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Compartmentation of the cerebellar cortex of hummingbirds (Aves: Trochilidae) revealed by the expression of zebrin II and phospholipase Cβ4

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ABSTRACT

The parasagittal organization of the mammalian cerebellar cortex into zones has been well characterized by immunohistochemical, hodological and physiological studies in recent years. The pattern of these parasagittal bands across the cerebellum is highly conserved across mammals, but whether a similar conservation of immunohistochemically defined parasagittal bands occurs within birds has remained uncertain. Here, we examine the compartmentation of the cerebellar cortex of a group of birds with unique cerebellar morphology—hummingbirds (Trochilidae). Immunohistochemical techniques were used to characterize the expression of zebrin II (aldolase C) and phospholipase Cβ4 (PLCβ4) in the cerebellar cortex of two hummingbird species. A series of zebrin II immunopositive/immunonegative parasagittal stripes was apparent across most folia representing three major transverse zones: an anterior zone with a central stripe flanked by three lateral stripes on either side; a central zone of high/low immunopositive stripes; and a posterior zone with a central stripe flanked by four to six lateral stripes on either side. In addition, both folia I and X were uniformly immunopositive. The pattern of PLCβ4 immunoreactivity was largely complementary—PLCβ4 positive stripes were zebrin II negative and *vice versa*. The similarity of zebrin II expression between the hummingbirds and the pigeon indicates that the neurochemical compartmentation of the cerebellar cortex in birds is highly conserved, but species differences in the number and width of stripes do occur.

1. Introduction

The gross morphology of the cerebellum varies tremendously both within and among the vertebrate classes. Most of the variation within classes can be attributed to variations in the size and morphology of components of the cerebellar vermis (i.e., folia or lobules) and hemispheres. In mammals, for example, the paraflocculus of bats and whales is enlarged compared to other groups (Paulin, 1993) and the foliation pattern of the cerebellar hemispheres varies widely among primates (Larsell, 1970). This variation in gross cerebellar morphology does, however, belie a highly conserved parasagittal organization of the cerebellar cortex. Using zebrin II/aldolase C (Brochu et al., 1990; Ahn et al., 1994: hereafter referred to as simply as ZII) as an immunohistochemical marker, the cerebellar cortex can be subdivided into a series of parasagittal stripes that correspond to physiologically and hodologically defined zones in the cerebellum (Gravel et al., 1987; Gravel and Hawkes, 1990; Ji and Hawkes, 1994; Hallem et al., 1999; Voogd et al., 2003; Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004; Pijpers et al., 2005; Gao et al., 2006; Pijpers and Ruigrok, 2006). The pattern of ZII expression is highly conserved among mammalian taxa that vary in the size and morphology of their cerebella (reviewed in Sillitoe et al., 2005). Thus, diversity in cerebellar morphology does not appear to reflect changes in the pattern of cerebellar compartmentation as revealed by zebrin II immunoreactivity. Whether this is also true of other vertebrate groups, however, is unknown.

Recently, Pakan et al. (2007) showed that ZII immunoreactivity in the pigeon (*Columba livia*) is expressed in parasagittal stripes in a

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similar fashion to that of mammals. Cerebellar morphology in birds, however, varies widely among taxa with significant differences in the degree of foliation of the cerebellar cortex (Iwaniuk et al., 2006a) as well as the relative size of the individual folia (Iwaniuk et al., 2007). The most divergent cerebellar morphology observed in birds thus, far is the significant reduction of the anterior lobe in hummingbirds (Trochilidae) and swifts (Apodidae) (Larsell, 1967; Iwaniuk et al., 2006a,b, 2007). As shown in Fig. 1, hummingbirds have an anterior lobe that is characterized by an extreme reduction in the size of folia II and III compared to other birds (Iwaniuk et al., 2006b, 2007). Whether this significant evolutionary change in the hummingbird cerebellum has resulted in a change in the organization of the cerebellum is unknown. Here, we examine the pattern of cerebellar compartmentation in hummingbirds as revealed by ZII expression and compare it to previous results for the pigeon (Pakan et al., 2007). Based upon the highly conserved pattern of expression in mammals, we predict that ZII expression will be largely concordant between hummingbirds and the pigeon.

ZII is not, however, the only antigen that reveals parasagittal bands in the cerebellar cortex. Over 20 different molecules yield a striped pattern in mammalian cerebella including 5′-nucleotidase (Scott, 1963), acetylcholinesterase (Marani and Voogd, 1977), corticotropin-releasing factor (King et al., 1997), heat shock protein 25 (Armstrong et al., 2001), human natural killer cell antigen (Eisenman and Hawkes, 1993; Marzban et al., 2004) cadherins (Arndt and Redies, 1998; Vanhalst et al., 2005; Neudert and Redies, 2008) and phospholipase Cβ4 (PLCβ4, Sarna et al., 2006; Marzban et al., 2007). In order to gain a better understanding of how the cerebellar cortex is organized in birds as well as the correspondence between ZII and other antigens, we also examined the pattern of PLCβ4 labeling throughout the hummingbird cerebellum.

2. Materials and methods

The brains from a total of nine adult and sub-adult Anna's (*Calypte anna*, $n = 5$) and Rufous (*Selasphorus rufus*, $n = 4$) hummingbirds that were immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer were kindly provided to us by Drs. Kenneth Welch Jr. and Raul Suarez (University of California, Santa Barbara). All animal procedures conformed to institutional regulations at the University of California, Santa Barbara (Institutional Animal Care and Use Committee Approval #672) and the *Guide to the Care and Use of Experimental Animals* from the Canadian Council of Animal Care.

2.1. Immunohistochemistry for serial sections

Following several weeks of fixation, eight of the hummingbird brains were cryoprotected, embedded in gelatin and sectioned on a freezing stage microtome in the coronal plane at a thickness of 40 μm. Every section throughout the cerebellum was collected in 0.1 M phosphate buffered saline (PBS, pH 7.4). The sections were then divided into four alternate series. One series from each bird was mounted onto gelatinized slides, stained for thionin, dehydrated through a graded series of ethanols and coverslipped with Permount. The remaining three series were used for immunohistochemistry.

Two different primary antibodies were used to examine the immunohistochemical pattern of cerebellar compartmentation in the hummingbirds. First, we used a monoclonal anti-mouse ZII antibody (1:200–400), which was produced by immunization with a crude cerebellar homogenate from the weakly electric fish *Apteronotus* (Brochu et al., 1990). This antibody recognizes a single polypeptide band in mouse with an apparent molecular weight of 36 kDa that cloning studies have identified as the isoenzyme aldolase C (Aldoc: Ahn et al., 1994; Hawkes and Herrup, 1995). Western blot analysis has shown that a single identical band is also recognized in pigeon cerebellar homogenate (Pakan et al., 2007). Second, we used an anti-rabbit PLCβ4 antibody that is raised against a synthetic peptide representing amino acids 15–74 of mouse PLCβ4 fused to GST protein and expressed in bacteria (Nakamura et al., 2004; Sarna et al., 2006; Marzban et al., 2007). Control immunohistochemistry using either antibody pre-absorbed with antigen polypeptides or cerebellar sections from a PLCβ4 knockout mouse yielded no significant immunostaining (Nakamura et al., 2004). Western blotting of this antibody in chicken (*Gallus gallus* f. *domesticus*) cerebellar homogenates has also revealed a band with the same molecular weight as murine PLCβ4 (Marzban et al., unpubl. data).

