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Heterogeneity of calretinin expression in the avian cerebellar cortex of pigeons and relationship with zebrin II

Douglas R. Wylie [a,b,*], Meagan Jensen [a], Cristian Gutierrez-Ibanez [a], David J. Graham [a], Andrew N. Iwaniuk [c]

[a] University Centre for Neuroscience, University of Alberta, Edmonton, AB T6G 2E9, Canada
[b] Department of Psychology, University of Alberta, Edmonton, AB T6G 2E9, Canada
[c] Department of Neuroscience, Canadian Centre for Behavioural Neuroscience, University of Lethbridge, Lethbridge, AB T1K 3M4, Canada

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ABSTRACT

The cerebellar cortex has a fundamental parasagittal organization that is reflected in the physiological responses of Purkinje cells, projections of Purkinje cells, afferent inputs from climbing and mossy fibres and the expression of several molecular markers. The most thoroughly studied of these molecular markers is zebrin II (ZII; a.k.a. aldolase C). ZII is differentially expressed in Purkinje cells, resulting in a pattern of sagittal stripes of high expression (ZII+ve) interdigitated with stripes of little or no expression (ZII−ve). The calcium binding protein calretinin (CR) is expressed heavily in mossy fibres terminating throughout the cerebellar cortex, but whether CR is heterogeneously expressed in parasagittal stripes, like ZII, is unknown. In this study, we examined CR expression in the cerebellum of pigeons and compared it to that of ZII. CR was expressed heavily in the granule layer in mossy fibres and their terminal rosettes. Moreover, CR is expressed heterogeneously in the granule layer such that there are sagittal stripes of heavy CR labelling (CR+ve) alternating with stripes of weaker labelling (CR−ve). The CR heterogeneity is most notable in folium IXcd and folia II–IV in the anterior lobe. In the anterior lobe, the central CR+ve stripe spanning the midline is aligned with the central ZII+ve stripe, whereas the other CR+ve stripes are aligned with the ZII−ve stripes. In IXcd, the CR+ve stripes are aligned with the ZII+ve stripes. This combination of aligned and unaligned CR+ve stripes, relative to ZII+ve stripes, differs from that of parvalbumin and other neurochemical markers, but the functional consequences of this is unclear. With respect to the posterior lobe, we suggest that the CR+ve mossy fibres to IXcd originate in two retinal recipient nuclei that are involved in the processing of optic flow.

1. Introduction

It is well established that the fundamental architecture of the cerebellar cortex consists of parasagittal zones (Voogd and Bigare, 1980). These zones are evident with respect to patterns of climbing and mossy fibre inputs, Purkinje cell efferents, and Purkinje cell response properties (Voogd, 1967; Voogd et al., 1969; Oscarsson, 1969; Ekerot and Larson, 1973; Andersson and Oscarsson, 1978a,b; Matsushita et al., 1984, 1991; Gerrits et al., 1985; Llinas and Sasaki, 1989; Sato and Kawasaki, 1991; Akintunde and Eisenman, 1994; De Zeeuw et al., 1994; Ji and Hawkes, 1994; Wylie et al., 1994, 1995, 2003; Voogd and Glickstein, 1998; Ruigrok, 2003; Winship and Wylie, 2003; Sugihara and Shinoda, 2004; Voogd and Wylie, 2004; Apps and Garwicz, 2005). In addition to hodology and physiology, a sagittal compartmentation of the cerebellar cortex is also shown by the expression of numerous molecular markers (for review, see Hawkes and Gravel, 1991; Herrup and Kuemerle, 1997; Armstrong and Hawkes, 2000; Apps and Hawkes, 2009), the most thoroughly studied of which is zebrin II (ZII) (aldolase C; Brochu et al., 1990; Ahn et al., 1994; Hawkes and Herrup, 1995). ZII is expressed by Purkinje cells in a heterogeneous manner such that there are parasagittal stripes of Purkinje cells with high ZII expression (ZII+ve stripes), alternating with stripes of Purkinje cells with little or no ZII expression (ZII−ve stripes) (Eisenman and Hawkes, 1993; Akintunde and Eisenman, 1994; Sillitoe and Hawkes, 2002; Voogd and Ruigrok, 2004; Sugihara and Shinoda, 2004, 2007; Larouche and Hawkes, 2006; Sugihara and Quy, 2007; Fujita et al., 2010). Alternating ZII+ve and ZII−ve parasagittal stripes occur in a range of mammalian (for review, see Sillitoe et al., 2005) and avian species (Pakan et al., 2007; Iwaniuk et al., 2009a; Marzban et al., 2010). The pattern of zebrin stripes in birds and mammals is remarkably similar (Pakan et al., 2007; Marzban and Hawkes, 2011), and thus likely important for cerebellar function.

* Corresponding author at: Department of Psychology, University of Alberta, Edmonton, AB T6G 2E9, Canada. Tel.: +1 780 492 7239; fax: +1 780 492 1768.
E-mail address: dwylie@ualberta.ca (D.R. Wylie).

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Recently, it has been shown in the pigeon vestibulocerbellum (folium IXcd) that the ZII stripes are correlated with the functional zones defined by Purkinje cell responses to optic flow stimuli (Pakan et al., 2011; Graham and Wylie, 2012).

Numerous other molecular markers are also expressed in parasagittal zones (e.g. Chan-Palay et al., 1981; Jaarsma et al., 1995; Van den Dungen et al., 1988; Cummings, 1989; Cummings et al., 1989; Eisenman and Hawkes, 1993; King et al., 1997; Marzban et al., 2004, 2007; Sawada et al., 2008). A few studies have compared the distribution of these other markers with that of ZII (Dehnes et al., 1998; Armstrong et al., 2000; Sawada et al., 2010; Sillitoe et al., 2010; Wylie et al., 2011). There is variation in the extent to which the stripes are coincident or complementary with ZII stripes. For example, the expression of phospholipase Cβ3 is concordant with that of ZII, whereas phospholipase Cβ4 shows a complementary expression pattern to that of ZII (Sarna et al., 2006; Iwaniuk et al., 2009a; Marzban et al., 2010). In contrast, the expression of corticotropin releasing factor is more complex, and neither concordant nor complementary to ZII (Sawada et al., 2008).

