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Research report

EphA4 is not required for Purkinje cell compartmentation

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Abstract

The Purkinje cells of both the adult and the developing cerebellar cortex are organized into parasagittal stripes or 'segments' expressing a variety of biochemical markers. We show that in the developing mouse cerebellar cortex, members of the Eph receptor gene family are expressed in mediolaterally alternating Purkinje cell segments. Since members of the Eph receptors family have been shown to play a role in hindbrain segmentation and boundary formation (Philos. Trans. R. Soc. Lond. B: Biol. Sci. 355 (2000) 993), we analyzed the effect of a null mutation of the EphA4 gene on Purkinje cell compartmentation. Using well characterized markers of Purkinje cell compartmentation in both the developing and the adult cerebellum, we observed no significant alteration in the banding pattern of these markers between the EphA4 knockout mice and their wild type controls. The ribboned pattern of migrating granule cells in the developing cerebellum also appears unaltered. The expression of other members of this gene family, including ephrin-B2, EphA2, and ephrin-A1, in a compartmentalized pattern within the Purkinje cell layer suggests a possible redundancy and/or a compensation of EphA4 function in the segmental patterning of cerebellar Purkinje cells.

Theme: Development and regeneration

Topic: Pattern formation, compartment, and boundaries

Keywords: Parasagittal; Purkinje cell; Eph; Mammal; Compartment; Boundary

1. Introduction

Morphologically, histologically, and anatomically, the cerebellum is a highly compartmentalized structure [51]. Most remarkable, however, is the molecular compartmentation of the cerebellar cortex evident in the biochemical heterogeneity of the cells comprising it [40]. A variety of molecular markers have shown a mediolateral (parasagittal) pattern of organization of Purkinje cells in both the developing and the adult cerebellar cortex (reviewed in Refs. [1,16,17,20,21,24,40]). Multiple studies have found a correlation between the boundaries of molecular compartments and the termination of afferent fiber projections in the cerebellar cortex [13,14,47,51,53,55]. While the development of parasagittal segments of Purkinje cells has been shown to be independent of afferent input [30,49,56], a correlation between Purkinje cell birth date and their parasagittal organization in stripes has been reported [10,26,27,39,44].

In the developing chick cerebellum, members of the Eph receptor family are expressed in parasagittal stripes on Purkinje cells [31,27]. The Eph receptor tyrosine kinases (RTK) and their ligands, the ephrins, are the largest family of RTKs, with 14 known receptors and nine ligands [11,37]. Since the ephrins are membrane bound, Eph–ephrin interaction is cell–cell mediated. The ephrins are subdivided into two groups based on their membrane anchorage—ephrin-A (GPI-linked) and ephrin-B (trans-

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membrane). Ephrin B ligands bind preferentially to EphB receptors, while the ephrin A ligands bind preferentially to EphA receptors [12]. EphA4, however, crosses subclasses by exhibiting appreciable affinity for ephrinB2 and ephrinB3 [12]. Perturbation studies have demonstrated a key role for the Eph receptors in segmental patterning in the developing nervous system (reviewed in Ref. [61]), by restricting the intermingling of cells at the boundaries between segments differentially expressing receptors and ligands [5,37,60]. In the developing chick cerebellum, we have shown that EphA4 is expressed in bands comprising early born Purkinje cells, while its ligand, ephrin-A5, is expressed in complementary bands comprising late born cells [27]. We hypothesized that, similar to the developing rhombomeres, compartmental boundaries within cerebellar Purkinje cells are maintained by Eph–ephrin interaction occuring at these boundaries [27]. In this study, we examine the expression of several Eph receptors and ephrins in the developing mouse cerebellum. Additionally, we test the hypothesis that EphA4 is involved in maintaining the boundaries of Purkinje cell segments by examining the effect of EphA4 null mutation on the compartmental distribution of molecular markers of Purkinje cell compartmentation of both the adult and developing cerebellar cortex.

