Distribution of Zebrin-Immunoreactive Purkinje Cell Terminals in the Cerebellar and Vestibular Nuclei of Birds

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ABSTRACT

Zebrin II (aldolase C) is expressed in a subset of Purkinje cells in the mammalian and avian cerebella such that there is a characteristic parasagittal organization of zebrin-immunopositive stripes alternating with zebrin-immunonegative stripes. Zebrin is expressed not only in the soma and dendrites of Purkinje cells but also in their axonal terminals. Here we describe the distribution of zebrin immunoreactivity in both the vestibular and the cerebellar nuclei of pigeons (*Columba livia*) and hummingbirds (*Calypte anna*, *Selasphorus rufus*). In the medial cerebellar nucleus, zebrin-positive labeling was particularly heavy in the "shell," whereas the "core" was zebrin negative. In the lateral cerebellar nucleus, labeling was not as heavy, but a positive shell and negative core were also observed. In the vestibular nuclear complex, zebrin-positive terminal labeling was heavy in the dorsolateral vestibular nucleus and the lateral margin of the superior vestibular nucleus. The central and medial regions of the superior nucleus were generally zebrin negative. Labeling was moderate to heavy in the medial vestibular nucleus, particulary the rostral half of the parvocellular subnucleus. A moderate amount of zebrin-positive labeling was present in the descending vestibular nucleus: this was heaviest laterally, and the central region was generally zebrin negative. Zebrin-positive terminals were also observed in the the cerebellovestibular process, prepositus hypoglossi, and lateral tangential nucleus. We discuss our findings in light of similar studies in rats and with respect to the corticonuclear projections to the cerebellar nuclei and the functional connections of the vestibulocerebellum with the vestibular nuclei. J. Comp. Neurol. 520:1532–1546, 2012.

INDEXING TERMS: aldolase C; cerebellum; pigeon; hummingbird

The cerebellar cortex is organized into "zones" that lie in the sagittal plane, perpendicular to the transverse lobules (see, e.g., Voogd and Bigaré, 1980). These sagittal zones are apparent in the distribution of climbing fiber and mossy fiber afferents, Purkinje cell (PC) projection patterns, and PC response properties (Voogd, 1967; Voogd et al., 1969; Ekerot and Larson, 1973; Andersson and Oscarsson, 1978; Llinas and Sasaki, 1989; De Zeeuw et al., 1994; Voogd and Glickstein, 1998; Wu et al., 1999; Ruigrok, 2003; Apps and Garwicz, 2005). Numerous molecular markers also show a parasagittal distribution in the cerebellum (for review see Hawkes and Gravel, 1991; Herrup and Kuemerle, 1997). The enzyme zebrin II (ZII; a.k.a. aldolase C; Brochu et al., 1990; Ahn et al., 1994; Hawkes and Herrup, 1995) is the most thoroughly studied in this regard. ZII is expressed heterogeneously by PCs such that ZII-immunopositive (ZII^+) PCs are distributed as a parasagittal array of stripes, separated by ZII-immunonegative (ZII^-) stripes (see Fig. 1C–E; see also, e.g., Sillitoe et al., 2004; Larouche and Hawkes, 2006). The pattern of ZII heterogeneity is remarkably similar in birds and mammals (Pakan et al., 2007; Iwaniuk et al., 2009; Marzban et al., 2010). Several studies have demonstrated that the ZII stripes are related to the sagittal distribution of climbing fiber and mossy fiber inputs and physiological

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response properties of PCs (Gravel and Hawkes, 1990; Matsushita et al., 1991; Hawkes and Gravel, 1991; Akintunde and Eisenman, 1994; Chockkan and Hawkes, 1994; Ji and Hawkes, 1994; Nagao et al., 1997; Voogd et al., 2003; Sugihara et al., 2004; Voogd and Ruigrok, 2004; Wadiche and Jahr, 2005; Gao et al., 2006; Pijpers et al., 2006; Sugihara and Shinoda, 2007; Sugihara and Quy, 2007; Pakan and Wylie, 2008; Ruigrok et al., 2008; Mostofi et al., 2010; Pakan et al., 2010, 2011; Paukert et al., 2010; Graham et al., 2011).

Although most of the emphasis in these studies has been on ZII expression in the cerebellar cortex, ZII immunoreactivity also occurs in the PC terminal fields. Sugihara and Shinoda (2007) described the distribution of ZII immunoreactivety in the cerebellar and vestibular nuclei in rats (*Rattus norvegicus*), noting that some areas were rich in ZII^+ terminals, whereas other areas were devoid of ZII^+ terminals. For example, the fastigial nucleus was clearly demarcated into a ZII^- rostrodorsal area and a ZII^+ caudoventral area. Thus, the functional compartmentalization of PCs defined by ZII immunoreactivity is not only apparent in the cerebellar cortex but also extends to their efferent targets. This study examines the distribution of ZII immunreactivity in the cerebellar and vestibular nuclei in birds. Because there is a remarkable degree of similarity between mammals and birds (Pakan et al., 2007; Marzban et al., 2010) with respect to the the location and pattern of ZII^+ and ZII^- PCs in the cerebellar cortex, we expected to observe similarities in ZII immunoreactivity at the level of the cerebellar and vestibular nuclei. In addition, whereas Sugihara and Shinoda (2007) concentrated on the cerebellar nuclei, we were able to examine the vestibular nuclei in great detail, because the reponse properties and projections of the PCs in the zones in the vestibulocerebellum (VbC) are well characterized (see, e.g., Wylie and Frost, 1991, 1993, 1999; Wylie et al., 1993, 1998, 2003a, b; Winship and Wylie, 2003; Pakan and Wylie, 2008; Pakan et al., 2010, 2011; Graham et al., 2011).

Abbreviations

Au	auricle
CbL	lateral cerebellar nucleus
CbM	medial cerebellar nucleus
ic	intercalated subnucleus
im	intermediate subnucleus
in	internal subnucleus
vm	ventromedial subnucleus
Inf	infracerebellar nucleus
La	nucleus laminaris
mc	nucleus magnocellularis
PC	Purkinje cell
pcv	cerebellovestibular process
ph	prepositus hypoglossi
rHA	rotation about horizontal axis
rVA	rotation about vertical axis
Ta	tangential nucleus
VbC	vestibulocerebellum
VDL	dorsolateral vestibular nucleus
VeD	descending vestibular nucleus
VeLd	lateral vestibular nucleus, pars dorsalis
VeM (mc/pc)	medial vestibular nucleus (magnocellularis/parvocellularis)
VeS	superior vestibular nucleus

MATERIALS AND METHODS

The procedures describing the use of animals for experimental purposes conformed to the guidelines established by the Canadian Council for Animal Care and were approved by the BioSciences Animal Care and Use Committee at the University of Alberta. We chose to use both pigeons and hummingbirds in this study for several reasons. The patterns of ZII immunoreactivity in PC somata have been described for both (Pakan et al. 2007; Iwaniuk et al., 2009). The pigeon has a "generic" cerebellum (Fig. 1B), but the hummingbird is somewhat unique in that is has a substantial reduction with respect to the size of the folia in the anterior lobe (Fig. 1A; Iwaniuk et al. 2006). Nevertheless, the distribution of ZII stripes in the cerebellar cortex is highly similar (Fig. 1C–E; Pakan et al. 2007; Iwaniuk et al., 2009). Moreover, hummingbirds and pigeons are distantly related to one another (see, e.g., Sibley and Ahlquist, 1990; Hackett et al., 2008), so we can (cautiously) apply our results to Aves in general. Six adult pigeons (*Columba livia*) obtained from a local supplier were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with phosphate-buffered saline (PBS; 0.9%, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PFA; pH 7.4). The brains were then removed and postfixed by immersion in the same fixative for several days. The immersion-fixed (also in 4% PFA) brains from hummingbirds (*Calypte anna* and *Selasphorus rufus*; two each) were kindly provided to us by Drs. Kenneth Welch Jr. and Raul Suarez (University of California, Santa Barbara) and stored in 4% PFA for several weeks.

