

# University of Alberta

General Principles of Cerebellar Organization: Correlating Anatomy, Physiology  
and Biochemistry in the Pigeon Vestibulocerebellum

by

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## **Abstract**

The cerebellum has historically been implicated solely in motor coordination. However, we now know it is a major associative center for sensory input. For example, the vestibulocerebellum (VbC) receives a large projection from retinal recipient nuclei in the Accessory Optic System and pretectum; these nuclei analyze visual optic flow information during self-motion and ultimately function in gaze stabilization. In birds, these nuclei project to the cerebellum directly, as mossy fibres, and indirectly as climbing fibres via the inferior olive. Therefore, there are parallel pathways carrying visual information to the cerebellum; these pathways show a remarkable modular organization. In fact, the pattern of cerebellar afferent and efferent connections, the physiological response properties of Purkinje cells, and a variety of molecular markers, all provide abundant evidence that the vertebrate cerebellum is organized into parasagittal zones. This modular organization exists across several different vertebrate species, suggesting that this basic principle of organization has been evolutionarily conserved and is necessary for fundamental information processing in the cerebellum. Although recent advances have increased our understanding of cerebellar organization, the relationship between these parasagittal patterns and the functional behavior of the cerebellum remains unknown. This dissertation seeks to tie together various features of the zonal organization of the cerebellum, using molecular, electrophysiological and neuroanatomical techniques, and to

relate this organization to visual motion processing in the VbC. To investigate the parasagittal biochemical organization we use the molecular marker, zebrin (aldolase C), which is expressed by a subset of Purkinje cells, creating a striped appearance in the cerebellar cortex. We provide the first evidence of the zebrin parasagittal expression pattern in an avian species and then further elucidated the relationship between zebrin stripes and the visual afferent climbing fibre input, mossy fibre input, and the physiological zones in the VbC. There is a substantial, and growing, body of evidence that now suggests a more fundamental cerebellar architecture is built around arrays of parasagittal modules. By understanding the basic principles underlying this organization, we gain insights that may lead to a better understanding of the principles of modular organization in the central nervous system in general.

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Science... it can be a bitch, but I love it.

“If you try and take a cat apart to see how it works, the first thing you have on your hands is a non-working cat.” ~Douglas Adams~

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**List of Abbreviations:**

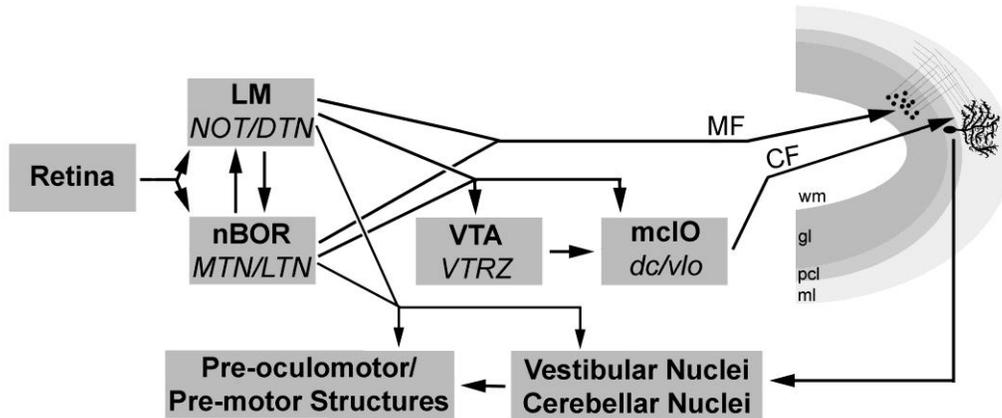
AOS	accessory optic system
Au	auricle
BDA	biotinylated dextran amine
Cb	cerebellum
CbL, CbM	lateral, medial cerebellar nucleus
CE	external cuneate nucleus
CF	climbing fibre
CP	posterior commissure
cp	cerebellar peduncle
dc	dorsal cap of Kooy
dc	dorsal cap of Kooy of the inferior olive
dl	dorsal lamella of the inferior olive
DLP	posterior dorsolateral nucleus of the thalamus
DTN, MTN, LTN	dorsal, medial, and lateral terminal nuclei
FLM	medial longitudinal fasciculus
FRL	lateral mesencephalic reticular formation
FRM	medial mesencephalic reticular formation
GCt	substantia grisea centralis
gl	granular layer of the cerebellum
GLv	ventral leaflet of the lateral geniculate nucleus
GT	tectal grey
Hsp25	heat-shock protein 25
Hy	hypothalamus
I	Folium I of the cerebellum
ICo	nucleus intercollicularis
Imc	nucleus isthmi, pars magnocellularis
IO	inferior olive
IPS	nucleus interstitio-prelecto-subprelectalis
IS	interstitial nucleus (of Cajal)
IXcd	folium IXcd of the cerebellum
LM (l, m, i)	nucleus lentiformis mesencephali, (lateral, medial, intercalated)
LP	lateral pontine nuclei
LPC	nucleus laminaris precommissuralis
mcIO	medial column of the inferior olive
MF	mossy fibre
ml	molecular layer of the cerebellum
MLd	lateral mesencephalic nucleus, pars dorsalis
MP	medial pontine nuclei
MST	middle superior temporal area

nBOR (d,p.l)	nucleus of the basal optic root (dorsal, proper, lateral)
NOT	nucleus of the optic tract
N-T	nasal-to-temporal
nVI	nucleus of the sixth cranial nerve (abducens)
NVI	sixth cranial nerve (abducens)
nX	nucleus of the vagus nerve
NXII	twelfth cranial nerve (hypoglossal nerve)
nXII	nucleus of the twelfth cranial nerve (hypoglossal)
OS	superior olive
pcl	Purkinje cell layer of the cerebellum
pl	posterolateral fissure
PPC	nucleus principalis precommissuralis
PST	tractus pretecto-subpretectalis
PT	pretectal nucleus
pvc	cerebellovestibular process
R	raphe
rH45	rotation about the vertical axis oriented 45° from midline
Rt	nucleus rotundus
Ru	nucleus ruber (red nucleus)
rVA	rotation about the vertical axis
SCE/I	stratum cellulare externum/ internum
SG	substantia gelatinosa Rolandi (trigemini)
SOp	stratum opticum
SP	nucleus subpretectalis
SpL, SpM	lateral, medial spiriform nucleus
TeO	optic tectum
TIO	tractus isthmo-opticus
T-N	temporal-to-nasal
TrO	tractus opticus (optic tract)
TT	tectothalamic tract
TTD	descending trigeminal nerve nucleus and tract
VbC	vestibulocerebellum
VeS	superior vestibular nucleus
vl	ventral lamella of the inferior olive
vlo	ventrolateral outgrowth of the inferior olive
VTA	ventral tegmental area
VTRZ	visual relay tegmental zone
wm	white matter of cerebellum
X	folium X of the cerebellum
zebrin+/-	zebrin II immunopositive, immunonegative

## Chapter 1: **Introduction**

The brain is a highly organized system that is capable of massive parallel processing across large numbers of interconnected regions, with exquisitely timed simultaneous and sequential operations. Although the individual neuron is the fundamental building block of the brain, the basic functional unit has been described as the “module” (Szentagothai, 1975; Chebkasov, 2000; Redies and Puelles, 2001; Buxhoeveden and Casanova, 2002). The concept of organized functional modules within parallel processing systems in the brain can be applied on many levels: from the organization of whole functional systems, to individual neurons forming functional and anatomical modules, such as in cortical cell columns. Many compelling examples of the modular organization within parallel processing streams can be seen in the visual system. From the specialization of photoreceptors and ganglion cells in the retina to the functional and anatomical separation of cortical visual streams, the visual system demonstrates many general principles of modular organization.

For example, on a larger systems level, there are at least three major parallel visual systems that are conserved across many species. These include the 1) geniculostriate pathway, 2) tectofugal pathway, and 3) accessory optic system (AOS) and associated pretectal pathway (Figure 1.1; see also section 1.1.2). Extensive study into the organization of the mammalian geniculostriate system has revealed parallel visual streams, each associated with specific visual functions (e.g. Livingstone and Hubel, 1988). Based on neuroanatomical, behavioural and



**Figure 1.1 Simplified wiring diagram of the afferent and efferent connections of the Accessory Optic System (AOS) and associated pretectal pathways to the cerebellum.** Avian and mammalian (in italics) nomenclature is included. LM, nucleus lentiformis mesencephali; nBOR, nucleus of the basal optic root; VTA, ventral tegmental area; mcIO, medial column of the inferior olive; VbC, vestibulocerebellum; MF, mossy fibre; CF, climbing fibre; wm, cerebellar white matter; gl, granule layer; pcl, Purkinje cell layer; ml, molecular layer; NOT, nucleus of the optic tract; DTN, dorsal terminal nucleus; MTN, medial terminal nucleus; LTN, lateral terminal nucleus; VTRZ, visual tegmental relay zone; dc, dorsal cap of Kooy of the inferior olive; vlo, ventrolateral outgrowth of the inferior olive.

neuropsychological studies, a dichotomy has been proposed for extrastriate visual cortices: the ventral (or temporal) stream processes colour and form and is related to perception and recognition of objects, whereas a dorsal (or parietal) stream processes motion and relative spatial relations and is important for visuomotor actions (e.g. Goodale and Milner, 1992; Milner and Goodale, 1993; Milner and Goodale, 2008).

In the tectofugal pathway, research in avian species has revealed similar parallel processing streams, which are also modularly organized. This is perhaps most evident in the anatomical and functional properties of the nucleus rotundus, which receives direct input from the optic tectum and is the homolog of the mammalian pulvinar nucleus (Karten and Revzin, 1966; Hunt and Kunzle, 1976; Karten and Shimizu, 1989; Mpodozis et al., 1996; Karten et al., 1997; Huang et al., 1998). Based on neurochemistry, cytoarchitecture, tectal innervation and physiology, nucleus rotundas has been divided into several subnuclei (Benowitz and Karten, 1976; Martinez-de-la-Torre et al., 1987; 1990; Mpodozis et al., 1996; Marin et al., 2003). Early electrophysiological studies showed that cells in the caudal subnuclei of the nucleus rotundus were motion sensitive (e.g. Revzin, 1979; Wang et al., 1993) and more recently cells in the rostral subnuclei were found to be responsive to, colour, luminance, simple motion, and looming (see also Wang et al., 1993; Sun and Frost, 1998). The nucleus rotundus projects to the telencephalon (Karten and Hodos, 1970) in a topographic manner (Laverghetta and Shimizu, 2003; Nguyen et al., 2004). Therefore, it has been shown that

parallel processing also occurs in the tectofugal system, with the analysis of visuomotion in caudal regions, and spatial-pattern vision and colour in rostral regions of the nucleus rotundus and telencephalic structures (Benowitz and Karten, 1976; Nixdorf and Bischof, 1982; Watanabe et al., 1986; Wang et al., 1993; Hellmann and Gunturkun, 2001; Nguyen et al., 2004).

Considerably less research has focused on the third visual system, the AOS and pretectum. This visual system is involved in various processes that analyze visual optic flow information – which is the pattern of motion that occurs across the retina as an organism moves through its environment (Gibson, 1954; see also section 1.1) – and, ultimately, this system functions to stabilize the visual image on the retina (Simpson, 1984; Simpson et al., 1988a; Simpson et al., 1988c; Cohen et al., 1992; Gamlin, 2006; Giolli et al., 2006). Visual acuity degrades when visual motion occurs across the retina, making retinal image stabilization essential for optimal visual acuity (Westheimer and McKee, 1975; Murphy, 1978; Barnes and Smith, 1981; Nakayama, 1981).

Like the other major visual pathways, the AOS and pretectal system also demonstrates principles of parallel processing and modular organization in both anatomical and functional aspects. For instance, the AOS and associated pretectal system begins with retinal ganglion cells which respond preferentially to visual stimuli that are moving in a particular direction, and at a particular speed, in a small portion of the visual field (Oyster et al., 1972). The retinal ganglion cells project to both the AOS and the pretectum, where nuclei in these regions respond

preferentially to different directions and speeds of visual motion (Karten et al., 1977; Reiner et al., 1979; Oyster et al., 1980; Fite et al., 1981; Simpson, 1984; Gamlin and Cohen, 1988b). These speed- and direction-selective responses are further integrated and modularly organized in the AOS and pretectal pathways. For instance, neurons further along this visual pathway in the cerebellum are organized in parasagittal modules or zones which respond to large, panoramic optic flow stimuli that are moving in a specific direction and at a specific speed (see section 1.1.4; Maekawa and Simpson, 1972; Simpson et al., 1979; Graf et al., 1988; Simpson et al., 1988b; Simpson et al., 1989; Wylie and Frost, 1991; Winship et al., 2005). One remarkable organizational feature of this visual system is that the axes of this direction-selectivity share common reference frames with the planes of the semicircular canals of the vestibular system and the extraocular muscles (Wylie and Frost, 1993; 1996; Wylie et al., 1998a). This allows visuovestibular convergence to take place in the cerebellum – specifically in the vestibulocerebellum (VbC) - and subsequent efferent vestibular and motor pathways to control head, neck and eye movements in order to establish gaze stabilization. Consequently, just as information is broken down into its main components (form, colour, motion, etc) in the geniculostriate and tectofugal systems and further integrated and processed in the telencephalon, various properties of movement across the retina as we travel through our environment are also modularly organized in the AOS and pretectum and further integrated and processed in the cerebellum (e.g. see section 1.3).

On a more subordinate level, the primary visual cortex is generally considered one of the best paradigms of modular organization. In seminal anatomical and physiological studies by Hubel and Wiesel (e.g. Hubel, 1959; Hubel and Wiesel, 1962; Hubel and Wiesel, 1963) a complicated organization of cortical columns was revealed. They described the basic unit of primary visual cortex: the *hypercolumn*. This consists of two *ocular dominance columns*, which are adjacent columns of cells receiving similar input from the same visual field but from either the left or right eye (Hubel and Wiesel, 1968; Wiesel et al., 1974; LeVay et al., 1975; Hubel and Freeman, 1977; Hubel et al., 1977; LeVay et al., 1980; Berman et al., 1982; Carlson et al., 1986). Organized orthogonally to the ocular dominance columns are *orientation columns* in which cells respond preferentially to lines of a particular orientation (Hubel and Wiesel, 1959; 1962; 1963; 1965; 1968; 1969). As one moves along the hypercolumn the orientation preference changes systematically (Hubel and Wiesel, 1974). Interspersed within the hypercolumns are columns of cortex that are responsive to color rather than line orientation (Hubel, 1971; Livingstone and Hubel, 1984; Hubel, 1986; Hubel and Livingstone, 1987; Livingstone and Hubel, 1988; Ts'o and Gilbert, 1988; Roe and Ts'o, 1995; 1999; Ts'o et al., 2001; Landisman and Ts'o, 2002a).