For ZII immunohistochemistry, free floating sections were washed several times in PBS and blocked with 10% normal donkey serum (in PBS, Jackson Immunoresearch Laboratories, West Grove, PA) and 0.4% Triton X-100 in PBS for 1–2 h at room temperature followed by incubation in the primary antibody for 48–72 h at room temperature. The sections were then rinsed several times in PBS and incubated in CY3- or CY2-conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, 1:200 in PBS, 2.5% donkey serum and 0.4% Triton X-100) for 2–3 h at room temperature. Following several rinses in PBS, the sections were then mounted onto gelatinized slides.

2.2. Double labeling

Double labeling followed a similar protocol to that of the ZII immunohistochemistry. Tissue sections were washed, blocked in PBS containing 10% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, PA) and incubated in blocking solution containing a combination of primary antibodies: anti-ZII (spent culture medium diluted 1:200: Brochu et al., 1990) and anti-PLCβ4 (1:1000) for 16–18 h at 4 °C. Following incubation in primary antibodies, sections were washed and then left in PBS containing CY3-conjugated goat anti-rabbit secondary antibody and CY2-conjugated goat anti-mouse secondary antibody (both diluted 1:1000, Jackson Immunoresearch Laboratories, West Grove, PA) for 2 h at 4 °C. After incubation in secondary antibody, the sections were washed in 0.1 M PBS buffer, mounted on chrome-alum gelatin subbed slides.

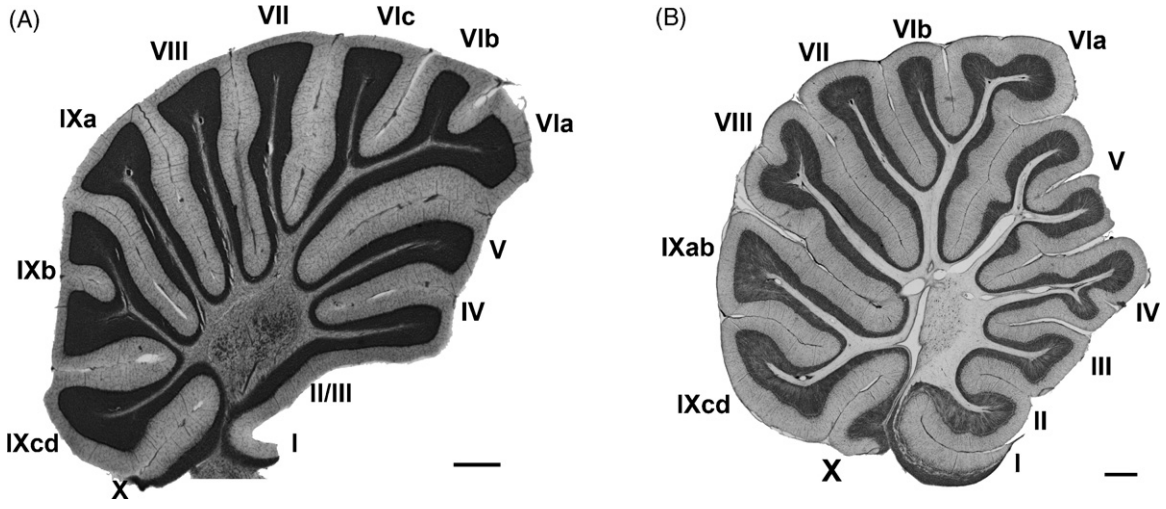

Fig. 1. Cerebellar midsagittal sections stained with thionin. (A) Anna's Hummingbird (*Calypte anna*) and (B) pigeon (*Columba livia*). The folia are numbered rostrocaudally from I to X following Larsell's (1967) terminology. Note the lack of foliated cerebellar cortex in the hummingbird corresponding to folia II/III in the pigeon. Scale bars = 800 μm.

2.3. Whole mount immunohistochemistry

The cerebellum of one male Rufous Hummingbird was removed from the rest of the brain by cutting through the cerebellar peduncles. It was subsequently immunostained as a whole mount using a protocol slightly modified from Sillitoe and Hawkes (2002) as used in Pakan et al. (2007). After incubating the cerebellum in fixative for 24–48 h, it was post-fixed overnight at 4 °C in Dent's fixative (Dent et al., 1989). Next, the cerebellum was incubated in Dent's bleach (Dent et al., 1989) for ~8 h then dehydrated in 2 × 30 min each 100% MeOH. The tissue was passed through 4–5 cycles of chilling to −80 °C and thawing to room temperature in 100% MeOH followed by overnight incubation in MeOH at −80 °C. For ZII staining, the cerebellum was rehydrated for 90 min each in 50% MeOH, 15% MeOH, and PBS then enzymatically digested in 10 μg/ml proteinase K (>600 units/ml; Boehringer Mannheim, Inc.) in PBS for 5 min at room temperature. After rinsing 3 × 10 min in PBS, the tissue was incubated in blocking buffer (Davis, 1993) for 6–8 h at room temperature. The tissue was then incubated for 48–96 h in ZII antibody (see details above), rinsed 3 × 2 h at 4 °C, and incubated overnight at 4 °C in goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories). Finally, the tissue was rinsed 4 × 3 h each at 4 °C followed by a final overnight rinse, incubated in 0.2% bovine serum albumin, 0.1% Triton X-100 in PBS for 2 h at room temperature and visualized with DAB.

2.4. Microscopy and image analysis

The serial sections were viewed with a compound light microscope (Leica DMRE) equipped with the appropriate fluorescence filters (rhodamine and FITC). Images were acquired using a Retiga EXi *FAST* Cooled mono 12-bit camera (Qimaging, Burnaby, BC, Canada) and analyzed with OPENLAB imaging software (Improvision, Lexington, MA). The images were compiled with PTGui v 6.0.3 (Rotterdam, Netherlands) and manipulated using Adobe Photoshop (San Jose, CA) to compensate for brightness and contrast.

2.5. Nomenclature

In general, we have adopted the avian cerebellar nomenclature developed by Larsell (1967) with eleven primary folia numbered in a rostrocaudal direction from I to X, but with a division of IX into IXab and IXcd for a total of eleven folia. Although Larsell (1967) intended this to reflect homologies between mammalian lobules and cerebellar folia, such a link has yet to be adequately demonstrated. As a result, we refer to the divisions of the avian cerebellum as folia instead of lobules to differentiate between birds and mammals and to prevent the inference that these subdivisions are indeed one and the same. This same nomenclature has been used for a wide array of species (Larsell, 1967; Iwaniuk et al., 2006b, 2007). The only exception in the hummingbirds concerns folia II and III. Because of the lack of a sulcus demarcating folia II from III in the hummingbirds (Fig. 1), we were unable to distinguish between the two using cytoarchitectonic criteria and therefore refer to them simply as folia II/III.

In our discussion of the topography of antigen expression, we use the same nomenclature for the ZII stripes adopted in mammalian studies (e.g., reviewed in Sillitoe et al., 2005) and previously applied to pigeon (Pakan et al., 2007). Briefly, the most medial immunopositive (i.e., strongly immunoreactive) stripe, straddling the midline, is designated P1+ and the number increases as the stripes move laterally to P7+. ZII immunonegative stripes are numbered P1− to P5− according to the P+ stripe medially. While this does not necessarily reflect stripe homologies among avian and mammalian species, it provides some consistency in labeling and discussing the labeling pattern.

