Original Paper

Brain, Behavior and Evolution

Brain Behav Evol 2015;85:94–106
DOI: 10.1159/000380810

Received: December 4, 2014
Returned for revision: January 20, 2015
Accepted after revision: February 9, 2015
Published online: April 2, 2015

Zebrin II Expression in the Cerebellum of a Paleognathous Bird, the Chilean Tinamou *(Nothoprocta perdicaria)*

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Key Words
Purkinje cells · Aldolase C · Sagittal zones · Brain evolution

Abstract
Zebrin II (ZII) is a glycolytic enzyme expressed in cerebellar Purkinje cells. In both mammals and birds, ZII is expressed heterogeneously, such that there are sagittal stripes of Purkinje cells with a high ZII expression (ZII+) alternating with stripes of Purkinje cells with little or no expression (ZII–). To date, ZII expression studies are limited to neognathous birds: pigeons (Columbiformes), chickens (Galliformes), and hummingbirds (Trochilidae). These previous studies divided the avian cerebellum into 5 transverse regions based on the pattern of ZII expression. In the lingular region (lobule I) all Purkinje cells are ZII+. In the anterior region (lobules II–V) there are 4 pairs of ZII+/– stripes. In the central region (lobules VI–VIII) all Purkinje cells are ZII+. In the posterior region (lobules VIII–IX) there are 5–7 pairs of ZII+/– stripes. Finally, in the nodular region (lobule X) all Purkinje cells are ZII+. As the pattern of ZII stripes is quite similar in these disparate species, it appears that it is highly conserved. However, it has yet to be studied in paleognathous birds, which split from the neognaths over 100 million years ago. To better understand the evolution of cerebellar compartmentation in birds, we examined ZII immunoreactivity in a paleognath, the Chilean tinamou *(Nothoprocta perdicaria)*. In the tinamou, Purkinje cells expressed ZII heterogeneously such that there were sagittal ZII+ and ZII– stripes of Purkinje cells, and this pattern of expression was largely similar to that observed in neognathous birds. For example, all Purkinje cells in the lingular (lobule I) and nodular (lobule X) regions were ZII+, and there were 4 pairs of ZII+/– stripes in the anterior region (lobules II–V). In contrast to neognaths, however, ZII was expressed in lobules VI–VII as a series of sagittal stripes in the tinamou. Also unlike in neognaths, stripes were absent in lobule IXab, and all Purkinje cells expressed ZII in the tinamou. The differences in ZII expression between the tinamou and neognaths could reflect behavior, but the general similarity of the expression patterns across all bird species suggests that ZII stripes evolved early in the avian phylogenetic tree.

Introduction

In both mammals and birds, the cerebellum can be divided into 10 transverse lobules [Larsell, 1967]. However, the basic units of the cerebellum are the sagittal zones that cut across the lobules [e.g. Voogd and Bigaré, 1980]. This

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sagittal organization is apparent with respect to several aspects of cerebellar anatomy and physiology including climbing and mossy fiber afferentation, Purkinje cell projections, and Purkinje cell response properties [Andersson and Oscarsson, 1978; Apps and Garwicz, 2005; De Zeeuw et al., 1994; Ekerot and Larson, 1973; Llinas and Sasaki, 1989; Ruigrok, 2003; Voogd, 1967; Voogd and Glickstein, 1998; Wu et al., 1999]. A sagittal organization is also apparent with respect to the expression of numerous molecular markers [for review, see Herrup and Kuemerle, 1997]. The most thoroughly studied marker in this regard is zebrin II (ZII), which is an epitope of the metabolic isoenzyme aldolase C [Ahn et al., 1994]. ZII is expressed in cerebellar Purkinje cells, but heterogeneously such that there are sagittally oriented stripes of Purkinje cells that strongly express ZII (ZII+) alternating with stripes that express ZII weakly or not at all (ZII–) [e.g. Brochu et al., 1990; Hawkes, 1992; Hawkes and Herrup, 1995].

The expression of ZII has been examined in the cerebella of several mammalian species, from marsupials to primates [Brochu et al., 1990; Eisenman and Hawkes, 1993; Fujita et al., 2010; Kim et al., 2009; Marzban et al., 2003, 2011, 2012; Sanchez et al., 2002]. Across these species, a consistent pattern of ZII expression has emerged. In lobules VI–VII (a.k.a. central region) and ventral IX and X (nodular region), ZII stripes are not apparent but rather most Purkinje cells are ZII+. However, lobules I–V (anterior region) and lobules VIII and dorsal IX (posterior region) consist of alternating sagittal ZII+ and ZII– stripes [Marzban and Hawkes, 2011; Sillitoe et al., 2005].

The expression of ZII has also been studied in a few species of birds: pigeons *(Columba livia)* [Pakan et al., 2007], hummingbirds *(Calypte anna* and *Selasphorus rufus)* [Iwaniuk et al., 2009], and chickens *(Gallus domesticus)* [Marzban et al., 2010]. In these birds, ZII is expressed heterogeneously such that there are ZII+ and ZII– sagittal stripes, and the pattern of expression is remarkably similar to that observed in mammals. Alternating ZII+ and ZII– stripes are apparent in lobules II–V (anterior region) and VIII and IX (posterior region), whereas most Purkinje cells in lobules VI, VII (central region), and X (nodular region) are ZII+. The only apparent difference between mammals and birds with respect to the pattern of ZII expression is in lobule I, which has stripes in mammals but is uniformly ZII+ in birds (lingular region). Note that in most mammalian species the ventral-most portion of IXcd has stripes, like birds, but in a few species it is all ZII+ [Marzban et al., 2011]. The similarity in cerebellar ZII expression patterns across these avian species is quite striking despite some obvious differences in gross cerebellar morphology [Iwaniuk et al., 2009; Marzban et al., 2010; Pakan et al., 2007].

The high degree of similarity between avian and mammalian cerebella, including the alternation of ZII+ and ZII– regions, suggests conservation of a similar cerebellar architecture, likely with a first evolutionary appearance in stem reptiles. However, in 2 stem reptiles, the red-eared slider *(Pseudemys scripta elegans)* and the diamondback rattlesnake *(Crotalus atrox),* a pattern of alternating ZII+ and ZII– sagittal stripes of cells is not present. Instead, Purkinje cells are uniformly ZII+, suggesting either that ZII stripes have been lost in both turtles and snakes or that they evolved convergently in birds and mammals [Aspden et al., 2015; Larsell, 1967; Sillitoe et al., 2005]. Therefore, what is needed is an examination of the ZII expression in species that represent basal clades within birds and mammals in order to track the development of ZII stripes through evolutionary history and to broaden our understanding of the evolution of cerebellar organization.