For more than 50 years since these original discoveries, the modular organization of the visual system has been established and further elucidated using anatomical and physiological methods. However, for the first 20 years of these investigations, classical morphological methods such as Nissl or myelin

stains failed to reveal any of these rich modular systems. It wasn't until the discovery by Margaret Wong-Riley (1979) of a pattern of regularly repeating *blob*-like structures in monkey striate cortex, that an aspect of this specific modular organization in the visual system could be visualized. Wong-Riley revealed this organization by staining for cytochrome oxidase (CO; Wong-Riley, 1979; Carroll and Wong-Riley, 1984; Kageyama and Wong-Riley, 1984; Wong-Riley and Carroll, 1984), which is a mitochondrial enzyme. CO *blobs* form parallel rows in cortical layers II and III in primary visual cortex and are aligned with, and centered on, the ocular dominance columns (Horton and Hubel, 1981; Landisman and Ts'o, 2002b). Using single-unit recording techniques, these blobs have since been correlated with functional aspects of colour vision (Livingstone and Hubel, 1984; Roe and Ts'o, 1995; Ts'o et al., 2001; Landisman and Ts'o, 2002a).

Since this discovery, the correlation of anatomical structures, physiological response properties and biochemistry has revealed complex, modular organizational principles in several other aspects of the geniculostriatal visual system (e.g. Kritzer et al., 1992). However, the application of these organizational principles need not be limited to the primary visual cortex. In fact, there are other brain regions that have traditionally shown a strikingly uniform morphology in Nissl staining, but a distinct underlying modular organization in anatomical and physiological investigations. The cerebellar cortex contains the same five main neuronal cell types, the same three laminae, and the same basic

circuitry throughout its extent (see section 1.2.1 and Figure 1.4). Although the basic anatomy of the cerebellar cortex has been largely established since the impressive work of Ramon y Cajal in the early 1900's (e.g. Ramon y Cajal, 1911), in the present day we know embarrassingly little about *how* the cerebellum works and, infact, what it actually does (see section 1.2.3). Early theories of cerebellar function were based on the assumption that there is structural and functional uniformity throughout the cerebellum (for a historical review see Glickstein et al., 2009); however, this conventional view of cerebellar organization has now been altered, leading to intriguing new research on the structural and functional complexity of this brain region. Researchers have realized that, far from being a homogenous, uniform structure, the cerebellum is highly organized into an array of distinct parasagittally oriented modules (i.e. in the medio-lateral dimension; see section 1.2.2; Arends and Voogd, 1989; Hawkes et al., 1993; Hawkes and Mascher, 1994; Tan et al., 1995b; Voogd et al., 1996; Hawkes, 1997; Herrup and Kuemerle, 1997; Voogd and Ruigrok, 1997; Voogd and Glickstein, 1998; Rivkin and Herrup, 2003; Pijpers et al., 2005; Ito, 2006). This modular organization has been observed anatomically, functionally, and most recently, biochemically (see section 1.2.2; for review see: Hawkes and Gravel, 1991; Hawkes, 1992; Tan et al., 1995b; Hawkes, 1997; Herrup and Kuemerle, 1997).

The pigeon VbC offers an exciting opportunity in which to study all aspects of cerebellar modular organization. Anatomically, the parasagittal

organization of the afferent climbing fibre input from the inferior olive to the VbC has been well documented (see section 1.3.1; Wylie et al., 1999c; Crowder et al., 2000; Pakan et al., 2005) and the topography has been confirmed with single-unit recording (Winship and Wylie, 2001). It is known that the major source of visual mossy fibre afferents to the VbC arises from the AOS and pretectum – nuclei that have been extremely well characterized (see section 1.1.4; see also Chapter 5; Wylie and Frost, 1990; Wylie and Frost, 1999b; Wylie and Crowder, 2000; Crowder and Wylie, 2001; Crowder and Wylie, 2002; Crowder et al., 2003b; Winship et al., 2006a). Functionally, as mentioned above, the visual response properties of the pigeon VbC differ with respect to the direction of visual optic flow and it has been shown that these response properties are organized into parasagittal zones (Wylie and Frost, 1991; Wylie et al., 1993; De Zeeuw et al., 1994; Wylie et al., 1994; Lau et al., 1998; Wylie et al., 1999c; Crowder et al., 2000; Winship and Wylie, 2003; Voogd and Wylie, 2004; see also Chapter 4). In fact, there is no other avian cerebellar system in which the response properties have been outlined in such detail (see section 1.3). Biochemically, the recent discovery of molecules that are expressed non-uniformly throughout the cerebellum, such as the metabolic enzyme aldolase C (zebrin; see section 1.2.2; Brochu et al., 1990), provide exciting opportunities to investigate the principles of the underlying cerebellar architecture by correlating the well known anatomical and functional organization of the pigeon VbC with novel molecular markers (see section 1.3.2). Like the formative studies in the geniculostriate pathway and

primary visual cortex, the cerebellum affords a unique opportunity to study principles of modular organization, which may lead to a better understanding of the principles of organization in the central nervous system in general.

This dissertation discusses the anatomical, functional and biochemical properties related to visual processing in the pigeon cerebellum. Anatomical tract tracing, electrophysiological recording techniques and immunohistochemistry are used to show that the apparently uniform cytoarchitecture of the cerebellum conceals a very elaborate underlying modular organization.

## **1.1 Optic Flow Processing in the Brainstem and Cerebellum**

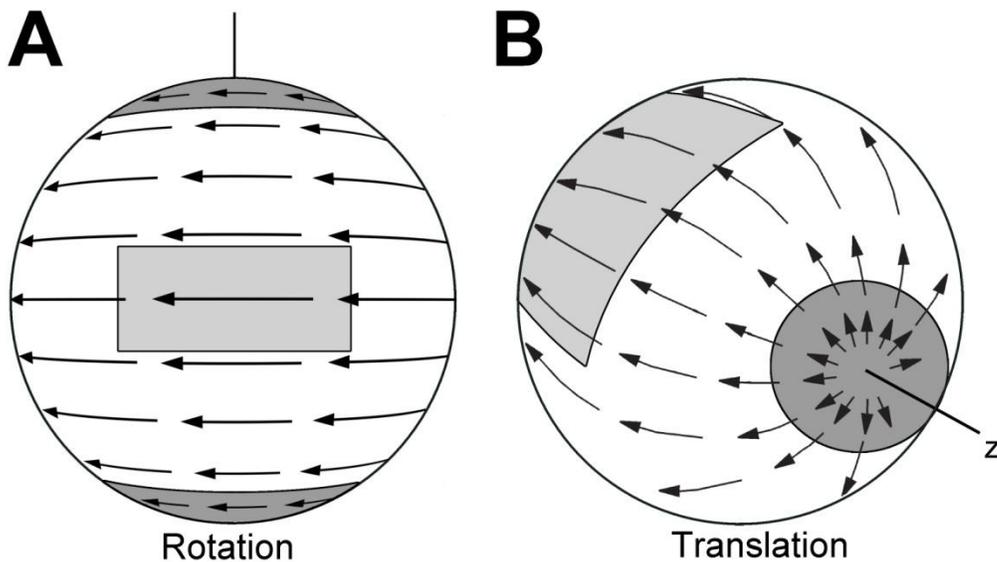
### **1.1.1 *Introduction to Optic Flow***

As organisms move through their environment distinct patterns of motion occur across the entire retina; this whole-field visual motion is known as optic flow (Gibson, 1954). Through the analysis of optic flow, a visual system can act as a proprioceptive sense, providing information about the animal's own self-motion relative to the surrounding environment. Specific patterns of optic flow provide information to an organism about its current position, speed, and trajectory through the environment (Gibson, 1966; Lee, 1980; Lishman, 1981; Koenderink and van Doorn, 1987; Harris and Rogers, 1999; Lappe et al., 1999; Warren et al., 2001; Collett, 2002; Frenz et al., 2003; Baumberger and Fluckiger, 2004; Galbraith et al., 2005; Nomura et al., 2005). This pattern of motion

occurring across the retina can be represented as a sphere surrounding the observer (a flow-field); Figure 1.2 shows two examples that depict the direction of motion across the retina created by self-motion. Figure 1.2A shows that head rotation to the left creates rightward optic flow across the equator (light grey) and circular optic flow would be present at the poles (dark grey). Figure 1.2B shows the pattern of optic flow created by forward translation. There is an expanding motion pattern in front of the observer with motion vectors that radiate outward from a *focus of expansion*. At the equator of this flow-field, the motion vectors are parallel to one another (creating laminar flow), and point backward relative to the observer. Directly behind the observer, the motion vectors converge at a *focus of contraction*.

A natural suggestion might be that the visual system could use this *focus of expansion* directly, to determine heading by analyzing the global optic flow structure. However, the neurophysiological analysis of optic flow is not so simple. Most naturally occurring optic flow patterns contain both translational and rotational components and, because the anatomy upon which the retina is placed is not fixed in space (e.g. eyeball, head, and torso can all move independently during self-motion; van den Berg, 2000), any natural self-motion might be composed of eye, head, or body movements that have different effects on the retinal image.

Optic flow-fields provide information vital for the generation of visuomotor behaviours, including the compensatory eye movements and head



**Figure 1.2** Optic flow-fields generated by self-rotation and self-translation. Arrows indicate the motion vectors in the optic flow-field. The diagram in **A** consists of a circular flow-field rotating about a vertical axis. The flow-field motion is opposite to the direction of head rotation. The flow-field in **B** is produced by forward translation along an axis marked 'z'. At the 'pole' (shaded dark grey) in the direction of translation, the arrows diverge from a point; the focus of expansion. Likewise, at the opposite pole (not shown) the vectors would converge to a point; the focus of contraction. At the "equator" (shaded light grey) of the sphere, the flow-field is laminar, with all vectors pointing in approximately the same direction.

movements necessary for retinal image stabilization and to control orientation and locomotion through the environment (Gibson, 1950). Specifically, neurophysiological systems can analyze optic flow in order to provide information on heading of self-motion, time to collision, object motion and object segmentation (Andersen and Atchley, 1997; Andersen, 1997; Lappe et al., 1999), the control of posture and locomotion (Warren et al., 2001), perception of egomotion, generation of the optokinetic response to facilitate gaze stabilization (Waespe and Henn, 1987) and path integration, a form of navigation (Kearns et al., 2002).

In vertebrates, numerous studies utilizing micro-stimulation, lesion, and electrophysiological methods have implicated the AOS and associated pretectal pathway, oculomotor nuclei, vestibular nuclei, as well as the inferior olive and the VbC, in the analysis of optic flow and the generation of optokinetic responses (for reviews see Simpson, 1984; Graf et al., 1988; Simpson et al., 1988c; Grasse and Cynader, 1990)

### ***1.1.2 Anatomy and Physiology of the AOS and Pretectum***

The anatomy of nuclei in the AOS and pretectum, and the role they play in the analysis of the visual consequences of self-motion, has been delineated in numerous mammalian and non-mammalian vertebrate species (see Figure 1.1; for review see Simpson, 1984). Briefly, in mammals the medial, lateral, and dorsal

terminal nuclei (MTN, LTN, and DTN, respectively) of the AOS receive direct retinal projections, as do optic flow sensitive neurons in the associated nucleus of the optic tract (NOT) of the pretectum (found immediately adjacent to DTN; Oyster et al., 1980; Ballas et al., 1981; Farmer and Rodieck, 1982; Weber, 1985). As in mammals, retinal recipient nuclei within the AOS and in the associated pretectum have been identified in birds. Within the AOS, the nucleus of the basal optic root (nBOR) receives direct retinal input from displaced ganglion cells in the retina (Karten et al., 1977; Reiner et al., 1979; Fite et al., 1981) and can be divided into three subdivisions: nBOR dorsal, lateral, and proper (nBORd, nBORl, and nBOR, respectively; Brecha et al., 1980). In the pretectum, the nucleus lentiformis mesencephali (LM) receives retinal input from ganglion cells in the ganglion cell layer proper and can be subdivided into two subnuclei, the LM pars lateralis and pars medialis (LMl and LMm, respectively; Gamlin and Cohen, 1988a; b).

The visual response properties of AOS and pretectal neurons have been examined in almost every vertebrate class. Across species, AOS and pretectal neurons have extremely large receptive fields, and exhibit direction-selectivity to large-field visual stimuli moving in the contralateral visual field (salamanders: Manteuffel, 1982, 1984; frogs: Cochran et al., 1984; Gruberg and Grasse, 1984; turtles: Rosenberg and Ariel, 1990; rabbits: Collewyn 1975; Maekawa et al., 1984; Simpson et al., 1979; rats: Natal and Britto, 1987, 1988; cats: Hoffmann and Schoppmann, 1981; Grasse and Cynader, 1984, 1990; opossum: Volchan et

al., 1989; monkey: Hoffmann et al., 1988; Mustari and Fuchs, 1989; Westheimer and Blair, 1974; chicken: Burns and Wallman, 1981; McKenna and Wallman, 1981, 1985b; pigeon: Britto et al., 1981; Morgan and Frost, 1981; Winterson and Brauth, 1985; Gioanni et al., 1984; Wylie and Frost, 1990a, 1999; Wolf-Oberhollenzer and Kirschfeld, 1994; Wylie, 2000; wallaby: Ibboston et al., 1994; Ibbotson and Price, 2001).