The calcium binding protein calretinin (CR) is expressed in several cell types in the adult avian cerebellar cortex including basket cells, mossy fibres and some climbing fibres originating in the dorsal lamella of the inferior olive (Rogers, 1989; De Castro et al., 1998; see Bastianelli (2003) for review). Both granule cells and some Purkinje cells express CR during development, but not in the adult (Bastianelli and Pochet, 1993; Gilbert et al., 2012). To the best of our knowledge, there is not a report suggesting that the CR expression in mossy fibres follows a parasagittal distribution. However a study from our lab (Pakan et al., 2010) suggests that this may be the case in folium IXcd: there is a mossy fibre projection to IXcd originating in the nucleus of the basal optic root (nBOR) and pretectal nucleus lentiformis mesencephali (LM) (Clarke, 1977; Brecha et al., 1980; Gamlin and Cohen, 1988; Wylie and Linkenhoker, 1996). Pakan et al. (2010) showed that mossy fibres from both LM and nBOR terminated in parasagittal bands aligned with the ZII+ve stripes. Previously, we showed that about half of the cerebellar projecting neurons in nBOR and LM express CR (Wylie et al., 2008; Iwaniuk et al., 2009b). In the present study, we examined the distribution of CR expression in mossy fibres in relation to the ZII stripes in the pigeon cerebellum. We predicted that the CR expression would be heterogeneous, with sagittal CR+ve stripes aligned with the ZII+ve stripes in folium IXcd.

2. 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. Adult pigeons obtained from a local supplier were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PFA, pH = 7.4). The brains were then removed and post-fixed by immersion in the same fixative for

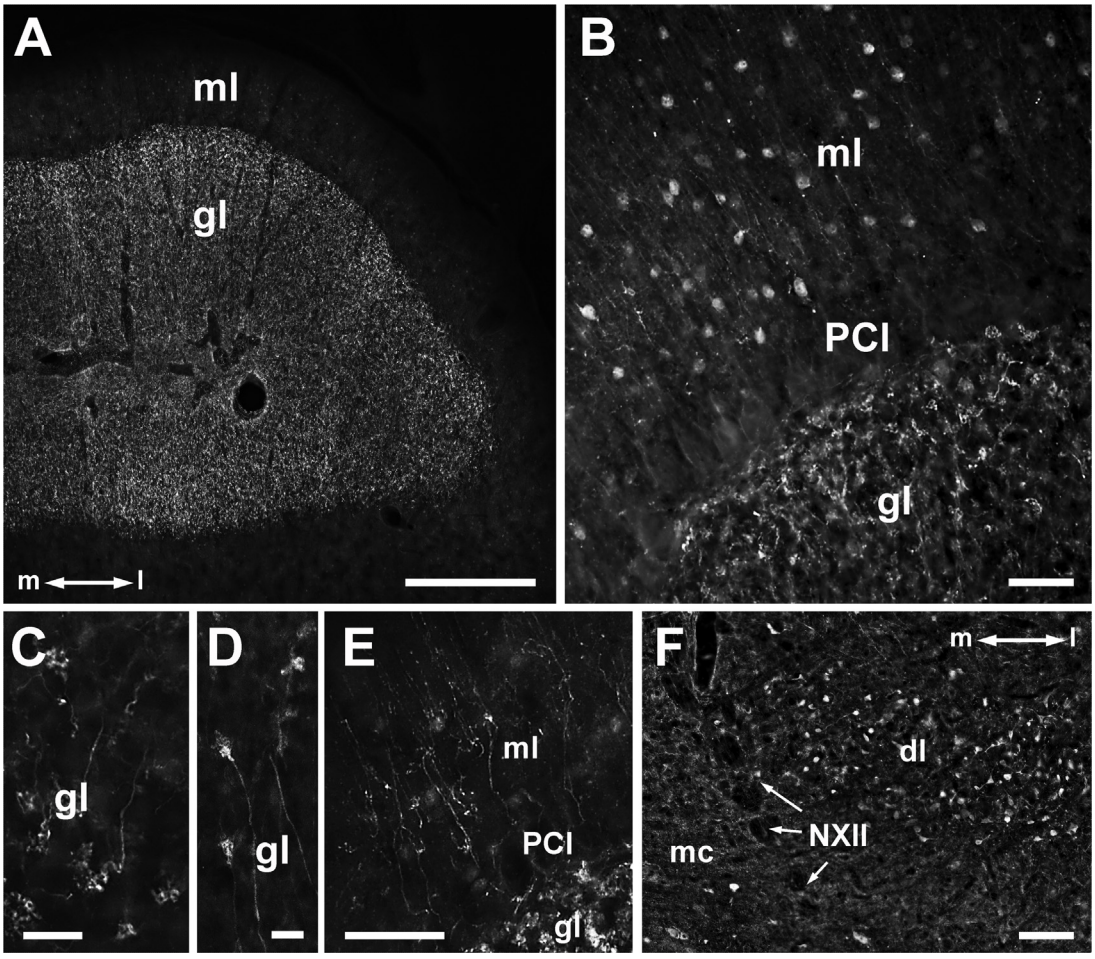

Fig. 1. Calretinin expression in the cerebellum. Photomicrographs of coronal sections are shown. (A) Shows a low power photomicrograph indicating that the heaviest calretinin immunopositive (CR+ve) labelling was seen in the granular layer (gl). Higher magnification images (C and D) show that these are labelled mossy fibres (MFs) and MF rosettes. (B) Shows a high magnification photomicrograph showing that interneurons in the molecular layer (ml) are also CR+ve. These are presumed basket and stellate cells. Note that the Purkinje cells are not CR+ve. (E) Shows labelled climbing fibres in the ml from folium VII. These originate in the dorsal lamella (dl) of the inferior olive. (F) Shows a section through the inferior olive. CR+ve cells were seen in the dorsal lamella (dl) but not the medial column (mc) or the ventral lamella (not shown). Other abbreviations: PCl, Purkinje cell layer; NXII, hypoglossal nerve; m, l, medial, lateral. Scale bars: 500 μm in (A); 50 μm in (B) and (E); 25 μm in (C) and (D); 100 μm in (F).

several days. 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 were collected into several series (in 0.1 M PBS).

2.1. Immunohistochemistry for calretinin and zebrin II

Free floating sections were washed several times in 0.1 M PBS and blocked with 10% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA) and 0.4% Triton X-100 in PBS for 1–2 h at room temperature. Sections were then incubated for 48–72 h at 4 °C in PBS containing 2.5% normal donkey serum, 0.4% Triton X-100 and a rabbit polyclonal anti-CR antibody (1:2000; 7699/3H, Swant, Switzerland). Sections were then rinsed in PBS and incubated for 2–3 h at room temperature in PBS, 2.5% normal donkey serum, and 0.4% Triton X-100 containing the appropriate secondary antibody; red (Cy3) or green (Cy2) conjugated donkey anti-rabbit (1:200, Jackson Immunoresearch Laboratories). The tissue was finally rinsed in PBS and mounted onto gelatinized slides for viewing.

