained 2 cell layers and at 7 months old, only 1 cell layer was identified. When we examined the cases containing 8 months-10 years old cerebellum specimens, we observed the migration of the cells from the EGL through the Molecular layer. The last examined case, a 12 year old cerebellum, did not contain any EGL at all.

Moreover, on the haematoxylin and eosin stained slides, we examined the foliation of the cerebellum and therefore, we considered the foliation in its evolution from 1 month old specimens up to the 10 months old specimens. Complete foliation was examined on the cases ranging from 1 year and 5 months, to the last 12 years old case.

The first step for immunohistochemical evaluation, made use of PROX1, which showed positivity in the EGL and Purkinje cells in case of the one month old cerebellum, up to the 5 months old cerebellum. Later on, only the cells from the Purkinje layer were cytoplasmic and nuclear positive.

Consequently, we noticed the fact that starting from the case containing the 5 months old cerebellum, EGL was negative for PROX1 until it completely disappeared.

We continued our study on the human cerebellum with another immunohistochemical marker, OCT ¾, which demonstrated EGL cells partial positivity starting from 1 month old until 7 months old specimens, but, when the cells from the EGL layer started to migrate (at the age of 8 months) in the molecular layer, the cells expressed negativity to OCT ¾. Nevertheless, we observed that on the last 3 cases, from 2 years and 7 months, until the age of 12, Purkinje neurons expressed positivity to OCT ¾.

Also, when we visualised all the slides on the NSE immunohistochmical reaction, we noticed that the cells from the external granular layer did not frequently present positivity to NSE; and neither did the Purkinje cells (except for the last 2 cases, when Purkinje cells were membrane positive)! Nonetheless, the molecular layer (ML) has showed positivity to NSE in all 14 cases, the expression being more intense in case of the 1 month old cerebellum and close to the surface in the cases containing 10 months and 10 years old specimens, however less positive near the foliation. Even more, the IGL cells presented frequent positivity to NSE on all the 14 cases included in our study on the human cerebellum.
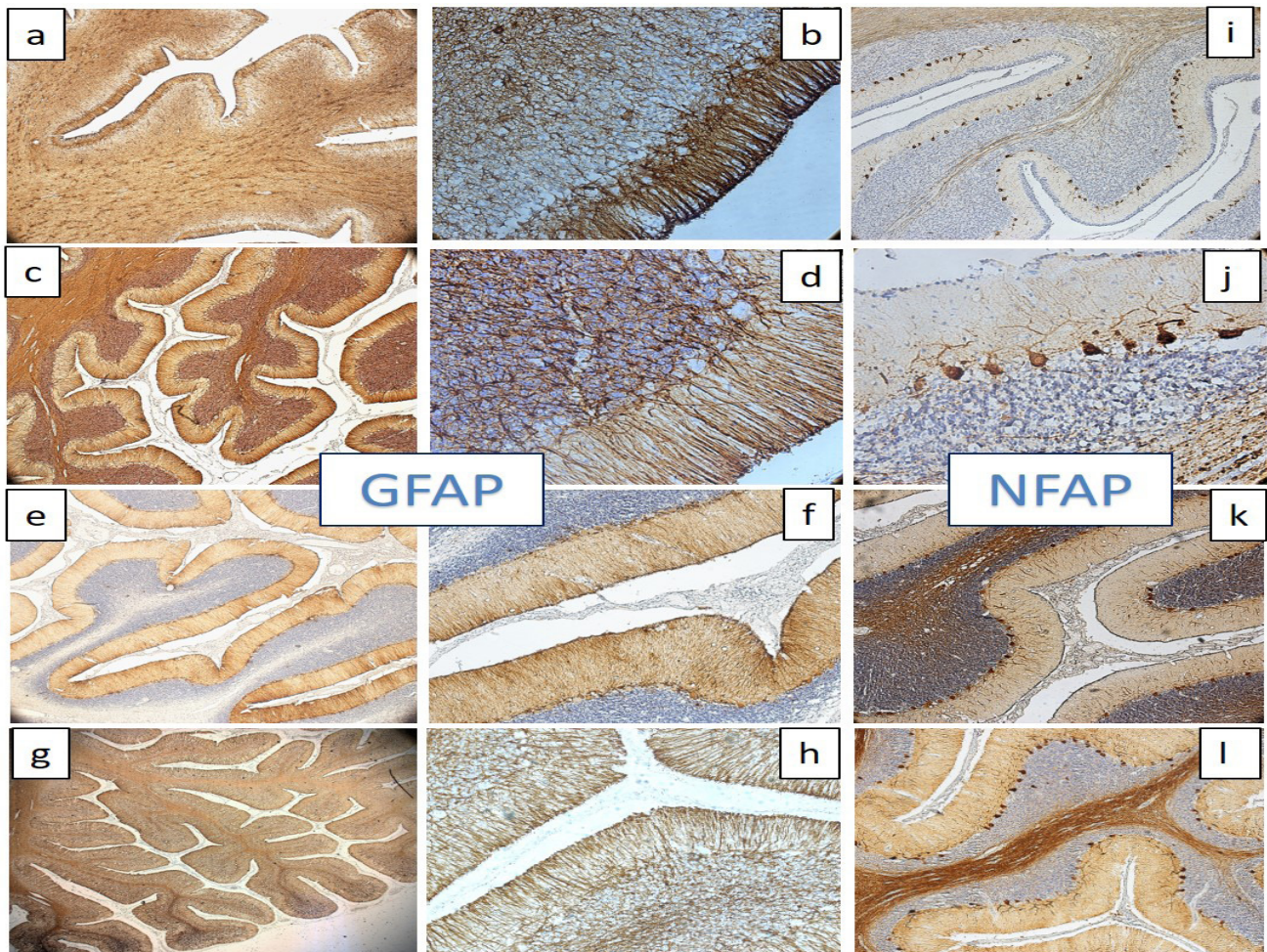
Fig.1. *GFAP AND NFAP expression in the human cerrebellum at different ages starting from 1 month(***a, b, i***), 4 months(***c, d, j***), 6 months(***e, f, k***) and 10 months(***g, h, l***)*