Although broadly tuned, most nBOR and LM neurons are maximally excited in response to motion in the *preferred* direction and strongly inhibited in response to motion in the opposite, *anti-preferred* direction (Winterson and Brauth, 1985; Wolf-Oberhollenzer and Kirschfeld, 1994; Wylie and Frost, 1996; 1999b; Wylie, 2000; Wylie and Crowder, 2000; Crowder and Wylie, 2002). Most LM neurons prefer forward (temporal-to-nasal) motion, with fewer neurons preferring up, down, and backward (nasal-to-temporal) motion (McKenna and Wallman, 1985; Winterson and Brauth, 1985; Fite et al., 1989; Fan et al., 1995; Wylie and Crowder, 2000). Interestingly, most neurons in nBOR prefer up, down or backward motion, while few prefer forward (Burns and Wallman, 1981; Morgan and Frost, 1981; Gioanni et al., 1984; Rosenberg and Ariel, 1990; Wylie and Frost, 1990). The nBOR is topographically organized in terms of direction preference, with up cells in the dorsal portion of the nucleus, down cells located ventrally compared to up cells, back cells along the most ventral and lateral surface of the nBOR, and forward cells in the posterior-dorsolateral margin of the nucleus (Burns and Wallman, 1981; Wylie and Frost, 1990). A consistent

functional topographical organization for the LM has yet to be discovered (see Chapter 2).

It is believed that the AOS and pretectum play a major role in the control of optokinetic nystagmus (OKN), which involves eye movements with an alternation of slow pursuit in one direction and a quick return in the opposite direction in order to stabilize a moving image on the retina. Lesions to the AOS or pretectum severely impair OKN, while lesions to geniculostriate or tectofugal structures leave OKN relatively unaffected (Gioanni et al., 1983a, b; Simpson, 1984; McKenna and Wallman, 1985; Simpson et al., 1988c). In birds, nBOR lesions abolished vertical OKN and monocular horizontal OKN in response to backward motion (Wallman et al., 1981; Gioanni et al., 1983b) and LM lesions abolished monocular horizontal OKN in response to forward motion (Gioanni et al., 1983a). These lesion results correspond well with the visual response properties of neurons in the nBOR and LM: as mentioned previously, most neurons in the nBOR prefer up, down, or backwards optic flow, whereas most LM neurons prefer forward optic flow.

### ***1.1.3 Efferent Projections of the AOS and Pretectum***

The efferent projections of the nBOR complex have been mapped extensively in pigeons using a variety of anterograde/retrograde tracers (Brecha et al., 1980; Casini et al., 1986; Wylie et al., 1997; Wylie, 2001; Wylie et al., 2007).

Brecha et al. (1980) provided a detailed investigation into the efferent projections of nBOR. Briefly, they found that anterograde injections into the nBOR complex demonstrate prominent bilateral projections upon the cerebellum (folium IV-VIII and IXcd), the medial column of the inferior olive (mcIO), the oculomotor nuclear complex (nucleus of cranial nerve III), and the nucleus interstitialis of Cajal, as well as a projection to the contralateral nBOR complex and a major ipsilateral projection upon the LM (Brecha et al., 1980). These projections have been confirmed using retrograde tracers and previously unreported bilateral projections have been identified to the nucleus Darkshewitsch, the red nucleus, the mesencephalic reticular formation, and the ventral tegmental area (VTA), as well as ipsilateral projections to the central grey, the pontine nuclei, the cerebellar and vestibular nuclei of the brainstem, the processus cerebellovestibularis, and the dorsolateral thalamus (Wylie et al., 1997; Wylie et al., 2007). Generally, the largest projections from nBOR were to the ipsilateral LM, the mcIO, and bilaterally to the VbC. The projection from nBOR to the ipsilateral LM has been observed to terminate mainly in LMI (Brecha et al., 1980; Wylie et al., 1997) and the projection to the mcIO has been observed to terminate mainly in rostral regions of the mcIO (Wylie, 2001). Wylie et al. (1997) suggested that projections from nBOR to the oculomotor complex, vestibular nuclei, cerebellar nuclei, VbC, LM, inferior olive, pontine nuclei, and interstitial nucleus of Cajal contribute to oculomotor function, projections to the interstitial nucleus of Cajal, reticular formation, and red nucleus contribute to opto-collic reflexes, and projections to

the interstitial nucleus of Cajal, reticular formation, red nucleus, nucleus Darkschewitsch, and central grey participate in the control of posture and locomotion.

Like nBOR, the pretectal nucleus LM also sends the majority of its efferent projections to pre-oculomotor structures (see Figure 1.1). Gamlin and Cohen (1988a) investigated the efferent projections of the LM using anterograde autoradiographic and retrograde tracer techniques in pigeons and found that projections were primarily ipsilateral and caudal. They described a projection from large, multipolar neurons in LM to folia VI through IX of the cerebellum; however, the specific topographical organization of this mossy fibre projection was not reported (see Chapter 5). Other efferent targets from neurons in LM include the mcIO, nBOR, VTA, lateral pontine nucleus, pedunculo-pontine tegmental nucleus, nucleus principalis precommissuralis, and the stratum cellulare externum. Wylie et al. (1998b) reported a projection from LM to the dorsolateral thalamus and a direct projection from the VTA to the hippocampal formation has also been reported in pigeons (Casini et al., 1986; Wylie et al., 1999a; Winship et al., 2006b).

Previous research has shown that neither nBOR nor LM can be regarded as a homogeneous nucleus, but instead consist of morphologically distinct neurons with differential distributions and projection patterns (Brecha et al., 1980; Gottlieb and McKenna, 1986; Zayats et al., 2002; Zayats et al., 2003; Pakan et al., 2006; Wylie et al., 2007; see also Chapter 2). Using double-retrograde labeling

with fluorescent tracers, Wylie et al. (2007) emphasized that the inferior olive- and VbC-projecting nBOR neurons differ with respect to size, morphology, and distribution in the nBOR complex. The VbC-projecting neurons are large multipolar neurons found throughout nBOR (Brecha et al., 1980; Wylie et al., 2007); in contrast, the inferior olive-projecting cells are much smaller in size, fusiform in shape, and localized to the dorsal margin of nBOR and the adjacent VTA (Brecha et al., 1980; Wylie, 2001; Wylie et al., 2007). Using injections of retrograde tracer into known LM projection sites, Pakan et al. (2006) found that large multipolar neurons in LM project only to the cerebellum, whereas a thin band of medium sized fusiform neurons project to the inferior olive, and small neurons project to nBOR and the dorsal thalamus. It has also been proposed that morphologically distinct neurons in LM and nBOR have differing electrophysiological properties and are associated with different functions (Tang and Wang, 2002a; b; Pakan et al., 2006; Winship et al., 2006a).

Therefore, previous research has shown that the AOS and associated pretectum have morphologically distinct cell populations that project in parallel fashion to various brain regions with potentially distinct functional consequences. However, aspects of the organization of these parallel projections have not yet been fully established. The remainder of this dissertation will focus on various organizational features of these visual pathways from the AOS and pretectum to the cerebellum.

#### 1.1.4 *Optic Flow Pathways to the Cerebellum*

A simplified wiring diagram of the AOS and pretectal pathways to the cerebellum is shown in Figure 1.1. As mentioned above, previous research in birds has shown that neurons in LM and nBOR provide direct mossy fibre projections to folia VI-VIII and IXcd and indirect olivocerebellar projections to the VbC (folia IXcd and X) via the mcIO (Clarke, 1977; Brecha et al., 1980; Gamlin and Cohen, 1988a; Wylie et al., 1997; Lau et al., 1998; Winship and Wylie, 2003). Therefore, visual optic flow information reaches the cerebellum through various routes in the pigeon.

The *direct* mossy fibre pathways from LM and nBOR to the cerebellum are not found in all vertebrates. Similar to pigeons, direct mossy fibre pathways from the AOS and pretectum to the VbC have been reported in turtles and fish, but not frogs (fish: Finger and Karten, 1978; turtle: Reiner and Karten, 1978; frogs: Montgomery et al., 1981; Weber et al., 2003). In mammals, there has been no report of a mossy fibre pathway from the NOT to the cerebellum, but a mossy fibre projection from the MTN to the VbC has been reported in some species (chinchilla: Winfield et al., 1978; tree shrew: Haines and Sowa, 1985), but not others (cats: Kawasaki and Sato, 1980; rats and rabbits: Giolli et al., 1984). There is evidence of several *indirect* mossy fibre pathways from NOT and the AOS to the cerebellum through which optic flow information can be conveyed. Most of the mossy fibre input to the VbC arises in the vestibular nuclei and the prepositus hypoglossi (Voogd et al., 1996; Ruigrok, 2003), but there are also projections

originating in the reticular formation, the raphe nuclei, a number of pontine regions, and neurons located within and around the medial longitudinal fasciculus (Blanks et al., 1983; Sato et al., 1983; Gerrits et al., 1984; Langer et al., 1985b; Mustari et al., 1994; Voogd et al., 1996; Nagao et al., 1997a; Ruigrok, 2003). The NOT and AOS project to many of these structures, including the vestibular nuclei, the medial and dorsolateral nuclei of the basilar pontine complex, the mesencephalic reticular formation, the prepositus hypoglossi, and the nucleus reticularis tegmenti pontis (NRTP; Itoh, 1977; Terasawa et al., 1979; Cazin et al., 1982; Holstege and Collewijn, 1982; Giolli et al., 1984; 1985; Torigoe et al., 1986b; a; Giolli et al., 1988; for review see Simpson et al., 1988a; Gamlin, 2006; Giolli et al., 2006). In mammals, information from the NOT also reaches folium VII of the oculomotor vermis via the dorsal, medial and dorsolateral pontine nuclei and the nucleus reticularis tegmenti pontis (Torigoe et al., 1986b; Yamada and Noda, 1987; Thielert and Thier, 1993; Voogd and Barmack, 2006).

In mammals and birds, visual optic flow pathways from the AOS and associated pretectum to the VbC are involved in generating compensatory eye movements (Ito et al., 1974; Miles and Lisberger, 1981; Simpson, 1984; Waespe and Henn, 1987; Nagao et al., 1991; Wylie et al., 1998a). The function of folia VI-VIII in pigeons has not been extensively investigated, however, these folia receive input from a tecto-pontine system (Clarke, 1977), which is implicated in avoidance behavior (Hellmann et al., 2004). In mammals, folia VI-VIII, in particular folium VII, are referred to as the “oculomotor vermis” and have been

linked to saccades and pursuit eye movements (for review see Voogd and Barmack, 2006). The organization and potential function of these optic flow projections to the cerebellum is the focus of Chapter 2.

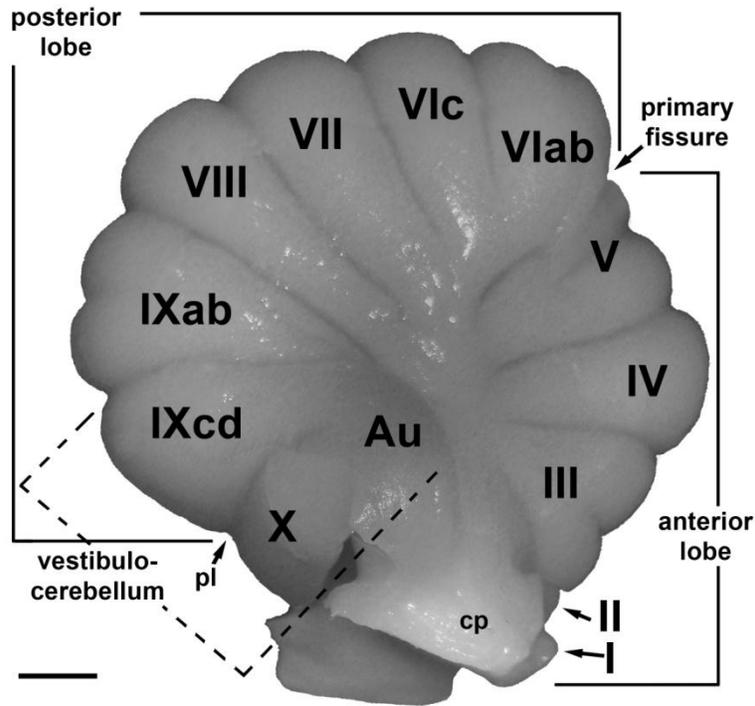
In birds, the AOS and pretectum provide large *indirect* projections to the mcIO, which, in turn, projects as climbing fibres to Purkinje cells in the VbC (Brecha et al., 1980; Gamlin and Cohen, 1988a; Wylie et al., 1997). Similarly, the nuclei of the AOS and the pretectal NOT of mammals provide a major input to the olivo-vestibulocerebellar pathway. NOT has been shown to provide a direct projection to the dorsal cap of Kooy (dc) and ventrolateral outgrowth (vlo) of the inferior olive (which are homologous to the avian mcIO; Takeda and Maekawa, 1976; Giolli et al., 1984; Giolli et al., 1985; Blanks et al., 1995). This region of the inferior olive then provides climbing fibre input to Purkinje cells in the VbC (Gerrits and Voogd, 1982; Hess and Voogd, 1986; Voogd et al., 1987a; Voogd et al., 1987b; Tan et al., 1995a; Sugihara et al., 2004; Sugihara and Shinoda, 2004). However, the projection from the AOS nuclei to the dc is primarily indirect. AOS nuclei project to a group of neurons in the VTA designated as the visual tegmental relay zone (VTRZ). In turn, AOS-derived input to the dc arises from the VTRZ (see Figure 1.1; Maekawa and Takeda, 1979). Wylie et al. (1999a) proposed that VTRZ may be a mammalian analogue of nBORD.

## **1.2 Functional Organization of the Cerebellum**

The cerebellum literally means “little brain”, yet some estimates suggest that cerebellar granule cells comprise up to 85% of the total number of neurons in the brain (Lange, 1975). The neuronal structure of the cerebellum is very highly conserved across species (Larsell and Jansen, 1967; Larsell, 1970) and the anatomical and functional organization of the cerebellum is now recognized to be far more precise and complex than its morphology would suggest.