Modern birds are divided into 2 superorders: Palaeognathae and Neognathae. Although phylogenetic relationships within each of these superorders have been contentious, it is clear that neognaths and paleognaths are sister taxa separated by 120–130 million years of evolution [Brown et al., 2008; Haddrath and Baker, 2012; Harshman et al., 2008; Phillips et al., 2010]. Most extant bird species are neognaths, with paleognaths currently represented by only the flightless ratites and the volant tinamous (see fig. 1 for an avian phylogenetic tree). The ratites are, for the most part, a highly derived group relative to their paleognathous ancestors and tinamous [Feduccia, 1999]. For example, kiwi *(Apteryx mantelli)* have evolved a number of unique sensory adaptations to function in a nocturnal ground-dwelling niche [Corfield et al., 2011, 2014a, b; Cunningham et al., 2013; Martin et al., 2007]. Similarly, the other ratites differ markedly in their anatomy and ecology from tinamous and neognathous birds [Davies, 2002]. Many of these anatomical traits in ratites relate to flightlessness, which likely evolved several times within the group [Baker et al., 2014; Feduccia, 1999; Leonard et al., 2005]. In contrast, tinamous (Tinamidae) are volant and share numerous traits with early paleognathous birds [Houde, 1986; Houde and Olson, 1981] as well as neognaths. Tinamous are therefore more likely to be representative of those species at the base of the modern bird phylogeny. In the present study, we examine the pattern of cerebellar compartmentation in tinamous as revealed by ZII expression and directly compare it to the pigeon to gain a better understanding of how the

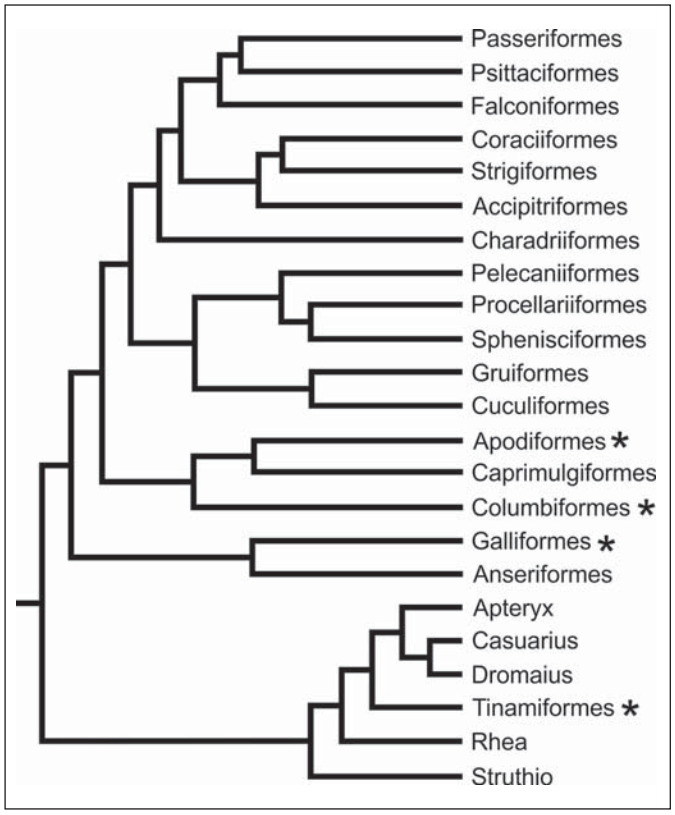

Fig. 1. Phylogenetic tree depicting the relationship among 18 orders of birds. The ratites have been further divided to the species level. The tree is based on Hackett et al. [2008]. * Indicates where ZII expression patterns have been described.

cerebellar cortex is organized in basal birds. Conserved characteristics in the Neognathae and Palaeognathae may represent the basal avian cerebellar condition that existed in a common ancestor at the base of the modern bird evolutionary tree, whereas differences would represent yet another trait that differentiates these superorders [see Corfield et al., 2013; Krabichler et al., 2014].

Materials and Methods

All animal procedures used in this study conformed to institutional regulations and the Guide to the Care and Use of Experimental Animals from the Canadian Council for Animal Care and were performed with the approval of the Biosciences Animal Care and Use Committee at the University of Alberta and the bioethics committee of the Facultad de Ciencias of the Universidad de Chile. Two adult Chilean tinamous *(Nothoprocta perdicaria)* were acquired from a Chilean breeder and 2 adult pigeons *(C. livia)* were obtained from a local supplier in Canada. Pigeons and tinamous 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 postfixed by immersion at 4°C in the same fixative for several days. The tinamous were perfused at the Universidad de Chile, and the brains were extracted and exported to Canada. The pigeon and tinamou brains were cryoprotected in 30% sucrose in 0.01 M phosphate-buffered saline (PBS) until they sunk and embedded in 10% gelatin. The embedded brains were frozen and then serially sectioned on the coronal plane on a sliding microtome at a thickness of 40 μm. Sections were collected in PBS with 0.01% sodium azide and divided into 4 alternate series. Sections from one series were mounted on gelatinized glass slides and Nissl-stained with thionin. The other 3 series were processed for ZII immunoreactivity.

The ZII expression patterns in the two tinamou specimens were nearly identical. Very little interindividual variation is also present in pigeons [e.g. Pakan et al., 2007, 2011]. Therefore, we are confident that the expression patterns we found in tinamous are an accurate indication of this species as a whole.

Immunohistochemistry

Floating sections were first rinsed thoroughly in PBS and blocked for 1 h in 10% normal donkey serum (Jackson Immunoresearch Laboratories) and 0.4% Triton X-100. Tissue was then incubated for 5 days at 4°C in 0.1 M PBS (pH 7.4) containing 0.1% Triton X-100, 2.5% normal donkey serum, and anti-ZII/aldolase C (1:1,000, goat polyclonal, catalog No. sc-12065; Santa Cruz Biotechnologies, Dallas, Tex., USA). Following this, sections were rinsed in PBS and then incubated for 3 h at room temperature in a PBS solution containing 2.5% normal donkey serum, 0.4% Triton, and Alexa Fluor 594 or 488 anti-goat antibody (1:200; Jackson Immunoresearch Laboratories). After the 3 h, sections were rinsed 5 times in PBS, mounted on gelatinized slides, and left to dry. In some cases an antigen retrieval protocol was employed, which consisted of pretreating sections with a sodium citrate buffer (10 mM, pH 8.5) preheated to 80°C for 30 min [Jiao et al., 1999].

Some sections were also processed for both ZII and calbindin (CB) immunoreactivity, as all Purkinje cells are CB immunopositive [Bastianelli, 2003]. Other sections were processed for both ZII and parvalbumin (PV) immunoreactivity because PV is expressed heterogeneously by Purkinje cells in the pigeon cerebellum such that the expression is generally complementary to that of ZII: ZII+ stripes are PV– and vice versa [Wylie et al., 2011]. For these sections the anti-CB (1:2,000, rabbit polyclonal, catalog No. CB38; Swant) or anti-PV (1:2,000, mouse monoclonal, catalog No. P3088; Sigma-Aldrich Co.) antibodies were combined with the ZII antibody in the primary solution. Alexa Fluor 488 or 594 anti-mouse or anti-rabbit secondary antibodies (1:200; Jackson Immunoresearch Laboratories) were used as appropriate such that the ZII and CB or PV were visualized with different colors.