2. Materials and methods

2.1. Animals and tissue preparation

The generation of the EphA4 knockout mice was described in Ref. [6]. Mice from different stages of development were taken from timed pregnant females with the date of birth designated P0 and embryo age E0 as the morning at which a vaginal plug is found. Mice were euthanized with an overdose of sodium pentobarbital (60 mg/kg i.p.), in accordance with institutional guidelines. At the time of kill, the brains were isolated and, for wholemount immunostaining, the meninges were removed. For embryonic tissue (P6 or younger), the tissue was fixed by overnight immersion in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS). For animals P6 or older, animals were transcardially perfused with 0.9% NaCl followed by 4% PFA. Dissected brains were immediately placed in the same fix for 12 h at 4 °C, rinsed in PBS, and cryoprotected through a graded series of sucrose (10–30%) in PBS. Wildtype littermate controls were included in all of the analyses. A sample of at least seven animals per group was used for analyzing the effect of EphA4 knockout on zebrin and calbindin expression

2.2. DNA extraction and PCR genotyping of mice

DNA was extracted from mouse tail tissue approximately 1.5 cm in length, using a protocol modified from Ref. [57]. The tissue was placed in 500 μl of lysis buffer (100 mM Tris–HCl pH 8.5, 400 mM NaCl, 0.2% SDS, 20 μg/ml RNase A, 500 μg/ml Proteinase K) and incubated at 37 °C overnight with constant rotation. EDTA was added to a final concentration of 5 mM and centrifuged at 13 000×*g* for 15 min. The supernatant was transferred to a clean tube, and two volumes of 100% ethanol were added, inverting the tube until DNA threads formed. The DNA threads were spooled onto a 10 μl sealed glass capillary tube and briefly rinsed by spinning in 70% ethanol. The DNA was resuspended in 300 μl of TE (10 mM Tris pH 8.0, 1 mM EDTA) by breaking off the tip of the capillary in a tube with TE and incubating the tube at 45 °C for 1 h and at 65 °C for 10 min. The DNA was then stored at 4 °C.

Mice were genotyped by PCR of tail DNA, using primer pairs P1 and P2, and P3 and P4 (GENSET, La Jolla, CA) as described in Ref. [6]. The final volume of the PCR was 25 μl and consisted of 50–500 ng DNA, 15 pmol of each primer, 2.0 mM MgCl, 100 μM dNTP's, 1 unit Taq polymerase (Promega, Madison, WI), and 1×Taq polymerase reaction buffer supplied by manufacturer. The cycling reaction was 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s.

2.3. Antibodies

The following affinity purified rabbit polyclonal antibodies were used: (1) anti-EphA4 antibody recognizing the 11 carboxy-terminal amino acids of the chick EphA4 receptor; (2) anti-EphA2 antibody made by injecting a GST fusion protein of the ligand-binding domain of EphA2 comprising approximately 200 amino acids of the mature N terminus (i.e. without the signal peptide), then affinity purified on a column containing the extracellular domain of EphA2 purified from CHO cell culture supernatants; (3) anti-ephrin-A1 antibody [41] (anti-ephrin-A1 antibody #2); and (4) anti-ephrin-B2 (Santa Cruz Biotechnology, Santa Cruz, CA). Specificity of EphA4, EphA2, and ephrin-A1 antibodies has been demonstrated [36,41]. The following mouse monoclonal antibodies were used: anti-calbindin-D_{28K} (Sigma, St. Louis, MO) and anti-Zebrin II (a gift from Dr Richard Hawkes, University of Calgary, Calgary, Alberta, Canada).

2.4. Immunostaining

For embryonic sections through postnatal day 7, tissue was embedded in Tissue Tek O.C.T. medium (Miles, Elkhardt, IN), frozen in liquid nitrogen, and 20 μm cryostat sections were collected on gelatin/poly-L-lysine coated slides. Brains used for Zebrin II analysis were sectioned either horizontally or coronally at 40 μm using a sledge microtome and collected as free floating sections. The protocol for Zebrin II immunostaining on adult sections is described in Ref. [29]. The protocol for immunostaining of frozen sections is described in Ref.

[27]. For peroxidase labeling, biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), followed by streptavidin HRP (Zymed Laboratories, San Francisco, CA), was visualized with 3,3′-diaminobenzidine (DAB) as the chromagen (Sigma, St. Louis, MO). For fluorescent labeling, the zebrin-II antibody was detected using a goat anti-mouse FITC (Jackson Laboratories, West Grove, PA).

Peroxidase labeled sections with DAB were viewed under a Nikon Eclipse E400 microscope (Nikon Inc., Melville, NY), and digital images were collected using a Nikon COOLPIX digital camera (Nikon Inc. Melville, NY). For fluorescent images, sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA), then analyzed and digitized with a confocal microscope (Bio-Rad Laboratories, Hercules, CA). Using Photoshop (Adobe, Mountain View, CA) and Canvas 6.0 (Deneba, Miami, FL), the images were cropped and corrected for brightness and contrast, but were not otherwise modified.