### **1.2.1 *Basic Structure and circuitry of the Cerebellum***

The cerebellum has been described as a “nest of paradoxes” (Herrup and Kuemerle, 1997), but even though its ultimate function may be mysterious, the basic structure of the cerebellum has been known for decades. A pioneering neuroanatomist, Lodewijk Bolk compared the structure of the cerebellum in 69 different mammals (Bolk, 1906). He identified a common plan in (virtually) all of them and described the major neuroanatomical divisions of the cerebellum in detail; many of his descriptive terms are still used today (for review see Glickstein et al., 2009). Figure 1.3 shows the main gross anatomical features of the avian cerebellum. In the rostral-caudal direction, three major subdivisions of the cerebellum are recognized: the anterior lobe (rostral to the primary fissure), the posterior lobe (caudal to the primary fissure), and the flocculonodular lobe (caudal to the posterolateral fissure). These anatomical divisions have functional significance, since the primary modalities subserved by these regions are



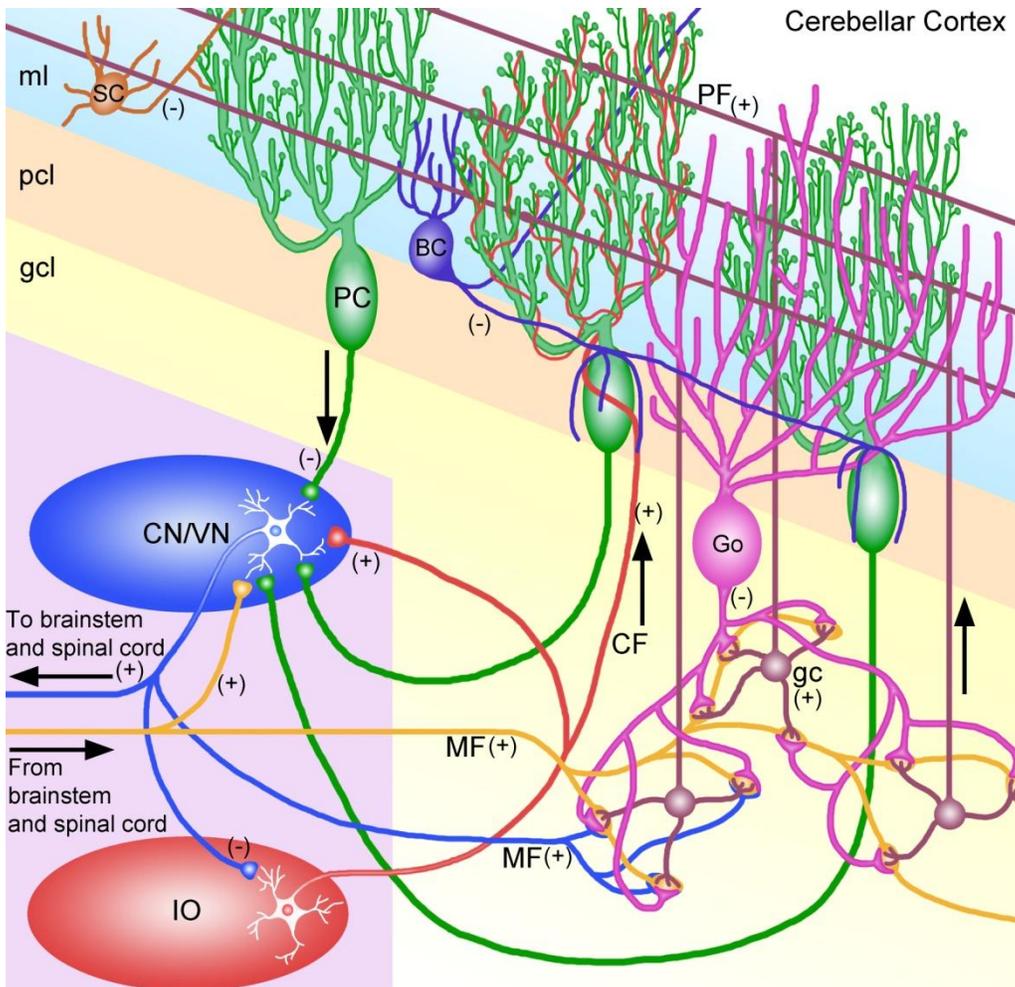
**Figure 1.3 Lateral view of the pigeon cerebellum.** The folia are numbered I-X (anterior to posterior) according to the nomenclature of Larsell (1967). Folia I and II are hidden behind the cerebellar peduncle (cp). Folia I-V comprise the anterior lobe which is separated from the posterior lobe (folia VI-IX) by the primary fissure. The posterior lobe is separated from folium X (nodulus) by the posterolateral fissure (pl). The vestibulocerebellum includes folia IXcd (ventral uvula) and X, which merge laterally and form the auricle (Au). Scale bar = 1mm.

different. The flocculonodular lobe is most closely associated with vestibular function; the anterior and posterior vermis are associated with spinocerebellar function (somatosensory); and the mammalian hemispheres are associated with “higher” functions, typically described as motor planning. In contrast to the mammalian cerebellum, the avian cerebellum consists primarily of a vermis, and the presence of a homolog of the mammalian hemispheres is contentious (e.g. Larsell, 1948; Larsell and Whitlock, 1952; Whitlock, 1952; see also Chapter 3).

The cerebellum is highly fissured in birds, mammals and some fish. These transverse fissures divide the cerebellum into lobules (generally used in reference to mammals) or folia (used in reference to avian species). Larsell distinguished ten folium in the vermis, depicted by the roman numerals I–X (see Figure 1.3). Folia I–V comprise the anterior lobe, folia VI–IX comprise the posterior lobe, and folia X is considered the flocculonodular lobe (Larsell and Whitlock, 1952; Larsell, 1967). Folia IXcd (uvula) and X comprise the vestibulocerebellum and merge laterally to form the auricle in birds (Voogd and Wylie, 2004).

The cerebellar cortex consists of three layers: the superficial molecular layer, a monolayer of Purkinje cells, whose dendrites extend up into the molecular layer, and the granular layer. There are only a few main cell types in the cerebellar cortex that comprise a single repetitive circuit that has been known since Ramon y Cajal (1911): granule cells; Purkinje cells and inhibitory interneurons (Golgi, stellate, and basket cells). Ito (1984) describes the basic anatomy, circuitry and functional properties of the cerebellar cortex in exquisite detail; what follows is a

brief description of this basic cerebellar circuitry, which is also illustrated in Figure 1.4. The cerebellar granule cells are widely held to be the most abundant class of neurons in the human brain (Lange, 1975). Axons of the granule cells ascend to the molecular layer of the cerebellar cortex and then bifurcate to form T-shaped branches called parallel fibres (because they run parallel to the long axis of the cerebellar folia) that relay information via excitatory synapses onto the dendritic spines of the Purkinje cells. Axons from a variety of brainstem, pontine, and spinal cord neurons project to the granular layer as mossy fibres - aptly named so because of the appearance of their synaptic terminals. Fine branches of the mossy fibre axons twist through the granule cell layer, and slight enlargements referred to as rosettes, give a knotted appearance indicating synaptic contacts. The Purkinje cells present the most striking histological feature of the cerebellum. Elaborate dendrites extend into the molecular layer from a monolayer of these giant cell bodies (Purkinje cell layer). Once in the molecular layer, the Purkinje cell dendrites branch extensively at right angles to the trajectory of parallel fibres (i.e. in the parasagittal plane). Therefore, each Purkinje cell is in a position to receive input from a large number of parallel fibres, and each parallel fibre can contact a large number of Purkinje cells (on the order of tens of thousands). In this way, the Purkinje cells receive indirect input from mossy fibre projections. Purkinje cells also receive direct modulatory input on their proximal dendritic shafts from climbing fibres, all of which arise in the inferior olive. Each Purkinje cell receives numerous synaptic contacts from a single climbing fibre.



**Figure 1.4 Representative neuronal components and connections of the cerebellar cortex.** Arrows indicate the direction of information flow. (+) and (-) signs represent excitatory and inhibitory synapses, respectively. BC, basket cell (purple); CF, climbing fibres (red); CN/VN, cerebellar nuclei and vestibular nuclei (blue); Go, Golgi cell (pink); gc, granule cell (maroon); gcl, granule cell layer; IO, inferior olive (red); MF, mossy fibre (orange/blue); ml, molecular layer; PC, Purkinje cells (green); pcl, Purkinje cell layer; PF, parallel fibres (maroon); SC, stellate cell (brown).

Purkinje cells project predominately to the deep cerebellar nuclei, although those in the flocculonodular lobe also project directly to the vestibular nuclei. Purkinje cells are the only output cells of the cerebellar cortex. Since these cells are GABAergic, the output of the cerebellar cortex is wholly inhibitory. However, the deep cerebellar nuclei also receive excitatory input from collaterals of the mossy and climbing fibres (shown in Figure 1.4). Therefore, there is constant, or tonic, excitation of neurons in the cerebellar and vestibular nuclei and Purkinje cell inhibitory projections serve to modulate the level of this excitation. Inputs from interneurons also modulate the inhibitory activity of Purkinje cells and occur on both dendritic shafts and the cell body. The most powerful of these local inputs are inhibitory complexes of synapses made around the Purkinje cell bodies by basket cells. Stellate cells receive input from the parallel fibres and provide an inhibitory input to the Purkinje cell dendrites. Golgi cell bodies are located in the granular cell layer and their apical dendrites are located in the molecular layer. The Golgi cells receive input from the parallel fibres and provide an inhibitory feedback to the cells of origin of the parallel fibres (the granule cells). Therefore, there are many potential feedback loops within the circuitry of the cerebellar cortex itself, as well as within the afferent and efferent projections patterns between the deep cerebellar and vestibular nuclei, and the inferior olive. This potential for feedback has been a driving force behind the theory that the cerebellum (and olivocerebellar system specifically) functions in the fine tuning of movements and motor coordination by providing error signals and/or precise

timing information during sensorimotor behaviours (e.g. Albus, 1971; Fujita, 1982; Strehler, 1990; Paulin, 1993; Bower, 1997; Braitenberg et al., 1997; Mauk and Donegan, 1997; Schweighofer et al., 1998). The histological structure of the cerebellum is virtually identical in all mammals and birds (Pearson, 1972). The structure of these connections is so invariant that Ramon y Cajal (1911) proposed it to be a “law of biology”.

### ***1.2.2 Modular Organization of the Cerebellum***

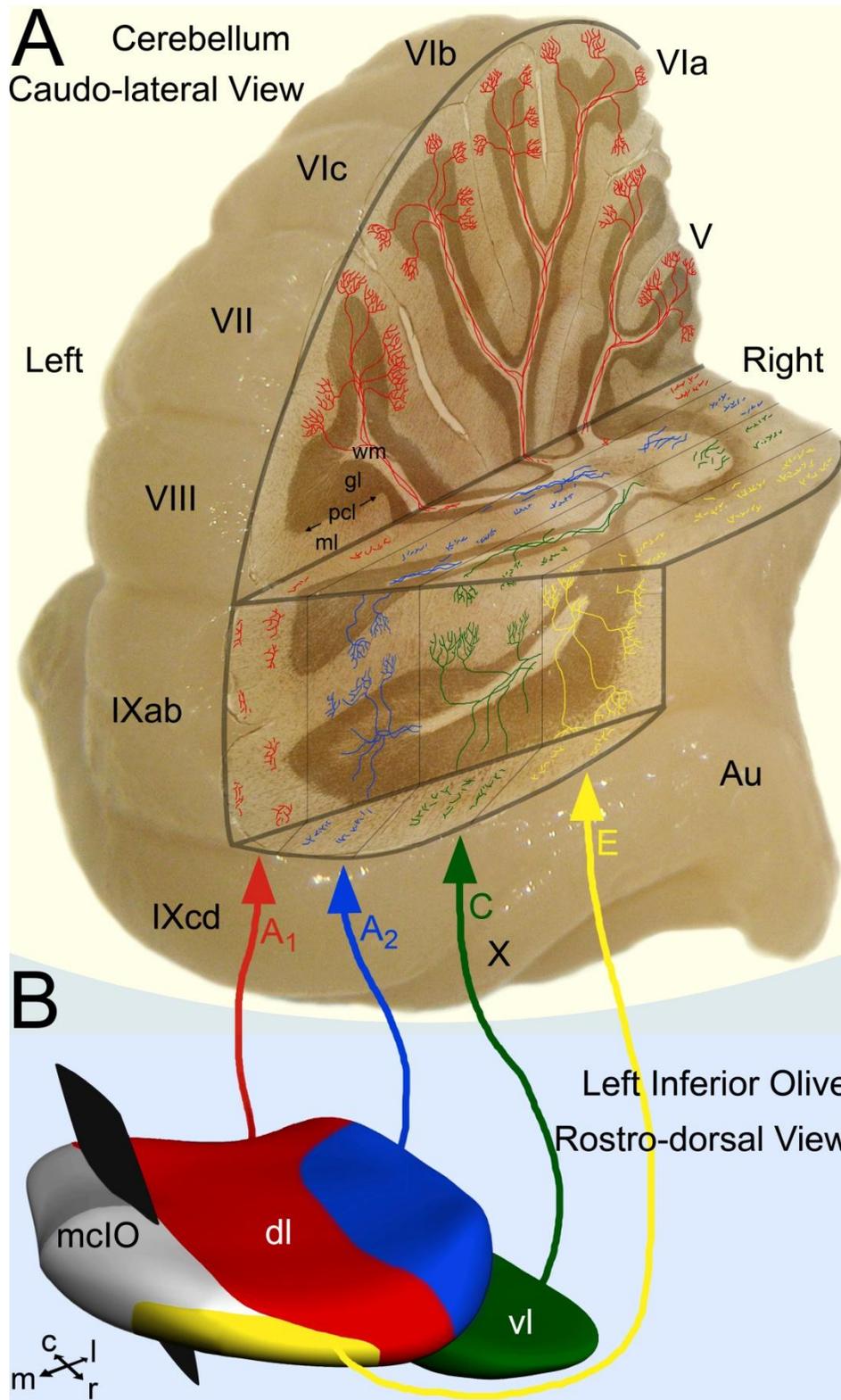
Although the neuronal circuitry is uniform across the entire cerebellar cortex, a fine modular organization is an essential functional feature of the cerebellum; this is exhibited in many aspects in the cerebellum. The transverse, or rostro-caudal lobular organization of the cerebellum was investigated quite extensively in classic anatomy (for review see: Voogd, 1975; Glickstein and Voogd, 1995; Herrup and Kuemerle, 1997; Glickstein et al., 2009). On the other hand, fibre degeneration studies performed by Jansen and Brodal in the 1940’s were probably the first to hint at the significance of the parasagittal component of cerebellar organization (Jansen and Brodal, 1940; Jansen and Brodal, 1942). However, it wasn’t until 30 years later that extensive work by Voogd and colleagues extended these observations and showed that there was a parasagittally banded organization of the afferent systems (i.e. oriented perpendicular to the long axis of the folia; see Figure 1.5), which applied to the mossy fibres as well as

to the climbing fibres (Verhaart and Voogd, 1962; Voogd, 1967; Voogd et al., 1969; Freedman et al., 1975; Kunzle, 1975; Groenewegen and Voogd, 1976; 1977; Groenewegen et al., 1979; Voogd and Bigaré, 1980; Voogd et al., 1981; Gerrits et al., 1984; Marani et al., 1986; Voogd et al., 1987b; Arends and Voogd, 1989; Voogd, 1992; Voogd and Ruigrok, 1997). Voogd also described alternating bundles of large and small diameter axons in the cerebellar white matter of numerous species, indicative of a longitudinal parcellation of cerebellar afferents. On the basis of the myeloarchitecture, a reproducible array of parasagittal compartments was proposed. Since then, it has become well established that olivocerebellar projections and several mossy fibre projections terminate in the cerebellum in the form of parasagittal bands (for review see: Herrup and Kuemerle, 1997; Ozol and Hawkes, 1997; Voogd and Ruigrok, 1997).