For ZII immunoreactivity, after washing and blocking as described above, the sections were incubated for 110–120 h at 4 °C in PBS containing 0.4% Triton X-100 and a goat polyclonal anti-aldolase C/zebrin antibody (1:1000, sc-12065, Santa Cruz Biotechnologies, Santa Cruz, CA). The sections were then rinsed several times in PBS and incubated for 2–3 h at room temperature in PBS, 2.5% donkey serum and 0.4% Triton X-100 and the appropriate secondary antibody, either red (Dylight 594 or Alexafluor-594) or green (Dylight 488 or Alexafluor-488) conjugated donkey anti-goat secondary antibody (1:200; Jackson Immunoresearch Laboratories). Following several rinses in PBS, the sections were then mounted onto gelatinized slides.

Double-labelling was performed by first incubating the tissue in the rabbit anti-CR primary antibody followed by the red (Cy3) or green (Cy2) donkey anti-rabbit secondary, and then incubated in goat anti-aldolase C/zebrin antibody followed by either red (Dylight 594 or Alexafluor-594) or green (Dylight 488 or Alexafluor-488) conjugated donkey anti-goat secondary antibody. The procedures for blocking, dilution factors, incubation periods, and the primary and secondary antibodies used are as described above.

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

2.2. Nomenclature

Compared to mammals, the avian cerebellum is best described as a vermis without hemispheres (Larsell, 1967), although Pakan et al. (2007) suggested that small rudimentary hemispheres may exist. As is the case with the mammalian vermis, the avian cerebellum is divided into 10 lobules that are referred to as "folia", and labelled using Roman numerals I–X (anterior to posterior). Also as in mammals, the avian cerebellum is divided into an anterior lobe (folia I–V), a posterior lobe (VI–IX) and the nodulus (X). The possible homologies between parts of the avian and mammalian cerebellum are discussed in Marzban and Hawkes (2011), Pakan et al. (2007) and Larsell (1967).

3. Results

3.1. Distribution of calretinin immunoreactivity

CR expression was prevalent in the granule layer (Fig. 1A) and this labelling seemed to be exclusively due to CR immunoreactivity in mossy fibres and their terminal rosettes (Fig. 1C and D). CR immunopositive (CR+ve) interneurons (basket and/or stellate cells) were also observed in the molecular layer (Fig. 1B). The Purkinje cells did not express CR (Fig. 1B). In folium VII, some climbing fibres were CR+ve (Fig. 1E), and likely arise from neurons in the dorsal lamella of the inferior olive, which were also CR+ve (Fig. 1F).

The heavy CR immunoreactivity of mossy fibres was not uniformly distributed (Fig. 1A). This was most apparent in the caudal half of folium IXcd where clear sagittal bands of stripes of high immunoreactivity alternated with bands of lower immunoreactivity (Fig. 2A and B). Although the stripes of high and low immunoreactivity contain CR+ve mossy fibres, we refer to these as CR+ve and CR−ve stripes, respectively. To be clear, it is not that mossy fibres in a CR−ve stripe had a weaker expression of CR compared to those in the CR+ve stripes, rather there were more

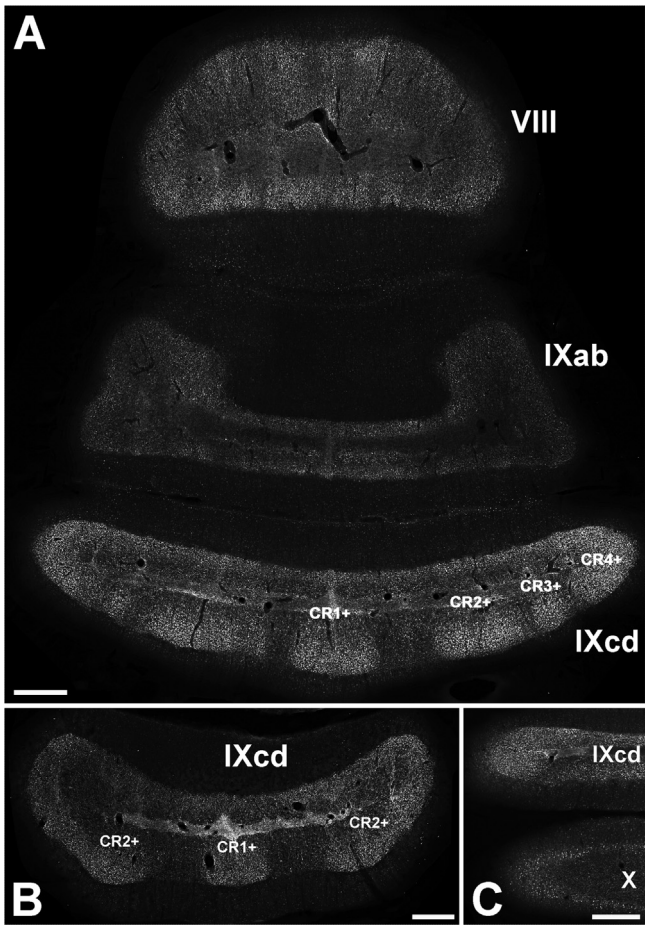

Fig. 2. Distribution of calretinin (CR) immunoreactive mossy fibres in the posterior lobe of the cerebellum. Photomicrographs of coronal sections are shown. Note the heterogeneous distribution of expression in folium IXcd (A and B). In IXcd there are clearly CR immunopositive (CR+ve) and CR immunonegative bands (CR−ve) (A). These were most apparent in caudal sections (B) where two CR+ve bands could be seen (CR1+, CR2+). More rostral, two more lateral bands could be seen in IXcd (A; CR3+, CR4+). (C) Shows that CR+ve mossy fibres were much less dense in folium X compared to IXcd. CR+ve and CR−ve stripes could be seen in folia VIII and IXab (A) but these were much thinner and the contrast was not as marked. Scale bars: 500 μm in (A)–(C).

CR+ve mossy fibres and rosettes in the CR+ve stripes compared to the CR−ve stripes.