Immunohistochemistry

Prior to sectioning, the brains were cryoprotected in sucrose (30% in PBS, pH 7.4), embedded in gelatin, and sectioned on a freezing stage microtome in the coronal plane at a thickness of 40 μm. Serial sections through the cerebellum, cerebellar nuclei, and vestibular nuclei were collected into 0.1 M PBS. Free-floating sections were washed several times in PBS and blocked with 10% normal donkey serum (in PBS; Jackson Immunoresearch, West Grove, PA) and 0.4% Triton X-100 in PBS for 1–2 hours at room temperature and then incubated in the primary antibody (1:200–400) for 48–72 hours at room temperature. Antizebrin II is a monoclonal antibody grown in mouse, produced by immunization with a crude cerebellar homogenate from the weakly electric fish

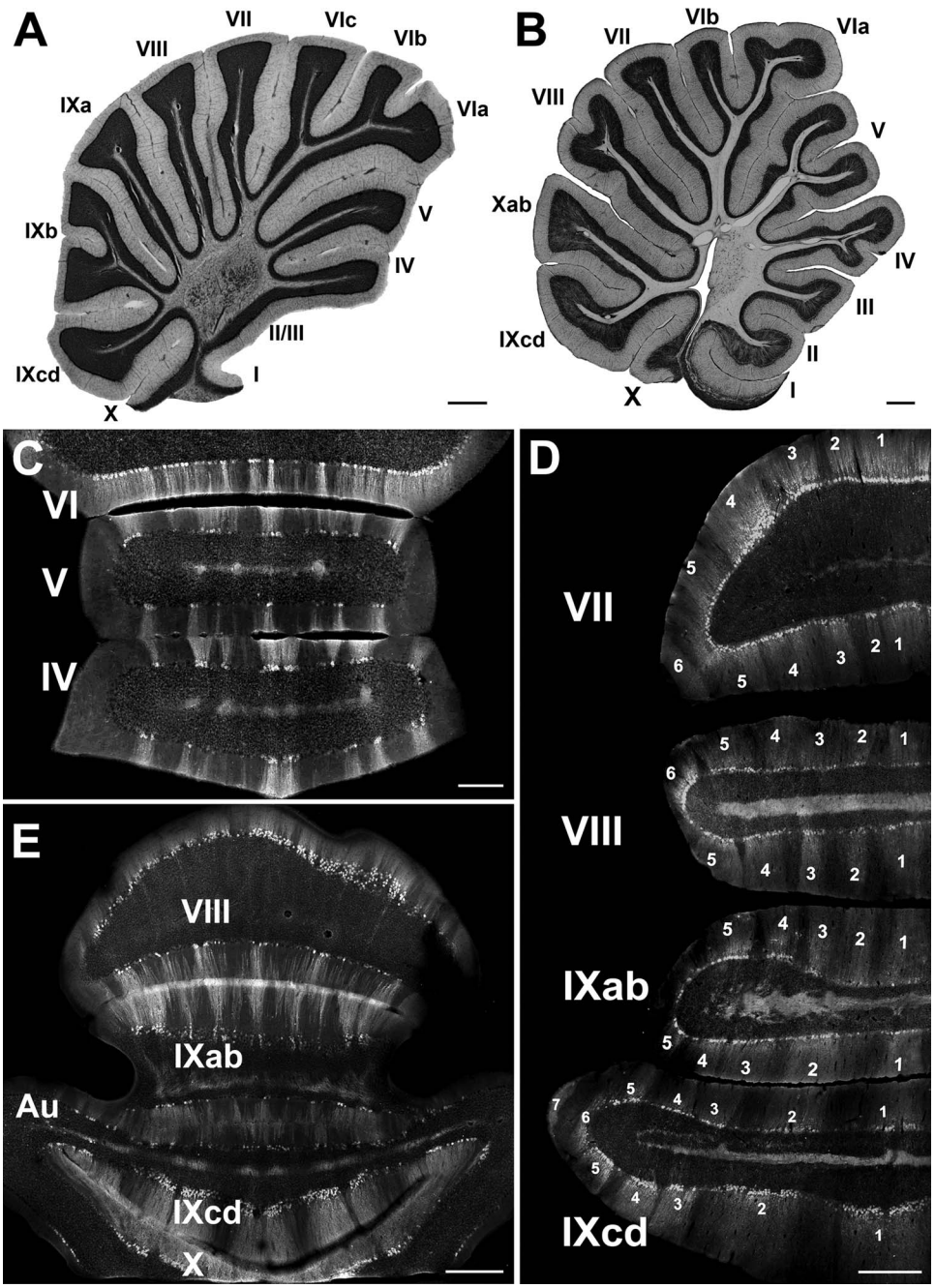

Figure 1. Photomicrographs of midsagittal sections through the cerebella of a hummingbird (**A**) and a pigeon (**B**). Coronal sections through the cerebella of hummingbirds (**C,E**) and pigeon (**D**) immunoreacted for ZII. These sections show the pattern of the sagittally oriented ZII stripes in the anterior (C) and posterior lobes (D,E). The Roman numerals indicate the folia (I–X). The numbers in E (i.e. 1–6) indicate the ZII^{+} stripes. Au, auricle. Scale bars = 800 μm in A,B; 300 μm in C; 400 μm in D; 500 μm in E.

Apteronotus (Brochu et al., 1990), and 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). Antizebrin II Western blot analysis of homogenate of pigeon cerebellum also detects a single immunoreactive polypeptide band, identical in size to the band detected in extracts from the adult mouse cerebellum (Pakan et al., 2007). It was used directly from spent hybridoma culture medium. The sections were then rinsed several times in PBS and incubated in Cy3-conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch; 1:200 in PBS, 2.5% donkey serum and 0.4% Triton X-100) for 2–3 hours at room temperature. After several rinses in PBS, the sections were then mounted onto gelatinized slides.

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

For a few sections, after images of the distribution of ZII immunoreactivity had been obtained, the sections were stained for Nissl with neutral red. This was done to ensure that we could accurately localize the boundaries of the cerebellar and vestibular nuclei and thus describe the distribution of ZII immunoreactivity in these areas (see Fig. 3).

Nomenclature

Compared with mammals, the avian cerebellum is best described as a vermis without hemispheres (Larsell, 1967), although there is evidence that there are small, rudimentary hemispheres (Pakan et al., 2007). Like the mammalian vermis, the avian vermis is divided into 10 lobules that are more often referred to as "folia" in birds and labeled using Roman numerals I–X (anterior to posterior; Fig. 1A,B). Also as in mammals, the cerebellar cortex is divided into an anterior lobe (folia I–V), a posterior lobe (VI–IX), and the nodulus (X). The hummingbird cerebellum is somewhat unique among birds because folia II and III are quite reduced (Fig. 1A; Iwaniuk et al., 2006). The VbC includes folia IXcd and X. We consider the lateral half of the VbC to be the flocculus and the medial half to be the the uvula/nodulus based on functional properties (Voogd and Wylie, 2004; Pakan and Wylie, 2008; Pakan et al., 2008, 2010, 2011). The flocculus also includes the auricle (Au), which is the lateral protrusion of IXcd, and X (Fig. 1D). The striking similarity between birds and mammals with respect to the functional properties and anatomical connections of the flocculus is discussed by Voogd and Wylie (2004). Possible homologies between other parts of the cerebellar cortex have been discussed by Pakan et al. (2007) and Larsell (1967).

For the nomenclature of the cerebellar nuclei, we relied on Arends and Zeigler (1991a,b) in addition to Karten and Hodos (1967). The cerellar nuclei consist of the medial and lateral nuclei (CbM, CbL), which are purportedly homologous to the fastigial and interposed nuclei in mammals. CbM is further subdivided into the ventromedial (vm), internal (in), intermediate (im), and intercalated (ic) subnuclei (Arends and Zeigler, 1991a,b). Arrends and Zeigler (1991a,b) described a small third cerebellar nucleus, the infracerebellar nucleus (Inf), consisting of a small leaflet of cells laterally and considered this to be homolgous to part of the dentate nucleus. The Inf lies dorsal to the dorsolateral vestibular nucleus (VDL), but its specific borders are difficult to delineate.

For the vestibular nuclei, we generally used the nomenclature of Karten and Hodos (1967), with a few exceptions. The vestibular nuclear complex consists of the medial, superior, descending, and lateral vestibular nuclei (VeM, VeS, VeD, VeL) as well as the VDL. VeL is divided into pars dorsalis (VeLd), a clear group of very large cells, and pars ventralis (VeLv). Dickman and Fang (1996) considered the VDL to be the dorsal extension of the VeLv, but VDL has been compared with the mammalian "group y" based on its oculomotor connections (Arends et al., 1991; Wylie et al., 2003a). Most of the VeM consists of parvocellular neurons (VeMpc) and lies dorsal and medial to the stria acoustica, but part of the VeM lies ventral to this fiber bundle. Although traditionally considered part of the VeLv, following the mammalian literature, we refer to this region as the *magnocellular VeM* (VeMmc; Epema et al., 1988). Dickman and Fang (1996) also identified groups A and B in pigeons, based on earlier studies in chickens (*Gallus domesticus*, Wold, 1976). In our material, we could not reliably identify these groups A and B. Therefore, following Diaz et al. (2003), we included groups A and B with the VeS and tangential nucleus (Ta), respectively. The Ta is a collection of large neurons that lies medial to the root of the vestibular nerve. The indistinct regions between the CbM, CbL, and the vestibular complex are collectively referred to as the *cerebellovestibular process* (pcv).

