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# Purkinje Cell Compartmentation as Revealed by Zebrin II Expression in the Cerebellar Cortex of Pigeons (*Columba livia*)

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ABSTRACT

Purkinje cells in the cerebellum express the antigen zebrin II (aldolase C) in many vertebrates. In mammals, zebrin is expressed in a parasagittal fashion, with alternating immunopositive and immunonegative stripes. Whether a similar pattern is expressed in birds is unknown. Here we present the first investigation into zebrin II expression in a bird: the adult pigeon (*Columba livia*). Western blotting of pigeon cerebellar homogenates reveals a single polypeptide with an apparent molecular weight of 36 kDa that is indistinguishable from zebrin II in the mouse. Zebrin II expression in the pigeon cerebellum is prominent in Purkinje cells, including their dendrites, somata, axons, and axon terminals. Parasagittal stripes were apparent with bands of Purkinje cells that strongly expressed zebrin II (+ve) alternating with bands that expressed zebrin II weakly or not at all (−ve). The stripes were most prominent in folium IXcd, where there were seven +ve/−ve stripes, bilaterally. In folia VI–IXab, several thin stripes were observed spanning the mediolateral extent of the folia, including three pairs of +ve/−ve stripes that extended across the lateral surface of the cerebellum. In folium VI the zebrin II expression in Purkinje cells was stronger overall, resulting in less apparent stripes. In folia II–V, four distinct +ve/−ve stripes were apparent. Finally, in folia I (lingula) and X (nodulus) all Purkinje cells strongly expressed zebrin II. These data are compared with studies of zebrin II expression in other species, as well as physiological and neuroanatomical studies that address the parasagittal organization of the pigeon cerebellum. J. Comp. Neurol. 501:619–630, 2007. 

**Indexing terms: Purkinje cell; whole mount immunohistochemistry; compartmentation; zebrin; avian cerebellum**

The gross anatomy of the cerebellum varies from a single leaf in amphibians to the elaborately foliated structure of birds, mammals, and some fish (e.g.,Voogd and Glickstein, 1998). Although the cerebellum is divided into clearly defined lobes and lobules (generally referred to as "lobules" in mammals and "folia" in birds), there is substantial evidence to suggest that a more fundamental cerebellar architecture is built around arrays of parasagittal zones of Purkinje cells that cut across the folia. These parasagittal stripes can be defined by climbing and mossy fiber input, Purkinje cell projection patterns, Purkinje cell response properties and topography, and cerebellar interneurons (Voogd, 1967; Hawkes and Gravel, 1991; Hawkes, 1992; Voogd et al., 1996; Hawkes, 1997; Herrup and Kuemerle, 1997; Oberdick et al., 1998; Voogd and Glickstein, 1998; reviewed in Armstrong and Hawkes, 2000). A para-

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sagittal organization has also been revealed with molecular markers. The most thoroughly studied marker is zebrin II (Brochu et al., 1990), which cloning studies demonstrate is the metabolic isoenzyme aldolase C (Ahn et al., 1994; Hawkes and Herrup, 1995). In mammals, zebrin II is expressed strongly by subsets of Purkinje cells, whereas other Purkinje cells, and other cell types, express zebrin II either very weakly or not at all (e.g., Walther et al., 1998). In all mammals studied thus far, zebrin II-immunopositive Purkinje cells are distributed as an array of immunoreactive parasagittal stripes—more than a dozen in some places—separated by intervening zebrin II-immunonegative or only weakly immunopositive stripes (Brochu et al., 1990; Eisenman and Hawkes, 1993; Sillitoe and Hawkes, 2002). These stripes occur in both the vermis and the cerebellar hemispheres and the basic organization of these stripes within the vermis is highly conserved among mammals (Sillitoe et al., 2005).

Mammalian and avian cerebella are very similar in terms of their gross morphology, histology, and local circuitry (for review, see Llinás and Hillman, 1969). In contrast to the mammalian cerebellum, the avian cerebellum consists primarily of a vermis, and the presence of homologs of the mammalian hemispheres is contentious (e.g., Larsell, 1948; Larsell and Whitlock, 1952; Whitlock, 1952). Like the mammalian vermis, the avian cerebellum is also organized into parasagittal stripes, as shown by studies of climbing fiber inputs, Purkinje cell projections, and Purkinje cell response properties (e.g., Arends and Voogd, 1989; Arends and Zeigler, 1991; Wylie and Frost, 1999; Winship and Wylie, 2003; Wylie et al., 2003a,b; Pakan et al., 2005). Whether zebrin II expression is also restricted to parasagittal stripes, as it is in mammals, has yet to be established in birds. Here, we provide the first study of zebrin II expression in the avian cerebellum.

## MATERIALS AND METHODS

Animal procedures conformed to institutional regulations and the *Guide to the Care and Use of Experimental Animals* from the Canadian Council for Animal Care.

Adult pigeons (*Columba livia*) were obtained from a local supplier. Pigeons were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were then removed and postfixed by immersion at 4°C in the same fixative for several days.

### Immunohistochemistry

***Whole mount immunohistochemistry.*** A whole mount of the cerebellum was immunostained using a protocol slightly modified from one originally designed for the mouse cerebellum (Sillitoe and Hawkes, 2002). The cerebellum was dissected from the brain by cutting through the cerebellar peduncles. After incubating the pigeon cerebellum in fixative for 24-48 hours it was postfixed 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 hours and then dehydrated in 2 × 30 minutes each 100% methanol. The tissue was passed through 4–5 cycles of chilling to −80°C and thawing to room temperature in 100% methanol followed by overnight incubation in methanol at −80°C. Anti-zebrin II is a mouse monoclonal antibody produced by immunization with a crude cerebellar homogenate from the weakly electric fish *Apteronotus* (Brochu et al., 1990) and subsequently shown to bind the respiratory isoenzyme aldolase c (Aldoc: Ahn et al., 1994: it was used directly from spent hybridoma culture medium diluted 1:200). For zebrin II staining the cerebellum was rehydrated for 90 minutes each through 50% methanol, 15% methanol, and phosphate-buffered saline (PBS), then enzymatically digested in 10 μg/mL proteinase K (>600 units/mL; Boehringer Mannheim, Germany) in PBS for 5 minutes at room temperature. After rinsing 3 × 10 minutes in PBS the tissue was incubated in blocking buffer (Davis, 1993) for 6–8 hours at room temperature. The tissue was then incubated for 48–96 hours in anti-zebrin II antibody (1:200), rinsed 3 × 2 hours at 4°C, and incubated overnight at 4°C in secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA). Finally, the cerebella were rinsed 4 × 3 hours each at 4°C in PBS followed by a final overnight rinse, incubated in 0.2% bovine serum albumin (BSA), 0.1% Triton X-100 in PBS for 2 hours at room temperature, and antibody binding sites were revealed with diaminobenzidine (DAB).