An example of the well established parasagittal organization of climbing fibre projections in birds is shown in Figure 1.5. This organization was revealed by Arends and Voogd (1989) using both anterograde and retrograde anatomical tract tracing methods. A third aspect of this modular organization in the cerebellum is revealed with respect to the corticonuclear projections. Purkinje cells within parasagittal zones of climbing fibres project to different region in the deep cerebellar and vestibular nuclei (Arends and Zeigler, 1991; Wylie et al., 1999b; Pardoe and Apps, 2002; Wylie et al., 2003a; Wylie et al., 2003b; Voogd and Ruigrok, 2004). Finally, Oscarsson (1969) was one of the first to produce electrophysiological evidence that the projections from the inferior olive to the

anterior lobe of the cerebellum were organized into discrete parasagittal bands of axons. Since then, detailed electrophysiological experiments have revealed the parasagittal organization of complex spike activity (CSA) of Purkinje cells in various regions of the cerebellum (Robertson and Laxer, 1981; Bloedel and Kelly, 1991; Garwicz, 2000), implying that the cerebellar parasagittal zones are basic units of cerebellar function.

Studies have shown that these cerebellar zones can be further divided into *microzones*. These *microzones* are 0.1 to 0.3mm wide, sagittally oriented cortical strips that receive climbing fibre inputs with similar receptive fields (Andersson and Oscarsson, 1978; Ito et al., 1982; Ekerot et al., 1991; Schweighofer, 1998; Sugihara et al., 2003; Sugihara, 2005; Herrero et al., 2006). Ensembles of functionally related *microzones* have been proposed to constitute the operational unit, or *module*, of cerebellar systems. (Oscarsson, 1979; Ito, 1982; Gerrits et al., 1985; Welker, 1990; Garwicz and Ekerot, 1994; Ekerot et al., 1995; Feirabend et al., 1996; Ekerot et al., 1997; Herrup and Kuemerle, 1997; Garwicz et al., 1998; Garwicz, 2000; Garwicz et al., 2002; Pijpers et al., 2005). Each *module* is proposed to consist of one or several *microzones* projecting onto a common efferent cell group in the deep cerebellar nuclei. By definition, a *module* receives homogeneous climbing fibre input from a specific motor region, and in turn, controls a specific motor output system (e.g. Garwicz, 2000). In recent research by Hawkes and colleagues, and in this dissertation, the general term “modular organization” is used in a more liberal sense to represent the



**Figure 1.5 Parasagittal organization of climbing fibre afferents from the inferior olive to the cerebellar cortex.** **A** shows a pigeon cerebellum from a caudo-lateral view. A midsagittal cut has been made through folia VIII-V in order to expose the medial aspect of the cerebellum (right side). Another cut has been made approximately 45° to the coronal plane in order to expose the internal portions of folium IXab. A Nissl stained sagittal section through the cerebellum was then overlaid onto the exposed folia as a schematic so that the layers of the cerebellar cortex could be differentiated (gl, granular layer; ml, molecular layer; pcl, Purkinje cell layer; wm, white matter). The parasagittal orientation of the climbing fibre projections from the inferior olive are shown as a schematic. The various colors represent projections from different regions of the inferior olive, shown on a 3-D schematic of this nucleus in **B**; the left (contralateral) inferior olive is shown, viewed from a rostro-dorsal angle. The inferior olive is made up of a dorsal lamella (dl), ventral lamella (vl) and the medial column of the inferior olive (mcIO; grey regions; which project in a parasagittal manner to folia IXcd and X, not shown). The pattern of the dl and vl projections to parasagittal zones in the cerebellar cortex (A<sub>1</sub> - red, A<sub>2</sub> - blue, C - green, and E - yellow) are according to Arends and Voogd (1989; see also Chapter 2).

compartmentalization of anatomical, electrophysiological and biochemical properties of the cerebellar cortex (e.g. Hawkes, 1992; Hawkes et al., 1993; Hawkes and Mascher, 1994; Hawkes, 1997; Herrup and Kuemerle, 1997; Redies and Puelles, 2001; Rivkin and Herrup, 2003; Sarna et al., 2006). However, we do wish to emphasize that there is a more traditional definition of the word *module* in relation to functional motor processing in the cerebellum specifically.

Thus, it has been established in the past 40 years that the cerebellar cortex is divided by its afferent and efferent circuitry, as well as its electrophysiological response properties, into a series of bilaterally symmetric bands that are stacked parasagittally (for review see Voogd and Glickstein, 1998). The similarity across different vertebrate species suggests that this basic pattern of cerebellar modular organization is evolutionarily conserved (Ito, 1984) and is most likely necessary for the proper topographic organization and function of the cerebellum. As important as the parasagittal arrangement of circuits may be to the physiological function of the cerebellum specifically, organized patterns of connections are not at all unusual in the nervous system (e.g. see previous discussion on ocular dominance columns in cerebral cortex). The importance of the modular pattern is magnified, however, by the fact that the afferents respect the expression of a variety of independent molecular markers. Thus, in addition to the anatomy and physiology, numerous gene products have been identified whose expression reveals a nearly identical parasagittal pattern of organization (for review see:

Hawkes and Gravel, 1991; Hawkes, 1992; Wassef et al., 1992; Hawkes et al., 1993; Hawkes and Herrup, 1995; Herrup and Kuemerle, 1997).

Scott (1963) was one of the first to recognize the biochemical pattern of the parasagittal organization of the cerebellum when he found that the enzyme, 5'-nucleotidase had a consistent parasagittal expression pattern in the cerebellum. Following this initial discovery, it was nearly two decades before additional molecules were identified that shared the property of a parasagittal distribution (for review see Hawkes, 1992). In recent years, over twenty other markers have now been described that are expressed in the cerebellar cortex of many species in a bilaterally symmetric and parasagittally oriented banded pattern. The identity of these markers varies widely and includes glycolipids as well as proteins. The proteins themselves have diverse functions: some are metabolic enzymes, while others are membrane-bound receptors. Some examples of these markers include cysteine sulfinic acid decarboxylase (Chan-Palay et al., 1982), the-low affinity NGF receptor (Koh and Higgins, 1991), glutamic acid decarboxylase (Chan-Palay et al., 1981), motilin (Chan-Palay et al., 1981), cytochrome oxidase (Leclerc et al., 1990), acetylcholinesterase (Marani and Voogd, 1977a; b; Feirabend and Voogd, 1986; Voogd et al., 1987a; Voogd et al., 1987b), synaptophysin (Leclerc et al., 1989), the HNK-1 antigen (Eisenman and Hawkes, 1993), the B1 antigen (Ingram et al., 1985), the Ppath antigen (Leclerc et al., 1992); excitatory amino acid transporter 4 (EAAT4; Welsh et al., 2002; Wadiche and Jahr, 2005), and Hsp25, (Armstrong et al., 2001). For many of these markers very few studies have been

done and the level of anatomical detail in the published descriptions is poor. Nevertheless, for each marker the levels of expression clearly vary along the medio-lateral axis of the cerebellar cortex. Some of these markers show similar expression patterns with common borders (e.g. zebrin, EAAT4, Ppath, Map-1a), and other show either a complimentary expression pattern (eg, zebrin and Hsp25) or a seemingly random relationship (e.g. HNK-1, NADPH; for review see: Hawkes and Gravel, 1991; Hawkes, 1992; Hawkes et al., 1993; Herrup and Kuemerle, 1997). Clearly, further studies are needed with respect to all these molecular markers; by examining the precise relationship between the various expression patterns, perhaps the functional significance of this organization can be elucidated.

One molecular marker whose pattern of cerebellar expression has been extensively studied is the antigen defined by the zebrin II antibody (Brochu et al., 1990). This antibody recognizes the Purkinje cell-specific intracellular antigen aldolase C, a glycolytic enzyme (Ahn et al., 1994; Caffè et al., 1994). Zebrin identifies a pattern of cerebellar bands in numerous species, ranging from fish to mammals (e.g. Brochu et al., 1990; Lannoo et al., 1991; Meek et al., 1992). The zebrin staining pattern is apparently conserved in evolution as it is nearly identical in all mammals studied (Leclerc et al., 1990; for review see Sillitoe et al., 2005).

Zebrin is expressed almost exclusively by Purkinje cells - including their dendrites, somata, axons and axon terminals. In many mammalian species, the zebrin expression pattern identifies at least four distinct *transversely* oriented

zones that are apparently independent of lobulation: the anterior region (lobules I–V), the central region (lobules VI and VII), the posterior region (lobules VIII and IX) and the nodular region (lobule IX and X; Hawkes and Eisenman, 1997; Herrup and Kuemerle, 1997; Oberdick et al., 1998; Ozol et al., 1999). Each region is then subdivided *mediolaterally* into parasagittal stripes. Seven alternating zebrin II-immunopositive (zebrin+) and zebrin II-immunonegative (zebrin-) Purkinje cell stripes extend through the anterior region, posterior region, and hemispheres - dividing the cerebellar cortex on each side into long, narrow parasagittally oriented stripes. The stripes themselves are numbered following an established mammalian nomenclature: the most medial positive stripe is designated P1+, followed by the most medial negative stripe P1-, and the number increases as the stripes move laterally to P7+ and P7- (Brochu et al., 1990; Ozol et al., 1999; Sillitoe and Hawkes, 2002; Sillitoe et al., 2005). In mammals, zebrin does not differentiate between Purkinje cells in the central region, nodular region or paraflocculus/flocculus, with the majority of cells in these regions being zebrin+ (Brochu et al., 1990; Hawkes et al., 1993). Interestingly, the small heat shock protein Hsp25 is expressed in the adult mouse cerebellum precisely in these regions where zebrin is expressed uniformly: parasagittal stripes of Hsp25-immunopositive Purkinje cells are confined to the central region, nodular region, and paraflocculus/flocculus (Armstrong et al., 2000).

Although the expression of aldolase C in the cerebellum has been extensively studied (with the zebrin II antibody), the function of this metabolic

enzyme in the brain is largely unknown (see Chapter 6 for further discussion). Briefly, it is known that aldolase C is a glycolytic isoenzyme that catalyzes the aldol hydrolysis of fructose-1,6-biphosphate into dihydroxyacetone phosphate and glycerol-3-phosphate. There are three aldolase isoenzymes in mammals, aldolase A-C. Aldolase A is found throughout most tissues (including some brain regions) and is especially rich in skeletal muscle, aldolase B is a liver-specific isoform, and aldolase C is the only brain-specific isoform. Recent studies have provided some evidence to suggested that zebrin+ and zebrin- Purkinje cells are physiologically different with respect to their role in synaptic plasticity (Nagao et al., 1997b; Wadiche and Jahr, 2005) and that the zebrin + Purkinje cells are more resistant to cell death (Sarna et al., 2001). Regardless of the specific functional consequences of the parasagittal striped pattern presented by zebrin/aldolase C expression, zebrin visualization in the cerebellum is useful for - and often used as - a positional landmark in the cerebellar cortex (Hawkes and Gravel, 1991; Hawkes, 1992; Hawkes et al., 1993; Ozol et al., 1999) and as a representative antigen for the parasagittal pattern of many other molecular markers that have the same expression pattern in the cerebellum (for review see: Hawkes et al., 1993; Voogd et al., 1996; Hawkes and Eisenman, 1997).

Chapter 3 investigates the aldolase C expression pattern in the pigeon cerebellum. This is the first demonstration of zebrin labeling in an avian species and - while the expression pattern of aldolase C in the pigeon is interesting in and of itself - the experiment discussed in this chapter provides an important

foundation for further exploration into the modular organization of the pigeon cerebellum.

### 1.2.3 *Cerebellar function*

One might imagine that sheer size and number of neurons in the cerebellum must indicate that it would be of critical importance to the overall function of the nervous system. Yet complete agenesis of the cerebellum is compatible with life (although, in the cerebellum's defense, normal behavior is severely impaired; Glickstein, 1994), and massive trauma to this region usually carries a good clinical prognosis for recovery. So what is the function of the cerebellum?

The proposed function of the cerebellum started off a bit sordid; the phrenologists Gall and his followers viewed the cerebellum as the organ of sexuality - philoprogenitiveness (love of making babies). Although this theory gained a surprising amount of support, at the beginning of the nineteenth century animal experiments began to give a more accurate functional understanding of the cerebellum (for review see Glickstein et al., 2009). As early as 1824, Flourens made the fundamental observation that movements are effected but not completely lost after cerebellar ablation, which led to the idea that it is involved in the *coordination* of movement, a property which had not previously been considered by physiologists (Flourens, 1824). While this pioneering work was clearly considerable - especially given the time period and experimental

techniques – for more than a century, the coordination of movement was the sole function attributed to the cerebellum. It wasn't until Marr (1969), more than 100 years later, that the theory of cerebellar function was offered a more sophisticated role. Marr proposed that the cerebellar cortex acts as a learning device; however, the underlying assumption that this was a means for controlling motor coordination was still held. Because, clinically, cerebellar damage and degeneration is largely associated with overt signs of the loss of fine motor control (e.g. De La Torre, 1977; Glickstein, 1992; Lewis and Zee, 1993; Thach, 1998; Earhart and Bastian, 2001; Bastian, 2002; Morton and Bastian, 2007), the traditional view of the cerebellum as solely part of the motor system has been perpetuated - and it is still classified as such in modern neuroscience textbooks (e.g. Kandel et al., 2000). This is not to say that the cerebellum is *not* involved in motor control, it is of course, however the cerebellum is also a major associative center for sensory input and it is, in fact, developmentally derived from the alar plate - the dorsal half of the neural tube that is the source of sensory structures (Muller and O'Rahilly, 1988).