3. Results

Purkinje cells were the only neurons immunoreactive for ZII in the cerebellar cortex of both hummingbird species and no differences in immunoreactivity were present between the two species. In ZII immunopositive Purkinje cells, the somata, dendrites and axons were all labeled. Also readily apparent was a pattern of parasagittal stripes, consisting of Purkinje cells that strongly express ZII alternating with those that weakly express, or do not express, ZII (see below, Figs. 2–4). In the wholemount (Figs. 2A, 3A and B), the contrast between the ZII immunopositive and immunonegative stripes was not as marked as is typically seen in rodents (e.g., Brochu et al., 1990; Eisenman and Hawkes, 1993), but was slightly higher than that we observed in pigeons (Pakan et al., 2007) and reminiscent of what has been reported in cats (Sillitoe et al., 2003) and primates (Sillitoe et al., 2004). In the serial sections, the contrast between stripes was much stronger (Figs. 2–4).

3.1. Zebrin II expression in the anterior lobe

As shown in Fig. 2, the anterior lobe of the hummingbird cerebellum expresses ZII in an immunopositive/immunonegative striped pattern across folia II–V. Although the number of stripes is conserved across these folia, one medial and three lateral stripes, the width and position of the stripes varies depending upon which folium is examined. In folia II/III, there is a broad central stripe flanked by three lateral stripes that are quite narrow (1–2 Purkinje cells in width, Fig. 2A and D). Four stripes were usually observed in folia IV and V (Fig. 2A–C), but sometimes a fifth could be delineated (Fig. 4B), and overall, stripes were somewhat broader than those in folia II/III. Stripes P2+ and P3+ could be traced from the P3+ and P4+ stripes of folia II/III (Fig. 2A–C). The number of stripes then increases in the dorsal part of V before transitioning into the largely positively labeled folium VI (Fig. 2B and E). Folium I (the lingula) is the sole exception to the striped pattern of ZII expression in the anterior lobe; all Purkinje cells are ZII+ and no stripes are apparent (Fig. 2A, C and E).

3.2. Zebrin II expression in the posterior lobe and vestibulocerebellum

Fig. 3 shows the expression of ZII across the posterior lobe of the hummingbird cerebellum. The dorsal part of the posterior lobe (folia VI and VII) does not have a positive/negative pattern of stripes in the same fashion as folia II–V of the anterior lobe or the rest of the posterior lobe. Instead, most Purkinje cells in folia VI and VII express ZII with 'negative' zones identified by lower levels of immunoreactivity rather than no immunoreactivity. This parallels a similar pattern observed in both pigeons (Fig. 3E and F; Pakan et al., 2007) and mammals (Brochu et al., 1990) whereby a 'central zone' in the cerebellum is defined by the presence of Purkinje cells that are weakly or strongly immunoreactive for ZII rather than positive and negative stripes.

In contrast to the central zone, folia VIII-IXcd comprise an expression domain with alternating ZII positive/negative Purkinje cell stripes. As with the anterior lobe (Fig. 2), one central and three lateral stripes are ZII-positive in folia VIII, IXa and IXb (Fig. 3A–D). The number of stripes within folium IXcd is increased by two additional stripes bilaterally (Fig. 3C and D). More medially, several weakly immunopositive stripes ('?') are interspersed between the more prominent and broader P+ stripes (Fig. 3B–D). These narrow, faintly immunoreactive stripes in IXcd were typically only 1–2 Purkinje cells in width, but were nevertheless apparent in both serial sections (Fig. 3C) and in wholemounts (Fig. 3B).

Finally, the most caudal folium of the posterior lobe, folium X, was generally uniformly immunopositive in the hummingbirds (Fig. 5A and B), the exception being the lateral margin where folia IXcd and X merge to form the auricle (Au), which was ZII immunonegative.

3.3. PLCβ4 expression

Anti-PLCβ4 immunoreactivity was expressed in the somata of the Purkinje cells, but not in the axonal processes (Fig. 4A). In a similar fashion to ZII, PLCβ4 revealed alternating immunopositive/immunonegative stripes in Purkinje cells of the cerebellar cortex (Fig. 4B–H). Generally, the pattern of PLCβ4 immunoreactivity was opposite to that of ZII; PLCβ4 positive stripes were negative for ZII and *vice versa* (Figs. 4 and 5). Similarly, This could be observed most clearly in the anterior lobe (Fig. 4B, D and F) and in folia IXcd (Fig. 5). Folium X is almost entirely negative for PLCβ4 and positive for ZII except for the lateral edge of X, where it joins with IXcd to form the auricle, in which a stripe of Purkinje cells is clearly positive for PLCβ4 and negative for ZII (Fig. 5). Within the 'central