***Immunohistochemistry for serial sections.*** For section immunohistochemistry the brain was postfixed in 4% paraformaldehyde (in 0.1 M PB) for several days. The tissue was equilibrated in sucrose (30% in 0.1 M PB) and serial (40-μm thick) coronal or sagittal sections were cut through the extent of the cerebellum using a cryostat. Immunohistochemistry was carried out as described previously (Marzban et al., 2003b). Briefly, tissue sections were rinsed thoroughly, blocked with 10% normal goat serum (Jackson Immunoresearch Laboratories), then incubated overnight at room temperature in 0.9% NaCl in 0.1 M PBS (pH 7.4) containing 0.1% Triton X-100 and the primary antibody, anti-zebrin II (diluted 1:200). For the secondary, either horseradish peroxidase (HRP)-conjugated or fluorescent-tagged antibodies were used. For the HRP-conjugated antibodies the sections were incubated in HRP-conjugated goat antimouse antibodies (Jackson Immunoresearch Laboratories; each diluted 1:1,000 in blocking solution) for 2 hours at room temperature, and antibody binding was revealed by DAB. Sections were dehydrated through an alcohol series, cleared in xylene, and coverslipped with Entellan mounting medium (BDH Chemicals, Toronto, ON). In some cases an epitope retrieval protocol was employed (Namimatsu et al., 2005; Yamashita and Okada, 2005). The antigen distributions revealed by the different methods were not, however, different. When fluorescent secondary antibodies were used the initial processing was as described as above, except that the brain was embedded in gelatin prior to sectioning and was incubated in the primary for 72 hours at room temperature. Tissue was then rinsed in PBS and sections were incubated in Cy3 donkey antimouse antibody (Jackson Immunoresearch Laboratories: diluted 1:100 in PBS, 2.5% normal horse serum, and 0.4% Triton X-100) for 2 hours at room temperature. In some cases sections were washed with 50 mM Tris HCl, pH 7.6, and incubated twice for 5 minutes in boiling 1 mM EDTA previous to incubation in blocking serum. With the addition of this step the results were comparable to normal methods. The tissue was then rinsed in PBS and mounted onto gelatinized slides for viewing.

***Double labeling of cerebellar sections for calbindin and zebrin II.*** Cerebellar sections for double fluorescence immunohistochemistry were processed as described previously (Marzban et al., 2003b). Briefly, tissue sections were washed, blocked in PBS containing 10% normal goat serum (Jackson Immunoresearch Laboratories), and incubated with gentle agitation in blocking solution containing a combination of primary antibodies: anti-zebrin II (spent culture medium diluted 1:200; Brochu et al., 1990) and anti-calbindin (1:1,000; C7354; Sigma Immunochemicals, St. Louis, MO) overnight at 4°C. Anti-calbindin D-28K (KD-15) is a synthetic peptide corresponding to the C-terminal region of rat calbindin D-28K (amino acids 185–199) and is developed in rabbit. This antibody recognizes a band of 28 kDa on Western blots. The sequence is identical in the corresponding human, mouse, and bovine calbindin D-28K sequences and is highly conserved (single amino acid substitution) in chicken and frog calbindin D-28K (manufacturer's information). Following incubation in primary antibodies, sections were washed and then left in PBS containing Cy3-conjugated goat antirabbit secondary antibody and Cy2-conjugated goat antimouse secondary antibody (both diluted 1:1,000, Jackson Immunoresearch Laboratories) for 2 hours at 4°C. The sections were then washed in 0.1 M PBS buffer, mounted onto chrome-alum and gelatin subbed slides, air-dried for 2 hours, and coverslipped in nonfluorescing mounting medium (Fluorsave Reagent, Calbiochem, La Jolla, CA).

## Microscopy and image analysis

For the whole mounts, the brains were examined and images obtained using a Spot digital camera (Diagnostics Instruments, Sterling Heights, MI) mounted on a Zeiss Stemi SV6 microscope. For the serial sections where zebrin II expression was visualized using the DAB-peroxidase reaction product, the sections were examined by using a Spot CCD camera (Diagnostics Instruments, La Jolla, CA) mounted on a Zeiss Axioplan II microscope. For fluorescent immunochemistry, the 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) and analyzed with OPENLAB imaging software (Improvision, Lexington, MA). The images were compiled and manipulated using Adobe Photoshop (San Jose, CA) to compensate for brightness and contrast.

## Western blotting

Western blot analysis of both pigeon and mouse tissue was carried out using a conventional protocol (Marzban et al., 2003b). Briefly, mice and pigeons were deeply anesthetized with a lethal dose of sodium pentobarbital (see above), decapitated, and the cerebellum quickly removed from the skull, diced, rinsed in PBS, and homogenized in RIPA (1× PBS, 1% Nonidet P-40) (Amresco, Cedarlane Laboratories, Hornby, ON) 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate buffer containing the protease inhibitor pepstatin (1 μg/mL: Bioshop Canada, Burlington, ON). Cerebellar homogenates were separated by using polyacrylamide gel electrophoresis through a 10% gel (Gibco BRL, Burlington, ON) and transblotted onto a nitrocellulose membrane (Bio-Rad, Mississauga, ON) for 1 hour by using a semidry blotting apparatus. Nonspecific binding sites on the membrane were blocked for 2 hours in 5% skim milk powder / 1% BSA in PBS. The membrane was incubated overnight at 4°C in anti-zebrin II (1/1,000 in 5% skim milk powder / 1% BSA in PBS). After several rinses in 2% Tween 20 in PBS, the membrane was incubated for 1 hour in 1:5,000 HRP-conjugated rabbit antimouse secondary antibodies (Jackson ImmunoResearch Laboratory) in blocking solution. Several rinses in 2% Tween 20 in PBS and a final rinse in Tris-buffered saline (10 mM Tris–HCl, pH 7.6, and 150 mM NaCl) followed antibody incubation. The reaction was visualized directly on the nitrocellulose membrane using 0.5 mg/mL DAB and 0.5 μL/mL 30% $H_2O_2$ in PBS until the desired color intensity was achieved. Membranes were scanned using a flatbed scanner (UMAX Astra 1220s) operating under Vistascan (UMAX Data Systems, Dallas, TX) and thereafter images were transported into Adobe Photoshop.

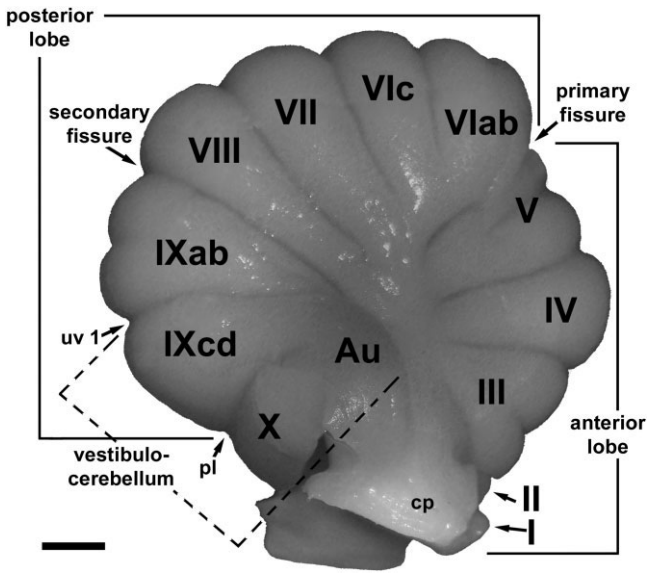



Fig. 1. A lateral view of the pigeon cerebellum. The folia are numbered I–X (anterior to posterior) according to the nomenclature of Larsell (1967). Folia I and II are hidden behind the cerebellar peduncle (cp). Folia I–V comprise the anterior lobe, which is separated from the posterior lobe (folia VI–IX) by the primary fissure. The posterior lobe is separated from folium X (nodulus) by the posterolateral fissure (pl). The vestibulocerebellum includes folia IXcd (ventral uvula) and X, which merge laterally and form the auricle (Au). uv 1, uvular sulcus 1. Scale bar = 1 mm.

## Nomenclature

The pigeon cerebellum consists of a vermis without evident hemispheres (Fig. 1). Ten primary folia are recognized according to Larsell (1967). Larsell (1967) suggested an explicit homology between folia I–X of birds and lobules I–X of the mammalian cerebellum. Homologies between avian cerebellar folia and the lobules of mammalian cerebella are conventionally drawn as follows: folium I in the pigeon corresponds to the lingula; folia II and III to the lobulus centralis; folia IV and V to the culmen; folium VI to the declive; folium VII to the folium plus tuber vermis; folium VIII to the pyramis; folium IX to the uvula; and folium X to the nodulus (Fox and Snider, 1967). There are no prominent cerebellar hemispheres in birds, but the unfoliated cortices covering the basis cerebelli may repre-

sent their rudimentary homologs (e.g., Larsell, 1948; see below).