An interesting function of visual sensory input into the VbC (specifically the lateral portion, the flocculus) comes from the adaptation of the vestibulo-ocular reflex (VOR). The VOR helps maintain a stable retinal image by generating compensatory eye movements to offset the effects of head rotation (i.e. when the head moves to the right, the eyes move to the left). Since slight head movements are present all the time, the VOR is very important for stabilizing

vision. Ito (1972; 1982) proposed that the flocculus was the main brain region involved in the adaptation of the VOR based on earlier learning theories of cerebellar cortical function (Marr, 1969; Albus, 1971). The vestibuloocular reflex (VOR) is a useful behaviour with which to probe cerebellar function, primarily due to the well-defined and economical neural architecture of VOR pathways, and the accessibility of these circuits for study. Modulation in the gain of this reflex (eye velocity/head velocity) represents a form of motor learning. A dramatic demonstration that this reflex is plastic and adaptable is demonstrated by observations of human and animal subjects wearing vision-reversal prisms (e.g. Haddad et al., 1980). When a subject wears glasses with prism lenses that reverse the perception of the environment in the horizontal plane, making everything appear upside down. The person is at first unable to move about because any rotation of the head results in apparent movement of the environment in the wrong direction. However, over a few days normal mobility gradually returns. During this time, the VOR is at first diminished in amplitude and then is reversed. Removal of the prisms results in a rapid return to the normal state (Baizer and Glickstein, 1974; Baizer et al., 1999; Redding and Wallace, 2003). The cerebellum is intimately involved in this process, since the flocculus projects directly to brain stem structures that are critical participants in the VOR neuronal arcs (Langer et al., 1985a), and removal or inactivation of the flocculus precludes further changes in VOR gain (Zee et al., 1981; Lisberger et al., 1984).

Further, there is an increasing amount of recent research that implicates the cerebellum in a significant number of higher cognitive functions, such as emotion, speech, and memory (Leiner et al., 1991; Akshoomoff and Courchesne, 1992; Paulin, 1993; Courchesne et al., 1994; Glickstein, 1994; Fiez, 1996; Frith et al., 2000; Blakemore and Sirigu, 2003; Kelly and Strick, 2003; Ito, 2005; Limperopoulos et al., 2005). Although its size is impressive and its neuronal circuitry and anatomical interconnections with the rest of the brain are well known, presently, we are unsure of what the cerebellum does. The uniformity of the neuronal architecture of the cerebellar cortex suggests that each small region must operate in a similar way, but it is also clear that different regions control different functions. To contribute to the understanding of the function of the cerebellum, this dissertation focuses on various aspects of the specific modular organization seen in the cerebellum, specifically with respect to the visual anatomical and functional projections from the AOS and associated pretectum to the VbC.

### **1.3 Functional Organization of the Vestibulocerebellum**

The pigeon vestibulocerebellum consists of the two most ventral folia of the posterior vermis: folia IXcd and X using the nomenclature of Karten and Hodos (1967). Generally, folia IXcd and X are referred to as the uvula and nodulus, respectively (Larsell, 1948; Larsell and Whitlock, 1952; Whitlock,

1952). These folia extend laterally and rostrally to form the auricle of the cerebellum, which has been referred to as the paraflocculus and/or flocculus (see section 2.1.2 for further discussion; Larsell, 1948; Larsell and Whitlock, 1952). The optic flow pathways to the VbC are involved in the generation of the optokinetic response, which minimizes optic flow across the retina, by producing eye and/or head movements in the direction of motion. The optokinetic response works together with the VOR to maintain a stable retinal image (Simpson, 1984; Waespe and Henn, 1987; Simpson et al., 1988c).

### 1.3.1 *Visual Afferents and Electrophysiological Response Properties*

As described previously (section 1.1.4), nBOR and LM project to the mcIO. Wylie (2001) used small iontophoretic injections of retrograde tracers in the mcIO to delineate the topographical organization of these projections. Injections into the caudal mcIO resulted in a greater proportion of retrogradely labeled cells in the LM, whereas a greater proportion of cells were found in the nBOR from injections into the rostral mcIO (see also Chapter 5). The projections from LM and nBOR to different regions of the mcIO are consistent with the optic flow preferences of the neurons in the mcIO (Wylie, 2001; Winship and Wylie, 2003). A pattern of connectivity from the AOS and associated pretectum to the inferior olive similar to that of birds has been found in mammals: the caudal dc receives input from the NOT (Mizuno et al., 1973; Takeda and Maekawa, 1976; Holstege and Collewyn, 1982) whereas most of the visual input to the rostral dc

and vlo of the inferior olive arrive indirectly from the MTN and LTN via the VTRZ (Maekawa and Takeda, 1979).

Previous studies have shown that the CSA of Purkinje cells in the VbC is modulated in response to particular patterns of optic flow (Simpson et al., 1981; Graf et al., 1988; Leonard et al., 1988; Simpson et al., 1989; Kano et al., 1990a; Kano et al., 1990b; Kusunoki et al., 1990; Shojaku et al., 1991; Wylie and Frost, 1991; 1993; Wylie et al., 1993; Wylie et al., 1998a; Wylie and Frost, 1999a).

These Purkinje cells have binocular, panoramic receptive fields, the CSA responds to optic flow simulating particular directions of rotation or translation and they have been shown to be topographically organized in terms of optic flow preference (Wylie et al., 1993; Wylie and Frost, 1999a; Wylie et al., 1999c; Crowder et al., 2000). The VbC is divided into a medial zone that prefers translational optic flow (the ventral uvula and nodulus), and a lateral zone that prefers rotational optic flow (the flocculus). Unlike the gross anatomical distinction between the flocculus and uvula/nodulus in mammals (Voogd, 1975), the distinction in pigeons is based solely on optic flow preference (Wylie et al., 1993).

In the uvula/nodulus, there are four types of translation neurons: *ascent* and *descent* neurons respond best to upward and downward translation along the vertical axis, respectively, and *contraction* and *expansion* neurons respond to backward and forward translation along horizontal axes oriented 45° from midline, respectively (Wylie and Frost, 1999b). The translation neurons are

zonally organized in the VbC and each zone receives climbing fibre input from a specific region in the lateral half of the mcIO (Crowder et al., 2000). Lau et al. (1998) used injections of retrograde tracers into the VbC to localize the origin of climbing fibre input to Purkinje cells in the flocculus and nodulus to the dorsomedial and ventrolateral mcIO, respectively. Subsequently, Wylie and colleagues (Wylie et al., 1999c; Crowder et al., 2000; Pakan et al., 2005) used smaller iontophoretic injections of retrograde tracers to correlate the topographical origins of the inputs to each individual type of neuron in the flocculus and uvula/nodulus.

The flocculus contains two types of neurons that are sensitive to rotational optic flow: neurons that prefer rotational optic flow about a vertical axis (rVA neurons), or about a horizontal axis oriented 45° to the midline (rH45 neurons; Graf et al., 1988; Wylie and Frost, 1993). The zonal organization of the rotation-sensitive Purkinje cells of the pigeon flocculus has been extensively studied (Wylie et al., 1999c; Winship and Wylie, 2003; Wylie et al., 2003a; Schonewille et al., 2006) and shows remarkable conservation across species (for review see Voogd and Wylie, 2004).

#### *1.3.1.1 Organization of the Flocculus*

The organization of optic flow sensitive neurons in the flocculus has been investigated using anterograde tracers injected in the inferior olive and retrograde tracers injected in the VbC in a number of mammalian species (Ruigrok et al.,

1992; De Zeeuw et al., 1994; Wylie et al., 1994; Tan et al., 1995a; Sugihara et al., 2004; Sugihara and Shinoda, 2004; Schonewille et al., 2006). Collectively, the results of these experiments indicate that Purkinje cells in the mammalian VbC receiving input from dc and vlo are organized into distinct, interdigitated, parasagittal bands, though the number of bands may vary by species (Voogd and Wylie, 2004). In several species, including non-human primates, the rVA and rH45 neurons are organized into 4-7 interdigitated zones (Voogd and Wylie, 2004). In pigeons, there are four parasagittal zones: two rVA zones and two rH45 zones. The caudo-medial most zone is an rVA zone, zone 0. This is followed successively by rH45 zone 1, rVA zone 2, and lastly the rostro-lateral most zone 3, an rH45 zone. These correspond to zones 0-3 of the rat cerebellum (Sugihara et al., 2004; Voogd and Wylie, 2004). The climbing fibre input to the rVA and rH45 zones arises from separate rostro-caudal regions in the medial half of the mcIO; neurons in the caudal mcIO send projections to rVA zones in the flocculus and neurons in the rostral mcIO send projections to rH45 zones in the flocculus (Wylie et al., 1999c; see also Chapter 4).

### 1.3.2 *Correlating Anatomical, Physiological, and Biochemical Patterns*

It is well established that the cerebellum is highly organized into parasagittal zones with respect to the anatomical and functional properties of the afferent inputs to the cerebellar cortex (for review see Voogd and Glickstein,

1998). However, only recently has the parasagittal organization been discovered with respect to molecular properties of the cerebellar cortex (for review see Hawkes and Gravel, 1991; Hawkes and Herrup, 1995; Herrup and Kuemerle, 1997). Naturally, an interesting progression is to investigate the relationship between these organizational patterns to determine if there is an underlying functional significance.

The relationship between the organization of the olivocerebellar climbing fibre system and the zebrin pattern of Purkinje cells has recently been described in considerable detail in mammals (Gravel et al., 1987; Hawkes and Leclerc, 1989; Gravel and Hawkes, 1990; Hawkes et al., 1993; Sugihara and Shinoda, 2004; Voogd and Ruijgrok, 2004; Pijpers et al., 2005; Sugihara and Quay, 2007). For example, a study by Voogd et al. (2003) on climbing fibre collateralization used small injections of a retrograde tracer into electrophysiologically identified climbing fibre zones of the cerebellar cortex and established that climbing fibre zones and zebrin banding reflect a common organizational scheme in the rat cerebellar hemisphere. In a comprehensive study of the cerebellum, Sugihara and Shinoda (2004) correlated the olivocerebellar projections to the aldolase C expression pattern by making discrete anterograde tracer injections into various small areas within the inferior olive in rats and examining the resulting climbing fibre labeling. They found that climbing fibre's from particular subnuclei of the inferior olive projected to either aldolase C positive or negative Purkinje cell zones, but not to both. They classified olivocerebellar projections and aldolase C

compartments into groups based on differences in the aldolase C expression pattern (positive or negative) and region of climbing fibre origin within the inferior olive. From this comprehensive study, Sugihara extended an earlier hypothesis of Voogd et al., (2003), that the aldolase C negative stripes receive input from climbing fibres conveying somatosensory information whereas the aldolase C positive stripes receive input from climbing fibres conveying information from visual, auditory and other sensory systems (see also Voogd et al., 2003; Voogd and Ruigrok, 2004; Sugihara and Quy, 2007; Sugihara and Shinoda, 2007). However, this hypothesis has yet to be investigated in non-mammalian species and the various functional cerebellar systems (see Chapter 4).

Fewer studies have investigated the organization of mossy fibre terminals in the cerebellum. The studies that have been done, principally in the spinocerebellar, cuneocerebellar, reticulocerebellar, and pontocerebellar projections, have found that the mossy fibres also terminate in a parasagittal organization in the granular layer (for review see Voogd and Ruigrok, 1997; Voogd and Glickstein, 1998). However, the relationship of these mossy fibre terminals to the zebrin expression pattern appears to be more complicated. For example, in the anterior lobe in rodents, alternating stripes of spinocerebellar and cuneocerebellar mossy fibre terminal fields either align with stripes defined by zebrin expression or subdivide homogenous zebrin- stripes into smaller units (Gravel and Hawkes, 1990; Akintunde and Eisenman, 1994; Ji and Hawkes, 1994; 1995). Pijpers et al., (2006) investigated mossy fibre collaterals resulting from

retrograde tracer injections in confined regions of the cerebellar cortex and found that the distribution of subpopulations of mossy fibre rosettes with a common lobular and zonal target (but with heterogeneous origin), show close relationships to that of the overlying zebrin pattern. However, a number of studies investigating the spinocerebellar projections from the lumbar spinal cord have found conflicting results with respect to the relationship between the mossy fibre terminal labeling and the zebrin expression pattern; some boundaries of the labeled mossy fibre fields correlate well with the zebrin bands, while others do not (Gravel and Hawkes, 1990; Matsushita et al., 1991; Ji and Hawkes, 1994).

The parasagittal organization of the cerebellar cortex is also seen in the topography of cerebellar function (Oscarsson and Sjolund, 1977; Cicirata et al., 1992; Chockkan and Hawkes, 1994; Wylie et al., 1995; Cheron et al., 1996; Escudero et al., 1996; Peeters et al., 1999). The comparison of the functional somatotopic maps in the cerebellum and the zebrin expression pattern demonstrates that the boundaries frequently align. For example, there is a close relationship between zebrin expression domains and boundaries in the tactile receptive field map (Chockkan and Hawkes, 1994; Bower, 1997; Hallem et al., 1999). However, the functional significance of cerebellar heterogeneity for the processing of afferent information still remains unknown.

The importance of the parasagittal pattern revealed by these molecular markers is emphasized by its close correspondence to the specific projection patterns of the cerebellar circuitry. Hawkes and collaborators (Gravel et al., 1987;

Hawkes and Leclerc, 1989; Gravel and Hawkes, 1990) were the first to combine zebrin immunohistochemistry with anterograde tracer techniques. They showed that the climbing fibre organization is coincident with the antigenic boundaries between zebrin+ and zebrin- Purkinje cell subsets (Gravel et al., 1987); this organizational correspondence is now well established in mammals (Voogd et al., 2003; Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004; Sugihara and Quy, 2007). The mossy fibre organization is less clear cut; however, Hawkes (1992) suggests that the lack of a perfect congruency in all instances may indicate a finer level of organization within the basic zebrin pattern. Overall, there seems to be a highly compelling link between the patterns of cerebellar afferent organization and the parasagittal zebrin expression in mammals, suggesting a profound functional significance to the basic pattern. The last two chapters of this dissertation investigate the relationship between the visual climbing fibre projections to the flocculus and the Purkinje cell response properties (Chapter 4), as well as the organization of the direct visual mossy fibre projection to the VbC (Chapter 5), and the zebrin expression pattern in the pigeon.