Microscopy

The sections were mounted onto glass slides, air-dried, and then water-stained prior to viewing using a compound light microscope (Leica DMRE) equipped with the appropriate fluorescence filters for visualization. Images were acquired using a Retiga EXi FAST Cooled Mono 12-bit camera (QImaging) and analyzed with Openlab imaging software (Improvision). Photos were then stitched together in PTGui (New House Internet Services BV) for visualization of the entire sections. Adobe Photoshop (San Jose, Calif., USA) was used to adjust for brightness and contrast.

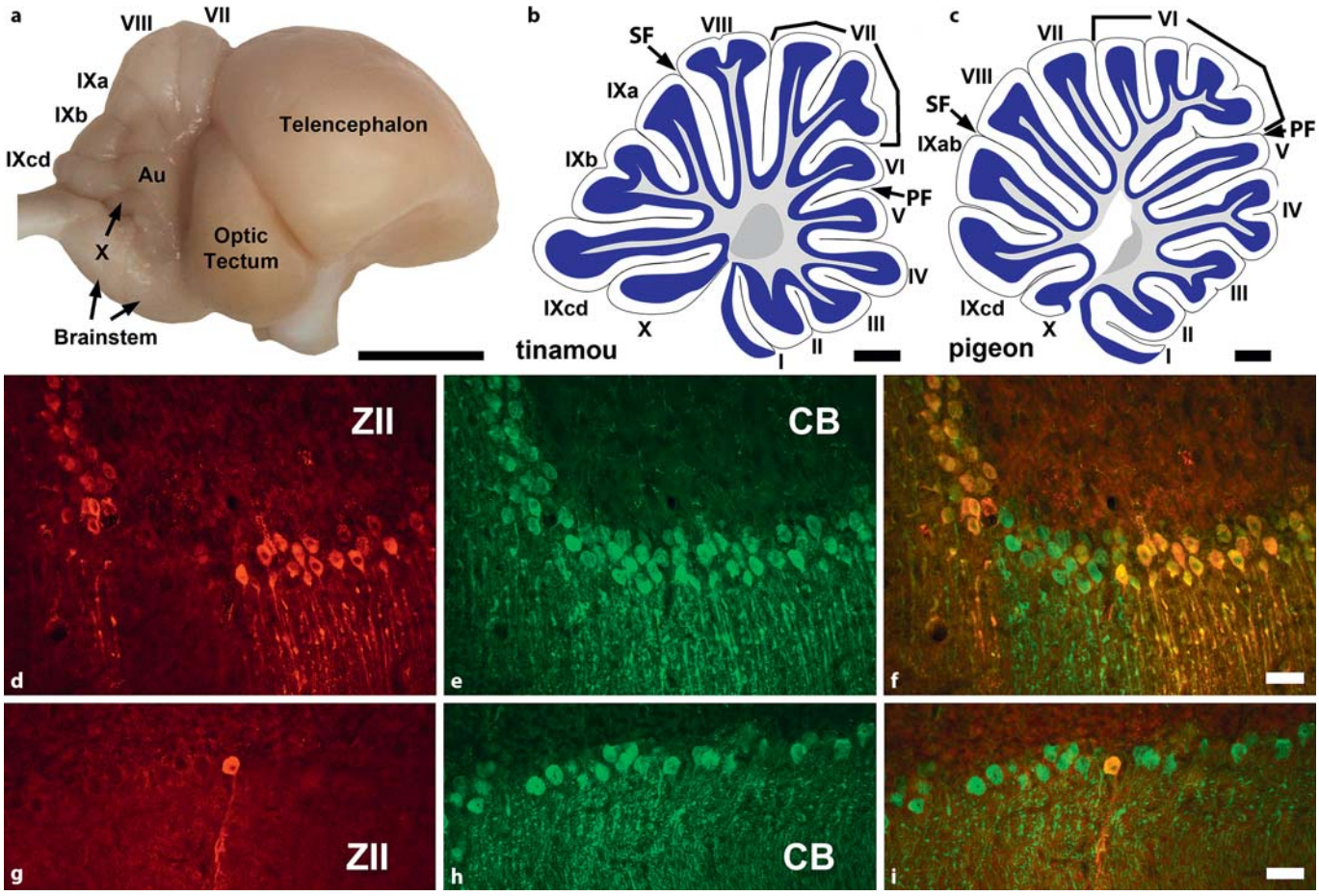

Fig. 2. Cerebellum and expression of ZII in the Chilean tinamou *(N. perdicaria)*. **a** Lateral view of the brain of the Chilean tinamou showing lobules VII–X. Lobules I–VI are hidden behind the telencephalon and optic tectum. **b**, **c** Schematic representations of a midsagittal section of tinamou and pigeon *(C. livia)* cerebellum. The lobules are numbered I–X (anterior to posterior) according to the nomenclature of Larsell [1967]. **d**, **g** ZII expression in the cerebellum of the tinamou. **e**, **h** CB expression in the cerebellum of the tinamou. **f**, **i** Overlay of ZII (red) and CB (green). Note that all Purkinje cells expressed CB, whereas only a subset expressed ZII. **g–i** represent a ZII+ stripe that is only 1 cell wide. PF = Primary fissure; SF = secondary fissure. Scale bars = 5 mm (**a**), 1 mm (**b**, **c**), and 50 μm (**d–i**).

Lobule Classification

The 10 primary lobules of the cerebellum are more easily identifiable from sagittal sections in birds. Therefore, to aid in identifying each of the 10 primary lobules in the tinamou, a virtual midsagittal section was reconstructed from the Nissl-stained coronal sections using Amira (v 5.2; Visage Imaging, San Diego, Calif., USA). For this, images of coronal sections were loaded into Amira and aligned based on anatomical features of the cerebellar lobules using the AlignSlices module. When these images are viewed in the LabelField module, there is an option to resample the images so that they can be viewed on either the sagittal or the axial plane. Lobules were identified from this section and numbered I–X (anterior to posterior) with reference to Iwaniuk et al. [2006, 2007] and Larsell [1967]. The primary fissure was used to separate lobules V and VI, and the secondary fissure was used to separate lobules VIII and IXab (fig. 2a–c) [Larsell, 1967].