A central CR+ve stripe spanned the midline (CR1+), and up to three more CR+ve stripes (CR2+, CR3+, CR4+) alternating with CR−ve stripes could be seen bilaterally. The CR−ve stripe between CR1+ and CR2+ was quite wide, but the other CR−ve stripes were thinner than their CR+ve counterparts (Fig. 2A and B). These stripes did not appear to continue into adjacent folia. In IXab and VIII, sagittal stripes of heterogeneous CR expression were still apparent, but the contrast between those with high and low immunoreactivity was much less and the number of stripes difficult to ascertain (Fig. 2A). In IXab, overall CR immunoreactivity was less relative to the adjacent folia, and in X, CR immunoreactivity was very low, and stripes were not apparent (Fig. 2C).

Sagittal CR+ve and CR−ve stripes were observed in folia II–IV, particularly in the anterior sections. As shown in Fig. 3A, there were three major CR+ve stripes (CR1+, CR2+, CR3+). CR1+, on the midline, was thin, but strongly immunopositive. The intensity of the labelling of CR2+ was less than that of the CR1+ and CR3+ stripes, but in some sections a CR−ve stripe divided the CR2+ stripes into two stripes, CR2+ a and CR2+ b. We consider the CR2+ a and b as subdivisions rather than individual stripes because the CR−ve space between them is small in extent, of low contrast, and

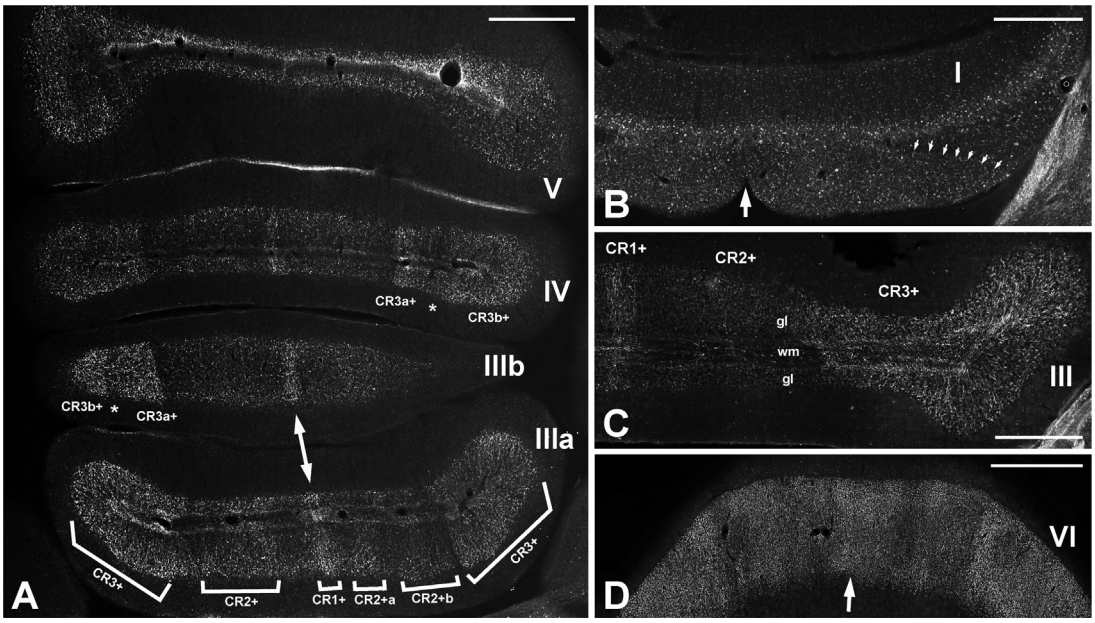

Fig. 3. Distribution of calretinin (CR) immunoreactive mossy fibres in the anterior lobe of the cerebellum. Photomicrographs of coronal sections are shown. (A) Shows the sagittal stripes that were visible in folia II–IV (II not shown) particularly in the anterior sections. There were three major CR+ve stripes: CR1+, CR2+, CR3+. The asterisks indicate CR−ve stripes that were apparent within the CR2+ and CR3+ stripes in some sections. These divided CR2+ into CR2+ a and CR2+ b, and CR3+ into CR3+ a and CR3+ b. (B) Shows a section through the lingula (folium I). The small arrows in (B) indicate an apparent boundary between areas of higher and lower CR expression. (C) Shows a section through the posterior part of folium III, where the CR3+ stripe occupied almost half the extent of the folium. (D) Shows a section through folium VI. The midline is indicated in (A), (B) and (D) by the vertical arrows. See text for details. gl, granule cell layer; wm, white matter. Scale bars: 500 μm in (A)–(D).