#### **1.4 Summary and Outline of Chapters**

The majority of the neurons in the brain are found in the cerebellum (Lange, 1975). The cerebellum integrates information from various sensory systems and, traditionally is regarded as critical for motor coordination. However, recent research indicates that it is involved in other processes including learning,

cognition and emotion (see section 1.2.3). How the cerebellum organizes and synthesizes information from the various senses is poorly understood. This dissertation investigates the complex modular organization of the cerebellum, specifically in relation to the visual optic flow pathways to the cerebellum from the AOS and associated pretectum. The visual connections to the cerebellum are discussed in light of the anatomical, physiological, and molecular organization of the cerebellum. Previous research has shown that the cerebellum has a unique and complex organization: molecular markers are expressed as “stripes” throughout the cerebellum, but the significance of these stripes is not known (for review see: Hawkes and Gravel, 1991; Hawkes and Eisenman, 1997; Herrup and Kuemerle, 1997). How the molecular stripes relate to the organization of the inputs from the visual system and the functions associated with these inputs is investigated using the pigeon VbC as a model. These experiments will provide much needed information about the general principles underlying the organization of the fundamental architecture of the cerebellum.

This dissertation includes four studies of the morphology, physiology, and molecular organization of the visual pathways from the AOS and pretectum to the cerebellum. In Chapter 2, using fluorescent tracer techniques, we seek to determine if the visual mossy fibre input from the pretectal nucleus LM to folium IXcd of the VbC differs from that to folia VI-VIII of the posterior cerebellum. Previous research has shown that the VbC is involved in visual-vestibular integration supporting gaze stabilization but the function of folia VI-VIII in

pigeons is not well understood, but these folia may be involved in analyzing local motion as opposed to optic flow. The functions of these two optic flow pathways to the cerebellum are discussed in relation to the results of this study. In Chapter 3, the expression of the molecular marker zebrin is used to investigate the parasagittal organization in the pigeon cerebellum with respect to the biochemistry of the Purkinje cells. Previous research in many vertebrate species has shown that zebrin is expressed in a parasagittal fashion with alternating immunopositive and immunonegative stripes (see section 1.2.2) but this study is the first to investigate zebrin expression in an avian species. In Chapter 4, we investigate the relationship between the climbing fibre afferent input, the Purkinje cell electrophysiological response properties and the zebrin parasagittal expression pattern in the flocculus of the VbC. Several studies in rodents suggest that, in general, climbing fibres to the zebrin negative stripes convey somatosensory information, whereas climbing fibres to the zebrin positive stripes convey information from visual and other sensory systems (Voogd et al., 2003; Sugihara and Shinoda, 2004; Sugihara and Quy, 2007). The pigeon flocculus consists of four pairs of zebrin<sup>+/-</sup> stripes (P4<sup>+/-</sup> through P7<sup>+/-</sup>; see Chapter 3), however the climbing fibre input consists entirely of visual inputs (e.g. Winship and Wylie, 2003). Thus, because the correspondence of zebrin expression and climbing fibre information must be different from that proposed for rodents, we examine this relationship in the pigeon flocculus. Finally, in Chapter 5, we examine the concordance of zebrin expression with visual mossy fibre afferents in

the VbC (folium IXcd) of pigeons. Visual afferents from the AOS and associated pretectum project directly to folium IXcd as mossy fibres and indirectly as climbing fibres via the inferior olive (Brecha et al., 1980; Gamlin and Cohen, 1988a; Pakan et al., 2006; Wylie et al., 2007; see also Chapter 2). Although it has been shown previously that LM and nBOR project directly to folium IXcd in the pigeon, the detailed organization of these projections has not been reported. Using anterograde tracers we examine the organization of mossy fibre terminals from LM and nBOR and subsequently related this organization to the parasagittal zebrin expression pattern.

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Chapter 2: **Projections of the Pretectal Nucleus Lentiformis Mesencephali to the  
Cerebellum in Pigeons**

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As organisms move through their environment, optic flow occurs across the retina (Gibson, 1954). Optic flow subserves many behaviors: control of posture and locomotion, determination of heading, generation of compensatory movements, and navigation (Lee and Lishman, 1977; Simpson, 1984; Lappe and Rauschecker, 1994; Bardy et al., 1999; Zanker et al., 1999; O'Brien et al., 2001; Warren et al., 2001). Optic flow is analyzed by retinal-recipient nuclei in the accessory optic system (AOS; Simpson, 1984; Giolli et al., 2006) and pretectum (Simpson et al., 1988; Gamlin, 2006). In birds, this pretectal nucleus is known as the lentiformis mesencephali (LM), the homologue of the mammalian nucleus of the optic tract (NOT; Collewijn, 1975b; Hoffmann and Schoppmann, 1975; McKenna and Wallman, 1985). The nucleus of the basal optic root (nBOR) of the avian AOS is the homolog of the terminal nuclei of the mammalian AOS (Simpson, 1984; Giolli et al., 2006).

LM is a retinal-recipient structure that has been divided into medial and lateral subnuclei (LMm and LMl, respectively) based on cytoarchitecture (see also, Gottlieb and McKenna, 1986; Gamlin and Cohen, 1988b). LM neurons respond best to moving largefield visual stimuli (i.e. optic flow; McKenna and Wallman, 1985; Winterson and Brauth, 1985; Wylie and Crowder, 2000) and have been explicitly implicated in the generation of the optokinetic response (Gioanni et al., 1983; Simpson et al., 1988). In pigeons, the efferent projections of LM include ipsilateral projections to the medial column of the inferior olive (mcIO), nBOR, parts of the anterior dorsal thalamus, and structures along the

midline in the mesencephalon (Clarke, 1977; Gamlin and Cohen, 1988a; Wild, 1989; Wylie et al., 1998b; 1999; Wylie, 2001; Pakan et al., 2006). In addition, neurons in LM provide a bilateral mossy fibre projection to folia VI-VIII and IXcd. Together, folia IXcd and X comprise the vestibulocerebellum but X does not receive input from LM (Clarke, 1977; Brecha et al., 1980; Gamlin and Cohen, 1988a; Pakan et al., 2006). The vestibulocerebellum (uvula, nodulus and flocculus) is involved in generating compensatory eye movements (Ito et al., 1974; Miles and Lisberger, 1981; Simpson, 1984; Waespe and Henn, 1987; Nagao et al., 1991; Wylie et al., 1998a). In contrast, the function of folia VI-VIII in pigeons has not been extensively investigated, however, these folia receive input from a tecto-pontine system (Clarke, 1977), which is implicated in avoidance behavior (Hellmann et al., 2004). In mammals, folia VI-VIII, in particular folium VII, are referred to as the “oculomotor vermis” and have been linked to saccades and pursuit eye movements (for review see Voogd and Barmack, 2006).

Previously we have shown that there are some differences with respect to the projection of LMm and LMI: the projection to nBOR, and preoculomotor structures along the midline of the mesencephalon is primarily from LMI as opposed to LMm (Pakan et al., 2006). The aim of the present study was to determine if the mossy fibre input from LM to folium IXcd differs from that to folia VI-VIII. We injected fluorescent retrograde tracers into these folia and observed the pattern of labeling in LM and throughout the brain.

## 2.1 Methods

### 2.1.1 *Surgical Procedures*

The methods reported herein conformed to the guidelines established by the Canadian Council on Animal Care and were approved by the Biosciences Animal Care and Policy Committee at the University of Alberta. Five Silver King and Homing pigeons (*Columba livia*), obtained from a local supplier, were anesthetized by intramuscular injection of a ketamine (65 mg/kg) /xylazine (8 mg/kg) cocktail with supplemental doses administered as necessary. Animals were placed in a stereotaxic device with pigeon ear bars and a beak bar adapter so that the orientation of the skull conformed to the atlas of Karten and Hodos (1967). To access folia VI, VII, and VIII, bone was removed from the dorsomedial surface of the cerebellum, lateral to the mid-sagittal sinus. To access the flocculus, the bone surrounding the semicircular canals was removed, as the dorsal surface of the flocculus (folium IXcd) lies within the radius of the anterior semicircular canal. The dura was removed and a glass micropipette (4-5  $\mu\text{m}$  tip diameter) containing 2M NaCl was advanced into the cerebellum using a hydraulic microdrive (Fredrick Haer & Co.). This enabled extracellular recordings to be made from Purkinje cell complex spike activity and the depth of the granular layer of the targeted folium was determined. Once the desired depth was established, the recording electrode was replaced with a micropipette (tip diameter 20-30 $\mu\text{m}$ ) containing green or red fluorescent latex microspheres (Lumafluor

Corp, Naples, FL). The tracers were pressure injected using a Picospritzer II (General Valve Corporation; 40psi, 100ms duration/puff). After surgery the craniotomy was filled with bone wax and the wound was sutured. Birds were given an intramuscular injection of buprenorphine (0.012mg/kg) as an analgesic.

After a recovery period of 2-3 days, the animals were deeply anesthetized with sodium pentobarbital (100mg/kg) and immediately perfused with phosphate buffered saline (0.9% NaCl, 0.1M phosphate buffer). The brains were extracted then flash-frozen in 2-methylbutane and stored at -80°C until sectioned. Brains were embedded in optimal cutting temperature medium and 40µm coronal sections were cut through the cerebellum, brainstem and thalamus with a cryostat, and mounted on electrostatic slides.

### 2.1.2 *Microscopy*

Sections were viewed with a compound light microscope (Leica DMRE) equipped with the appropriate fluorescence filters (rhodamine and FITC). Images were acquired using a Retiga EXi *FAST* Cooled mono 12-bit camera (Qimaging, Burnaby BC), analyzed with OPENLAB imaging software (Improvision, Lexington MA). Images were adjusted using Adobe Photoshop to compensate for brightness and contrast.

### 2.1.3 *Nomenclature*

For the nomenclature of LM, we relied on Gamlin & Cohen (1988a,b) who divided the LM into a medial and a lateral subdivision (LMm, LMI respectively). Both subnuclei contain large multipolar cells, which project to the cerebellum (Gottlieb and McKenna, 1986; Gamlin and Cohen, 1988a; Papan et al., 2006). Continuous with the LMI at its lateral and caudal aspects is the tectal grey. The LMm, LMI and the rostral part of tectal grey all receive retinal input (Gamlin and Cohen, 1988b). The LMm is bordered medially by the nucleus laminaris precommissuralis, a thin strip of cells that do not receive retinal input. Medial to the nucleus laminaris precommissuralis is the nucleus principalis precommissuralis, which is lateral to the nucleus rotundus (see Fig. 2.3E-I). In Nissl stained sections and in fresh tissue viewed under the fluorescent microscope, the layers of the pretectum are relatively easy to distinguish, although the border between tectal grey and LMI can be difficult to localize. Previously the LMm was known as the LM magnocellularis (LMmc), and the LMI and tectal grey were included as the LM parvocellularis (LMpc; Karten and Hodos, 1967).

For the nomenclature of the subdivisions of the inferior olive we relied on Arends and Voogd (1989). The inferior olive consists of dorsal and ventral lamellae that are joined medially by the medial column of the inferior olive (mcIO). The mcIO projects topographically to the vestibulocerebellum (Wylie et al., 1999; Crowder et al., 2000). For the zonal projection of the ventral lamella

and the dorsal lamella of the inferior olive to folia VI-VIII we also relied on Arends and Voogd (1989).

For the nomenclature of the cerebellar folia, we used Karten and Hodos (1967). As in mammals, the cerebellum in birds is highly foliated but is generally considered to be restricted to a vermis without hemispheres. Folia IXcd (uvula) and X (nodulus) comprise the vestibulocerebellum and merge rostromedially to form the auricle. Larsell (1967) considered the lateral extensions of folium IXcd and X as the paraflocculus and flocculus, respectively. In recent years we (Wylie and Frost, 1999; Winship and Wylie, 2003; Wylie et al., 2003a; Wylie et al., 2003b) divided the vestibulocerebellum into the flocculus, nodulus and ventral uvula based on function and homology with mammals. Purkinje cells throughout the vestibulocerebellum respond to optokinetic stimulation (e.g. Wylie et al., 1993). In the medial half, Purkinje cells CSA responds best to patterns of optic flow resulting from self-translation (Wylie et al., 1993; 1998a). In the lateral half of IXcd and X, they respond best to rotational stimuli about the vertical axis (rVA neurons) or a horizontal axis oriented 45 degrees to the midline (rH45 neurons). These responses are essentially identical to those observed in the mammalian flocculus (Graf et al., 1988; Wylie and Frost, 1993). Thus, we consider these zones in the lateral half of both IXcd and X as the flocculus. These floccular zones are labeled 0-3 from caudo-medial to rostral-lateral and follows that used for rats and rabbits (Voogd and Wylie, 2004). In mammals, a similar phenomenon has occurred: parts of the cerebellum traditionally included in the ventral

paraflocculus are now considered part of the “floccular region”, “lobe” or “complex” (see Voogd and Barmack, 2006). For example, the four optokinetic zones of the flocculus, as well as the C2 zone extend significantly into folium p, traditionally considered part of the paraflocculus (DeZeeuw et al. 1994; Tan et al. 1995). In keeping with Larsell (1967), we refer to the medial half of folia IXcd and X as the uvula and nodulus, respectively.