ZII Stripe Reconstructions

The ZII stripes were reconstructed on an 'unfolded' cerebellar cortex (fig. 5). ZII+ and ZII– stripes in the pigeon and tinamou were measured from coronal sections using Openlab imaging software and using CB and PV labeling to verify ZII-negative stripes. Stripe widths were measured from every eighth section (320 μm). Positive and negative stripes were measured starting at the midline, which was identified by a notch or gap in the Purkinje cell layer [Pakan et al., 2007; Wylie, 2013]. Separate measurements were made of the ventral and dorsal lamellae for each lobule. The ZII labeling was examined in serial sections and assigned to one of 3 categories; a ZII+ stripe had intense Purkinje cell labeling, whereas a ZII– stripe had little to no labeling. In nearly all cases, the contrast between the ZII+ and ZII– Purkinje cells was obvious. A third labeling intensity was distinguished where the Purkinje cells clearly expressed ZII, much more than

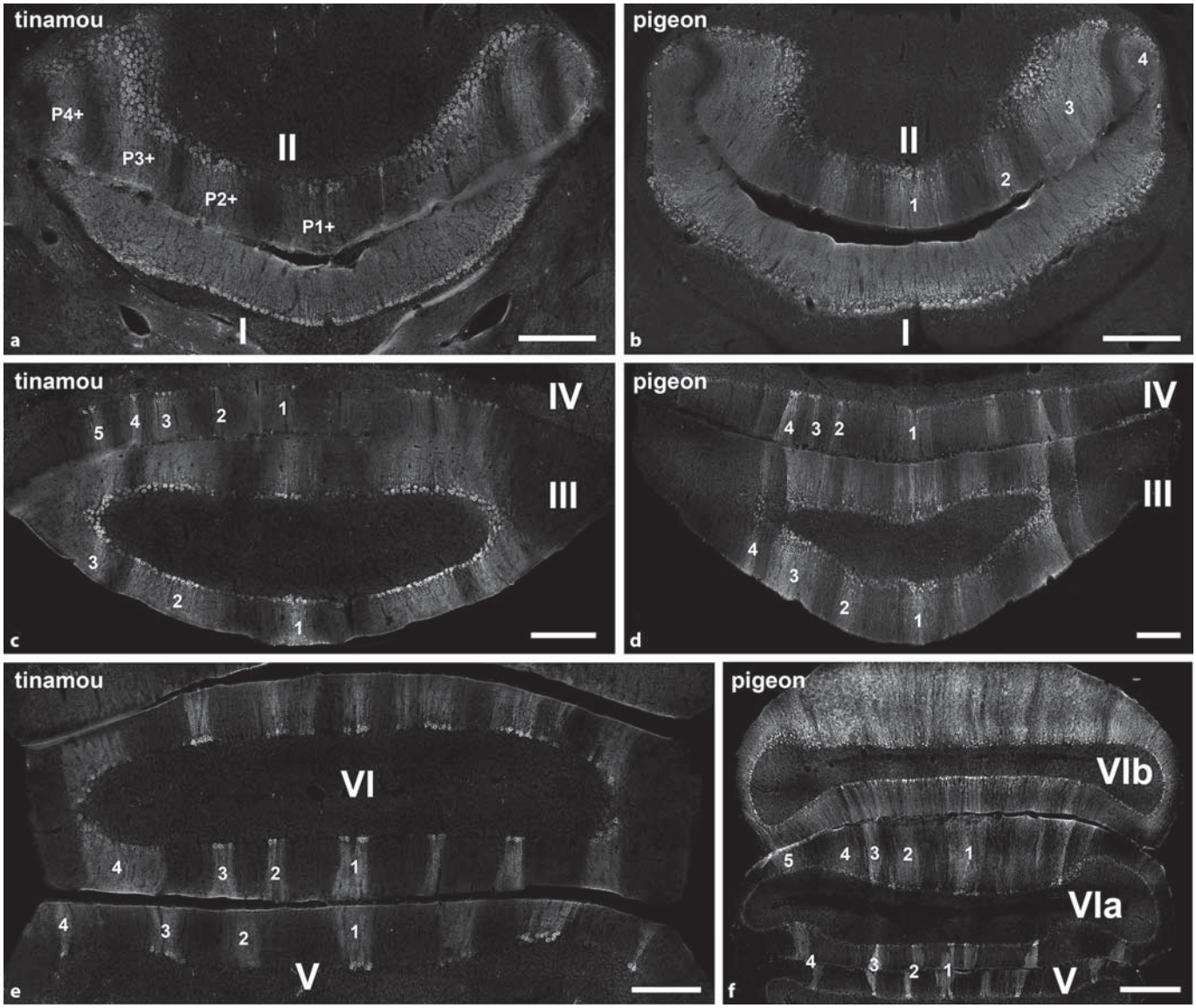

Fig. 3. ZII expression in the anterior cerebellum and lobule VI in the Chilean tinamou *(N. perdicaria)*. **a–d** Coronal sections through lobules I–IV in the tinamou and pigeon *(C. livia)* illustrating the ZII+ and ZII– stripes spanning the lobule. The ZII+ bands are numbered 1–5 in ascending order from the midline. In **a** and **b** stripes can be seen in lobule II as opposed to the uniform immunopositive labeling in lobule I. **e**, **f** ZII immunoreactivity in lobules V and VI in the tinamou and pigeon. Note the ZII+ and ZII– stripes in lobule VI of the tinamou compared to the largely ZII+ labeling present in the pigeon. Scale bars = 400 μm.

the ZII– cells, but clearly less intensely than the adjacent ZII+ cells [Pakan et al., 2007]. To remove observer bias, a subset of sections were also measured by a second observer; no appreciable differences were observed. All measurements were obtained from both halves of the cerebellum and then averaged. The stripes themselves were numbered following the nomenclature used in pigeons, whereby the most medial positive stripe is designated P1+, or simply 1, and the number increases as the stripes move laterally to P7+ [Pakan et al., 2007]. The widths of the stripes, as well as their position relative to the midline, were plotted using graphing software (SigmaPlot v11; Systat sSoftware, Inc.) and shading and labels were added using Coreldraw X5 (Corel Corporation). The resulting schematic depicts the entire cerebellar cortex unfolded, showing the ZII parasagittal striped pattern.