RESULTS

In the cerebellum, ZII is expressed in PCs. Although it is most clearly seen in the somata and proximal dendrites of PCs, it is also evident throughout the axons and their terminals fields. Consistent with our previous studies (Pakan et al., 2007; Iwaniuk et al., 2009), we found a heterogenous distribution of ZII in the cerebellar cortex. Figures 1 and 2 show comparisons of ZII labeling in the hummingbird (left) and the pigeon (right). There are clear parasagittal bands of alternating ZII^{+} and ZII^{-} PCs in the cerebellar cortex of the hummingbirds (Figs. 1C,E, 2C) and pigeon (Figs. 1D, 2D). Following convention, the most medial ZII stripe pair, which is on the midline, is designated $P1^{+}/P1^{-}$ (Brochu et al., 1990). The pattern of the ZII stripes is virtually identical in pigeons and hummingbirds and has been described in detail by Pakan et al. (2007) and Iwaniuk et al. (2009), respectively. Briefly, there are four ZII^{+} stripe pairs ($P1^{+/-}$ to $P4^{+/-}$; Fig. 1C) in folia II–V of the anterior lobe and up to seven stripes in folia VIII and IX of the posterior lobe (Fig. 1D,E). In folia VI and VII most of the PCs are ZII^{+}, and, rather than clear $ZII^{+/-}$ stripes, there appear to be more ZII^{+} stripes separated by few PCs that do not show as much ZII immunoreactivity (Fig. 1C,E). Finally, both folia I and X are uniformly ZII^{+}.

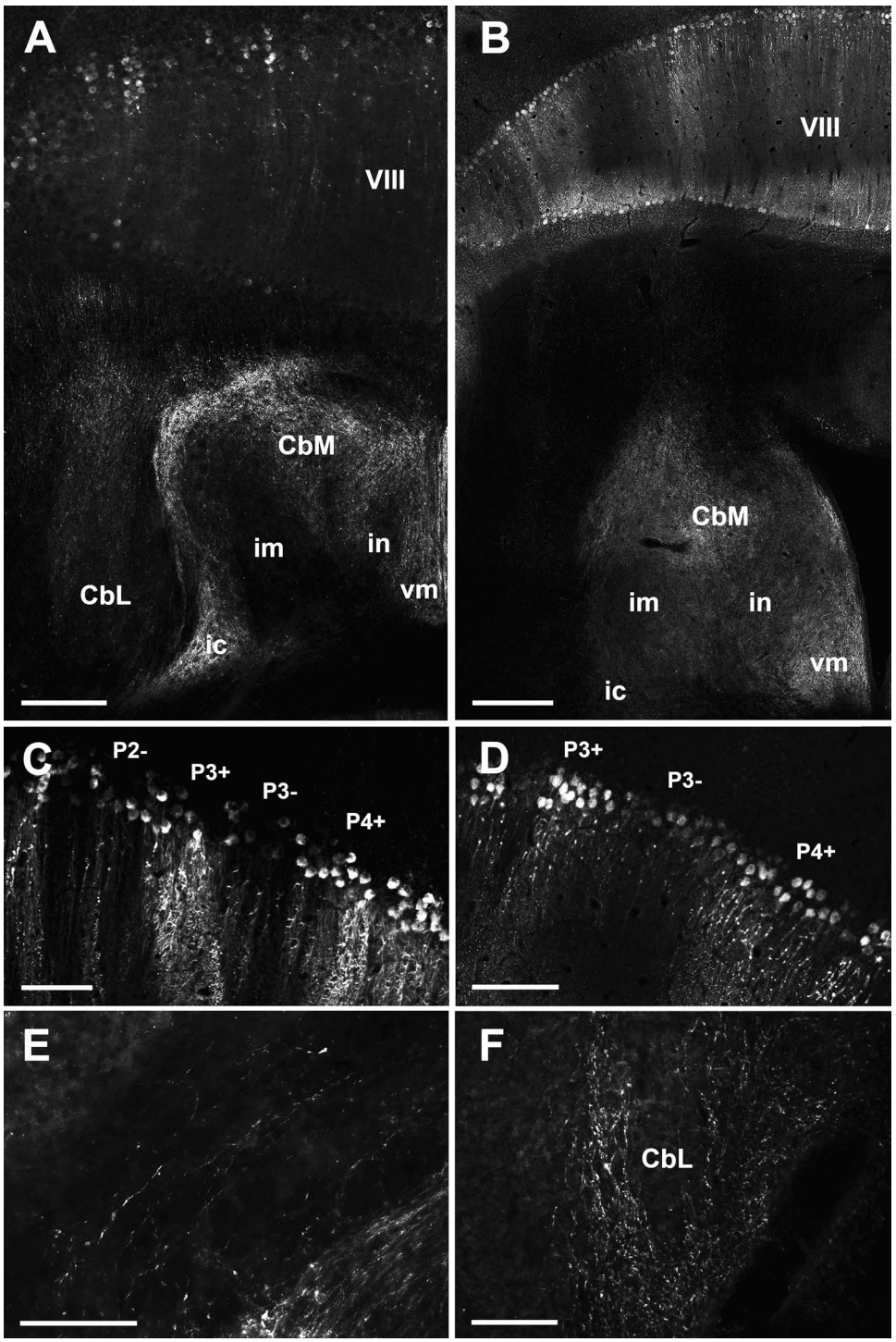

Figure 2. Zebrin II labeling is shown in photomicrographs of coronal sections through the cerebella of hummingbird (**A,C,E**) and pigeon (**B,D,F**). A and B are low-power magnifications to show labeling of Purkinje cells (in folium VIII) and terminal labeling in the subregions of the medial cerebellar nucleus (CbM). C and D are higher magnification photomicrographs of labeled PCs in the posterior lobe, whereas E and F are higher magnification photomicrographs ZII^{+} terminal labeling. The terminal labeling shown in E was in the cerebellovestibular process (pcv). The left side of each panel is lateral. For abbreviations see list. Scale bars = 600 μm in A,B; 100 μm in C,E,F; 200 μm in D.

ZII immunoreactivity was also apparent in the terminals of PC axons in the cerebellar and vestibular nuclei (Fig. 2A,B,E,F). This terminal labeling was much more intense in the hummingbird sections, where it appeared as strong as that in the PC somata (Figs. 2A, 3). In pigeons, the ZII^{+} terminal labeling was not as intense as that in the PC somata (Figs. 2B, 4). With respect to the distribution of ZII^{+} terminal labeling, however, there were few appreciable