Folia I–V comprise the anterior lobe, which is separated from the posterior lobe (folia VI–IX) by the primary fissure. The posterolateral fissure separates folium X (nodulus) from the posterior lobe. Folia IXcd (uvula) and X comprise the vestibulocerebellum (Voogd and Wylie, 2004) and merge laterally to form the auricle. Larsell (1967) considered the extensions of IXcd and X to be the paraflocculus and flocculus, respectively. However, based on physiological properties, olivary input, and Purkinje cell projection patterns, the lateral half of folia IXcd and X is considered to be equivalent to the mammalian flocculus (e.g., Wylie and Frost, 1999; Wylie et al., 1999b, 2003a,b; Crowder et al., 2000; Wylie, 2001; Winship and Wylie, 2003; Voogd and Wylie, 2004). The homolog of the paraflocculus remains uncertain.

# RESULTS

## Western blot analysis

Anti-zebrin II recognizes in mouse a single polypeptide band with an apparent molecular weight of 36 kDa, which cloning studies have shown to be the metabolic isoenzyme aldolase C (Ahn et al., 1994; Hawkes and Herrup, 1995). Western blot analysis of pigeon cerebellum homogenate also detects a single immunoreactive polypeptide band, identical in size to the band detected in extracts from the adult mouse cerebellum (Fig. 2A).

## Zebrin II expression

Purkinje cells were the only neurons that were immunoreactive for zebrin II in the cerebellar cortex of the pigeon (Fig. 2B–D). Immunoreactivity was present in the dendritic arbors and in the somata of Purkinje cells. Purkinje cell axons in the white matter tracts and in the granular layer were strongly immunoreactive (Fig. 2C,D) but, as in mammals, there was no zebrin II immunoreactivity in the Purkinje cell nuclei (Fig. 2B,E,F). Anti-zebrin II immunostaining was also detected in Purkinje cell axon terminals located within the cerebellar and vestibular nuclei. In some cases, weak zebrin II immunoreactivity was also associated with astrocytes—for example, the somata of Bergmann glial cells and glial end feet on blood vessels. Although we cannot exclude background and/or nonspecific staining, a similar low level of apparently specific, anti-zebrin II immunoreactivity has also been reported in mouse glia (e.g., Walther et al., 1998). In Figure 2F, a section reacted for zebrin (green) and calbindin (red) is shown. Note the presence of calbindin labeled Purkinje cells in the P1− stripe, where zebrin II reactivity was weak. That is, the zebrin II −ve stripes were not simply devoid of Purkinje cells.

In the pigeon cerebellum most Purkinje cell somata are zebrin II-immunoreactive but vary in the strength of their immunoreactivity, with alternating stripes of high and low immunoreactivity. The stripes are revealed most clearly through different levels of expression in the dendritic arbors. For example, Figure 2E,F shows coronal sections double immunofluorescence-stained for zebrin II (green) and calbindin (red: a marker of all Purkinje cells in both rodents (Baimbridge et al., 1982; Celio, 1990) and birds (Pasteels et al., 1987; de Talamoni et al., 1993; Sechman et al., 1994)). Clusters of strongly zebrin II-immunoreactive Purkinje cells alternate with clusters of Purkinje cells that only express zebrin II immunoreactivity weakly. In what follows, the zebrin II-immunopositive/immunonegative terminology will be used to refer to the strong and weak subsets. The stripes themselves are numbered following the nomenclature used in mammals whereby the most medial positive stripe is designated P1+ and the number increases as the stripes move laterally to P7+ (Brochu et al., 1990; Eisenman and Hawkes, 1993; Ozol et al., 1999; Sillitoe and Hawkes, 2002; reviewed in Sillitoe et al., 2005), but no formal homology between individual stripes in birds and mammals should be inferred. Alternating zebrin II+/− stripes were apparent in the posterior (Figs. 3, 4) and anterior lobes (Fig. 5). In the whole mounts, the contrast between the stripes was not as clear as in rodents, but resembled that seen in other mammals (e.g., cat: Sillitoe et al., 2003b; primate: Sillitoe et al., 2004; reviewed in Sillitoe et al., 2005).

## Posterior lobe

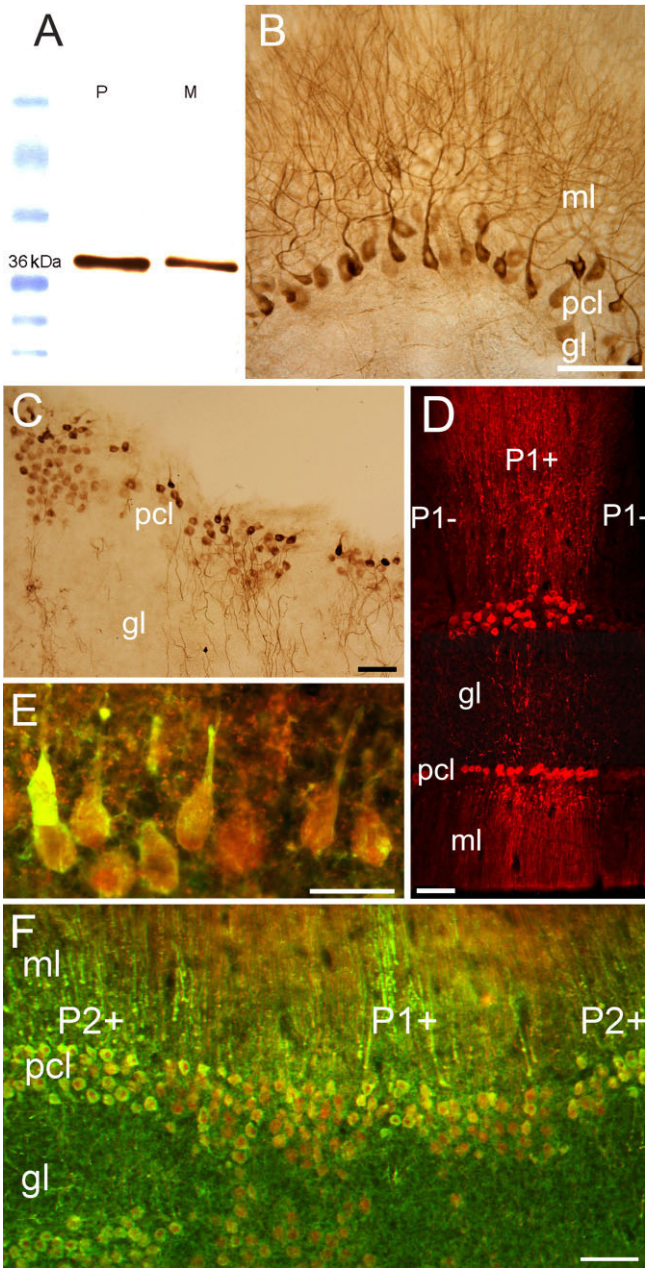
The most consistent and clear stripes were observed in folium IXcd (Fig. 3). In both whole mounts (Fig. 3B) and serial sections (Fig. 3C,D), a zebrin II-immunopositive stripe, numbered P1+, straddled the midline. Six other zebrin II-immunopositive stripes, P2+ to P7+, were located laterally on either side of the cerebellar midline and extended into the auricle. The two medial stripe pairs (P1+/− and P2+/−) were wider than the five lateral stripe pairs (P3+/− to P7+/−). In some cases, a narrow immunopositive stripe (indicated by "?" in Fig. 3) was present in folium IXcd, in the middle of P1−. This stripe was most often seen in the most caudal (and superficial) aspects of the folium.