2.5. Wholemount immunostaining

For wholemount immunostaining, the tissue was fixed in 4% paraformaldehyde for 2 h at 4 °C. This was followed by three washes in ice cold PBS and graded dehydration into 100% methanol. Following overnight incubation at −20 °C in 100% methanol, the tissue was treated with 0.3% H_2O_2 in methanol for 1 h at room temperature and was then gradually rehydrated to tris-buffered saline (TBS). To reduce non-specific binding of antibodies, specimens were incubated for a minimum of 3 h at room temperature in a blocking solution consisting of 10% horse serum with 0.2% Triton-X in TBS. The specimens were then incubated in primary antibody appropriately diluted in the blocking solution for 48 h at 4 °C. The next day, specimens were washed extensively in TBS, followed by an overnight incubation at 4 °C in the respective HRP-conjugated secondary-goat anti-mouse HRP for calbindin immunostaining (Jackson Laboratories) and goat anti-rabbit HRP for EphA4 and ephrin-B2 immunostaining (Vector Laboratories). The specimens were again washed extensively in TBS, and visualized with DAB as chromagen. Wholemount stained cerebella were viewed under a Leica microscope (Leica Inc., Heidelberg, Germany), and digital images were collected using a Kodak DC290 ZOOM digital camera (Kodak, Rochester, NY).

3. Results

3.1. EphA4 expression in the developing mouse cerebellum

EphA4 expression in the developing cerebellum is illustrated in a horizontal section of a P3 cerebellum (Fig. 1D) and in a wholemount view of an E19 cerebellum (Fig. 2A). Mediolateral stripes of EphA4 expression extend throughout the cerebellum (Figs. 1D and 2A). The expression appears membranous and is localized to the Purkinje