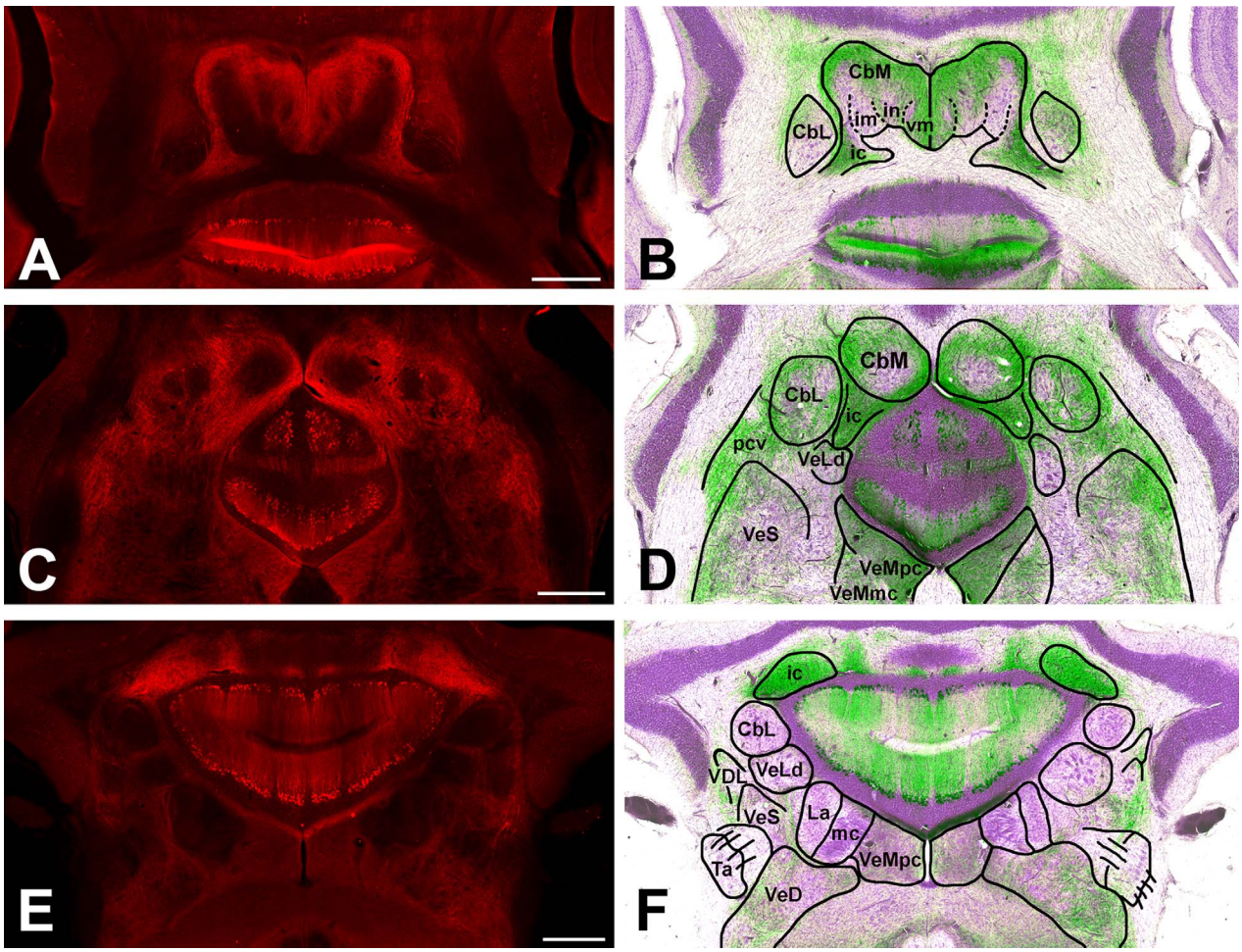

Figure 3. Zebrin II terminal labeling in the cerebellar and vestibular nuclei of hummingbird. Three coronal sections (rostral [top row] to caudal [bottom row]) are shown to provide a general overview of the overall distribution of ZII terminal labeling. ZII terminal labeling is shown at left (**A,C, E**). The same sections subsequently stained for Nissl are shown at right (**B,D,F**), and the ZII labeling, pseudocoloured green, is superimposed. Scale bars = 400 μm.

differences between the hummingbirds and the pigeons. An overview of the ZII$^+$ terminal labeling is shown for the hummingbird in Figure 3. At three different rostrocaudal levels, the terminal labeling is shown throughout the vestibular and cerebellar nuclei. On the left, the ZII$^+$ terminal labeling is shown (red). On the right, the same sections subsequently stained for Nissl are shown, with lines demarcating the borders of the nuclei. The ZII$^+$ labeling pseudocoloured as green is superimposed. Below, the ZII$^+$ terminal labeling is described in detail for each subnucleus for both pigeons and hummingbirds.

Cerebellar nuclei and cerebellovestibular process (pcv)

ZII$^+$ terminal labeling was most striking in the hummingbird CbM, particularly in the vm and ic subnuclei (Figs. 2A, 3A–F). Although not as intense, the same pattern was consistently observed in the pigeon CbM (Figs. 2B, 4A-C). Generally speaking, the ZII$^+$ terminal labeling was highly concentrated in the boundaries of CbM, such that it appeared as a ZII$^+$ shell with ZII$^-$ core (Figs. 2A,B, 3A–D, 4A,B). When stained for Nissl, it was clear that the ZII$^-$ core region of the CbM consisted of larger cells than the ZII$^+$ shell (Fig. 3B,D). In both the pigeon and the hummingbird, there was heavy ZII$^+$ labeling in the dorsal aspect of the CbM complex, and it was also heavy in the vm but most intense in the ic subnucleus (Figs. 3A–F, 4A–C). The ic contains densely packed, smaller cells, and ZII$^+$ labeling was very heavy throughout the rostrocaudal extent of ic (Fig. 3).

Labeling was also heavy in the CbL, but not as intense as in the CbM. In hummingbirds, ZII$^+$ labeling in the rostral CbL was confined to the rostrolateral region. At the middle of the rostrocaudal range of CbL (Fig. 3A,B), labeling was heavier in the medial, dorsal, and ventrolateral regions such that the pattern of a ZII$^+$ shell$^-$ core emerged, similar to what was seen for CbM (Fig. 3C,D). Again, the Nissl

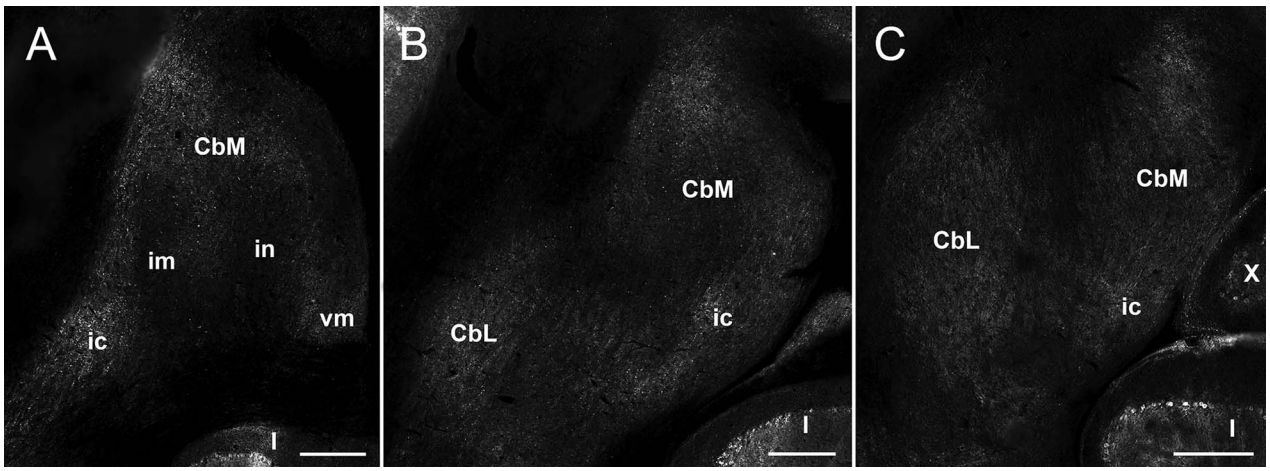

Figure 4. Zebrin II terminal labeling in the cerebellar nuclei of pigeon. Three coronal sections, rostral to caudal (**A–C**), are shown. The left side of each panel is lateral. Scale bars = 400 μm.

staining suggested that larger cells were found in this ZII$^-$ core region (Fig. 3D). At the most caudal extreme of the hummingbird CbL, labeling was absent (Fig. 3E,F). In pigeon, this pattern of labeling in CbL was not as apparent. Rather, the CbL appeared uniformly covered by ZII$^+$ terminals in the middle of the rostrocaudal range (Fig. 4B,C). ZII$^+$ labeling was also seen in the pcv surrounding the CbL in both pigeons and hummingbirds. Clearly, this was more intense laterally and ventrolaterally (Figs. 3C,D, 5A,D,F).

Vestibular nuclei complex

Some ZII$^+$ terminals were present in all of the subnuclei of the vestibular nuclei complex. As shown in Figure 3, most of this labeling was found laterally, the exception being the moderate amount of labeling in the rostral VeM.

Superior vestibular nucleus

There was a strip of heavy labeling in the lateral VeS in the hummingbird (Figs. 3C,D, 5B) and pigeon (Fig. 5D,G) that is continuous with the ZII$^+$ labeling in the lateral margin of the pcv. The heavy labeling within this lateral strip was in stark contrast to the central and medial parts, where labeling was sparse (central) or absent (medial).

Dorsolateral vestibular ucleus

Moderate to heavy terminal ZII$^+$ labeling was seen in the VDL in both hummingbirds (Figs. 3E,F, 5A) and pigeons (Fig. 5C,E). In hummingbirds the labeling was heaviest in the lateral half, and in pigeons most of the labeling was confined to the ventrolateral margin. When clearly visible in the sections, the Inf was similarly labeled (Fig. 5A).

Tangential nucleus

At first glance, the ZII$^+$ terminal labeling appeared rather sparse in the hummingbird Ta (Fig. 3E,F). However, in the pigeon sections, ZII$^+$ terminals could clearly be seen among the large cells in the lateral Ta (Fig. 6F). These terminal fields were rather dense and appeared to encapsulate the large cells in the lateral Ta (Fig. 6D). Upon closer examination, these terminals could also be seen in the hummingbird Ta but were not as striking (Fig. 6A,C).

Medial vestibular nucleus

There was a substantial amount of ZII$^+$ terminal labeling in the rostral half of the VeMpc (Figs. 3C,D, 7A,C), although this was not as striking in pigeons (Figs. 5D, 6F-H, 7B,D). A moderate amount of ZII$^+$ labeling was also seen in the VeMmc (Figs. 3C,D, 6E–H, 7A,D) and in the prepostitus hypoglossi (ph; Fig. 7E). In contrast, ZII$^+$ labeling in the caudal VeMpc was sparse (Fig. 3E,F).

Descending vestibular nucleus

A moderate amount of ZII$^+$ terminal labeling occurred in the VeD, where the emphasis was in the lateral margin, at levels where VeD is caudal to Ta (Figs. 3E,F, 6A,B). However, some was also observed dorso- and ventromedially, leaving a central region with sparse ZII immunoreactivity (Figs. 3E,F, 6E–H).