In the ventral lamella of folium IXab the continuation of the P1+/− to P5+/− stripes from IXcd was apparent (Fig. 4A,B); however, in folium VII and VIII six +/− stripes were observed in coronal sections (Fig. 4B). The dorsal lamella of folium IXab represented a transition between the two patterns. P1+ and P1− were narrower in folia VII–IXab than in folium IXcd, and P2+, which was wide in folium IXcd, became quite narrow, such that all stripes in folia VI were of approximately the same width. P3 and P4 of folium IXcd were aligned reasonably well with P4 and P5 of dorsal IXab and VIII. In folium VI the six +ve/−ve stripes were apparent in the molecular layer, but the contrast between the +ve and −ve stripes was reduced. This was because the overall expression of zebrin II was greater in VI than in the other folia, and most Purkinje cells were zebrin II-immunopositive. The transition from the pattern in folium VII to that in VI began in the dorsal lamella of folium VII and was more gradual than indicated in Figure 4A. The pattern continued into the dorsal lamella of folium V (see Fig. 5).

There were also three +ve/−ve stripe pairs that traversed the lateral surface of the cerebellum (Fig. 4C–G). This region of the cerebellum is formed by the lateral extensions of folia VI–VIII and has been called the lateral unfoliated cortex (Larsell, 1967; Arends and Zeigler, 1991). These stripes, 1L–3L, were clear in coronal sections that transected the middle of the cerebellum through the cerebellar nuclei (Fig. 4E–G), and sagittal sections through the lateral edge of the cerebellum (Fig. 4D).

## Anterior lobe

In the anterior lobe, an array of zebrin II immunoreactive parasagittal stripes spanning folia II–V was clearly seen (Fig. 5A) in both whole-mount preparations (Fig. 5B) and coronal sections (Fig. 5C). There were four immunopositive (P1+ to P4+) alternating immunonegative stripes. The P1+ and P3+ zones were more distinct. Overall, the −ve stripes were slightly wider that the +ve stripes. In the dorsal lamella of folium V the stripes appeared less distinct as in folium IV because the overall zebrin II immunoreactivity in the Purkinje cell somata was higher, but stripes were still apparent in the molecular layer.




## Folia I and X

All Purkinje cells in folia I (lingula) and X (nodulus) were immunopositive. This is shown in Figure 6B in a ventral view of whole-mount preparation. The stripes can clearly be seen in folium II as opposed to the uniformly intense immunopositive labeling in folia I and X. This was also apparent in coronal sections (Fig. 6C,D). Figure 6E shows a sagittal section through folia I, II, and X, through the P1− stripe. Note the immunopositive labeling in I and X opposed to the absence of labeling in folium II.

## Other molecular markers

Several other neurochemical markers reveal stripes in mammalian cerebella including motilin (Chan-Palay et al., 1981), acetylcholinesterase (Jaarsma et al., 1995), corticotropin-releasing factor (van den Dungen et al., 1988; Cummings, 1989; Cummings et al., 1989; King et al., 1997), heat shock protein 25 (Hsp25; Armstrong et al., 2000), human natural killer cell antigen (HNK)-1 (Eisenman and Hawkes, 1993; Marzban et al., 2004), and phospholipase cβ4 (Sarna et al., 2006). In fact, over 20 such antigens can produce banding patterns in juvenile or adult mammals. In addition to zebrin II, we also processed the pigeon cerebellum for acetylcholinesterase using a histochemical reaction and Hsp25 and phospholipase cβ4 using immunohistochemistry. None of these were effective in revealing a banding pattern in the pigeon cerebellum.

## DISCUSSION

In the present study we have shown that zebrin II is expressed in alternating immunopositive and immunonegative stripes that are arranged parasagittally across the mediolateral extent of the pigeon cerebellum. Two lines of evidence suggest that zebrin II/aldolase C expression is evolutionarily conserved. First, Western blots of cerebellar tissue from numerous species ranging from fish to primates reveal a single immunoreactive polypeptide band of identical apparent molecular weight (36 kDa; rat: Brochu et al., 1990; opossum, *Monodelphis domestica:* Dore et al., 1990; squirrel monkey, *Saimiri sciureus:* Leclerc et al., 1990; weakly electric fish: Lannoo et al., 1991a; rabbit: Sanchez et al., 2002; hamster, *Mesocricetus*

Fig. 2. Zebrin II expression in the adult pigeon cerebellar cortex. **A:** Western blot of pigeon (P) and mouse (M) cerebellar homogenates probed with anti-zebrin II. A single immunoreactive band is detected in both cases, apparent molecular weight 36 kDa. **B,C:** Sections through the vermis, immunoperoxidase stained with anti-zebrin II. DAB reaction product is prominent in the Purkinje cell somata in the Purkinje cell layer (pcl) and the Purkinje cell dendrites in the molecular layer (ml) and in the axon fragments coursing through in the granular layer (gl) and the white matter. The tissue in C had been boiled, which removed most of the molecular layer. **D:** Coronal section through the vermis, processed for zebrin II using a fluorescent secondary antibody. Zebrin II-immunopositive (P1+) and -immunonegative (P1−) Purkinje cells can be clearly distinguished. **E,F:** Section through pigeon cerebellum double immunofluorescence stained for calbindin (red) and zebrin II (green). Zebrin II-immunoreactive Purkinje cells form a symmetrical array of stripes (P1+ at the midline: for stripe terminology, see Sillitoe and Hawkes, 2002). Purkinje cell somata are strongly zebrin II-immunoreactive in the "immunopositive stripes" and express immunoreactivity at lower levels or not at all in the intervening zebrin II "immunonegative" Purkinje cells. Scale bars = 100 μm B–D; 250 μm in F; 50 μm in E.