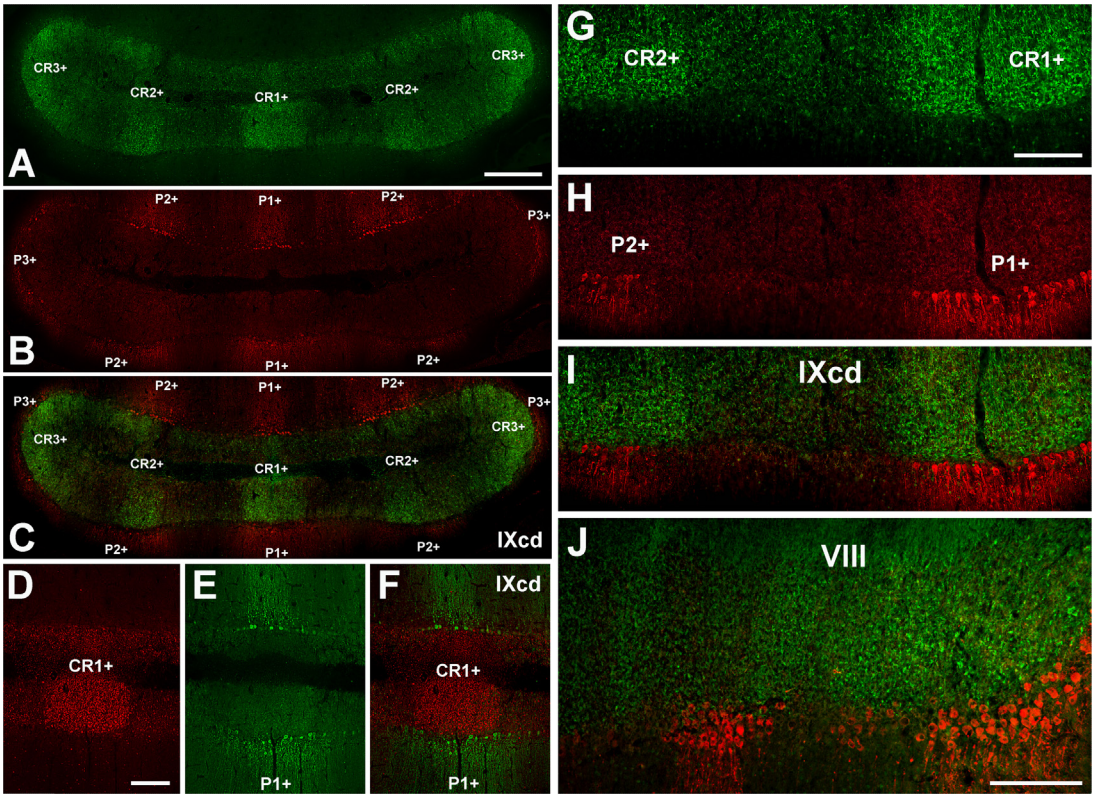

Fig. 4. Relationship between zebrin II (ZII) and calretinin (CR) immunopositive stripes in folium IXcd. Photomicrographs of coronal sections are shown. In (A)–(I) triptychs show CR immunoreactivity (A, D, G), ZII immunoreactivity (B, E, H) and the overlays (C, F, I). In (A)–(C) a section through IXcd shows the correspondence of the CR1+, CR2+ and CR3+ stripes with the P1+, P2+ and P3+ ZII stripes, respectively. From different cases, (D)–(F) show a higher magnification of the correspondence of the P1+ and CR1+ stripes, and (G)–(I) show the correspondence of the CR1+ and CR2+ stripes with the P1+ and P2+ stripes. (J) Shows a photomicrograph through folium VIII with CR immunoreactivity shown in green, and ZII immunoreactivity shown in red. A relationship between ZII and CR was not apparent in folia V-IXab. Scale bars: 500 μm in (A); 250 μm in (D), (G) and (J).

not always apparent. (The same applies to the CR3+ stripe (see below)). The intensity of CR2+ was greatest in the anterior sections through folia II and III and diminished in more posterior sections through folium III and IV (Fig. 3C). The CR immunoreactivity in CR3+ was quite intense. CR3+ could also be subdivided into two stripes in some sections, CR3+ a and CR3+ b (Fig. 3A). In more anterior sections through folium III and IV, although the intensity of the stripe remained high, and the stripe occupied almost half the extent of the folium, the division between CR3+ a and CR3+ b became less obvious (Fig. 3C). The CR stripes do not persist into folium V, VI and VII, but there is heavier expression laterally suggesting an extension of CR3+ into these folia (Fig. 3D). Otherwise there was a series CR stripes of low contrast as in folia VIII and IXab. In the lingula (folium I), the overall CR expression was lower than in the other folia in the anterior lobe. Although distinct stripes were not seen, in some sections the medial half of the lingula had higher CR expression than the lateral half (Fig. 3B). The distribution of labelled interneurons in the molecular layer appeared to be uniform throughout the cerebellar cortex, and not related to the sagittal CR+ve stripes.

3.2. Calretinin distribution in relation zebrin II immunoreactivity

In Figs. 4–7, the relationship between the CR stripes and ZII stripes is shown. Consistent with previous studies (Pakan et al., 2007), the cell bodies, dendrites and axons of Purkinje cells were