Lateral vestibular nucleus

Whereas the VeLd is easily identifiable based on the large cells of Dieter's, VeLv can be difficult to distinguish from the ventral parts of VeS. In the VeLd, ZII$^+$ labeling was sparse (Figs. 3C–F, 5A,C).

DISCUSSION

ZII is expressed heterogeneously in the mammalian cerebellar cortex as a series of parasagittal bands or stripes of PCs that express ZII strongly, alternating with

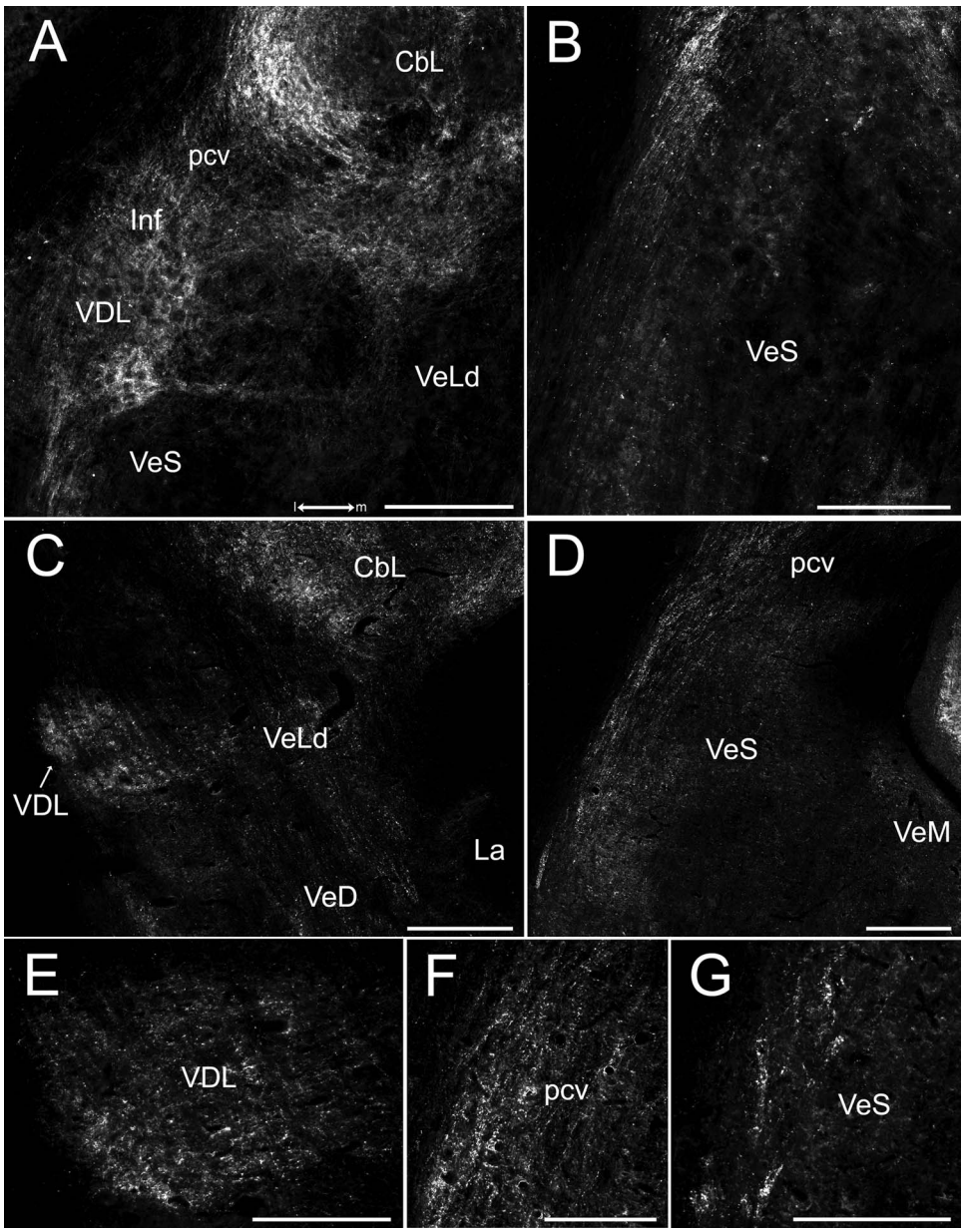

Figure 5. Zebrin II terminal labeling in the rostral vestibular nuclei. Photomicrographs of coronal sections from both hummingbird (**A,B**) and pigeon (**C–G**) are shown to illustrate ZII terminal labeling in the superior vestibular nucleus (VeS) and dorsolateral vestibular nucleus (VDL). Labeling in the cerebellovestibular process (pcv), infracerebellar nucleus (Inf), and ventrolateral margin of the lateral cerebellar nucleus (CbL) is shown. The left side of each panel is lateral. Scale bars = 200 μm in A,B,E–G; 400 μm in C,D.

stripes containing PCs that express ZII weakly or not at all (Brochu et al., 1990). This banding is seen with respect to the expression of both mRNA and protein (Rivkin and Herrup, 2003). Recent studies have shown that there is a highly similar pattern of expression in avian species, and the overall pattern is largely conserved across species (Pakan et al., 2007; Iwaniuk et al., 2009; Marzban et al., 2010). In rats, PCs are initially all ZII^{+}, but at postnatal days 8–10 a transition occurs, and the ZII^{+} and ZII^{-} stripes emerge in adulthood (Leclerc et al., 1988). In mice, the stripes are apparent at birth (Rivkin and Herrup, 2003). It is not known when the stripes develop in birds.

In this study, we have shown that there is a heterogeneous distribution of ZII^{+} terminal labeling in the vestibular and cerebellar nuclei in birds. Our data suggest that PCs in the ZII^{+} and ZII^{-} stripes project to different regions within the cerebellar and vestibular nuclei. However, we did not directly identify the terminals of ZII^{-} PCs. We used only an antibody to ZII and examined the