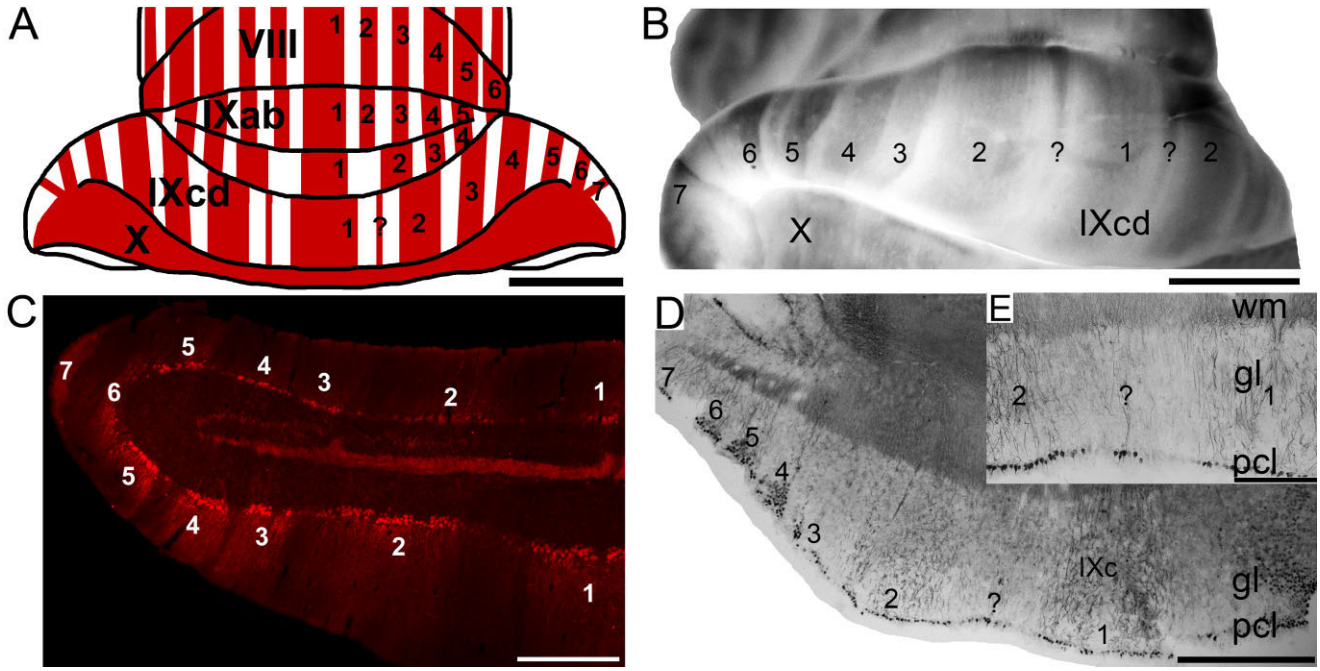



Fig. 3. Topography of Zebrin II (ZII) expression in folium IXcd of the pigeon cerebellum. **A:** Schematic of the ZII-positive (+ve; red) and ZII negative (–ve; white) bands in IXcd shown from a posteroventral view. The +ve bands are numbered (1–7) in ascending order from the midline. The thin band (?) between 1 and 2 was observed in the dorsal lamella in some but not all cases. **B:** Whole mount of the cerebellum from a posterolateral view, emphasizing the stripes in IXcd. **C:** Coronal section through IXcd illustrating the stripes spanning the folium. Note the absence of the thin band between 1 and 2. **D,E:** Adjacent horizontal sections through the dorsal lamella of IXcd. This tissue was boiled, which destroyed most of the molecular layer. gl, granular layer; pcl, Purkinje cell layer; wm, white matter. Scale bars = 2.5 mm in A,B,D; 500 μm in C,E.

*auratus:* Marzban et al., 2003a; cat: Sillitoe et al., 2003b; tenrec: Sillitoe et al., 2003a; primate: Sillitoe et al., 2004; reviewed in Sillitoe et al., 2005). This is consistent with cloning studies that have revealed that in mice the zebrin II antigen is the metabolic enzyme aldolase C (Ahn et al., 1994). Second, as in all other animals studied to date, except amphibians, in which no zebrin II immunoreactivity has been detected (Sillitoe et al., 2005), the zebrin II antigen is prominently expressed in pigeon Purkinje cells. Not all Purkinje cells in pigeons express zebrin II immunoreactivity and this differential expression reveals the elaborate underlying cerebellar topography. Zebrin II immunocytochemistry has revealed a pattern of zones and stripes in all mammalian species examined thus far (>20), and the same is also true in chicken (unpubl. data). In fish, two expression patterns have been reported—either all Purkinje cells are zebrin II-immunoreactive (e.g., zebrafish, *Danio rerio:* Lannoo et al., 1991b), or there are both zebrin II+/− phenotypes, but not organized into stripes (e.g., *Eigenmannia*: Lannoo et al., 1991a; *Gnathonemu:* Meek et al., 1992).

## Comparison of zebrin II stripes in pigeons and mammals

In rodents, the fundamental cerebellar architecture consists of four transverse zones: the anterior zone (AZ: ≈lobules I–V), the central zone (CZ: ≈lobules VI–VII), the posterior zone (PZ: ≈lobules VIII–IX), and nodular zone (NZ: ≈lobules IX–X) (Ozol et al., 1999). Within the AZ and PZ, zebrin II-immunopositive Purkinje cells are distributed as an array of immunoreactive parasagittal stripes—more than a dozen in some places—separated by intervening zebrin II-immunonegative stripes (Brochu et al., 1990; Eisenman and Hawkes, 1993; Sillitoe and Hawkes, 2002). Based on the expression pattern of zebrin II in the pigeon cerebellum we can tentatively identify four transverse zones reminiscent of those in mammals—two striped (folia II–V and folia VII–IX), and two essentially zebrin II-immunopositive (folium VI and folium X). We therefore suggest that folia II–V are homologous with the mammalian AZ, folium VI with the CZ, folia VII–IX with the PZ, and folium X with the NZ. In addition, we identify a transverse expression domain associated with the lingula (folium I) that seems to have no mammalian homolog.

***Anterior zone.*** The mammalian AZ is characterized by an array of thin stripes of Purkinje cells that express zebrin II separated by broad stripes that either express no zebrin II-immunoreactivity (e.g., rat, mouse) or express it more weakly (e.g., cat, primate). Parasagittal stripes of zebrin II-immunopositive Purkinje cells are also evident in the pigeon AZ. These stripes are not as narrow as those in the mammalian AZ, but the zebrin −ve stripes are slightly broader than the +ve stripes. The homology between pigeon folia II–V and the mammalian AZ is supported by the similar distribution of afferent terminal fields: both zones are prominent targets of the spinocerebellar projection. The avian spinocerebellar tract has terminal fields in folia I–VIa,b. The projections from the neck, is primarily to folia II–IV, whereas the cervical enlargement, representing the wings, projects primarily