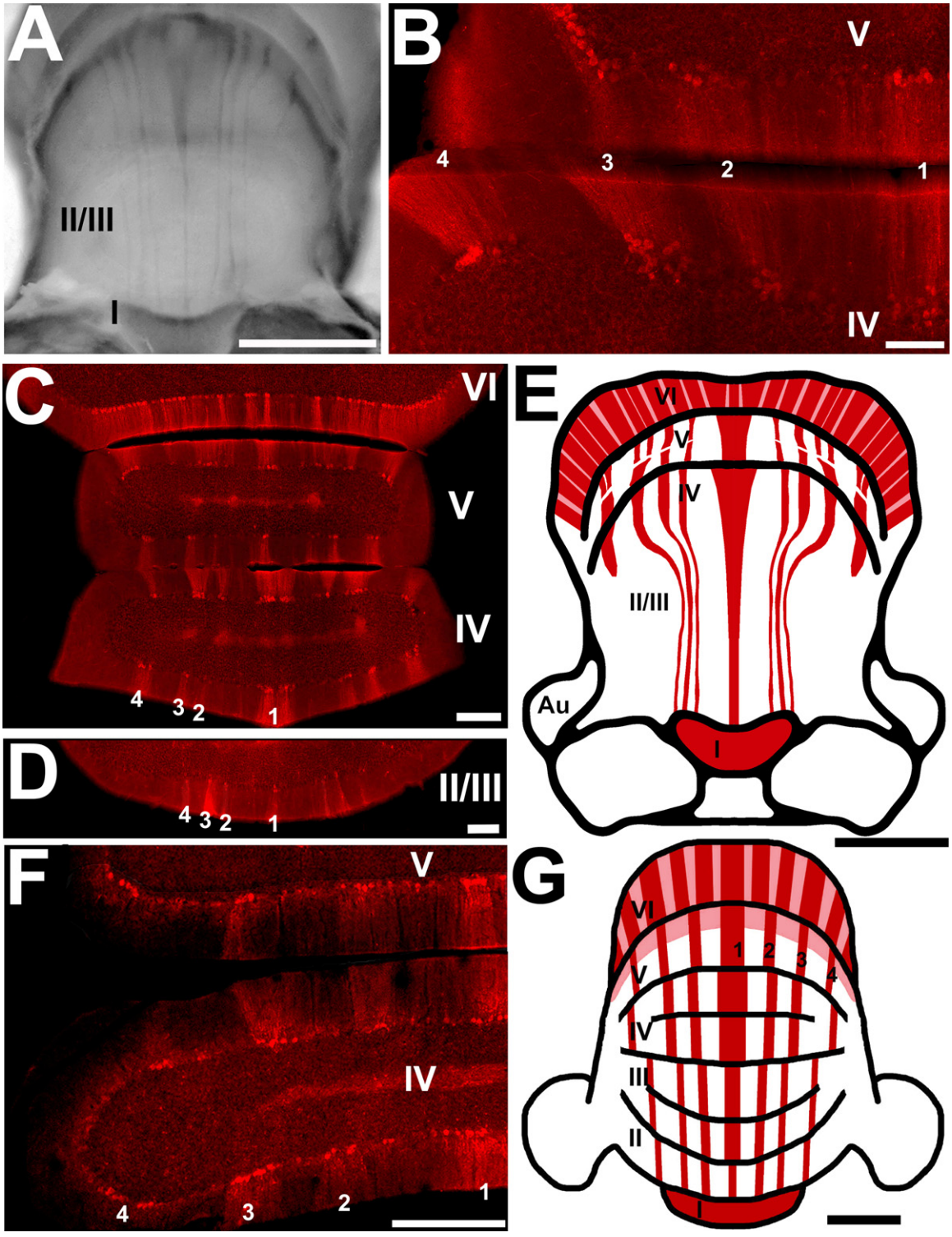

Fig. 2. The expression of zebrin II (ZII) in the anterior lobe of Anna's Hummingbird (*Calypte anna*) and pigeon (*Columba livia*) cerebella as revealed by immunohistochemistry. (A) A photo of the anterior lobe of a hummingbird cerebellum prepared as a wholemount, with folia I and II/III indicated by Roman numerals. Scale bar = 1 mm. (B) A coronal section taken through folia IV and V of a hummingbird cerebellum showing the striped pattern of ZII expression with labeling of the somata and dendritic arbors of Purkinje cells. Four stripes are shown: a medial positive stripe (1) flanked by three lateral stripes (2–4). Scale bar = 100 μm. (C) A coronal section taken through the anterior lobe of a hummingbird cerebellum that shows the striped pattern of ZII expression across folia IV–VI. Note that the ventral (bottom) half of folium V corresponds more closely to the stripes of folium IV, whereas the dorsal (top) half of V is more similar to that of folium VI. In addition, folium VI is largely immunopositive with weakly delineated stripes of low/high immunoreactivity as opposed to the immunonegative/immunopositive stripes of folia IV and V. Scale bar = 200 μm. (D) Zebrin II expression in folia II/III of a hummingbird is shown. As with folia IV and V, a central stripe (1) is flanked by three lateral stripes (2–4), but in folia II/III they are only 1 or 2 Purkinje cells wide. Scale bar = 100 μm. (E) Schematic of ZII expression across the entire anterior lobe of the hummingbird with immunopositive stripes/regions indicated in red and the auricle by 'Au'. The pale pink stripes in folium VI represent the weakly immunopositive stripes characteristic of this folium. Scale bar = 1 mm. (F) A coronal section through the anterior lobe of the pigeon cerebellum showing a similar striped pattern of ZII immunoreactivity with stripes labeled 1–4. Scale bar = 500 μm. (G) Schematic of ZII expression across the entire anterior lobe of the pigeon with immunopositive stripes/regions indicated in red and the weakly immunopositive stripes of folia V and VI in pink. Scale bar = 2.5 mm.