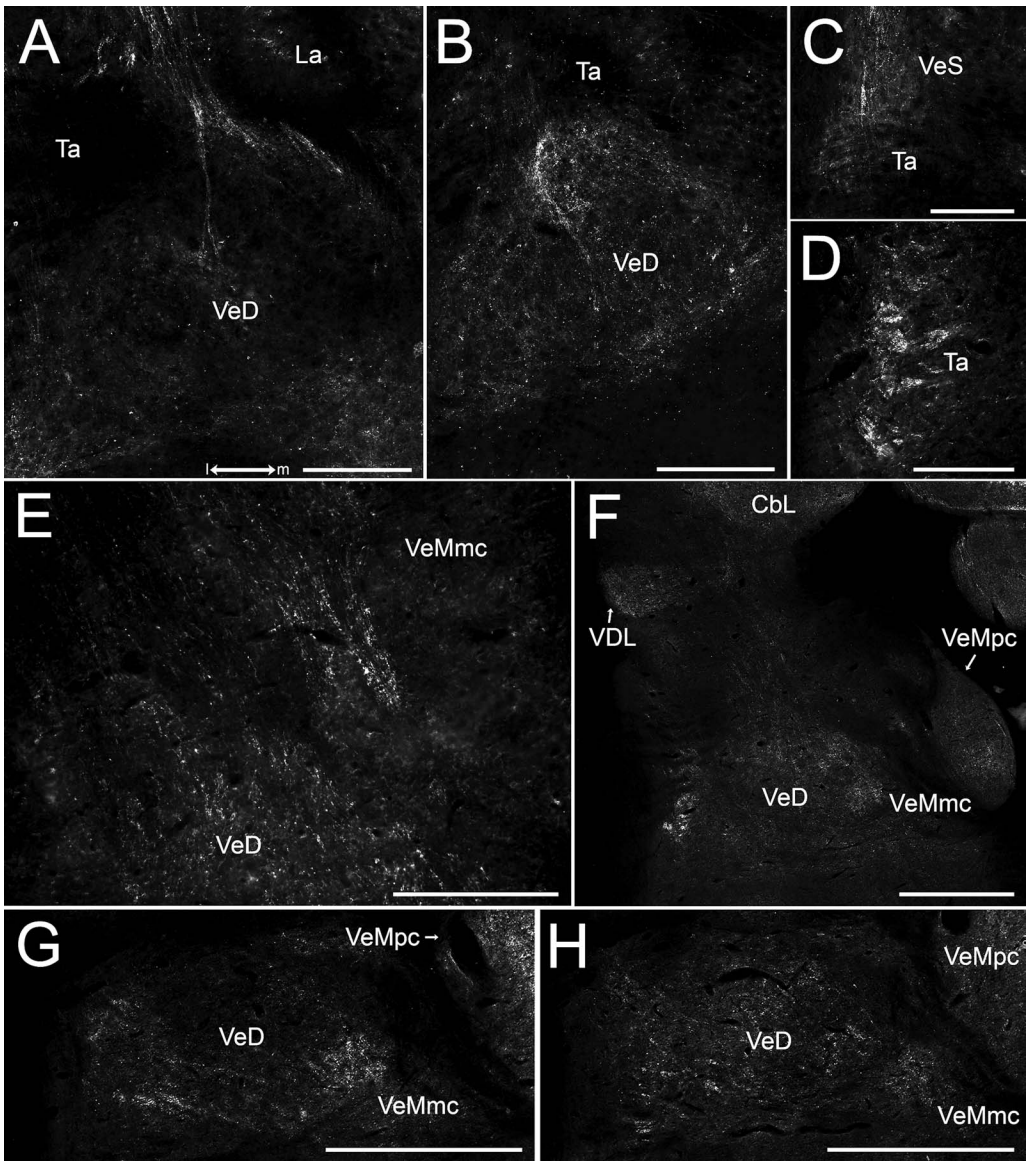

Figure 6. Zebrin II terminal labeling in the caudal vestibular nuclei. Photomicrographs of coronal sections from both hummingbird (**A–C**) and pigeon (**D–H**) are shown to illustrate ZII terminal labeling in the descending and medial vestibular nuclei (VeD, VeM) as well as the tangential nucleus (Ta). The left side of each panel is lateral. Scale bars = 200 μm in A–D; 400 μm in E; 800 μm in F–H.

distribution of immunoreactive terminals in the cerebellar and vestibular nuclei. Because ZII labels only PCs (Brochu et al. 1990), we can be sure that the ZII$^+$ PCs project to discrete areas in the cerebellar and vestibular nuclei. We assume that the areas that do not contain ZII$^+$ terminal labeling, but are known to receive PC input, receive their input from ZII$^-$ PCs. This assumption is supported by the findings of Sugihara et al. (2009). They traced labeled PC axons to the cerebellar nuclei in rats. Those PC axons that originated in ZII$^-$ stripes terminated in areas of the cerebellar nuclei that showed little or no ZII immunoreactivity. Likewise, PC axons that originated from ZII$^+$ stripes terminated in areas of the cerebellar nuclei that showed abundant ZII$^+$ terminal labeling.

Given that our study represents the first comprehensive description of ZII expression in both cerebellar and vestibular nuclei, we will discuss the three main implications of our findings. First, we compare the results of the present study with a mammalian study in which the distribution of ZII$^+$ terminal labeling in the cerebellar and vestibular nuclei was examined in rodents (Sugihara and Shinoda, 2007). Second, we discuss the implications of the heterogeneous distribution of ZII$^+$ terminals in CbM and CbL in light of studies of corticonuclear projections to these nuclei. Finally, we offer an extensive discussion of the heterogeneous distribution of ZII$^+$ terminals in the vestibular nuclei, which receive projections from the vestibulocerebellum (VbC; folia IXcd and X).

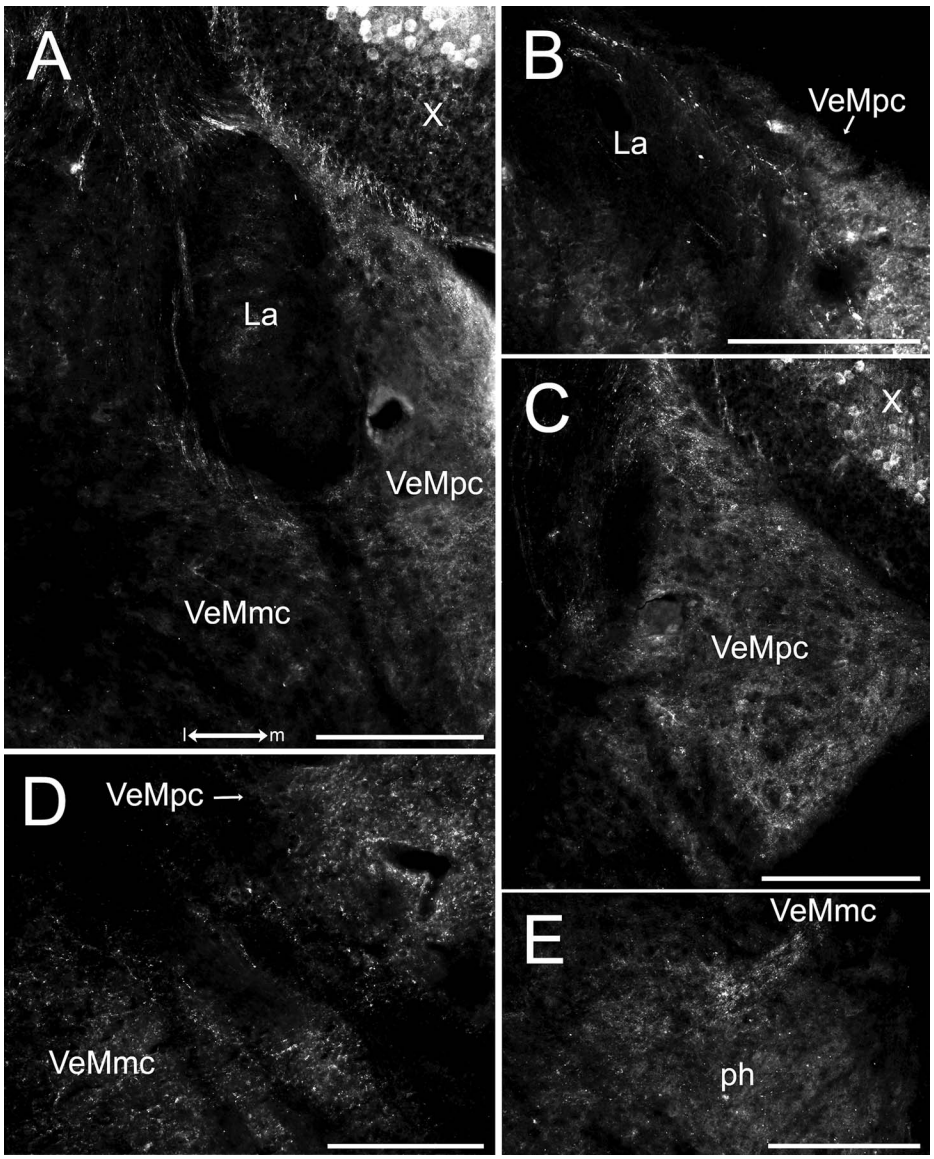

Figure 7. Zebrin II terminal labeling in the medial vestibular nucleus (VeM) and adjacent prepositus hypoglossi (ph). Photomicrographs of coronal sections from both hummingbird (**A,C,E**) and pigeon (**B,D**) are shown. See text for detailed description. The left side of each panel is lateral. Scale bars = 400 μm in A; 200 μm in B–E.

Comparison with mammals

Sugihara and Shinoda (2007) described the distribution of ZII immunoreactivity in the cerebellar and vestibular nuclei in rats. With respect to the vestibular nuclei, VeM, VeS, VeL, and VeD (a.k.a. the inferior vestibular nucleus) all have homologs in mammals with the same nomenclature (Butler and Hodos, 2005). With respect to the cerebellar nuclei, it is generally agreed that CbL and CbM are homologous to the interposed and fastigial nuclei in mammals, respectively (Larsell, 1967; Arends and Zeigler, 1991a,b). Birds do not have a dentate nucleus, although Arends et al. (1991) considered the Inf and VDL together to be homologous to the ventral dentate and/or group Y in mammals. In rodents, the fastigial nucleus is clearly demarcated into a ZII^- rostrodorsal area and a ZII^+ caudoventral area. In birds, the distribution in CbM is similar insofar as caudally and ventrally the CbM is heavily ZII^+, but the dorsal regions are also ZII^+. It is a central core of the avian CbM that is ZII^- (Figs. 2–4). ZII labeling in the interposed nuclei in rats has a distribution of $ZII^+/^-$ areas similar to that of the fastigial nucleus, a ZII^- rostrodorsal area and a ZII^+ caudoventral area. In birds, the ZII^- region in the CbL was rostroventral to medial, and the ZII^+ region was caudolateral (Figs. 3, 4B,C, 5A,C). Finally, ZII distribution in the dendate nucleus might be the most similar to that of the avian homologs. The dentate nucleus was ZII^+, with the densest staining ventrally, and group Y was also ZII^+. We also

found that the VDL (and the Inf when demarcated) was ZII$^+$, with the most dense staining ventrally (Figs. 3E,F, 5A,C, 6F). If ZII immunoreactivity is used as an indicator to help determine the mammalian homolgs of the cerebellar and vestibular nuclei in birds, then our data reinforce data from previous studies (Larsell, 1967; Arends and Zeigler, 1991a,b; Arends et al., 1991). That is, the homologs are as follows: CbL, interposed; CbM, fastigial; Inf/VDL, dentate/group y.