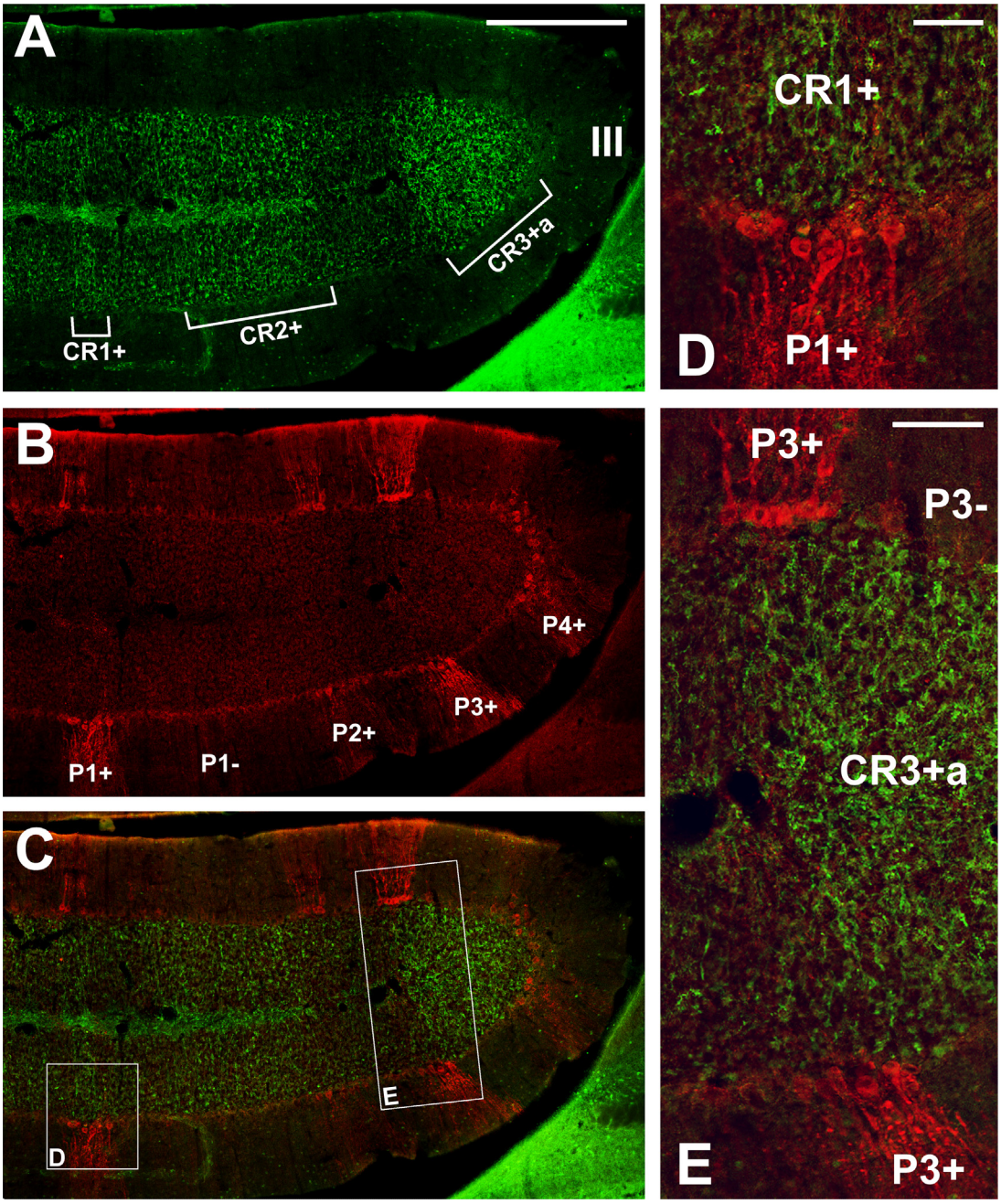

Fig. 5. Relationship between zebrin II (ZII) and calretinin (CR) immunopositive stripes in the anterior lobe. Photomicrographs of a coronal section through folium III are shown. (A)–(C) shows a triptychs with the CR labelling on top (A), ZII reactivity in the middle (B), and the overlay below (C). CR immunoreactivity was heterogeneous such that there were several sagittal stripes of labelled mossy fibres (CR1+, CR2+, CR3+ a). ZII was also heterogeneous such that there were four sagittal stripes of immunopositive Purkinje cells (P1+, P2+, P3+, P4+). Shown at higher magnification in (D), the midline P1+ stripe is aligned with CR1+, and in (E), the medial edge of CR3+ a abutted the lateral edge of the P3+ stripe. Scale bars: 500 μm in (A); 50 μm in (D); 100 μm in (E).

ZII immunoreactive. ZII expression was heterogeneous such that there were sagittal ZII+ve stripes alternating with ZII−ve stripes. By convention the ZII+ve stripes are indicated with Arabic numerals, increasing from the ZII+ve midline stripe (P1+). ZII+ve stripes were not observed in folia I, V–VII and X, because almost all Purkinje cells in these folia strongly express ZII (Pakan et al., 2007).

The relationship between the CR and ZII stripes was most clear in folium IXcd, where the CR+ve stripes in the granular layer aligned with the ZII+ve stripes. Fig. 4A–I shows examples of the clear correspondence of the P1+, P2+ and P3+ ZII stripes with the CR1+, CR2+ and CR3+ stripes in IXcd. In VIII (Fig. 4J) and IXab (not shown) a relationship between the ZII and CR stripes was not apparent.

As shown in Figs. 5–7, there was a clear relationship between the CR and ZII stripes in folia II–IV. Confirming the findings of Pakan et al. (2007), these folia are characterized by four ZII+ve/−ve stripe pairs (Figs. 5B, 6B and 7B). There is a central ZII+ve stripe along the midline (P1+), and three more lateral ZII+ve stripes (P2+, P3+, P4+). The ZII+ve stripes are thinner than their component ZII−ve stripes. The midline CR1+ stripe was aligned with the P1+ ZII stripe (Fig. 5D; see also Figs. 5C, 6C and 7C), however the other CR+ve stripes were not aligned with ZII+ve stripes. The CR2+ stripe was found within the P1− stripe. It did not occupy the extent of the P1− stripe, but rather the lateral edge of CR2+ b stripe abutted the medial edge of the P2+ stripe (Fig. 6D; see also Figs. 5C, 6C and 7C). Similarly, but more striking, the CR3+ a stripe was aligned with the P3− stripe. This is shown with higher magnification photomicrographs in Figs. 5E, 6D and 7D. In many sections, originating from the medial region of the P3+ stripe, a band of strongly ZII+ve axons could be seen in the granule layer (Fig. 7B). It is clear that this band of axons lies along the medial edge of the CR3+ a stripe (Fig. 7D; see also Fig. 6D). Finally, the area of weak CR-immunoreactivity between the CR3+ a and CR3+ b stripes was concordant with the P4+ ZII stripe (Fig. 7C and D; see also Fig. 5C).

4. Discussion

The expression of the calcium-binding proteins in the central nervous system has been described for several species (e.g., Celio, 1990; Van Brederode et al., 1990; Resibois and Rogers, 1992; Pfeiffer and Britto, 1997; Pritz and Siadati, 1999). CR is expressed in the cerebellar cortex of various species (see Bastianelli, 2003 for review), including birds (Rogers, 1989; Bastianelli and Pochet, 1993; De Castro et al., 1998; Gilbert et al., 2012). Previous studies of birds primarily described the development of CR expression in the cerebellum. CR is not expressed in most Purkinje cells (Bastianelli and Pochet, 1993), but Gilbert et al. (2012) found that some presumptive Purkinje cells in the nodulus express CR transiently between embryonic days 8–12 (E8–E12). CR is also expressed transiently in some cells in the granule layer from E11 to as late as E20, and these may include granule cells, Golgi cells, and unipolar brush cells (Bastianelli and Pochet, 1993; De Castro et al., 1998). In the molecular layer, CR is expressed in interneurons late in development (E18–20) (Gilbert et al., 2012), but these are described as 'scarce' by Bastianelli and Pochet (1993). Our observations in the adult pigeon are consistent with these, insofar