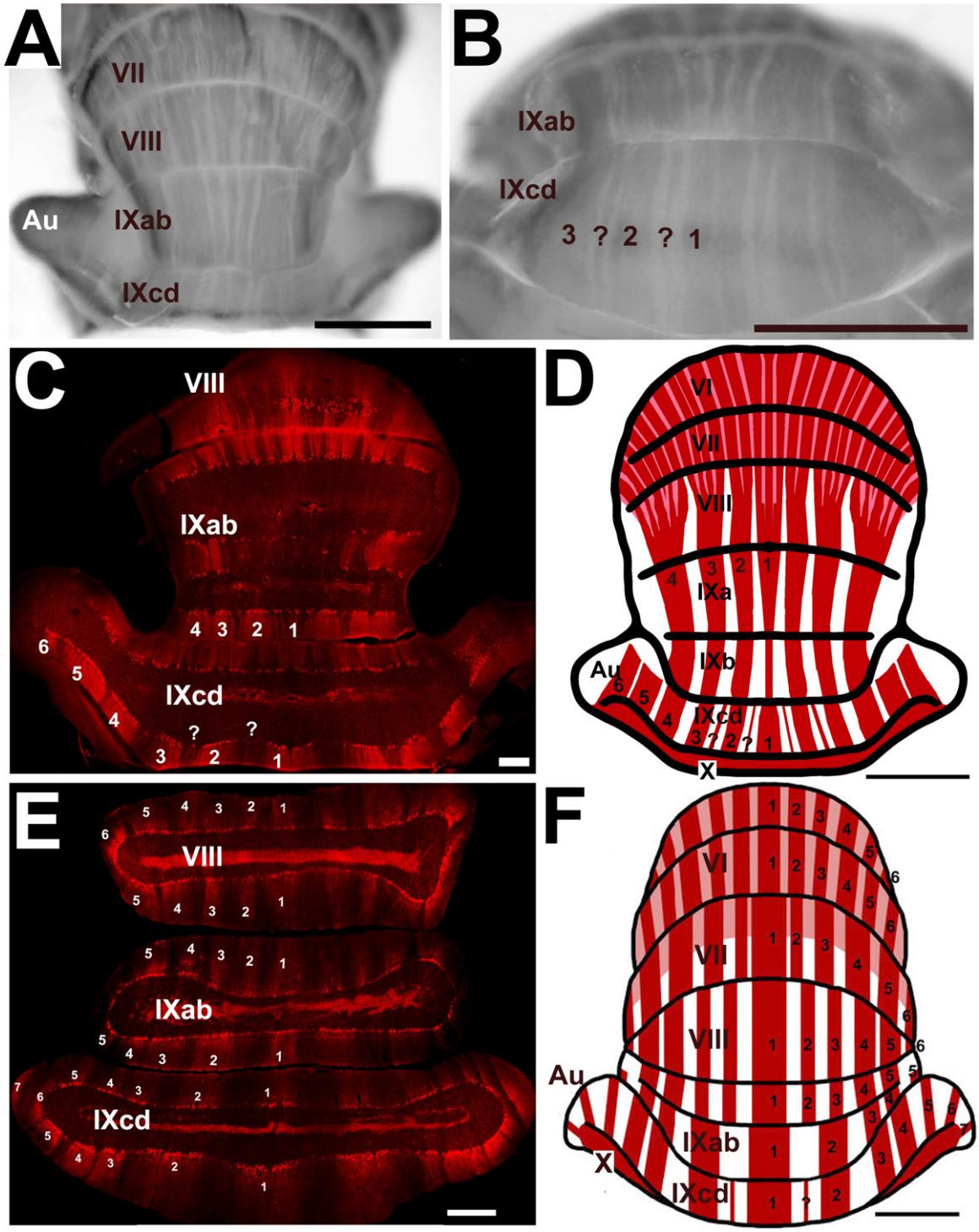

Fig. 3. The expression of zebrin II (ZII) in the posterior lobe of the Anna's Hummingbird (*Calypte anna*) and pigeon (*Columba livia*) cerebella as revealed by immunohistochemistry. (A) A photo of the posterior lobe of a hummingbird cerebellum prepared as a wholemount with folia VII–IXcd and the auricle (Au) indicated. As with the anterior lobe, a parasagittal series of immunpositive/immunonegative ZII stripes are clearly present. Scale bar = 1 mm. (B) A magnified view of folia IXab and IXcd of the wholemount showing the faint, narrow ZII immunopositive stripes that are interspersed between the strongly ZII immunopositive and broader 1–3 stripes. Scale bar = 1 mm. (C) A coronal section taken through the posterior lobe of a hummingbird cerebellum. As with the anterior lobe, ZII stripes are clearly present across the folia. Note that folium VIII is largely immunopositive with stripes of low immunoreactivity rather than immunonegative stripes. Several stripes are indicated (1–6) as well as several narrow and faintly immunopositive stripes in folium IXcd (indicated by '?'). Scale bar = 200 μm. (D) Schematic of ZII expression across the entire posterior lobe of the hummingbird with immunopositive stripes (1–6) indicated in red, the weakly immunopositive stripes of folia VI–VIII in pink, and the auricle by 'Au'. The '?' refers to narrow and faint immunopositive stripes characteristic of folium IXcd. Scale bar = 1 mm. (E) A coronal section through the posterior lobe of the pigeon cerebellum (folia VIII–IXcd) showing a similar striped pattern of ZII immunoreactivity with stripes labeled 1–7. Scale bar = 500 μm. (F) Schematic of ZII expression across the entire posterior lobe of the pigeon cerebellum with immunopositive stripes (1–7) indicated in red, the weakly immunopositive stripes of folia VI and VII in pink and the auricle by 'Au'. As with the hummingbird in (D), the '?' refers to the narrow and weakly immunopositive stripes characteristic of folium IXcd. Scale bar = 2.5 mm.

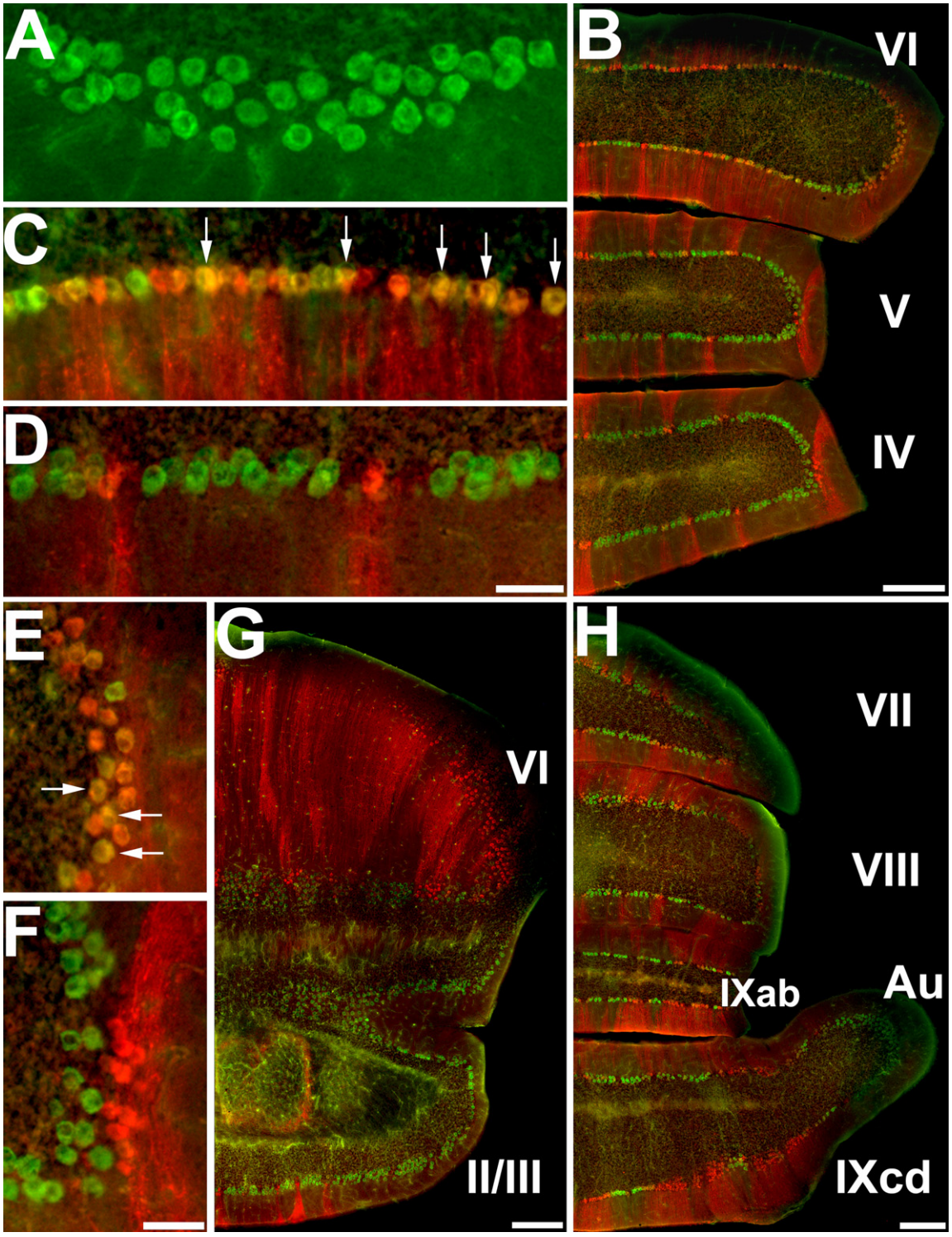

Fig. 4. The expression of phospholipase Cβ4 (PLCβ4) in the Rufous Hummingbird (*Selasphorus rufus*) cerebellum as revealed by immunohistochemistry. (A) The pattern of PLCβ4 expression in the cerebellar cortex of the hummingbird; the somata, but not the axonal processes, are labeled. Scale bar = 50 μm. (B) A coronal section taken through the anterior lobe of a hummingbird cerebellum showing the topography of PLCβ4 (green) and zebrin II (ZII, red) immunolabeling across folia IV–VI. In general, the PLCβ4 stripes are complementary to those of ZII. Scale bar = 250 μm. (C) A magnified view of PLCβ4 and ZII immunolabeling in folium VI (from Fig. 3B). The arrows indicate double-labeled cells. Scale bar = 50 μm. (D) A magnified view of folium IV (from Fig. 4B). Scale bar = 50 μm. (E) A magnified view of PLCβ4 and ZII immunolabeling in lateral folium VI (from Fig. 4B). The arrows indicate double-labeled cells. Scale bar = 50 μm. (F) A magnified view of lateral folium IV (from Fig. 4B). Scale bar = 50 μm. (G) A coronal section taken through folium VI and II/III showing the topography of PLCβ4 and ZII. Note that the majority of folium II/III is immunopositive for PLCβ4 with only narrow ZII immunopositive stripes. Folium VI has a mix of both PLCβ4 and ZII immunopositive cells. Scale bar = 250 μm. (H) A coronal section taken through the posterior lobe (folia VII–IXcd) of the hummingbird cerebellum showing the topography of PLCβ4 and ZII. Scale bar = 250 μm.