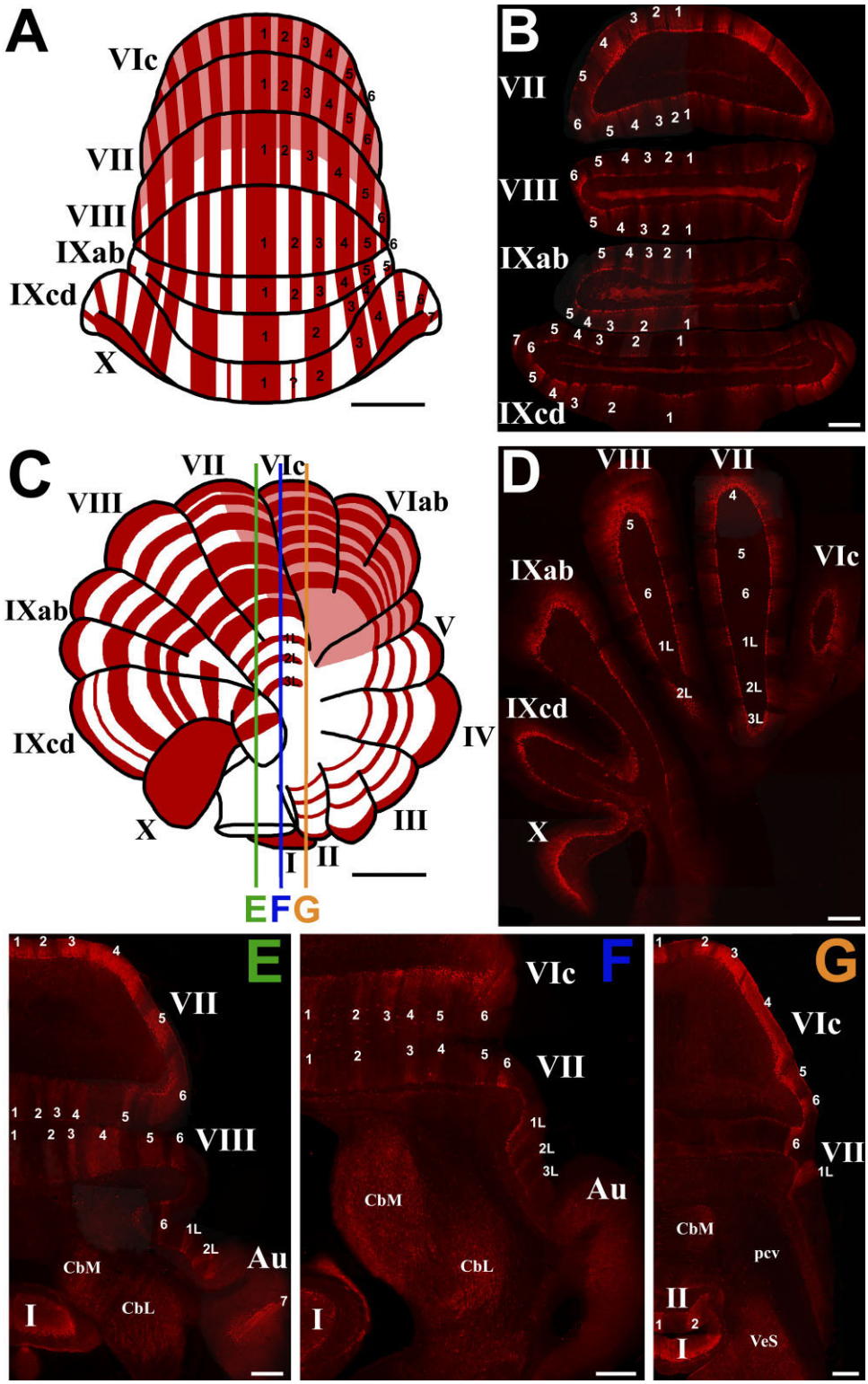



Fig. 4. Topography of zebrin II (ZII) expression in the posterior lobe of the pigeon cerebellum. **A:** Schematic of the ZII-positive (+ve; red), ZII weakly positive (pink), and ZII-negative (–ve; white) bands in folia VI–IXcd, shown from a posterior view. The pink in the immunonegative bands in folia VI is to represent that the overall zebrin II expression was higher and the contrast between the +ve and −ve bands was less. **B:** Photomicrograph of ZII expression in a coronal section through folia VII–IXcd. **C:** Schematic of ZII expression from a lateral view. The lettered vertical lines through the cerebellum in C represent coronal sections shown in **E–G**, taken from their representative location in the anterior-posterior plane. **D:** Photomicrograph of a parasagittal section taken about 3 mm from midline, illustrating the lateral stripes in folia VII and VIII. Au, auricle; gl, granular layer; pcl, Purkinje cell layer; wm, white matter; CbM/CbL, medial/lateral cerebellar nucleus; pcv, cerebellovestibular process; VeS, superior vestibular nucleus. Scale bars = 2.5 mm in A,C; 500 μm in B,D–G.

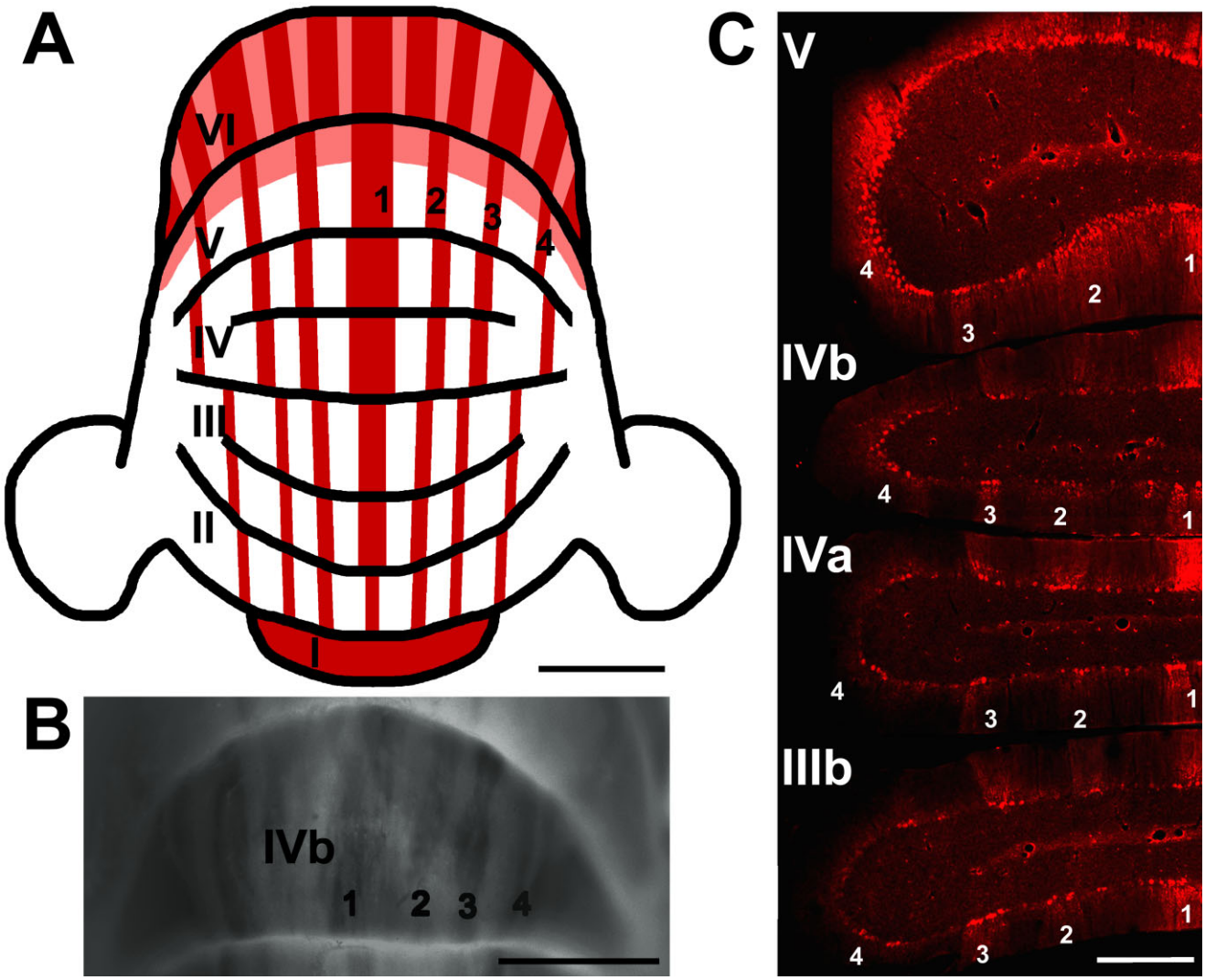



Fig. 5. Topography of Zebrin II (ZII) expression in the anterior lobe (folia I–V) of the pigeon cerebellum. **A:** Schematic of the ZII-positive (+ve; red), ZII weakly positive (pink), and ZII-negative (–ve; white) bands in folia I–VI shown from an anterior view. The pink in the immunonegative bands of the dorsal part of folium V and throughout folium IV (posterior lobe) is to represent that the overall ZII expression was higher and the contrast between the +ve and −ve bands was less. **B:** ZII expression in folium IVb from a whole mount. **C:** Coronal section through folia IIIb to V. Note the four (1–4) immunopositive stripes, but the greater overall expression in dorsal V (C). Scale bars = 2.5 mm in A,B; 500 μm in C.

to folia III–V and the lumbosacral enlargement, representing the legs, projects primarily to folia III–VIa,b (Fox and Snider, 1967; Schulte and Necker, 1998; Necker, 2001). In rats and mice, the spinocerebellar and cuneocerebellar tracts project strongly to lobules II–V of the anterior lobes (e.g., reviewed in Voogd et al., 1996; Voogd and Ruigrok, 1997) where they segregate into parasagittal stripes in register with the overlying Purkinje cell stripes (Gravel and Hawkes, 1990; e.g., Akintunde and Eisenman, 1994; Ji and Hawkes, 1994, 1995).