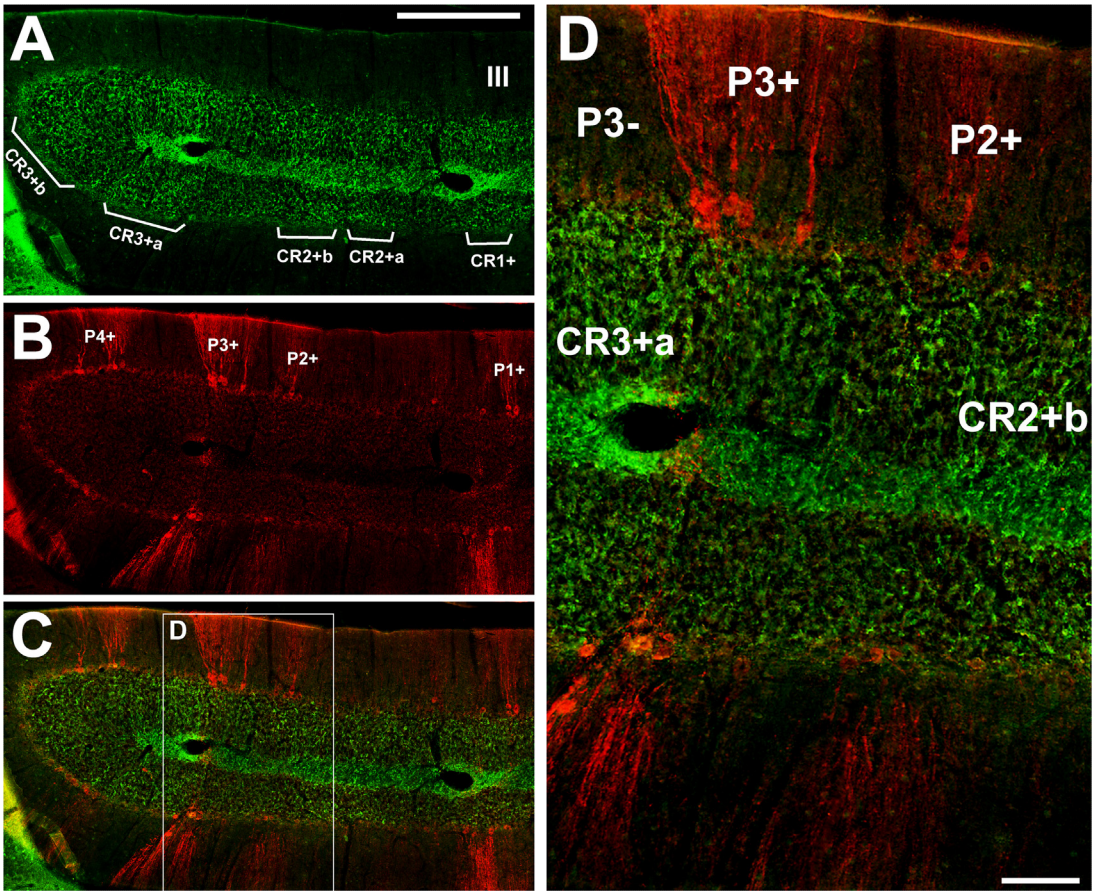

Fig. 6. Relationship between zebrin II (ZII) and calretinin (CR) immunopositive stripes in the anterior lobe. Photomicrographs of a coronal section through folium III are shown. Trypitchs are shown with the CR labelling on top (A), ZII reactivity in the middle (B), and the overlay below (C). CR immunoreactivity was heterogeneous such that there were several sagittal stripes of labelled mossy fibres (CR1+, CR2+ a, CR2+ b, CR3+ a, CR3+ b). ZII was also heterogeneous such that there were four sagittal stripes of immunopositive Purkinje cells (P1+, P2+, P3+, P4+). Shown at a higher magnification in (D), the lateral edge of CR2+ b abutted the medial edge of the P2+ stripe and the medial edge of CR3+ a abutted the lateral edge of the P3+ stripe. Scale bars: 500 μm in (A); 100 μm in (D).

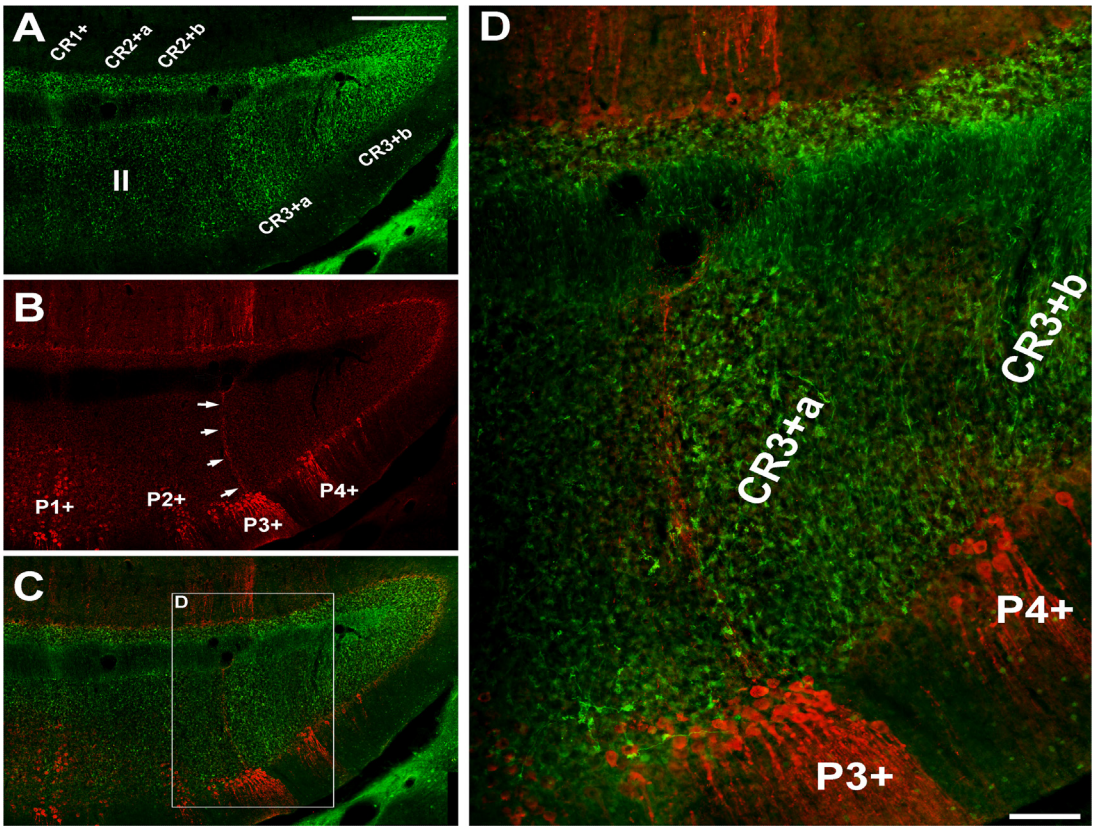

Fig. 7. Relationship between zebrin II (ZII) and calretinin (CR) immunopositive stripes in the anterior lobe. Photomicrographs of a coronal section through folium II are shown. Trypitchs are shown with the CR labelling on top (A), ZII reactivity in the middle (B), and the overlay below (C). CR immunoreactivity was heterogeneous such that there were several sagittal stripes of labelled mossy fibres (CR1+, CR2+ a, CR2+ b, CR3+ a, CR3+ b). ZII was also heterogeneous such that there were four sagittal stripes of immunopositive Purkinje cells (P1+, P2+, P3+, P4+). Shown at a higher magnification in (D) the ZII positive axons from P3+ that course through the granular layer (indicated by the white arrows in (B)) can be seen containing the CR labelled MFs of CR3+ a. Also in (D), the CR sparse region separating CR3+ a and CR3+ b is coincident with the P4+ stripe. Scale bars: 500 μm in (A); 200 μm in (D).

as we found that most of the CR immunoreactivity was in mossy fibres in the granular layer, however the labelling of interneurons in the molecular layer was abundant (see Fig. 1B). We also found some CR+ve climbing fibres in folium VII, which likely arise from CR+ve neurons in the dorsal lamella of the inferior olive. In chicks, De Castro et al. (1998) also noted that some neurons in the rostral pole of the dorsal lamella of the inferior olive were CR+ve and retrogradely labelled from injections in folia V and VI.