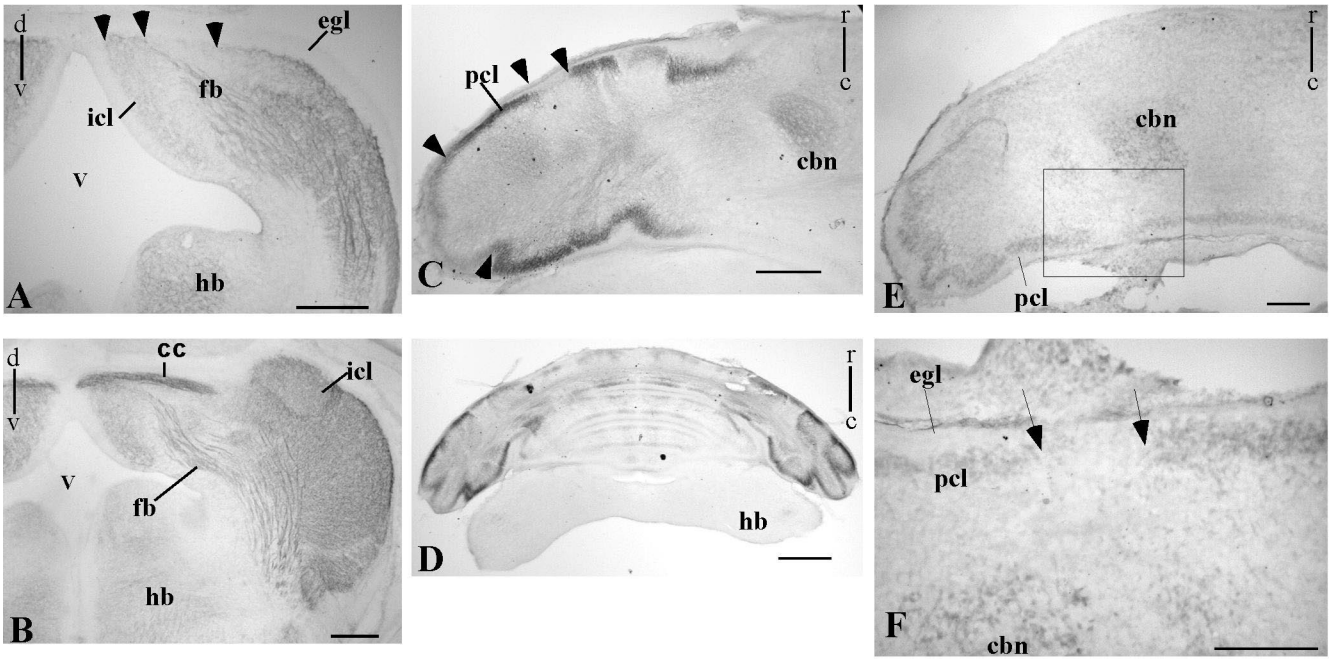

Fig. 1. (A–D) EphA4 immunostaining on mouse cerebellar sections from stages E16 (A) E17 (B), P0 (C), and P3 (D). Arrowheads point to borders of EphA4 expression domains. Heterogeneous expression of EphA4 is observed in the cerebellar Purkinje cells. (E) ephrin-B2 immunostaining on P0 mouse cerebellar sections. (F) high magnification of boxed region in (E). Arrows point to borders of expression domains. c, caudal; cbn, cerebellar nuclei; cc, cerebellar commissure; d, dorsal; egl, external granule cell layer; fb, fibers; hb, hindbrain; icl, inner cortical layer; pcl, Purkinje cell layer; r, rostral; V, ventricle; v, ventral. Scale bar=200 μm.

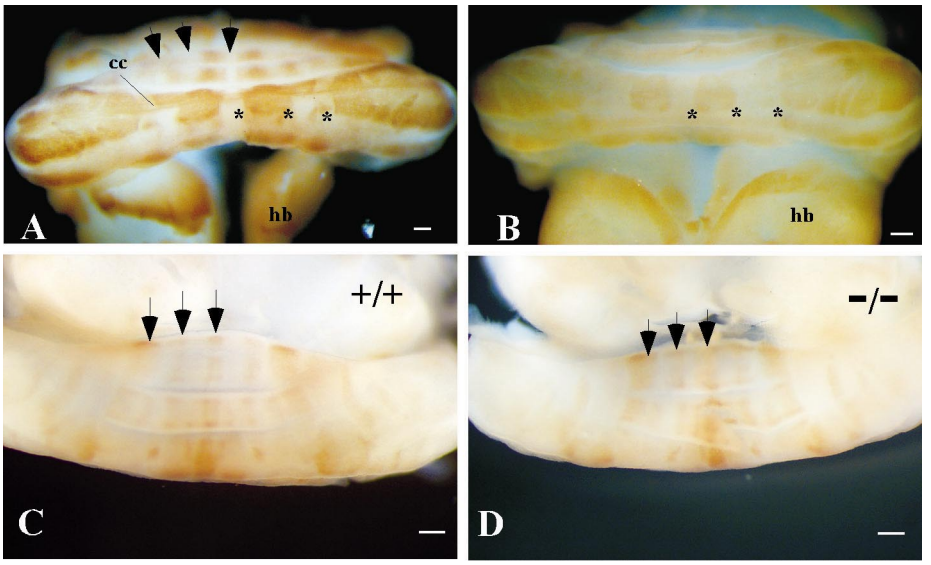

Fig. 2. (A) Wholemount immunostaining of EphA4 in an E19 mouse cerebellum showing striped pattern of expression. The cerebellar comissure (cc) is also stained. (B) Wholemount immunostaining of ephrin-B2 in a P0 mouse cerebellum. Asterisks in (A, B) point to ephrin-B2 bands that are distributed similarly to those of EphA4. (C, D) Wholemount immunostaining of calbindin in P0 cerebella of wild type littermate control (C) and EphA4 homozygote. Arrows point to calbindin bands anteriorly (C, D) and their corresponding location (A). cc, cerebellar commissure; hb, hindbrain. Scale bar=200 μm.

cell layer and cells of the deep cerebellar nuclei (Figs. 1C,D and 5G). Spatially the expression varies between the lobules (Fig. 2A). In the anterior lobe, the expression appears complementary to that of calbindin (compare arrows in Fig. 2A and C). Parasagittal stripes of EphA4 are observed as early as E16 in the inner cortical layer where Purkinje cells and cells of the deep cerebellar nuclei reside (arrows in Fig. 1A). The cerebellar commissure and corticonuclear fibers appear labeled with EphA4 (Figs. 1B and 2A). Heterogeneous expression of EphA4 expression continues to P7 (data not shown). In the adult cerebellum, while EphA4 expression appears mainly homogenous, areas of heterogeneity are observed in the hemispheres (Fig. 5H).