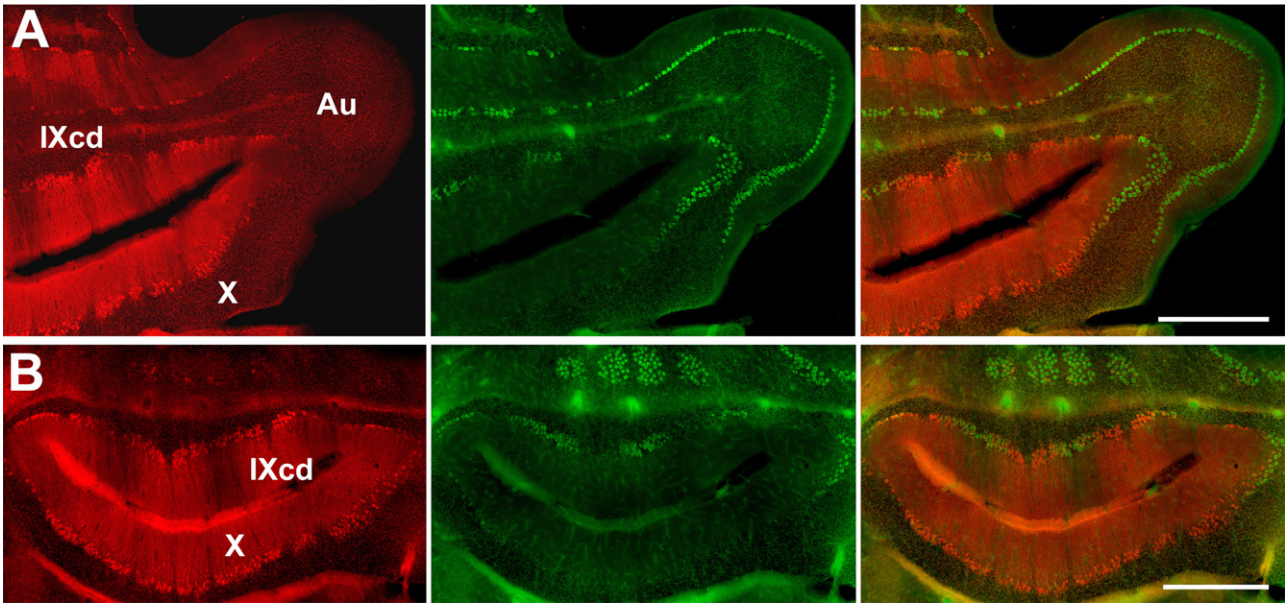

Fig. 5. Immunolabeling for zebrin II (ZII, red, left) and phospholipase Cβ4 (PLCβ4, green, centre) through two sections of folia IXcd and X of the posterior lobe of Rufous Hummingbird (*Selasphorus rufus*). The overlay is shown on the right. Folia IXcd and X merge laterally to form the auricle ('Au'). The section in (A) is posterior to that in (B). Scale bars = 500 μm.

zone' of folia VI and VII, cells expressed PLCβ4 in a series of weak and strong stripes (Fig. 4B, G and H). Most of these weak/strong stripes were opposite to that of the ZII immunolabeling, but some the cells appeared to be double-labeled for both PLCβ4 and ZII (Fig. 4C and E). The remainder of the posterior lobe was heavily striped in a largely complementary fashion to that of ZII, with some Purkinje cells double-labeled for PLCβ4 and ZII, particularly at the borders of the stripes. Finally, the lingula (folium I) was entirely negative for PLCβ4.

4. Discussion

Overall, ZII is expressed in the cerebellar cortex of hummingbirds in a striped pattern of areas of low/none and high immunoreactivity. These stripes are grossly similar to those of the pigeon, but with some variations. Furthermore, this pattern of ZII immunolabeling is largely complementary to that of PLCβ4; ZII immunopositive zones tend to be immunonegative for PLCβ4. This complementary labeling is not, however, exact and there are populations of Purkinje cells that are double-labeled for both ZII and PLCβ4. These results have significant implications for understanding cerebellar evolution in birds by providing insight into the neurochemical compartmentalization of the cerebellar cortex.

4.1. Zebrin II expression in birds and other vertebrates

The overall topography of ZII expression in the hummingbird cerebellum is similar to that observed in the pigeon (Pakan et al., 2007). In both taxa, five main transverse zones can be identified: a lingula zone restricted to folium I, in which all Purkinje cells are ZII+; an anterior zone with one large medial stripe flanked by several narrow stripes; a central zone that is largely immunopositive but within which numerous stripes displaying higher and lower immunoreactivity can be discerned; a posterior zone with prominent stripes; and a nodular zone in which almost all Purkinje cells are ZII immunopositive.

Within each of the zones, the pattern of ZII expression in pigeons (Pakan et al., 2007) and hummingbirds is generally similar. The lingular zone (folium I), which is unique to birds (Pakan et al., 2007), is entirely immunopositive. In the anterior zone, a large medial stripe extends from folium II to IV and is flanked by three lateral stripes on either side. Folium V then represents an area of transition between the anterior and central zones; its ventral aspect is similar to that of folium IV, but the dorsal aspect is more similar to folium VI and the rest of the central zone. Folia VI and VII, the central zone, are largely immunopositive with numerous thin stripes of low/high immunoreactivity. Folium VIII is the second area of transition whereby the pattern of expression in dorsal VIII is similar to that of folium VII, but the ventral VIII is similar to that of IXab. Thus, ventral VIII has a central stripe flanked by several broad positive stripes on either side. Folia IXcd even expresses thin weakly positive stripes adjacent to the medial stripe in hummingbirds and the pigeon. Finally, the nodular zone (folium X) was entirely immunopositive.

Despite this similarity in overall pattern, several differences between the hummingbirds and the pigeon are worth noting. First, the lateral stripes in the anterior zone of the hummingbirds are far narrower than those of the pigeon; in most cases, they are only 1–2 Purkinje cells wide (Fig. 2D). These narrow stripes are also far more medial in the hummingbirds than in the pigeon, at least in folia II/III. Second, there are fewer immunopositive stripes in folia VIII-IXab of hummingbirds compared to the pigeon. In the pigeon, the central stripe is followed by up to five lateral stripes (Fig. 3E and F), whereas only three such lateral stripes were identified in the hummingbird (Fig. 3C and D). This sort of variation in the orientation, width and number of ZII stripes in the cerebellar cortex also occurs in mammals. For example, the central zone of the Rhesus macaque (*Macaca mulatta*, Sillitoe et al., 2004) is striped whereas in rodents it is largely immunopositive (Eisenman and Hawkes, 1993; Sillitoe and Hawkes, 2002). Similarly, there are other subtle variations in the number and width of stripes across mammals (reviewed in Sillitoe et al., 2005). The functional implications of having fewer or more stripes or thinner/wider stripes is unknown, but given the correspondence between ZII immunolabeling and physiology and hodology (e.g., Gravel et al., 1987; Gravel and Hawkes, 1990; Ji and Hawkes, 1994; Hallem et al., 1999; Voogd et al., 2003; Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004; Pijpers et al., 2005; Gao et al., 2006; Pijpers and

Ruigrok, 2006), these variations could reflect species differences in the strength of cerebellar connections or the role of the cerebellum in different behaviours. Thus, the observed differences between the hummingbirds and the pigeon, particularly in folia II/III, could reflect species differences in cerebellar afferents and/or behaviour. Folia II/III are much smaller in hummingbirds (and swifts) than other birds (Iwaniuk et al., 2006b, 2007) and Larsell (1967) postulated that this reflected the poor hindlimb musculature of hummingbirds, so it is possible that this reflects weak connections with nerves innervating the hindlimbs.