While examining the GFAP stained slides, we focused on the fibres of Bergmann glial cells and also on the presence of astrocytes in the human cerebellum layers during its development.

Thus, on the cases ranging between 1 month and 7 months, we encountered an increased density of fibres in the molecular layer, but from 7 months onward, the number of fibres began to decrease. Therefore, in the ML of the 12 years old cerebellum we only identified interrupted fibres of small calibre. Even more, GFAP proved to be useful for the identification of astrocytes, which were constantly present in the IGL.

NFAP staining caught our attention regarding its expression in the Purkinje cells and in the climbing fibres as well. Hence, we discovered that Purkinje cells expressed positivity for NFAP in every examined case, from 1 month to 12 years old cerebellum specimens. Also, the climbing fibres expressed positivity for NFAP, but apart from being homogeneously observed in all slides, as Purkinje cells did, we only observed small calibre fibres in the ML from 1 month, and then, from 2 months onward, the climbing fibres were thicker, the molecular layer being traversed by it.

Expression of GFAP is highlighted in Figure 1.

DISCUSSIONS

Most of the studies that focused on the developmental and pathological issues of the cerebellum were conducted on rodents [1, 2, 3]. It appears that the mouse cerebellum undergoes several modification in both size and number of the cell population depending on the presence and/or absence of several molecular factors [1]. Additionally, various genetic diseases such as Down Syndrome seem to influence the expression of some nervous tissue markers, namely S100 and GFAP [2], thus further study is needed in the field. The age related changes in the human cerebellum identified in our study are somehow supported by experimental studies in which the Purkinje cells from rodent cerebellum specimens appear to be affected by epigenetic changes [3].