Sugihara and Shinoda (2007) did not offer as detailed an analysis of the vestibular nuclei in rats; it was not the focus of their study. Nonetheless, they described the VeS as "moderately or weakly" ZII$^+$ and the VeM as ZII$^+$ except at its rostral margin. They refer to "patches" in the lateral and central VeD as strongly ZII$^+$, although they attributed these to bundles of axons heading to the VeM and described the VeD as "weakly" ZII$^+$. Generally, we also found that the VeM was ZII$^+$ in birds, but the labeling was heavier rostrally and more pronounced in the VeMpc than in the VeMmc (Figs. 3C–F, 6E–H, 7). The VeS in birds could be described as weakly or moderately ZII$^+$, but clearly the lateral VeS was strongly ZII$^+$ (Figs. 3C,D, 5A,B,D,G, 6C). We would describe the avian VeD as weakly to moderately labeled overall, and, although ZII$^+$ terminals were found throughout the VeD with the exception of the central core, this labeling was most prominent laterally (Figs. 3E,F, 5C, 6A,B,E–H). Thus, the overall pattern described for mammals appears to be broadly similar to that observed in birds.

Purkinje cell projections to the medial and lateral cerebellar nuclei

Arends and Zeigler (1991a) studied the cortionuclear projections to the cerebellar nuclei in pigeons using horseradish peroxidase. They described heavy projections from folia II–IXab to CbM and CbL, whereas the projections from IXcd to X were sparse, and those of folium I were not determined. Arends and Zeigler (1991a) identified, based on PC projections to the vestibular and cerebellar nuclei, four parasagittal zones spanning folia I–IXab: zones A, B, C, and E. Zones A and C project to the CbM and CbL, whereas zones B and E, which are much thinner than A and C, project to the vestibular nuclei. Arends and Zeigler (1991a) also found that, generally speaking, zone A in the folia of the anterior lobe projected more rostrally in CbM, whereas the posterior lobe projected more caudally in CbM. A similar rostrocaudal topography was observed for the CbL, although it was not as marked. How zones A and C relate to the ZII stripes in folia II–IXab of the cerebellar cortex is not known, but data from the present study can speak to this. To review, there are four ZII$^+$ stripes in the anterior lobe extending from folium II to the ventral parts of folium V (Pakan et al., 2007; Iwaniuk et al., 2009). P1$^+$ spans the midline and is quite a bit thicker than the other three ZII$^+$ stripes, which are separated by thicker ZII$^-$ zones (see Fig. 1C). In the other folia, ZII immunoreactivity is generally greater, with the five to seven ZII$^{+/-}$ band pairs in VIII and IXab (see Fig. 1D,E). In folia VI and VII, the majority of PCs are ZII$^+$ (Fig. 1C). In the present study, we found that the core of CbM was ZII$^-$, whereas ZII immunoreactivity was heavy in the shell and in the ic and vm subnuclei. This suggests that the P1$^+$ and P1$^-$ zones are within the A zone. Furthermore, we suggest that the medial portions of the A zone, corresponding to the P1$^+$ ZII stripe, project to the shell, whereas the lateral A zone, corresponding to P1$^-$, projects to the core. Arends and Zeigler (1991a) found that CbMvm was labeled after injections in II and III but not after injections in other folia. Based on ZII expression, the CbMvm likely receives its projection from P1$^+$ in II and III. The heaviest ZII$^+$ terminal labeling we observed was in CbMic (Figs. 2A,B, 3, 4). Arends and Zeigler (1991a) found that the input to CbMic was largely from from VI, with some from VII and VIII. These data are consistent with the fact that the majority of PCs in VI and VII are ZII$^+$. The CbMic is actually different from the other subnuclei of the CbM. Axons from the CbMin, CbMim,. and CbMvm all travel in the fasciculus uncinatus and project caudally in the brainstem. Those from CbMic, however, join with those from CbL in the brachium conjuctivum and have ascending projections. CbMic seems to target the intercollicular nucleus, the midbrain central gray, and the stratum cellulare externum (Arends and Zeigler, 1991b).

We cannot make as strong predictions about the CbL. We did find ZII$^+$ and ZII$^-$ regions in CbL, indicating that zone C spans both ZII$^+$ and ZII$^-$ stripes. Which ZII stripes these might include is largely speculative. In comparing the figures from Arends and Zeigler (1991a) with those from Pakan et al. (2007), it appears that zone C would encompass P3$^{+/-}$ and perhaps parts of P2$^{+/-}$ and/or P4$^{+/-}$ in the anterior lobe, and zones P3$^{+/-}$ to P5$^{+/-}$ in folia VIII and IXab. With more confidence, we can state that the projection from the anterior lobe to the rostral CbL is from ZII$^+$ stripes to the dorsolateral regions and that from the posterior lobe to the caudal CbL is from ZII$^+$ stripes to the shell and ZII$^-$ stripes to the core.

Purkinje cell projections to the vestibular nuclei

The projection from the cerebellar cortex to the vestibular nuclear complex originates mainly from the VbC (IXcd and X), although there are some inputs from zones B and E (Arends and Zeigler, 1991a). The exception is VeLd, which receives PC input only from folia II–VII. Much is known about the VbC from electrophysiological recordings of PC response properties (Wylie and Frost, 1993,