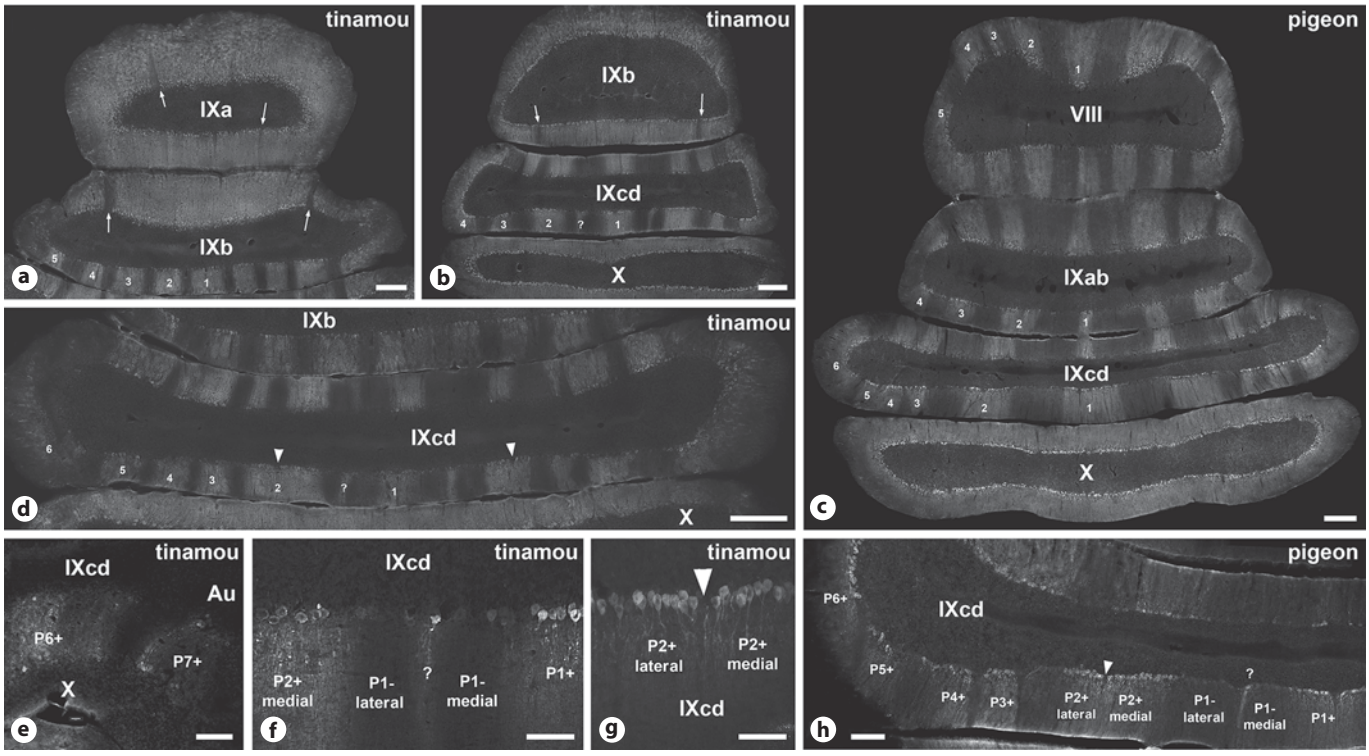

Fig. 4. ZII expression in the posterior and vestibulocerebellum in the Chilean tinamou *(N. perdicaria)*. **a–d** Coronal sections through lobules IX–X in the tinamou and pigeon *(C. livia)*. Note the mostly uniform ZII+ labeling in lobules IXa and IXb in the tinamou (**a**, **b**), except in the rostral ventral lamella in IXb (**a**, **d**). The arrows in **a** and **b** indicate the small weakly ZII+ stripes in IXa, b. In pigeons, ZII+/– stripes are observed in IXab (**c**). Purkinje cells in lobule X are uniformly ZII+ in both tinamous (**b**) and pigeons (**c**). The remarkable similarity in the pattern of ZII stripes in IXcd in pigeons and tinamous is shown in **c–h** (see text for details). The arrowheads in **d**, **g**, and **h** indicate the 'notch' in the P2+ stripe that divides stripe 2 into medial and lateral halves. The thin band ZII+ stripe (?) between 1 and 2 was observed in all cases (**b**, **d**, **f**, **h**). **e** highlights stripes 6 and 7 in lateral regions of IXcd and in the auricle (Au) of the tinamou. Scale bars = 400 (**a–d**), 200 μm (**e**, **h**), and 100 μm (**f**, **g**).

Results

All 10 primary lobules were identified in the tinamou cerebellum and labeled according to Larsell [1967] (fig. 2a–c). The cerebellum was smaller in tinamous (196.04 mm^3) compared to pigeons (301.08 mm^3), and the cerebellar foliation index (a measure of the degree of folding) was similar (pigeon, 3.41; tinamou, 3.62) [see Iwaniuk et al., 2006, for a description of cerebellar foliation indices]. The anterior lobe (lobules I–V) in the tinamou appeared smaller and less foliated than in pigeons, especially lobules III and IV, which lacked the branching found in pigeons (fig. 2b, c). Lobule VI in the tinamou consisted of a single lobule, lacking the 3 secondary lobules that are present in pigeons. In contrast to the anterior lobe, the remaining posterior lobe appeared more foliated than in pigeons, with lobule VII having 2 secondary lobules. In addition, lobule IXab is clearly separated into IXa and IXb in the tinamou, unlike in the pigeon.

ZII labeling was present in dendritic arbors, somata, and axons of Purkinje cells in the tinamou cerebellum (fig. 2d, g). As in the pigeon, ZII immunoreactivity was heterogeneous with sagittally oriented ZII+ stripes alternating with ZII– stripes (fig. 2d, g). These alternating stripes of high and low immunoreactivity produced caudo-rostral stripes throughout the cerebellum. As illustrated in figure 2g–i, the presence of CB-labeled Purkinje cells where ZII reactivity was weak demonstrates that the ZII– stripes were not simply devoid of Purkinje cells. In some areas of the cerebellum, ZII+ stripes consisted of a single Purkinje cell (fig. 2g–i). The pattern of the ZII+ and ZII– stripes is described in more detail below with reference to photomicrographs (fig. 3, 4) and the unfolded reconstruction of the stripes (fig. 5).