***Central zone.*** The alternating zebrin II-immunopositive and -immunonegative stripes that characterize the AZ in pigeons are replaced in folium VI by a more uniform pattern of zebrin II expression, not unlike the CZ (lobules VIa,b,c) in rodents. Although zebrin II does not reveal CZ heterogeneity, in rodents a parasagittally striped organization has been shown in the afferent terminal field distributions (e.g., Serapide et al., 1994; Voogd and Ruigrok, 1997) and by the expression of the small heat shock protein Hsp25 (Armstrong et al., 2000: no Hsp25 expression is seen in pigeon Purkinje cells, unpubl. data). Other features lend support to the notion that folium VI in birds might be homologous with lobule VI in mammals. In both mammals and birds lobule/folium VII is considered part of the oculomotor vermis, where there are both visual and trigeminal inputs (Gross, 1972; Clarke, 1977; Williams, 1995; for review, see Voogd and Barmack, 2006). Moreover, folium/lobule VI is a site of heavy pontocerebellar inputs in both birds and mammals (Gerrits and Voogd, 1986; Yamada and Noda, 1987; Voogd and Barmack, 2006).

***Posterior zone.*** In the mouse the transition from the CZ to the PZ lies in the prepyramidal fissure between lobules

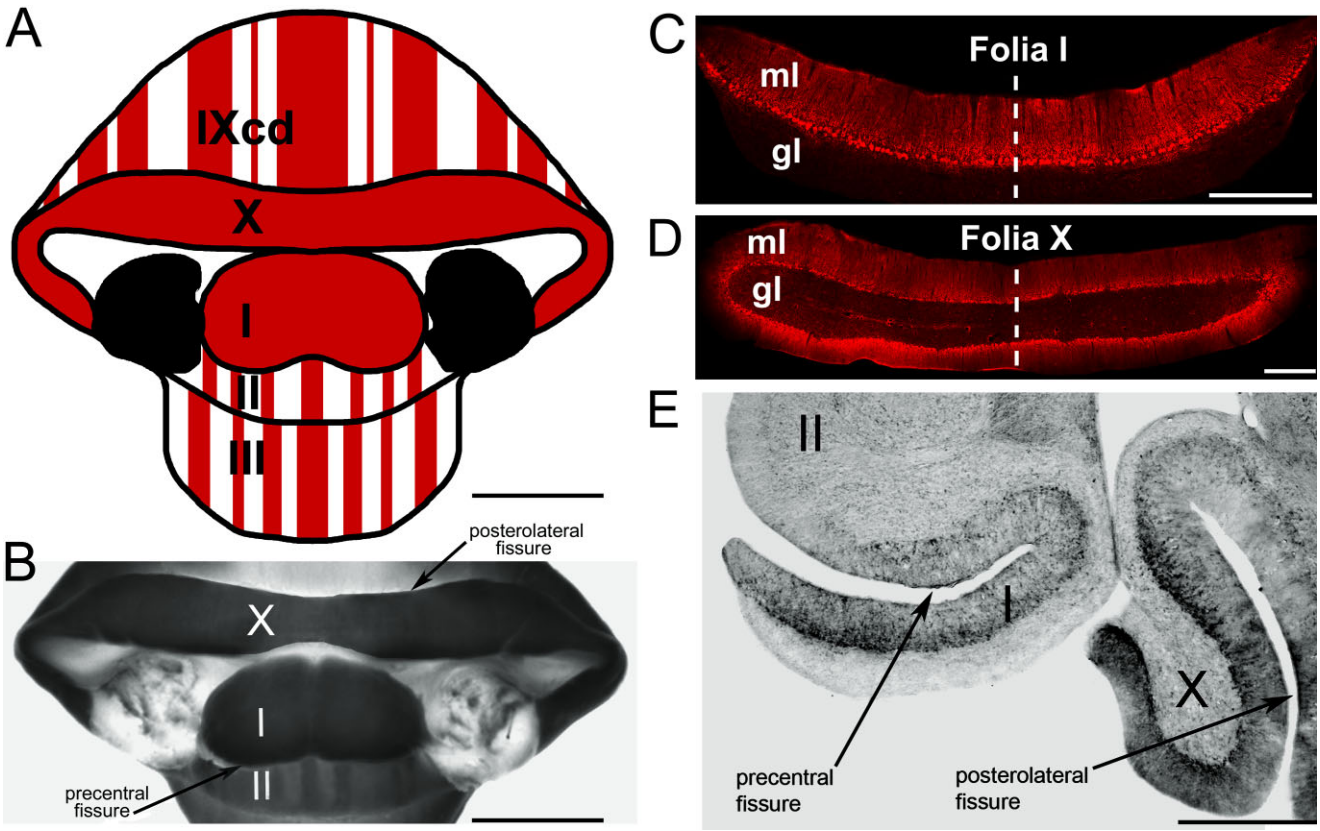



Fig. 6. Zebrin II (ZII) expression in the folium I (lingula) and folium X (nodulus) of the pigeon cerebellum. **A:** Schematic of the ZII-positive (+ve; red) and ZII-negative (–ve; white) bands of the cerebellum shown from a ventral view. The cerebellar peduncles are shaded black. **B:** Photograph of the same view of a whole-mount cerebellum. The bands are apparent in folium II, but folium I and folium X are uniformly immunopositive. **C,D:** Coronal sections through folium I and folium X, respectively, illustrating this uniform immunopositive expression. E: Sagittal section through folia I, II, and X. Purkinje cells are immunonegative through folium II, but immunopositive throughout folium I and folium X. Scale bars = 2.5 mm in A,B; 500 μm in C–E.

VII and VIII: on the dorsal aspect of lobule VII all Purkinje cells express zebrin II and on the ventral aspect of lobule VIII a prominent array of stripes is apparent (e.g., Eisenman and Hawkes, 1993). In the pigeon a similar transition, albeit less striking, is seen between folia VII and VIII (Fig. 4A,C). A pattern of zebrin II expression in which stripes of high-expressing Purkinje cells are separated by equal-width stripes of anti-zebrin II-unreactive cells characterizes the PZ in many mammals, including mouse (e.g., Sillitoe and Hawkes, 2002), rat (e.g., Brochu et al., 1990), rabbit (Sanchez et al., 2002), guinea pig (*Cavia porcellus:* Larouche et al., 2003), hamster (Marzban et al., 2003a), cat (Sillitoe et al., 2003b), and primates (Sillitoe et al., 2004). In the pigeon cerebellum, stripes of zebrin II expressing Purkinje neurons are separated by narrow immunonegative stripes in folia VII and VIII, and the dorsal lamella of IXab, and by wider stripes in folia IXcd. Zebrin II compartmentation is clearly distinguished in lobule IXcd of the pigeon cerebellum as broad parasagittal P1+ to P3+ stripes of highly immunoreactive Purkinje cells alternating with Purkinje cells that are either zebrin II-immunonegative or express the antigen at lower levels. In addition, within the vermis a pair of narrow zebrin II+ stripes is seen in pigeon either side of P1+ (Fig. 3C–E) that is only seen irregularly in the rodent cerebellum (so-called "satellite bands," e.g., Hawkes and Leclerc, 1987). The P4+ to P7+ stripes in folia IXcd in pigeons are present on the lateral extension of folia IXcd into the auricle (flocculus). Thus, based on the pattern of zebrin expression, it appears that the PZ of mammals (lobules VII and VIII) corresponds with folia VII–IXcd. A similar striped pattern of expression is also found in folium IX of the chick cerebellum, supporting the hypothesis that folium IX constitutes the PZ of the avian cerebellum (Hawkes et al., unpubl. data). In mammals, lobule IX is part of the NZ, where there is uniform expression of zebrin (see below). Thus, folium IX in pigeons appears to correspond to lobule VIII in mammals. This postulation is consistent with data on mossy fiber afferent terminal field distributions. For example, spinocerebellar pathways to the posterior cerebellum predominantly terminate in lobule VIII (pyramis) in mammals, but folium IX (uvula) in the chicken (Vielvoye and Voogd, 1977) and pigeon (e.g., Necker, 1992).