3.2. Effect of EphA4 gene deletion on Purkinje cell compartmentation

To analyze the effects of loss of EphA4 function on cerebellar compartmentation, the expression of calbindin and zebrin was analyzed in the EphA4 homozygous mice. Zebrin and calbindin are chosen as two markers of the adult and developing Purkinje cell compartmentation, respectively, since their expression is well characterized [9,15,18,19,45], and their boundaries have been shown to correlate with afferent input [13,14,47,51,53,55]. Moreover, BrdU birthdating revealed a correlation between the localization of late born cells and calbindin expression and that of early born cells and zebrin expression [44]. Comparison of calbindin and EphA4 expression patterns in the P0 cerebellum reveals a complementary expression between the two in the anterior lobules (Fig. 2A and C) consistent with the finding that EphA4 marks early born cells in chicken [27]. Finally, there has been no evidence to suggest that zebrin or calbindin is downstream of Eph–ephrin signaling, thus reducing the possibility that an effect would be the result of regulation of gene expression rather than of alteration in compartmental boundaries.

In general, the cerebellum of the knockout mouse appears morphologically and histologically normal (data not shown). Fig. 2 compares the expression of calbindin in the EphA4 knockout cerebellum to that of the wild type control. In wholemount view, calbindin stripes in the knockout cerebella appear similar to those of the wild type control (Fig. 2C, D). Although the overall pattern of the calbindin stripes and the sharpness of the bands appear comparable to that of the wildtype littermate, some subtle changes may be present. The calbindin bands of the knockout cerebellum (Fig. 2D) appear more closely spaced compared to those of wild type littermate control (Fig. 2C). However, the analysis is complicated by the fact that the EphA4 knockout mice and their cerebella are significantly smaller in size at any age examined than their wildtype or heterozygote littermate controls. In the developing cerebellum, migrating granule cell ribbons occur at the boundaries of Purkinje cell compartments [34,28]. Fig. 3 shows lack of effect of EphA4 null mutation on the migrating granule cell ribbons. Finally, in the adult cerebella, no major changes in the compartmental expression of zebrin are observed in Purkinje cells. Fig. 4 shows representative sections of the zebrin stripes in the knockout cerebella. Overall, the boundaries appear sharp and no bands seem to be missing (Fig. 4). Like the calbindin bands, the zebrin bands in the knockout cerebella (Fig. 4A,D) appear more closely spaced than those of the wild type or heterozygote littermate controls (Fig. 4). However,

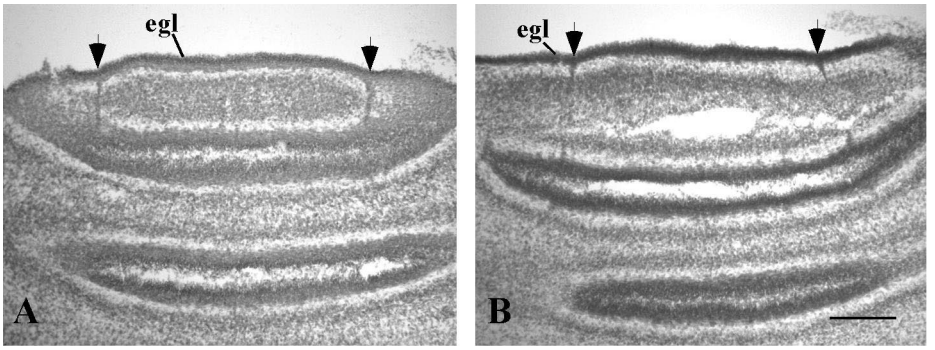

Fig. 3. Cresyl violet nuclear stain of horizontal sections of a P3 cerebella of EphA4 homozygote (A) and wildtype littermate control. Arrows point to granule cell ribbons. The granule cell ribbons are present in both. egl, external granule cell layer. Scale bar=200 μm.

this may simply reflect the size difference between cerebella of knockout mice and those of their wildtype or heterozygote littermate controls. Moreover, this size difference makes it difficult to precisely align the sections from these two groups. In Fig. 4, the qualitative difference observed in the banding pattern near the midline of the central lobe (IV,V) of the knockout cerebellum compared to that of the wildtype or the heterozygote littermate controls (Fig. 4D–F) is not consistently observed in sections taken from the EphA4 knockout mice. It probably represents an alignment artifact.

3.3. Expression of other members of the Eph receptor gene family in the mouse cerebellum

The lack of major effects of EphA4 deletion on molecular compartmentation indicates that EphA4 is not essential for Purkinje cell patterning. To address whether redundant or compensated function of multiple Eph receptors might account for this function, we examined the expression profiles of other Eph receptors and ephrins in the developing mouse cerebellum. Ephrin-B2, a ligand known to bind EphA4 [12], appears to be expressed in Purkinje cell stripes in the P0 cerebellum (Figs. 1E–F and 2B). A comparison of the two wholemounts shows that expression of ephrin-B2 is distributed similarly to EphA4 (asterisks in Fig. 2A, B). Histologically, the expression appears localized to the Purkinje cell layer and cells of the deep cerebellar nuclei (Fig. 1E–F). Consistent with published results [33], lower levels of ephrin-B2 expression are also observed in the upper layer of the external granule cell layer (Fig. 1E–F).