The present study has extended these previous studies by showing that CR is not uniformly expressed in the granular layer. Rather CR+ve mossy fibres form sagittal stripes in the cerebellar cortex as described above. We are not aware of any previous reports demonstrating this in any species. However, it is not surprising given that mossy fibre systems terminate in sagittal stripes (Voogd et al., 1969, 2003; Ekerot and Larson, 1973; Matsushita et al., 1984; Gerrits et al., 1985; Arends and Zeigler, 1989; Matsushita et al., 1991; Akintunde and Eisenman, 1994; Ji and Hawkes, 1994).

Previously we showed that another calcium binding protein, parvalbumin (PV), is also expressed in sagittal stripes in the pigeon cerebellum, but in Purkinje cells (Wylie et al., 2011). There are stripes of Purkinje cells that strongly express PV (PV+ve), alternating with stripes where PV expression is weak (PV−ve). The relationship of PV expression with ZII expression is complimentary: PV+ve stripes were ZII−ve and vice versa. In the present study, we found a more complex relationship between CR and ZII stripes. In the posterior lobe, particularly in IXcd, CR+ve stripes were aligned with ZII+ve stripes. However, in folia II–IV of the anterior lobe, the central CR+ve stripe spanning the midline was aligned with the central ZII+ve stripe, whereas the other CR+ve stripes were aligned with the ZII−ve stripes. Why CR+ve mossy fibres would target ZII+ve stripes in some parts of the cerebellum and ZII−ve stripes is unknown.

A few other studies have investigated the relationship between mossy fibre terminal organization and zebrin expression. Gravel and Hawkes (1990) investigated the parasagittal organization of spino-cerebellar mossy fibres in relation to zebrin I (ZI) in rats (*Rattus norvegicus*). Similar to our results, they found that in some folia the mossy fibre stripes were aligned with the ZI+ stripes, whereas in other folia they were aligned with ZI− stripes. Chockkan and Hawkes (1994) recorded from neurons in the granule layer of folium IXa in rats. Units that responded to vibrissal stimulation were found in sagittal bands that spanned the ZII−ve stripes, but spread into the adjacent ZII+ve stripes. Akintunde and Eisenman (1994) noted that the cuneocerebellar fibre termination pattern in the mouse did not always align with the ZII stripes. Matsushita et al. (1991) examined the topographic relationship between zebrin stripes and the distribution of spinocerebellar fibers originating from the central cervical nucleus in the rat. They found that in lobules I–V and VIII and the copula pyramidis, the labelled MF terminals were clustered beneath ZII+ve stripes. Thus, in sum, it appears that although there is a relationship between ZII bands and sagittal mossy fibre input patterns, the relationship is not straightforward (see also Ji and Hawkes, 1994; Hallem et al., 1999).

With respect to the CR+ve mossy fibre bands in folium IXcd that are aligned with the ZII+ve stripes, there is evidence that these mossy fibres convey visual information. Pakan et al. (2010)

investigated the mossy fibre inputs from the nucleus of the basal optic root (nBOR) and the pretectal nucleus lentiformis mesencephali (LM) to folia IXcd in pigeons. nBOR and LM are retinal-recipient nuclei that are involved in the processing of optic flow that results from self-motion (Burns and Wallman, 1981; Morgan and Frost, 1981; Winterson and Brauth, 1985; Wylie and Frost, 1990; Wylie and Crowder, 2000). Pakan et al. (2010) showed that these mossy fibres terminated in sagittal bands aligned with ZII+ve stripes in IXcd. Thus, the CR+ve mossy fibres to IXcd likely originate in LM and nBOR. There is evidence in support of this assertion: about half of the neurons in LM and nBOR retrogradely labelled from injections in IXcd are also CR+ve (De Castro et al., 1998; Wylie et al., 2008; Iwaniuk et al., 2009b). Although the vast majority of nBOR mossy fibres project to IXcd (Brecha et al., 1980; Pakan and Wylie, 2006), LM projects to other folia in the posterior lobe, particularly VI–VIII and IXab (Clarke, 1977; Gamlin and Cohen, 1988; Pakan and Wylie, 2006). Thus, the CR+ve mossy fibres to these folia could arise from LM. De Castro et al. (1998) showed that neurons in the medial spiriform nucleus, a visual nucleus of unknown function that projects to folia VI–IXab in pigeons (Clarke, 1977; Wild, 1992; Pakan and Wylie, 2006), are CR+ve and labelled after injections of retrograde tracer in the cerebellum. The CR+ve fibres to these folia could therefore arise from LM and the medial spiriform nucleus. Note that none of these visual inputs to the posterior cerebellum project to folium X, where we have suggested CR immunoreactivity is comparatively weak (Fig. 2C).

But what is the significance of the visual inputs to the cerebellum showing CR immunoreactivity? This is difficult to address given that the role of CR, and indeed calcium bindings proteins in general, is largely unknown at the systems level (see reviews by Schwaller et al. (2002), Bastianelli (2003) and Schwaller (2009)). Because LM and nBOR neurons respond to optic flow that results from self-motion (Burns and Wallman, 1981; Winterson and Brauth, 1985), these neurons would be constantly active as an animal moves through the environment. Thus, calcium buffering may be critical to prevent excitotoxicity in this system, although the neuroprotective roll of CR has been downplayed in favour of a role in calcium homeostasis, and timing of pre- and post-synaptic signals for plasticity (Schwaller et al., 2002). In the posterior cerebellum, there is an integration of visual, vestibular, spinal, and oculomotor signals (for review see Voogd and Barmack, 2006) and vision is important for the maintenance and adaptive modification of eye movements (e.g., Barash et al., 1999; Lisberger et al., 1994). Perhaps the CR+ve visual fibres are critical for altering the timing of the sensorimotor integration to mediate effective adaptation.

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