Literature data is sparse in that which considers research studies focused on human cerebellum specimens. Fernandez-Pol conducted a study that included cases containing human cerebellum and focused on the expression of ribosomal proteins that may influence the normal and pathological dynamic of this nervous structure [4]. Hence, we considered the use of such specimens as a highly challenging attempt in order to analyse and interpret the developmental changes of the cerebellum at an immunohistochemical level. Also, we made use of little known and/or less studied markers, such as OCT3/4 and Nestin, along with the commonly known markers of the nervous tissue, such as NSE and GFAP. Our study reveals the fact that the human cerebellum possesses a great plasticity in both early and later developmental phases. These changes are encountered at a morphological and immunohistochemical level. The heterogeneous expression of NSE found in our study supports its' implication in the maintenance of the cerebellar architecture as well as in the identification and distribution of granule cells along with their fibers [5]. The granule cells may represent one of the key players in the development of the human cerebellum. On the other hand, NSE represents a marker of neuronal injury as it has been revealed in a study conducted by Mueller et al [6]. Considering the fact that some of the cerebellum specimens included in our study presented associated pathologies, the differential and heterogeneous expression of NSE may be due to the morphological and molecular changes that occur in these diseases. In Friendreich's ataxia for instance, cerebellar specimens displayed a severe loss of NSE reactivity in the large neurons [7]. Unlike other experimental studies [8] we observed a positive, homogeneous reaction for NFAP in the Purkinje cells. In a study conducted by Milenkovic et al., the expression of NFAP in the Purkinje cells was found only in pathologic conditions, at least in case of phosphorilated NFAP [8]. Also, Garcia-Atares et al. have shown a differential age-dependant expression of NFAP which decreases in older animals [9]. These controversial results may be related to associated pathologies and require further investigation, considering the fact that the above mentioned studies made use of rodent specimens. However, Sawant et al. have shown that different NFAP fractions occur in different stages of development in the rat cerebellum, although the complexity of these events has not yet been fully understood [10]. The differential expression of NFAP subunits was not only stage – but also structure-dependent [10].

Unlike in our study, where a complete disappearance of PROX1 reactivity was noticed in the later stages of cerebellar development, Lavado et al. have shown that that PROX1 expression persists in the hippocampus and in the cerebellum in adult mice during the development of the central nervous system [11]. However, it may be stated that PROX1 presents a differential activity during embryogenesis in different species. We also noticed a differential expression in case of OCT ¾ which is supported by previous studies. OCT ¾ is known to regulate PAX2 enhancer, PAX2 being the earliest gene that is involved in cerebellar development [12]. These aspects may explain the partial loss of OCT ¾ in the later stages of embryogenesis found in our study.

The expression of GFAP was less examined in human cerebellar development and was mostly the focus of murine experimental studies that made use of disease-affected specimens [13]. Through the use of GFAP, we analyzed the dynamic formation of cerebellar fibres during the early and late developmental stages. The development of the human cerebellum seems to imply the participation of a wide range of molecular players that are differentially expressed depending on the evolutionary stage. This aspect is currently supported by research studies conducted on rodent specimens and is in need of further examination through the use of both normal and pathological human cerebellum specimens.

CONCLUSIONS

Considering the sparse literature data regarding the developmental aspects of the human cerebellum, our study is a challenging attempt to understand the basics of their mechanisms. Most of the experimental studies in this field made use of animal specimens and have pinpointed the morphologic changes and differential expression of various markers during cerebellar development. The dynamic of the human cerebellum during evolution is supported in our study, at an immunohistichemical level, through the use of nervous tissue-specific and non-specific markers. Markers such as NSE are implicated in the development of the normal cerebellum and are correlated with nervous cell injury in case of different diseases that affect this organ. It may be stated that a complete understanding of cerebellar development could open new perspectives in order to find appropriate prognostic and therapeutic factors for cerebellum associated pathologies. We support further research in this field by using comparative studies that are focused on normal and pathological human cerebellum specimens in order to fully elucidate the morphologic and molecular changes that occur in pathological conditions.

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