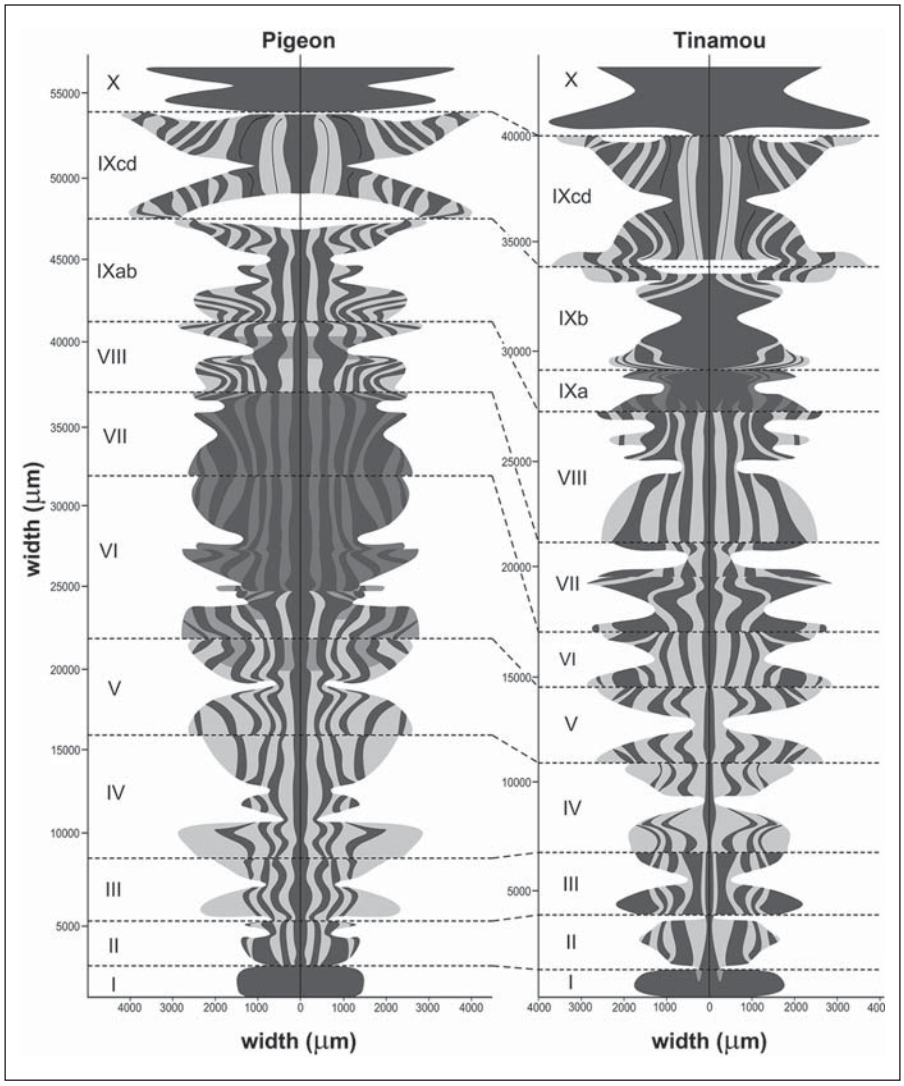

Fig. 5. Schematic of the ZII immunoreactivity throughout the entire cerebellum of the Chilean tinamou *(N. perdicaria)* and the pigeon *(C. livia)*. The schematic depicts the entire cerebellar cortex unfolded, showing the ZII+ (dark grey) and ZII– (light grey) parasagittal striped pattern. The intermediate grey color indicates stripes that were classified as weakly ZII+. In lobule IXcd, lines in stripe 1– and 2+ indicate the thin ZII+ band (?) and the ZII– notch, respectively. The plot is shown for one of the tinamou specimens but was virtually identical in both individuals.

Anterior Lobe

In the anterior lobe of the tinamou cerebellum, 4–5 ZII immunoreactive parasagittal stripes were seen spanning lobules II–V (fig. 3, 5). In lobule I (lingula), similar to what is seen in pigeons, all Purkinje cells were immunopositive (fig. 3a, b, 5), except in the rostral regions of lobule I in the tinamou, where 2 bands of weaker ZII immunoreactivity can be seen on either side of a ZII+ stripe on the midline (fig. 5). Figure 3a (tinamou) and b (pigeon) shows the ZII stripes in lobule II opposed to the uniform immunopositive labeling in lobule I. In lobules II and III, the ZII+ stripes were noticeably wider than in lobules IV and V. In lobule IV, some of the ZII+ stripes were reduced to a single Purkinje cell (fig. 2g). There are also two noteworthy differences between the pigeon and tinamou with respect to the anterior lobe. First, in lobule III the ZII+ stripes are thicker in the tinamou, and there is a thick P5+ stripe that is not apparent in the pigeon (fig. 5). Second, in pigeons, the stripes present in the dorsal lamella of lobule V are less distinct as the immunoreactivity in the ZII– stripes is quite high (fig. 5) [Pakan et al., 2007], whereas in the equivalent region in the tinamou clear ZII+ and ZII– stripes are present (fig. 5). In the tinamou, it was also not clear how the 4 ZII+ stripes in lobules II and IV lined up with the 5 ZII+ stripes in lobule III.

Posterior Lobe

In the pigeon, lobules VI and VII have a higher overall expression of ZII than the other lobules, with stripes only apparent in the molecular layer (fig. 3f, 5) [Pakan et al.,