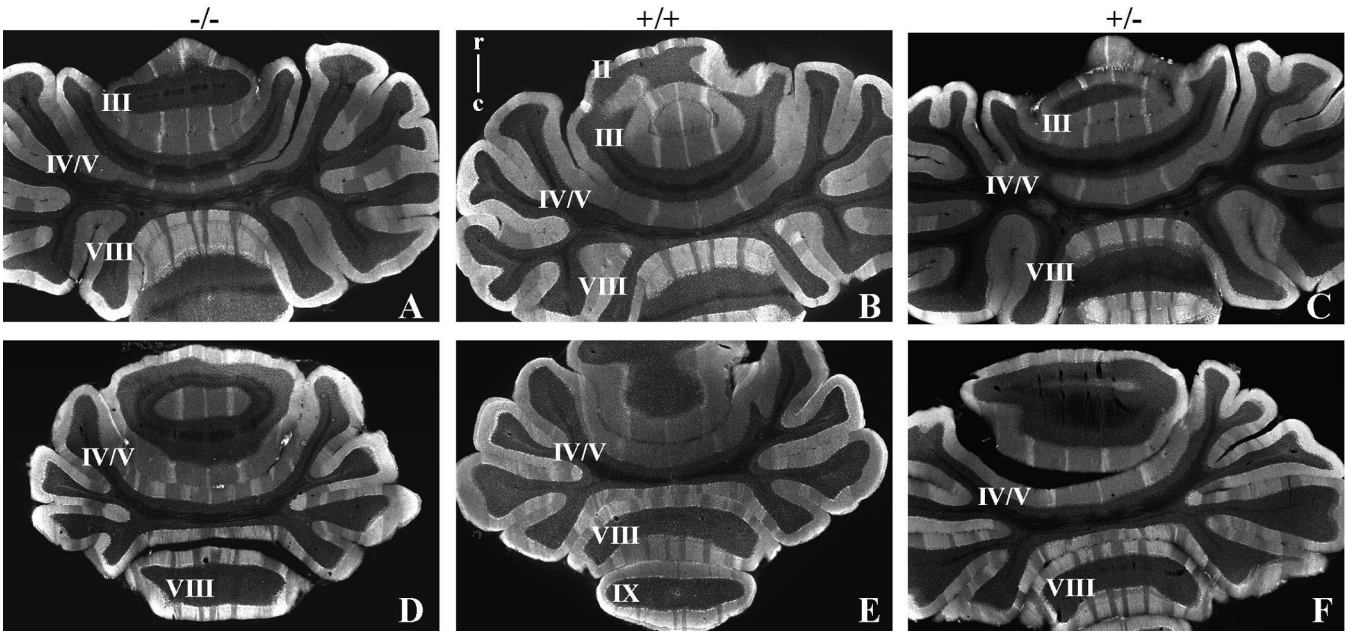

Fig. 4. Zebrin immunostaining of adult cerebellar sections from cerebella of EphA4 homozygote (A, D), wildtype littermate controls (B,E), and EphA4 heterozygotes (C,F). Sections C–D are more ventral than those shown in E–G. Intact zebrin bands are present in the EphA4 homozygote cerebella. Roman numeral refer to cerebellar lobules. r, rostral; c, caudal.

EphA2 and its ligand, ephrin-A1, are also expressed in the developing cerebellar cortex (Fig. 5A,D). Parasagittaly striped expression is observed for EphA2, while ephrin-A1's expression appears homogenous (Fig. 5A,D). Cells of the deep cerebellar nuclei, the external granule cell layer, and cells in the IGL also appear to express ephrin-A1 (Fig. 5D). The expression of EphA2 and ephrin-A1 continues in the adult cerebellar cortex, where heterogeneous expression of both receptor and ligand is observed in a manner that resembles EphA4 (Fig. 5B,E,H). However, the subcellular localization of these proteins on the Purkinje cell neuron is different (Fig. 5C,F,I). While EphA4 expression appears on the Purkinje cell bodies and their entire dendritic tree (Fig. 5I), ephrin-A1's immunostaining is localized to Purkinje cell bodies and the primary dendritic arbors (Fig. 5C). EphA2's immunostaining appears localized to surfaces of cells located in the Pukinje cell layer and their extensions. The unbranched nature of the processes in the molecular layer throughout the cerebellum suggest that the expression is present on Bergmann glial cell bodies and their processes (Fig. 5F).