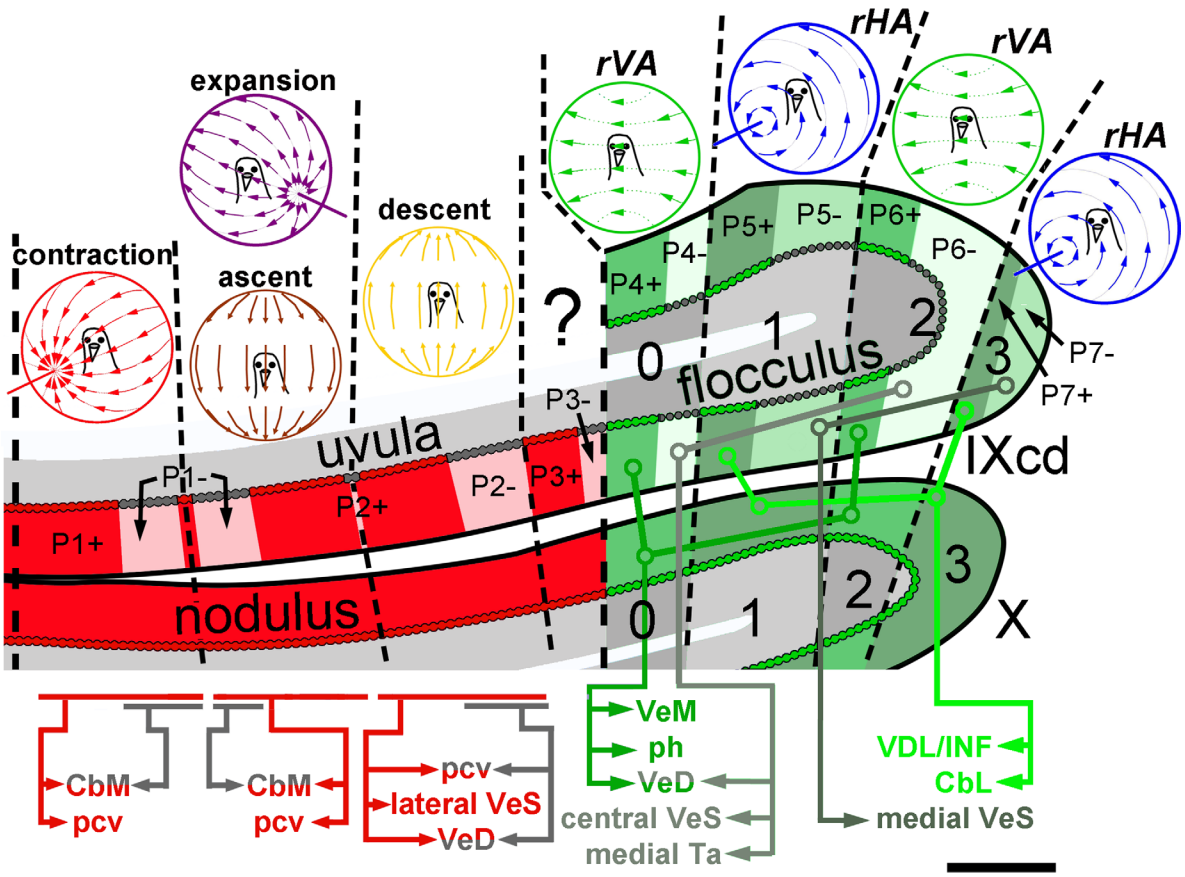

Figure 8. Projections of the Purkinje cells (PCs) in the ZII^{-} and ZII^{+} stripes of the vestibulocerebellum (VbC). Diagram of the optic flow zones in the VbC (folia IXcd and X). The lateral half of the VbC is the flocculus, shown in green shades. The medial half is the uvula/nodulus, shown in red shades. The location of the optic flow zones, as revealed in previous electrophysiological studies (Wylie et al., 1993, 2003a,b; Wylie and Frost, 1999; Winship and Wylie, 2003; Pakan et al., 2005), are shown with the ZII stripes in IXcd superimposed. Although the sagittal optic flow zones span folia IXcd and X, the ZII stripes do not: all PCs in X are uniformly ZII^{+}. There are six types of optic flow neurons organized into seven sagittal zones. In the flocculus, there are four zones: PCs in zones 0 and 2 respond best to rotational optic flow about either the vertical axis (rVA neurons), whereas PCs in zones 1 and 3 respond best to rotational optic flow about an horizontal axis oriented at 45° contralateral azimuth (rHA neurons; Wylie and Frost, 1993). rVA zones 0 and 2 span the P4$^{+/-}$ and P6$^{+/-}$ stripes, and the rHA zones 1 and 3 span the P5$^{+/-}$ and P7$^{+/-}$ stripes, respectively (Pakan and Wylie, 2008; Pakan et al., 2011). In the uvula/nodulus, PCs respond best to optic flow resulting from self-translation (Wylie and Frost, 1991; Wylie et al., 1998). There are three zones. In the most medial zone, PCs respond to optic flow resulting from backward translation (contraction). In the adjacent zone, two types of PCs are intermingled, those that repond to forward and upward translation (expansion and ascent cells). In the most lateral zone, PCs respond to optic flow resulting from downward translation (descent). The contraction zone spans P1^{+} and the medial half of P1^{-}; the expansion/ascent zone spans the lateral half of P1^{-} and the medial half of P2^{+}; and the descent zone spans the lateral half of P2^{+} and P2^{-}. PCs in ZII stripe P3$^{+/-}$ do not respond to optic flow (Graham et al., 2011). The projections of each of the optic flow zones are also indicated (from Wylie et al., 1999, 2003a,b). We speculate on the projections of each of the ZII^{+} stripes (colored arrows) and ZII^{-} stripes (gray arrows) based on the results of the present study. See text for additional details. Scale bar = 500 μm.

1999; Wylie et al., 1993, 1999), studies of efferent and afferent projections of the VbC (Lau et al., 1998; Wylie et al., 1999, 2003a,b; Crowder et al., 2000), and the relationship between the ZII stripes and the functional zones in the VbC (Pakan and Wylie, 2008; Pakan et al., 2011; Graham et al., 2011). The overall organization of the VbC is summarized in Figure 8. PCs in the VbC respond to optic flow, which is the pattern of visual motion that occurs during self-motion (Gibson, 1950; Wylie et al., 1993, 1999). In Figure 8, the optic flow-fields that preferentially excite PCs in each of the zones are depicted. PCs in the medial half of the VbC, which is refered to as the *uvula/nodulus*, respond to patterns of optic flow that result from self-translation (Wylie et al., 1993; Wylie and Frost, 1999). There are four types of cells organized into three functional zones. PCs in the most medial zone respond to radial contraction, which results from backward self-translation. In the adjacent zone, PCs respond best to flow-fields resulting from either forward translation (i.e., a flow-field with radial expansion) or upward

translation (ascent), and lateral to this is a zone where PCs respond best to optic flow resulting from downward translation (descent). PCs in the lateral half of the VbC, which is refered to as the *flocculus*, respond to patterns of optic flow that result from self-rotation (Wylie and Frost, 1993). rVA neurons respond best to rotation about the vertical axis (i.e., yaw), whereas rHA neurons respond best to rotation about a horizontal axis oriented 45° to the midline (Graf et al., 1988; Wylie and Frost, 1993). There are two rVA zones (zones 0 and 2) interdigitated with two rH45 zones (zones 1 and 3). Voogd and Wylie (2004) emphasized that the organization and connectivity of the flocculus is strikingly similar in mammals and birds. Similarly, data from monkeys suggest that the mammalian uvula/nodulus is involved in processing information related to self-translation (Yakusheva et al., 2010).

Recently, we have shown that each optic flow zone in the flocculus spans a ZII$^{+/-}$ stripe pair: the rVA zones 0 and 2 span the P4^{+}/P4^{-} and P6^{+}/P6^{-} ZII stripe pairs, respectively, and the rHA zones 1 and 3 span the P5^{+}/P6^{-} and P7^{+}/P7^{-} stripe pairs, respectively (Pakan and Wylie, 2008; Pakan et al., 2008, 2011). A similar pattern occurs in the ventral uvula: the contraction zone spans the P1^{+} and the medial half of the P1^{-} stripe. (The lateral half of P1^{-} is separated from P1^{-} med by a satellite ZII^{+} band that is only one to three PCs in width). The adjacent zone, which contains both ascent and expansion PCs, spans the lateral P1^{-} stripe and the medial half of the P2^{+} stripe. (The P2^{+} ZII stripe is separated into medial and lateral halves by a satellite ZII^{-} stripe that is about two PCs in width). The descent zone spans the lateral P2^{+} stripe and the P2^{-} stripe. PCs in P3^{+}/P3^{-} stripes are not modulated by optic flow, and their function is not known (Graham et al., 2011).

Previously, Wylie et al. (2003a,b) determined the projections of PCs in the optic flow zones to the vestibular and cerebellar nuclei, as indicated at the bottom of Figure 8. With the data from the present study, we can make predictions with regard to which structures receive input from ZII^{+} and ZII^{-} PCs. The rVA zones project to VeM, ph, VeD, the central VeS, and the medial Ta (Wylie et al., 2003a). In the present study, ZII labeling was not present in the medial Ta or central VeS, thus these areas likely receive input from the P4^{-} and P6^{-} stripes. In contrast, ZII^{+} terminals were present in the VeM and ph, so we predict that these areas receive input from the P4^{+} and P6^{+} stripes. ZII^{+} terminals were present in some parts of VeD, so the projection from the rVA zones may arise from ZII^{+} or ZII^{-} stripes in these zones. The rHA zones project to Inf, VDL, the lateral edge of CbL, and the medial VeS (Wylie et al., 2003b). With the exception of the medial VeS, these structures showed heavy ZII^{+} terminal labeling. Thus, the projection to the medial VeS is likely from the P5^{-} and P7^{-} stripes, whereas the projection to the Inf, VDL and lateral CbL is from the P5^{+} and P7^{+} stripes. The contraction and expansion/ascent zones both project to the CbM and pcv, albeit to different regions of these structures (Wylie et al., 2003b). Because these projections to CbM are not exclusive to the core or shell, we predict that they arise from both ZII^{+} and ZII^{-} stripes (i.e., P1^{+}, medial and lateral P1^{-}, medial P2^{+}). The projections to the pcv are localized to the regions abutting the CbM. Thus, these projections likely arise from P1^{+} and medial P2^{+}, because these regions of the pcv showed heavy ZII^{+} terminal labeling in the present study. The descent zone projects to the pcv, VeD, and lateral VeS (Wylie et al., 2003b). The projection to the pcv is to the region between the CbM and VeS. This projection may arise from PCs in both the lateral P2^{+} stripe and the P2^{-} stripe. The same can be said of the projection to VeD. The projection to the lateral VeS, however, is likely from the lateral P2^{+} stripe, because the lateral VeS contains ZII^{+} terminals.

In conclusion, we suggest that PCs in the ZII^{+} and ZII^{-} stripes within an optic flow zone have differential projections. A specific functional role distinguishing ZII^{+} and ZII^{-} PCs remains unknown, although it has been suggested that ZII^{+} and ZII^{-} PCs may differ in their role in synaptic plasticity (Nagao et al., 1997; Wadiche and Jahr, 2005; Paukert et al., 2010). The glutamate transporter EAAT4 is expressed in higher denstity in ZII^{+} PCs (Nagao et al., 1997). Wadiche and Jahr (2005) suggested that the abundance of EAAT4 results in a reduced likelihood that ZII^{+} PCs are subject to long-term depression of parallel fiber inputs during periods of sustained climbing fiber activity. Thus, for example, during optokinetic stimulation about the vertical axis, in the rVA zones PCs in the P4^{-} and P6^{-} stripes will be subject to long-term depression moreso than PCs in the P4^{+} and P6^{+} stripes. Furthermore, from the current study, we can state that the PCs that are more subject to this plasticity project to different areas in the vestibular and cerebellar nuclei. That is, the cerebellum may contain corticonuclear systems undergoing robust plasticity (i.e., ZII^{-} PCs and their recipient neurons) operating in parallel with corticonuclear systems that are not as plastic (i.e., ZII^{+} PCs and their recipient neurons).

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