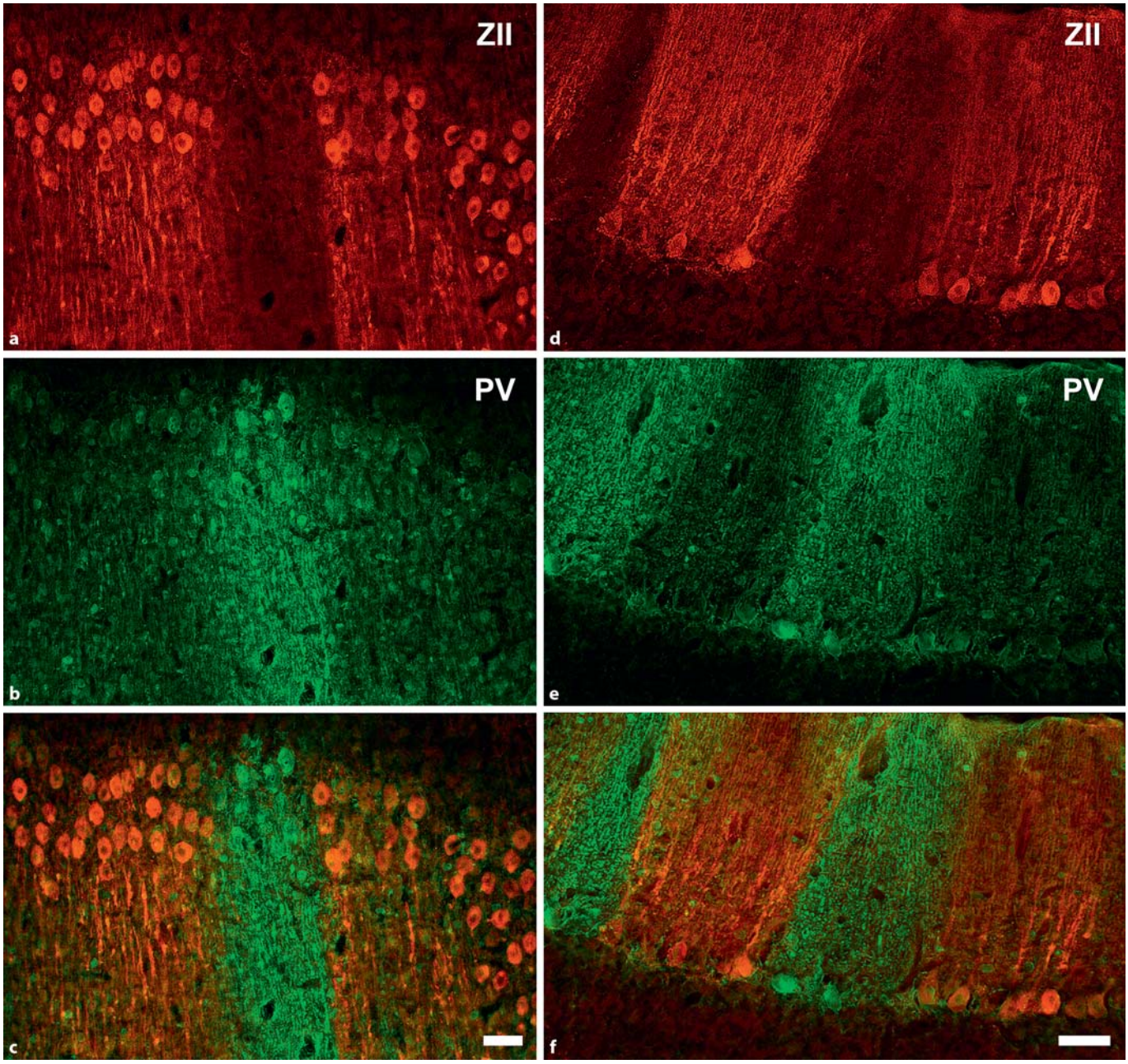

Fig. 6. ZII and PV expression in the Chilean tinamou *(N. perdicaria)* cerebellar cortex. **a**, **d** ZII expression. **b**, **e** PV expression. **c**, **f** Overlay of ZII and PV expression. **a–c** Labeling from medial VIII. **d–f** Labeling from lateral IXcd. ZII (red) and PV (green) show a complementary expression pattern in tinamous. Scale bars = 50 μm.

2007]. Because of this higher ZII expression, the contrast between the positive and negative stripes is lower than in other striped lobules. In the tinamou, lobules VI, VII, and VIII had 4–6 clear ZII+ stripes separated by ZII– stripes (fig. 3e, 5). The 6 ZII+ stripes in lobule VI of the tinamou cerebellum were all relatively thin, except for P5+, which was wider than the other stripes. Lobule VII was mostly ZII+, with 3 thick ZII+ stripes and a smaller P4+ stripe clearly separated by thinner ZII– stripes. Lobule VIII had 4 ZII+ stripes in the dorsal lamella and 6 stripes in the ventral lamella (fig. 5), whereas in pigeons there were 6 ZII+ stripes in both lamella (fig. 4c, 5).

There was also a clear difference between tinamous and pigeons with respect to the immunoreactivity in lobule IXa, b. In pigeons there are 4–6 clear ZII+/– stripe pairs (fig. 4c, 5). In tinamous, the overall expression of ZII is higher as most Purkinje cells are ZII+. In lobule IXa, stripes are only observable at the rostral extreme of the dorsal lamella, but the expression in the ZII– stripes is quite high and the contrast of the stripes is low. Otherwise lobule IXa is all ZII+ with only some very thin ZII– stripes laterally (fig. 4a, 5). Similarly, in lobule IXb most of the Purkinje cells are ZII+, but the rostral part of the ventral lamella has a series of ZII+/– stripes (fig. 4b, 5).

Vestibulocerebellum (Lobules IXcd and X)

Overall, the pattern of ZII expression in the vestibulocerebellum (VbC) is concordant between the pigeon and tinamou. In both species, lobule X (nodulus) is uniformly positive and there are 7 clear ZII+/– stripe pairs in lobule IXcd (fig. 4b–d, h, 5). The similarities in ZII expression even extended to minute details. For example, in pigeons, P2+ is considerably wider than the rest and contains a 'notch' that is devoid of Purkinje cells and effectively bisects the stripe into P2+ medial and P2+ lateral (fig. 4h). This was also observed in the tinamou (fig. 4d, g). Similarly, in pigeons the P1– stripe contains a satellite ZII+ stripe that is 1–2 Purkinje cells in width and bisects P1– into P1– medial and P1– lateral. Following Pakan et al. [2007], this is designated as '?' in figure 4h. This stripe was also present in the tinamou (fig. 4b, d, f). The P7+ stripe is quite thin and was only observed in a small area of the auricle in the ventral lamella of IXcd (fig. 4e).

PV Expression in the Tinamou Cerebellum

In addition to ZII and CB, we also processed a few sections of the tinamou cerebellum for PV, which is expressed in a complementary fashion to ZII in pigeons and other neognathou birds [PV+ stripes were ZII-ve and vice versa; Wylie et al., 2011]. In the tinamou, PV immunoreactivity was observed across the entire cerebellum and was mostly restricted to the Purkinje cell layer and molecular layer rather than in the granular layer (fig. 6). In pigeons it has been suggested that PV labeled the basket and stellate cells in the molecular layer [Wylie et al., 2011] and this also appears to be true for tinamous. Also similar to pigeons, PV immunoreactivity in the tinamou formed sagittal stripes of PV immunopositive Purkinje cells alternating with PV immunonegative Purkinje cells (fig. 6). Sections processed for both ZII and PV revealed a complementary expression of these two molecular markers (fig. 6).

Discussion

This is the first study to examine ZII expression in a paleognathous bird, the Chilean tinamou. The results demonstrate that ZII is expressed heterogeneously in Purkinje cells such that there are alternating sagittal ZII+ and ZII– stripes as has been observed in neognathous birds [Iwaniuk et al., 2009; Marzban et al., 2010; Pakan et al., 2007]. PV is also expressed heterogeneously by Purkinje cells, complementary to ZII expression, a pattern that is shared with neognathous birds [Wylie et al., 2011]. Together, this suggests that, despite an evolutionary gap of over 100 million years, pigeons and tinamous have retained many elements of the cerebellum parasagittal stripe organization that likely originated in a common ancestor. The similarity of the pattern of ZII expression was most striking in lobule IXcd of the VbC. In both tinamous and pigeons, 7 ZII +/– stripes were present [Pakan et al., 2007; Wylie, 2013]. Moreover, a thin ZII+ stripe (see '?' in fig. 4) that divides P1– into medial and lateral halves and also a thin ZII– notch (see the arrows in fig. 4) that divides the P2+ stripe into medial and lateral halves were present in both tinamous and pigeons. In pigeons, the ZII stripes within the VbC correspond to specific patterns of optic flow, such that each optic flow sagittal zone encompasses an adjacent ZII+/– stripe pair [Graham and Wylie, 2012; Pakan et al., 2010, 2011; Wylie, 2013]. The VbC is a site for the integration of vestibular [Wilson et al., 1974], optic flow [Wylie and Frost, 1991, 1993; Wylie et al., 1993], and cutaneous information [Schulte and Necker, 1998] and it is critical for mediating compensatory head, body, and eye movements to facilitate retinal image stabilization [Waespe and Henn, 1987]. Retinal image stabilization is necessary for optimal visual acuity [Westheimer and McKee, 1975] and velocity discrimination [Nakayama, 1981]. Given that this is critical for survival, and that circuitry is highly conserved [Voogd and Wylie, 2004], it is not surprising that ZII expression in the VbC is strikingly similar in paleognaths and neognaths. As discussed below, the differences in the pattern of ZII observed in other parts of the cerebellum may reflect differences in behavior.