4. Discussion

The conservation of a segmental expression pattern of EphA4 on cerebellar Purkinje cells from chick to primate [27,28], the complementary expression of its ligand, ephrin-A5 [27], the correlation between EphA4 expression and the localization of early born Purkinje cells and that of its ligand with late born cells [27], along with the well documented role of EphA4 in segmental patterning in the developing forebrain, rhombomeres, and somites [8,37,58–60], prompted a hypothesis that EphA4 interaction with its ligand at Purkinje cell compartmental boundaries is important in the formation and maintenance of these

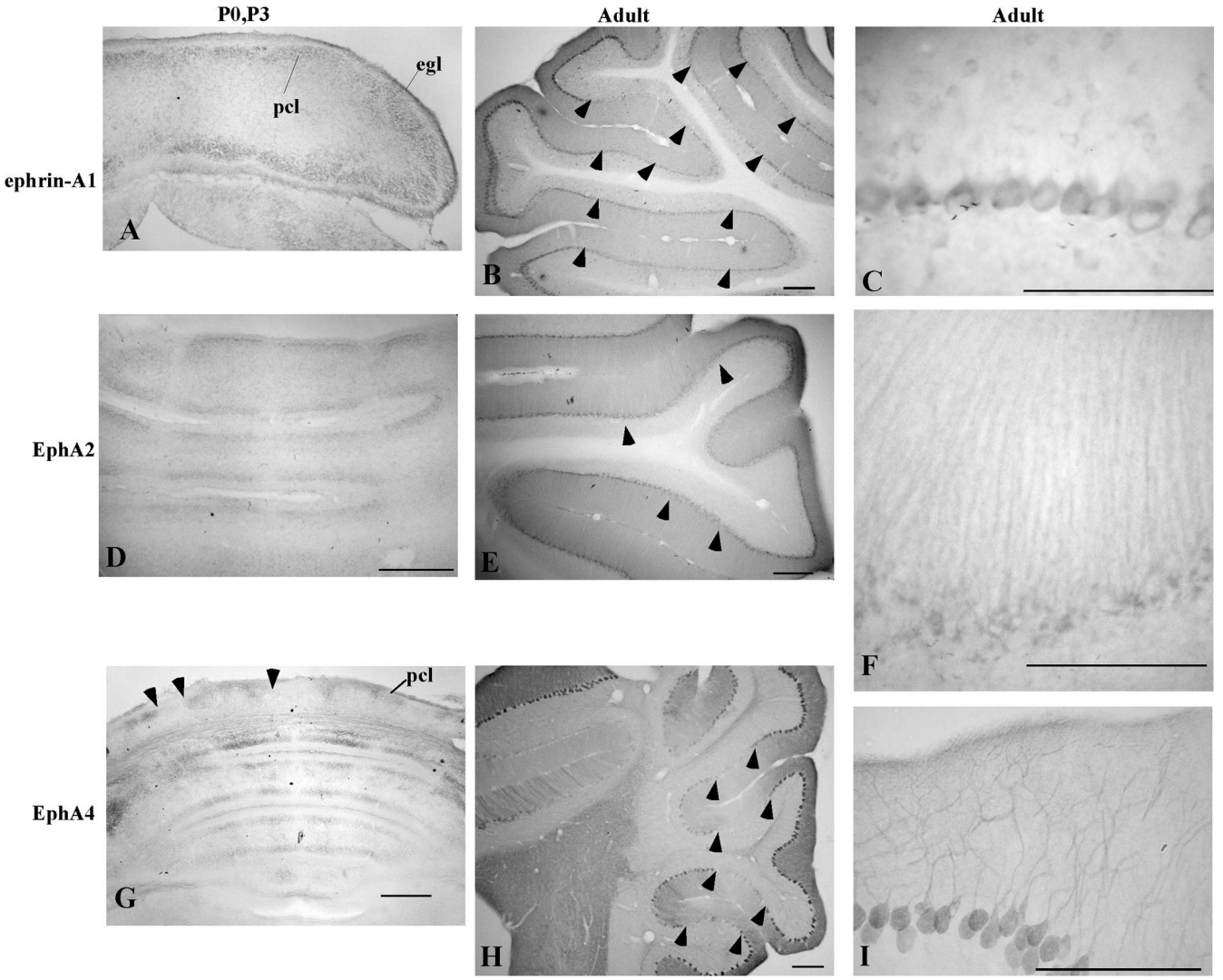

Fig. 5. Immunostaining of ephrin-A1 (A–C), EphA2 (D–F), and EphA4 (G–I) on horizontal cerebellar sections of P0 (A), P3 (D, G) and adult (B, C, E, F, H, I) mice. Arrowheads point to borders of expression domains. Scale bar (A, B, D, E, G, H)=200 μm. Scale bar (C, F, I)=100 μm.

boundaries. We tested this hypothesis in EphA4 knockout mice.