4.2. Expression of PLCβ4 and other antigens

In mammals, several immunohistochemical and histochemical markers have been used to explore the complex pattern of cerebellar compartmentation. Recently, PLCβ4 was shown to express a striped pattern that was complementary to that of ZII (Sarna et al., 2006; Marzban et al., 2007). That is, ZII positive zones are negative for PLCβ4 and *vice versa*. In the hummingbirds, we found a similar pattern of expression: ZII positive stripes were generally PLCβ4 negative and ZII negative stripes were PLCβ4 positive. PLCβ4 immunohistochemistry did, however, differ from that observed in mammals in two respects. First, PLCβ4 labeling within the cerebellar cortex was markedly different in the hummingbird compared to the mouse. In the mouse, the somata of Purkinje cells is only weakly stained and the dendritic arbors are darkly stained (Sarna et al., 2006). As shown in Fig. 4A, this is not the case in hummingbirds. In fact, the cell bodies were darkly stained and the dendritic arbors were only weakly stained. This more closely resembles the expression of phospholipase Cβ3 (PLCβ3) in the mouse. PLCβ3 is expressed in Purkinje cell bodies in the mouse, whereas PLCβ4 is only expressed in the dendrites (Sarna et al., 2006). However, the expression of PLCβ3 is coincident with that of ZII, whereas PLCβ4 is complementary. Thus, the expression of PLCβ4 in the hummingbird is similar to that of the PLCβ3 in the mouse at the cellular level, but its topography relative to ZII is more similar to that of PLCβ4.

The second main difference between the PLCβ4 labeling in the hummingbirds versus that of the mouse is the degree to which it is complementary to that of ZII. In the mouse, PLCβ4 positive stripes are exclusively ZII negative, but this was not true of the hummingbirds. Instead, double-labeled Purkinje cells were consistently found at the edges of stripes and many double-labeled cells in the central zone, which is predominantly ZII immunopositive. Thus, in general, the PLCβ4 positive stripes are complementary to the ZII positive stripes, but not exclusively so.

4.3. Evolutionary implications

Recent studies by Iwaniuk et al. (2006a,b, 2007) have demonstrated the degree of variation in cerebellar morphology among birds. Unlike mammals in which variation can occur both within the vermis and the cerebellar hemispheres, all of the interspecific variation in birds is in the vermis. For example, parrots (Psittaciformes) have highly folded cerebella characterized by large posterior folia (IXab and IXcd), whereas the hummingbirds and swifts have cerebella with extremely small folia II and III. This variation has been correlated with flying ability and, to a lesser extent, hindlimb strength among species (Iwaniuk et al., 2007), but whether this variation also extends to other features of the cerebellum, such as neurochemical organization, has remained unknown until now.

As described above, our results clearly indicate that the overall pattern of cerebellar compartmentation is largely similar between the pigeon, which represents an 'average' bird, and the hummingbird, which has one of the most divergent cerebellar morphologies of any species examined to date (Iwaniuk et al., 2006b, 2007). That is, both the pigeon and hummingbirds exhibit positive ZII immunolabeling throughout the lingula, anterior and posterior zones of positive/negative ZII stripes and a central zone of weak/strong ZII stripes. Given that the hummingbirds and pigeons are distantly related to one another (see reviews in Sibley and Ahlquist, 1990; Johansson et al., 2001; Livezey and Zusi, 2001; Cracraft et al., 2004; Hackett et al., 2008), we predict that the same pattern of ZII expression will be true of all birds. Thus, despite marked differences in the degree of foliation in cerebellar cortex (Iwaniuk et al., 2006a) and the relative size of individual folia (Iwaniuk et al., 2007), this overall pattern of ZII expression will persist. Consistent with this hypothesis, preliminary data for the chicken has shown that it too shares a similar groundplan (Marzban et al., unpubl data).

Although the overall pattern of expression may be consistent across avian species, our study indicates that species differences in the number and thickness of ZII stripes occur. Thus, within the confines of a broader pattern, species differences in ZII expression have evolved. As mentioned previously, differences in the number of stripes or stripe thickness could reflect differential projection patterns among species. The difference between hummingbirds and the pigeon in the size of ZII stripes in folia II/III could reflect species differences in hindlimb innervation. The examination of species with well developed hindlimb musculature, such as hawks and eagles (Accipitridae) or owls (Strigiformes) could aid in addressing this issue with respect to the anterior lobe. Iwaniuk et al. (2007) showed that the relative size of folia VI and VII are significantly expanded in strong fliers, such as waterfowl (Anseriformes), hawks and seabirds (Procellariiformes). If the size of ZII positive stripes reflects innervation patterns, then it is possible that stripes in these folia will be larger in strong fliers as a result of stronger pectoral muscles. By performing such comparisons, it will be possible to determine how conserved the anterior-central-posterior zone organization is across species as well as the degree to which stripe width or number of stripes reflects behavioural differences among species.

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References

Ahn, A.H., Dziennis, S., Hawkes, R., Herrup, K., 1994. The cloning of zebrin II reveals its identity with aldolase C. Development 120, 2081–2090.

Armstrong, C.L., Krueger-Naug, A.M., Currie, R.W., Hawkes, R., 2001. Expression of heat-shock protein Hsp25 in mouse Purkinje cells during development reveals novel features of cerebellar compartmentation. J. Comp. Neurol. 428, 7–21.

Arndt, K., Redies, C., 1998. Development of cadherin-defined parasagittal subdivisions in the embryonic chick cerebellum. J. Comp. Neurol. 401, 367–381.

Brochu, G., Maler, L., Hawkes, R., 1990. Zebrin II: a polypeptide antigen expressed selectively by Purkinje cells reveals compartments in rat and fish cerebellum. J. Comp. Neurol. 291, 538–552.

Cracraft, J., Barker, F.K., Braun, M., Harshman, J., Dyke, G.J., Feinstein, J., Stanley, S., Cibois, A., Schikler, P., Beresford, P., Garcia-Moreno, J., Sorenson, M.D., Yuri, T., Mindell, D.P., 2004. Phylogenetic relationships among modern birds (Neornithes). In: Cracraft, J., Donoghue, M.J. (Eds.), Assembling The Tree of Life. Oxford University Press, New York, pp. 468–489.

Davis, C.A., 1993. Whole-mount immunohistochemistry. Methods Enzymol. 225, 502–516.

Dent, J.A., Polson, A.G., Klymkowsky, M.W., 1989. A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in *Xenopus*. Development 105, 61–74.

Eisenman, L.M., Hawkes, R., 1993. Antigenic compartmentation in the mouse cerebellar cortex: zebrin II and HNK-1 reveal a complex, overlapping molecular topography. J. Comp. Neurol. 335, 586–605.

Gao, W., Chen, G., Reinert, K.C., Ebner, T.J., 2006. Cerebellar cortical molecular layer inhibition is organized in parasagittal zones. J. Neurosci. 26, 8377–8387.

Gravel, C., Eisenman, L.E., Sasseville, R., Hawkes, R., 1987. Parasagittal organization of the rat cerebellar cortex: a direct correlation between antigenic Purkinje cell bands revealed by mabQ113 and the organization of the olivocerebellar projection. J. Comp. Neurol. 263, 294–310.

Gravel, C., Hawkes, R., 1990. Parasagittal organization of the rat cerebellar cortex: direct comparison of Purkinje cell compartments and the organization of the spinocerebellar projection. J. Comp. Neurol. 291, 79–102.