Locomotion Mode May Be Influencing ZII Expression Patterns

Recent studies suggest that the ancestor of paleognathous birds could fly and that there have been at least 3 relatively recent losses of flight within this group [Hackett et al., 2008; Harshman et al., 2008; Phillips et al.,

2010]. Because the cerebellum plays a crucial sensorimotor role in coordinated movements, including control of flight [Feenders et al., 2008; Larsell, 1948, 1967], some of the difference in ZII expression present in tinamous could also be a result of reduced forelimb innervation and increased hind limb innervation. Although tinamous are the only extant paleognaths capable of flight, they do spend most of the time on the ground and only fly in short bursts [Cabot, 1992; Davies, 2002]. In birds, the majority of nerve fibers from the wing (cervical enlargement; CE) and leg (lumbosacral enlargements; LSE) project to the anterior cerebellum and to lobule IX [Furue et al., 2010; Necker, 1992; Okado et al., 1987; Schulte and Necker, 1998]. There is no clear separation of the representation of wing and leg in the anterior cerebellum, although terminals arising from the LSE were primarily concentrated in lobules II–IV, with CE terminals extending into lobule V [Furue et al., 2010]. In hummingbirds, it has been hypothesized that their poor hind limb musculature has resulted in an extreme reduction of the anterior lobe [Iwaniuk et al., 2006, 2007, 2009; Larsell, 1967]. This size reduction was most noticeable in lobules II and III, which also had ZII+ stripes that were narrower than those of pigeons and in most cases only 1–2 Purkinje cells wide [Iwaniuk et al., 2009]. In addition, Iwaniuk et al. [2009] described ZII+ stripes in lobules IV and V that are broader than those in lobules II and III. This expression pattern is the opposite of that observed in the tinamou, where large ZII+ stripes are present in lobule III and small stripes are found in lobule IV.

The contrasting expression patterns between the tinamou and hummingbirds provides some convincing evidence that locomotion mode may be influencing the functional organization of the anterior cerebellum in birds, but the implications of varying ZII+ widths are unknown. Further, complicating the interpretation of these species differences in ZII+ stripe sizes are the expression patterns in lobule IXab and ZII expression in the chicken. IXab receives projections predominately from the wing [Furue et al., 2010; Schulte and Necker, 1998] but is largely ZII+ in the tinamou. If the variation in ZII expression arises from having weak hind limb (hummingbird) or forelimb (tinamou) musculature, then the expression of ZII in IXab contradicts this functional link. Tinamous also exhibit several behavioral similarities with chickens and other gallinceous birds, such as being primarily terrestrial and only flying in short bursts, but the ZII expression pattern in chickens more closely resembles that of the pigeon [Marzban et al., 2010; Pakan et al., 2007]. Interpreting anything specifically functional about these species differences in ZII expression is therefore difficult at this stage. Examining ZII expression across a broader range of species, including flightless ratites and neognathous birds, might aid in resolving these issues.

A Novel ZII Expression Pattern in the Central Transverse Region in the Tinamou

In pigeons [Pakan et al., 2007], chickens [Marzban et al., 2010], and hummingbirds [Iwaniuk et al., 2009], ZII is expressed strongly in lobules VI and VII such that the contrast between the ZII– and ZII+ cells is rather low and clear stripes are difficult to discern. In mammals, too, a central region is characterized by uniform ZII+ Purkinje cells, except that perhaps there are thin ZII– stripes through this region in primates *(Macaca mulatta)* [Sillitoe et al., 2004]. Therefore, a uniformly ZII+ central region appears to be a feature present throughout birds and mammals, that is except for the tinamou, where clear stripes are present in lobules VI and VII. Interestingly, in lobule IXa and most of IXb in tinamous the opposite pattern is observed; most Purkinje cells are ZII+ and stripes are absent or of very low contrast, whereas in neognaths there are clear ZII+ and ZII– stripes in IXab. Thus, it is as if this central region of ZII+ cells has moved caudally in the tinamou relative to the pigeon cerebellum (fig. 5). If this central region has shifted caudally in the tinamou, one possibility is that the stripy anterior transverse region has expanded into lobules VI, VII, and VIII; the central region occupies lobule IXa and the dorsal lamella of IXb, and the posterior region has been reduced to IXcd and the rostral part of the dorsal lamella of IXb. In most mammals, the expression boundaries between transverse regions are tied to the pattern of lobulation, but this is not always the case, and transverse expression boundaries and fissures do not necessarily coincide in all species [Kim et al., 2009; Ozol et al., 1999; Sillitoe et al., 2003]. For example, in the Madagascan hedgehog tenrec *(Echinops telfairi),* an afrotherian species, the cerebellum has only 5 lobules, yet it still has the ZII transverse region and parasagittal stripe expression pattern that is typical of other mammals [Sillitoe et al., 2003]. Sillitoe et al. [2003] concluded that transverse boundaries between expression domains are not necessarily dependent on lobules and fissures. The tinamou could represent an avian example in which ZII boundaries and lobules are disjunct compared to other birds.

It is intriguing to speculate as to why the boundary of the anterior transverse regions may have shifted in tina-

mous; is this due to an expansion of the anterior region or a reduction of the posterior and central regions? Birds considered 'strong fliers', such as waterfowl (Anseriformes) and seabirds (Procellariiformes), have expanded lobules VI and VII [Iwaniuk et al., 2007]. This is also true in the bat cerebellum, where the large central region has been divided into anterior and posterior components, but this could be to service motor and cognitive aspects of echolocation [Kim et al., 2009]. In the tinamou it is possible, therefore, that flightlessness has led to a reduced central region and resulted in a shift of the anterior transverse region boundary. Because projections from the wing and legs are not separated in the anterior cerebellum, it is hard to determine if the anterior cerebellum has expanded in the tinamou, although there is some evidence correlating strong hind limbs with an enlargement of the anterior lobe in birds [Iwaniuk et al., 2007].

What Is the Basal Cerebellum Condition?

Although some aspects of the unique ZII expression pattern in the tinamou might reflect behavior, the question of whether the tinamou or neognath pattern of ZII expression is ancestral remains. Based on the data presented herein, ZII stripes likely arose early in avian evolution and many components of this expression pattern are highly conserved. Thus, the ancestral bird likely had the same number and pattern of ZII stripes across most of the anterior and central regions and in the vestibulocerebellum. Whether the ancestral bird had clear ZII stripes in IXab, the degree of ZII expression in the central region (lobules VI and VII), and the number and continuity of ZII stripes in lobule IV are, however, equivocal. As discussed above, this could reflect behavior or the ancestral state of all birds. To resolve this quandary of how ZII was expressed in the ancestral bird, it will be necessary to examine ZII expression in other paleognaths as well as other diapsids, especially crocodilians. In doing so, we can start to unravel the origins of cerebellar organization and its relationships with cerebellar morphology and behavior.

Acknowledgments

Funding for this study was provided by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant (372237) and Accelerator Supplement (380284-2009) to A.N.I., NSERC grants (446013) to D.R.W., and a National Fund for Scientific and Technological Research grant (FONDECYT, project No. 1110281) to G.J.M.

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