Data in the P0 and the adult mouse cerebellum reveal a generally unaltered expression of compartmental markers in the EphA4 knockout mice (Figs. 2 and 4). While multiple neuronal phenotypes are associated with the EphA4 knockout mice [6,22], the lack of a major effect of EphA4 knockout on Purkinje cell segmentation or on the ribboned pattern of granule cell migration (Fig. 3) indicates that EphA4 is not necessary for the gross morphological formation and/or maintenance of molecular compartmentation in the cerebellar cortex. It is important to emphasize, however, that while our results show that Purkinje cell compartmental boundaries remain sharp with no missing bands, some subtle changes might be present. Figs. 2 and 4 show that the spacing between the bands in knockout cerebella appears different from that of the wildtype littermate control. However, the difference in size of the cerebella of wildtype and EphA4 knockout animals confounds this analysis, preventing a clear-cut interpretation of subtle apparent differences. The lack of a major EphA4 effect on Purkinje cell compartmentation is consistent with reports of lack of obvious defects in the developing hindbrain of homozygous EphA4 mutant mice [22], where Eph–ephrin receptor signaling has been shown to be involved in the restriction of cell intermingling [37,59,60].

In the cerebellum, the striped expression of other members of the Eph receptor family members on Purkinje cells (Fig. 1 and 5) [31,46] and of splice variants [4] suggests that EphA4 might be playing a redundant role in Purkinje cell compartmentation. Reverse signaling by the ephrin-B subfamily could also account for the lack of effect on Purkinje cell patterning. While the ephrins were initially characterized as receptor-activating ligands, a number of studies have shown that cytoplasmic domains of ephrins of the B class are capable of mediating signal transduction, and that this signaling is stimulated by interaction with Eph receptors [3,23,25,33,37,50]. In this context, ephrins are receptors, and Ephs are ligands.

Fig. 2 shows that ephrin-B2 is expressed in Purkinje cell stripes that are distributed similarly to those of EphA4 in the developing cerebellum (asterisks in Fig. 2A,B). It is conceivable that in the absence of the receptor, reverse signaling through ephrin-B2 would compensate for EphA4's role in maintaining the boundaries of Purkinje cell domains. In vitro aggregation experiments have shown that bi-directional activation is required to prevent intermingling between cell populations [37]. Future studies with double knockouts under a neuronal promoter would address this question.

It is worth noting that in the cerebellum, the only gene manipulation known to result in diffuse zebrin compartmental boundaries consistent with a role in inhibiting the mixing of cells from different compartments concerns the ectopic expression of En-2 [2]. In the En-2 null mutation, although zebrin bands are reported missing, their boundaries remain sharp [29]. These data, along with the report that En-2 regulates ephrin expression [32], are consistent with a redundant role for the Eph–ephrin system in maintaining zebrin compartmental boundaries.

EphA4's role in cerebellar patterning may be redundant with other Eph receptors, or with other heterogeneously expressed guidance cues such as cadherins [34]. Interestingly, the apparently heterogeneous expression of EphA2 on Bergmann glia suggests that neuron–glia interactions may contribute to neuronal patterning events. Parasagittally banded heterogeneity of Bergmann glia has been previously observed [35,48], but heterogeneous expression of guidance proteins on cerebellar glia has not been noted before.

Alternatively, EphA4 function in the developing cerebellum may be limited to axon guidance, since this gene family has been shown to play a prominent role in axon pathfinding (reviewed by Refs. [7,11,42,43]). In the developing mouse cerebellum, EphA4 expression is observed on the cerebellar commissure through which the mossy fibers enter the cerebellum (Figs. 1B and 2A), on cells of the deep cerebellar nuclei (Fig. 1C), and on several hindbrain nuclei that represent targets for afferent and efferent cerebellar pathways (data not shown). EphA2, ephrin-A1, and ephrin-B2 are also expressed on cells of the deep cerebellar nuclei and on several hindbrain nuclei (Fig. 5 and data not shown).

5. Uncited references

[38]; [52]; [54]

Acknowledgements

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