Hackett, S.J., Kimball, R.T., Reddy, S., Bowie, R.C.K., Braun, E.L., Braun, M.J., Chojnowski, J.L., Cox, W.A., Han, K.-L., Harshman, J., Huddleston, C.J., Marks, B.D., Miglia, K.J., Moore, W.S., Sheldon, F.H., Steadman, D.W., Witt, C.C., Yuri, T., 2008. A phylogenomic study of birds reveals their evolutionary history. Science 320, 1763–1768.

Hallem, J.S., Thompson, J.H., Gundappa-Sulur, G., Hawkes, R., Bjaalie, J.G., Bower, J.M., 1999. Spatial correspondence between tactile projection patterns and the distribution of the antigenic Purkinje cell markers anti-zebrin I and anti-zebrin II in the cerebellar folium crus IIA of the rat. Neuroscience 93, 1083–1094.

Hawkes, R., Herrup, K., 1995. Aldolase C/zebrin II and the regionalization of the cerebellum. J. Mol. Neurosci. 6, 147–158.

Iwaniuk, A.N., Hurd, P.L., Wylie, D.R.W., 2007. Comparative morphology of the avian cerebellum: II. Size of folia. Brain Behav. Evol. 69, 196–219.

Iwaniuk, A.N., Hurd, P.L., Wylie, D.R.W., 2006a. Comparative morphology of the avian cerebellum. I. Degree of foliation. Brain Behav. Evol. 68, 45–62.

Iwaniuk, A.N., Hurd, P.L., Wylie, D.R.W., 2006b. The comparative morphology of the cerebellum in caprimulgiform birds: evolutionary and functional implications. Brain Behav. Evol. 67, 53–68.

Ji, Z., Hawkes, R., 1994. Topography of Purkinje cell compartments and mossy fiber terminal fields in lobules II and III of the rat cerebellar cortex: spinocerebellar and cuneocerebellar projections. Neuroscience 61, 935–954.

Johansson, U.S., Parsons, T.J., Irestedt, M., Ercison, P.G.P., 2001. Clades within the 'higher land birds', evaluated by nuclear DNA sequences. J. Zool. Syst. Evol. Res. 39, 37–51.

King, J.S., Madtes Jr., P., Bishop, G.A., Overbeck, T.L., 1997. The distribution of corticotropin-releasing factor (CRF), CRF binding sites and CRF1 receptor mRNA in the mouse cerebellum. Prog. Brain Res. 114, 55–66.

Larsell, O., 1967. The Comparative Anatomy and Histology of the Cerebellum from Myxinoids Through Birds. University of Minnesota Press, Minneapolis.

Larsell, O., 1970. The comparative anatomy and histology of the cerebellum from monotremes through apes. University of Minnesota Press, Minneapolis.

Livezey, B.C., Zusi, R.L., 2001. Higher-order phylogenetics of modern Aves based on comparative anatomy. Neth. J. Zool. 51, 179–206.

Marani, E., Voogd, J., 1977. An acetylcholinesterase band-pattern in the molecular layer of the cat cerebellum. J. Anat. 124, 335–345.

Marzban, H., Chung, S., Watanabe, M., Hawkes, R., 2007. Phospholipase cβ4 expression reveals the continuity of cerebellar topography through development. J. Comp. Neurol. 502, 857–871.

Marzban, H., Sillitoe, R.V., Hoy, M., Chung, S.H., Rafuse, V.F., Hawkes, R., 2004. Abnormal HNK-1 expression in the cerebellum of an N-CAM null mouse. J. Neurocytol. 33, 117–130.

Nakamura, M., Sato, K., Fukaya, M., Araishi, K., Aiba, A., Kano, M., Watanabe, M., 2004. Signaling complex formation of phospholipase Cbeta4 with metabotropic glutamate receptor type 1alpha and 1,4,5-triphosphate receptor at the perisynapse and endoplasmic reticulum in the mouse brain. Eur. J. Neurosci. 20, 2929–2944.

Neudert, F., Redies, C., 2008. Neural circuits revealed by axon tracing and mapping cadherin expression in the embryonic chicken cerebellum. J. Comp. Neurol. 509, 283–301.

Pakan, J.M.P., Iwaniuk, A.N., Wylie, D.R.W., Hawkes, R., Marzban, H., 2007. Purkinje cell compartmentation as revealed by zebrin II expression in the cerebellar cortex of pigeons (*Columba livia*). J. Comp. Neurol. 501, 619–630.

Paulin, M.G., 1993. The role of the cerebellum in motor control and perception. Brain Behav. Evol. 41, 39–50.

Pijpers, A., Ruigrok, T.J., 2006. Organization of pontocerebellar projections to identified climbing fibre zones in the rat. J. Comp. Neurol. 496, 513–528.

Pijpers, A., Voogd, J., Ruigrok, T.J., 2005. Topography of olivo-cortico-nuclear modules in the intermediate cerebellum of the rat. J. Comp. Neurol. 492, 193–213.

Sarna, J.R., Marzban, H., Watanabe, M., Hawkes, R., 2006. Complementary stripes of phospholipase cβ3 and cβ4 expression by Purkinje cell subsets in mouse cerebellum. J. Comp. Neurol. 496, 303–313.

Scott, T., 1963. A unique pattern of localization within the cerebellum. Nature 200, 793.

Sibley, C.G., Ahlquist, J.E., 1990. Phylogeny and Classification of Birds. Yale University Press, New Haven, CT.

Sillitoe, R.V., Hawkes, R., 2002. Whole-mount immunohistochemistry: a high throughput screen for patterning defects in the mouse cerebellum. J. Histochem. Cytochem. 50, 235–244.

Sillitoe, R.V., Hulliger, M., Dyck, R., Hawkes, R., 2003. Antigenic compartmentation of the cat cerebellar cortex. Brain Res. 977, 1–15.

Sillitoe, R.V., Malz, C., Rockland, K., Hawkes, R., 2004. Antigenic compartmentation of the primate and scandentid cerebellum: a common topography of zebrin II in *Macaca mulatta* and *Tupaia belangerie*. J. Anat. 204, 257–269.

Sillitoe, R.V., Marzban, H., Larouche, M., Zahedi, S., Affanni, J., Hawkes, R., 2005. Conservation of the architecture of the anterior lobe vermis of the cerebellum across mammalian species. Prog. Brain Res. 148, 283–298.

Sugihara, I., Shinoda, Y., 2004. Molecular, topographic and functional organization of the cerebellar cortex: a study with combined aldolase C and olivocerebellar labeling. J. Neurosci. 24, 8771–8785.

Vanhalst, K., Kools, P., Staes, K, van Roy, F., Redies, C., 2005. Delta-protocadherins: a gene family expressed differentially in the mouse brain. Cell Mol. Life Sci. 62, 1247–1259.

Voogd, J., Pardoe, J., Ruigrok, T.J., Apps, R., 2003. The distribution of climbing and mossy fiber collateral branches from the copula pyramidis and the paramedian lobule: congruence of climbing fibre cortical zones and the pattern of zebrin banding within the rat cerebellum. J. Neurosci. 23, 4645–4656.

Voogd, J., Ruigrok, T.J., 2004. The organization of the corticonuclear and olivocerebellar climbing fiber projections to the rat cerebellar vermis: the congruence of projection zones and the zebrin pattern. J. Neurocytol. 33, 5–21.