Other data, however, argue against the postulated homology of folia IX in birds and lobule VIII in mammals. An extensive literature has examined the physiology and climbing fiber afferents to folia IXcd and X in birds (Wylie and Frost, 1991, 1993; Lau et al., 1998; Wylie et al., 1998, 1999a; Wylie and Frost, 1999; Crowder et al., 2000; Winship and Wylie, 2001, 2003; Wylie, 2001). The climbing fibers carry optokinetic information to folia IXcd and X

and form parasagittal stripes spanning the two folia. In the lateral half of folia IXcd and X, Purkinje cells respond to rotational optokinetic stimuli in precisely the same manner as the flocculus in mammals (Graf et al., 1988). Moreover, the zonal organization is remarkably similar to the flocculus in rats and rabbits (Voogd and Wylie, 2004). Thus, based on response properties and the P4+/− to P7+/− stripes, folium IXcd in pigeons appears to be the homolog of the flocculus, but the flocculus in mammals is uniformly +ve for zebrin expression (e.g., Ozol et al., 1999; Sanchez et al., 2002; Sillitoe and Hawkes, 2002; Marzban et al., 2003a). In the medial parts of the ventral lamella of folium IXcd and in folium X of pigeons Purkinje cells respond to translational optokinetic stimulation (Wylie and Frost, 1999). These responses are reminiscent of those in the uvula (lobule IX) and nodulus (lobule X) of mammals, where Purkinje cells respond to either optokinetic stimuli or vestibular stimulation originating in the otolith organs (Barmack and Shojaku, 1995). Thus, despite the stripes, the medial parts of folium IXcd more resemble the uvula in mammals.

***Nodular zone.*** In mammals the NZ is characterized by uniform zebrin II expression in lobules IX and X. In mouse the interdigitated boundary between the PZ and the NZ lies in the ventral face of lobule IX (Ozol et al., 1999; Armstrong and Hawkes, 2000). In the pigeon an NZ in which all Purkinje cells uniformly express zebrin II is restricted to folium X (the nodulus). Folium X is separated from the rest of the cerebellar cortex by the posterolateral fissure—the first to form during cerebellar development (Larsell, 1967). The lateral extensions of folium X, the flocculi, are uniformly zebrin II-immunopositive in pigeon, as in mammals. Primary vestibular afferents are restricted to lobules IX and X in mammals (Fox and Snider, 1967; Voogd and Wylie, 2004) and folium IXcd and X in birds (Schwarz and Schwarz, 1983), perhaps indicative in birds of the same interdigitation of transverse zones also identified in mammals (e.g., Ozol et al., 1999). There is also a prominent external granular layer boundary in this region for Tlx-3 expression in chick (Logan et al., 2002). Granule cell lineage boundaries between the PZ and NZ have been described in mice (e.g., Hawkes et al., 1999). Vestibular afferents also terminate in lobules I and II, perhaps hinting at a relationship between the nodular zone and a putative lingular zone (see below).

***Lingular zone.*** The most obvious discrepancy between the cerebellar ground plan in mammals and birds, as reflected in the present data, is the strong uniform expression of zebrin II by all Purkinje cells in folium I. There is some reason to consider the lingula as a distinct transverse zone, with no equivalent in mammals. For example, the lingula is always separated from the more ventral lobulus centralis by a deep precentral fissure, and for this reason, together with its large size in birds, the lingula was regarded by Larsell (1948) as a distinct primary folium. Functionally, the avian lingula has been associated with the control of tail feathers and the tail musculature (Larsell, 1948), although Necker (2001) reported a high concentration of neck afferents in I. It is possible that lobule I in mammals is a rudimentary homolog of the avian lingula, but given their very different patterns of zebrin II expression, we favor the hypothesis that the avian lingula is a unique transverse zone with no mammalian homolog, and that lobule I in mammals is simply the anterior tip of the AZ, continuous with lobules II–V. A second possibility is that the lingula in pigeon is derived embryologically from the NZ, with which it shares a zebrin II expression profile. Although folium I is located at the extreme anterior of the cerebellum and X the posterior extreme, because of the way the cerebellum folds they end up located adjacent to one another, but separated by the recess of the fourth ventricle. This is consistent with afferent terminal field maps showing that mossy fiber afferent fibers from the reticular nucleus of the pons terminate in all folia of the vermis, except I and X (Kawamura and Hashikawa, 1981; Gerrits and Voogd, 1986) and with the observations that both I and X are vestibulocerebellar receiving areas (Fox and Snider, 1967; Williams, 1995). In this context, it is pertinent that the flocculonodular lobe, which receives mostly vestibular inputs, and the lingula, which receives spinocerebellar and vestibulocerebellar inputs, together form the oldest part of the cerebellum, known classically as the archicerebellum (Williams and Warwick, 1980).

## Are there hemispheres in the pigeon cerebellum?

Many previous researchers have regarded the large central body of the avian cerebellum as the homolog of the mammalian cerebellar vermis (Fox and Snider, 1967). However, Larsell (1948) directed attention to the fact that on either side of the base of the corpus cerebelli in birds there is a small swelling, the lateral unfoliated cortex. He proposed this swelling to represent the region which, in mammals, becomes the lateral cerebellar hemisphere (Larsell, 1948). This hypothesis received immediate support from the work of Brodal et al. (1950), who concluded, on the basis of the pontocerebellar projection in the chick, that the unfoliated lateral cortex and the adjacent lateral parts of folia V–VIII represent an avian homolog of the mammalian cerebellar hemispheres. We therefore examined this region for evidence of patterned zebrin II expression. In mammals the hemispheres express alternating striped and uniform zones; the lobulus simplex, crus II, and paramedian lobules are striped, whereas crus I, paraflocculus, and flocculus are uniformly positive. In the unfoliated lateral cerebellar cortex of pigeon, we were able to consistently identify a reproducible array of three zebrin II+ stripes that we labeled 1L–3L. This is similar to the organization of the cerebellar hemispheres in rodents, where there are four stripes, P4+ to P7+. This adds some support to the suggestion that the avian hemisphere may be a derivative of the lateral PZ.

## Evolutionary implications

Our results clearly demonstrate that zebrin II is expressed as a pattern of parasagittally oriented stripes in the cerebellar cortex of pigeons. As such, this is not only the first demonstration of zebrin II immunoreactivity in a bird, but also the first published study of zebrin II expression in a vertebrate other than fish (Brochu et al., 1990; Lannoo et al., 1991a,b; Meek et al., 1992) or mammals (Sillitoe et al., 2005). Although we suggest that the pattern of zebrin II labeling is largely consistent, and possibly homologous, with that of mammals, the prominent differences in the flocculus and lingula between birds and mammals also suggests that this may not be true for all folia and lobules. Whether these regions truly are homologous and whether the lateral stripes actually represent the hemispheres requires further examination of zebrin II

expression in other groups of vertebrates, especially in nonavian reptiles (i.e., snakes and lizards) and crocodylians. The cerebellum of nonavian reptiles consists of a single, leaf-shaped sheet in which the granule cell layer is inverted such that it is the dorsal most layer of the cerebellar cortex (Larsell, 1967). Crocodylians, on the other hand, possess an avian-like cerebellum with three folds that have been homologized with the avian cerebellar folia (Larsell, 1967) in the following combinations: I–V, VI–VIII, and IX–X. If zebrin-expressing zones are evolutionarily conserved among amniotes, as they are within mammals (Sillitoe et al., 2005), then similar transitions in stripe patterns between the AZ, CZ, PZ, and NZ should be apparent in both nonavian reptiles and crocodylians. If not, then the zonal organization of the cerebellum may be an example of convergent evolution between birds and mammals (i.e., homoplasy) and not necessarily a homologous trait.

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