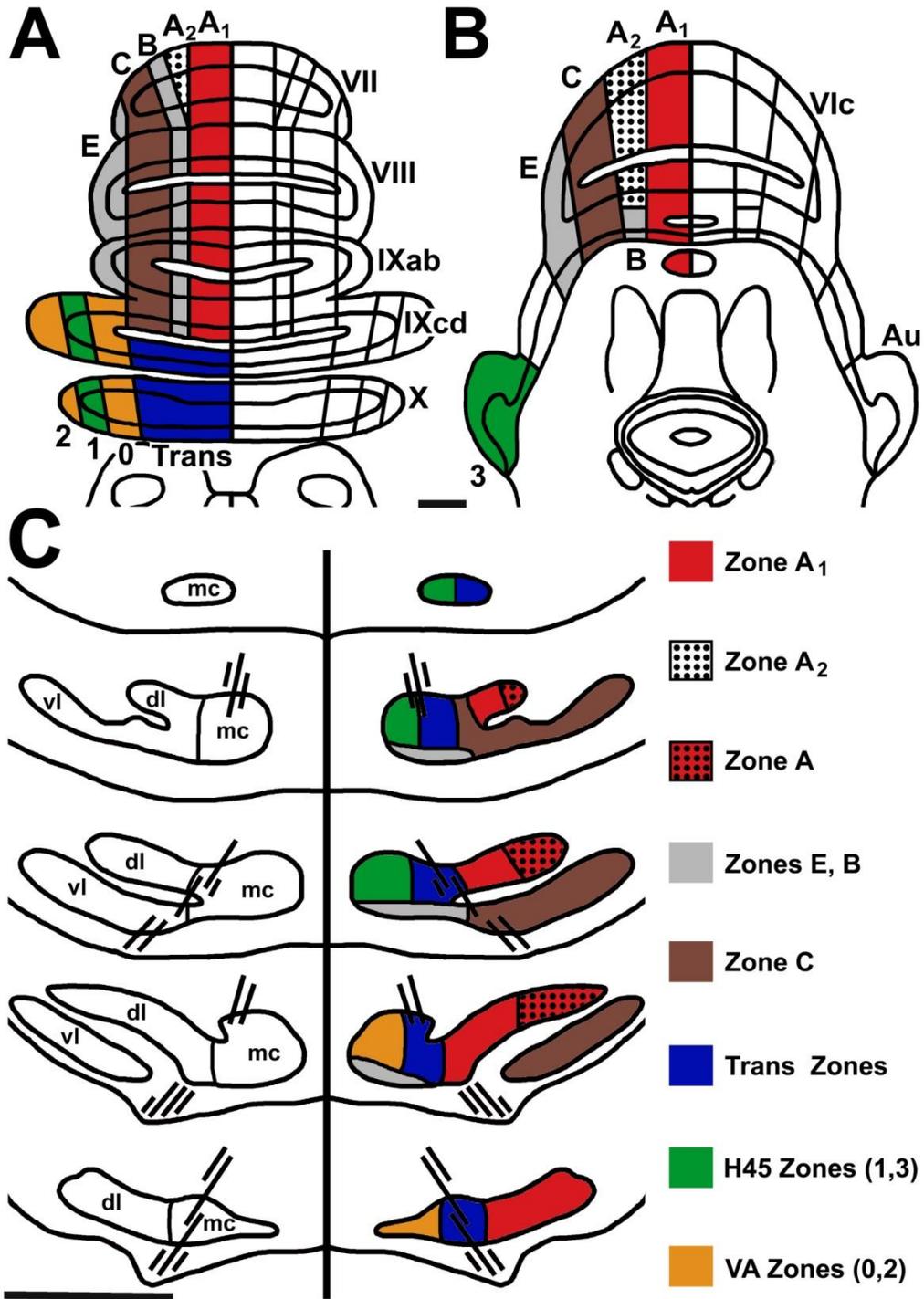
## **2.2 Results**

### ***2.2.1 Injection Sites and Retrograde Labeling in the Inferior Olive***

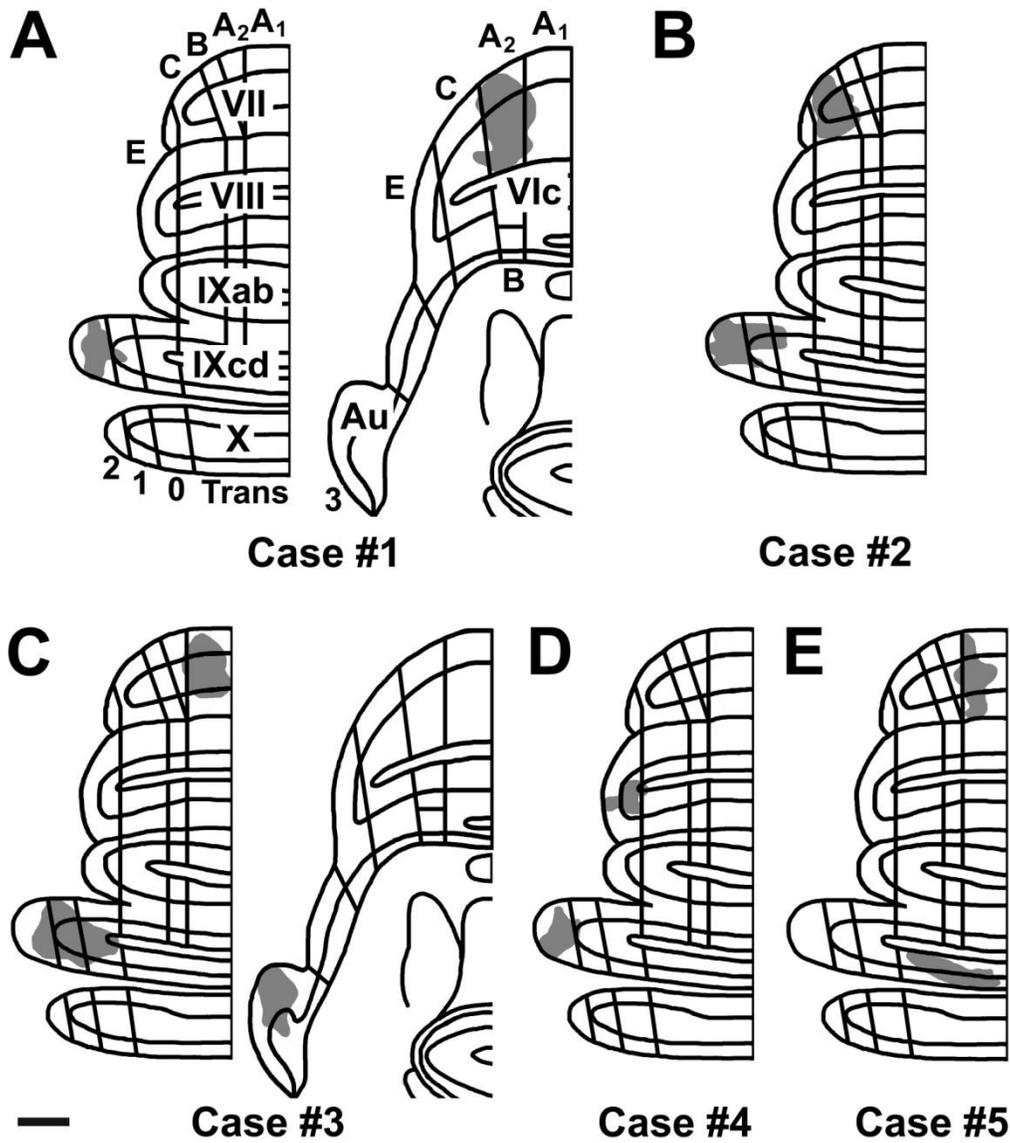
In all experimental animals, an injection of either red or green latex microspheres was made into folium IXcd and an injection of an alternate color was made into one of folia VI (1 case), VII (3 cases), or VIII (1 case). As the topography of the projection from the subnuclei of the inferior olive to the cerebellar cortex has been well documented (Arends and Voogd, 1989; Lau et al., 1998; Wylie et al., 1999; Crowder et al., 2000; Pakan et al., 2005), the pattern of retrograde labeling in the inferior olive served as a guide to verify the location and extent of the injection sites. Figure 2.1 shows a schematic of the olivo-cerebellar projections and zonal organization of the posterior vermis based on these aforementioned studies. Specifically, for injections in VI-VIII retrograde labeling in the dorsal lamella of the inferior olive (Fig. 2.4A,B) was indicative of an injection in zone A<sub>1</sub> and/or A<sub>2</sub>, labeling in the ventral lamella of the inferior olive

(Fig. 2.4C) was indicative of an injection in zone C, and labeling in the ventral strip of the mcIO was indicative of an injection in zones B or E. For the IXcd injections, labeling in the caudal half of the medial mcIO was indicative of injections in floccular zones 0 and 2 (rVA zones), whereas labeling in the rostral half of the medial mcIO was indicative of injections in floccular zones 1 and 3 (rH45 zones; Fig. 2.4B,C; Wylie et al., 1999; Pakan et al., 2005). Labeling in the lateral half of the mcIO, (i.e. lateral to the twelfth cranial nerve), was indicative of injections in the translation areas in the medial half of folium IXcd (ventral uvula; Fig. 2.1A; Lau et al., 1998; Crowder et al., 2000). Figure 2.2 shows drawings of the locations and extent of the injection sites from all cases. Based on these data, the zonal location of each injection is indicated in Table 2.1.

In four cases (cases #1-4), injections into folium IXcd were made just below the surface of the exposed auricle, i.e. the flocculus (Fig. 2.2A-D) and retrograde labeling was abundant in the medial mcIO, but absent in the lateral mcIO. In the remaining case (case #5) the injection in IXcd was more medial in the vestibulocerebellum; i.e. the uvula (Fig. 2.2E). Labeling was restricted to the lateral mcIO indicating the injection spared the flocculus. A few cells were observed ipsilateral to the exposed side, indicating that the injection crossed the midline. All five injections in IXcd appeared confined to that folium, with the possible exception of case #2. In this case there was some suggestion that the injection spread into the adjacent folium IXab, but there were no retrogradely labeled cells in the ventral or dorsal lamellae of the inferior olive.



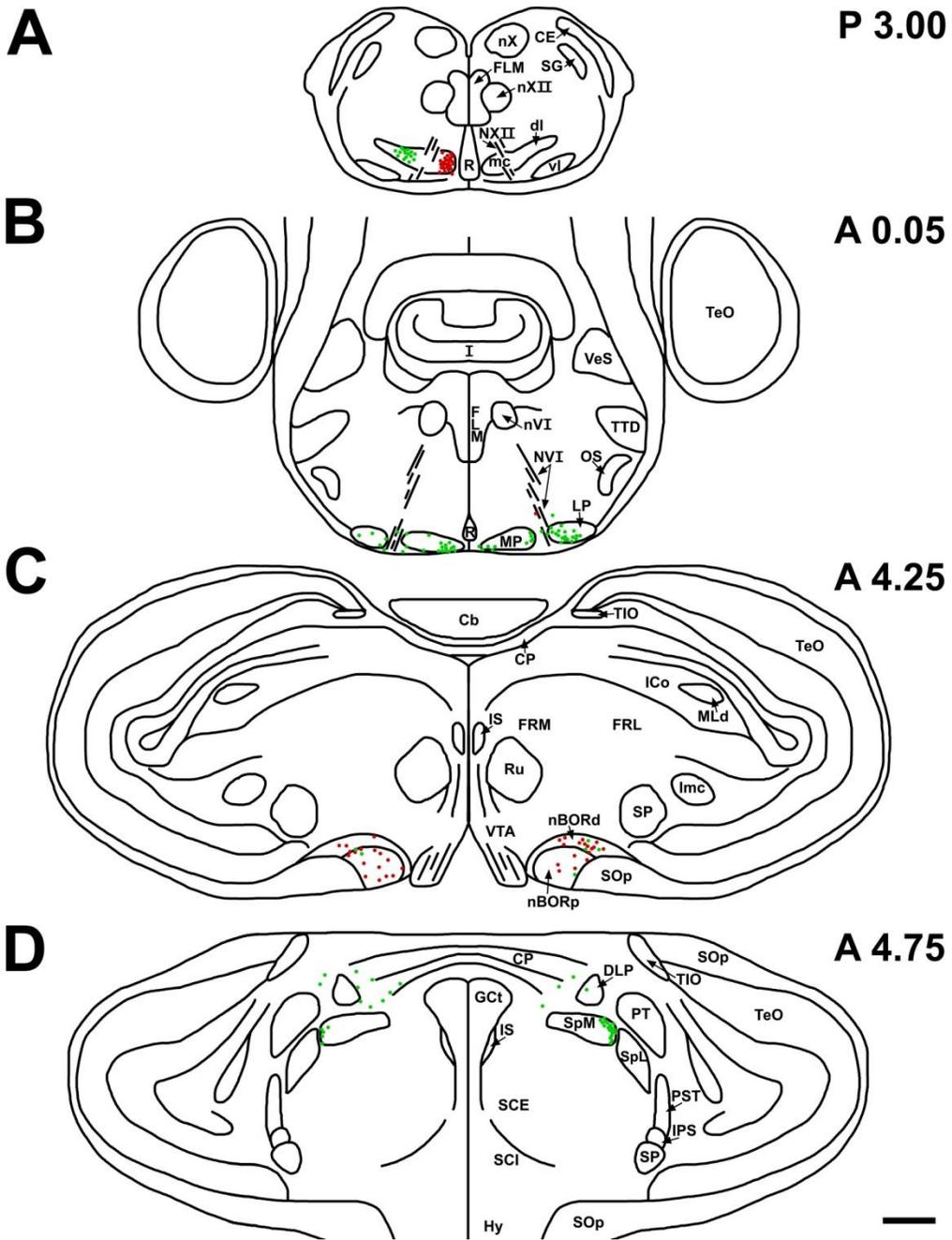
**Figure 2.1** **Olivo-cerebellar zones in the pigeon.** **A** and **B** show representations of coronal sections through the cerebellum (**A**, caudal; **B** rostral). **C** shows coronal sections through the inferior olive (top = rostral). The parasagittal zones shown on the left side in **A** and **B** are color coded to correspond with the region of the inferior olive providing climbing fibre input. The organization of the zones in the vestibulocerebellum (folia IXcd and X) is based on Wylie and colleagues (Lau et al., 1998; Wylie et al., 1999; Crowder et al., 2000; Pakan et al., 2005). Zones 0-3 comprise the flocculus and contain the rotation sensitive Purkinje cells (rVA and rH45 zones). The ‘trans’ zone includes the nodulus (X) and ventral uvula (IXcd) and contains Purkinje cells responsive to translational optokinetic stimuli. Zones A-E are based on Arends and Voogd (1989). For abbreviations see list. Scale bars = 1mm.

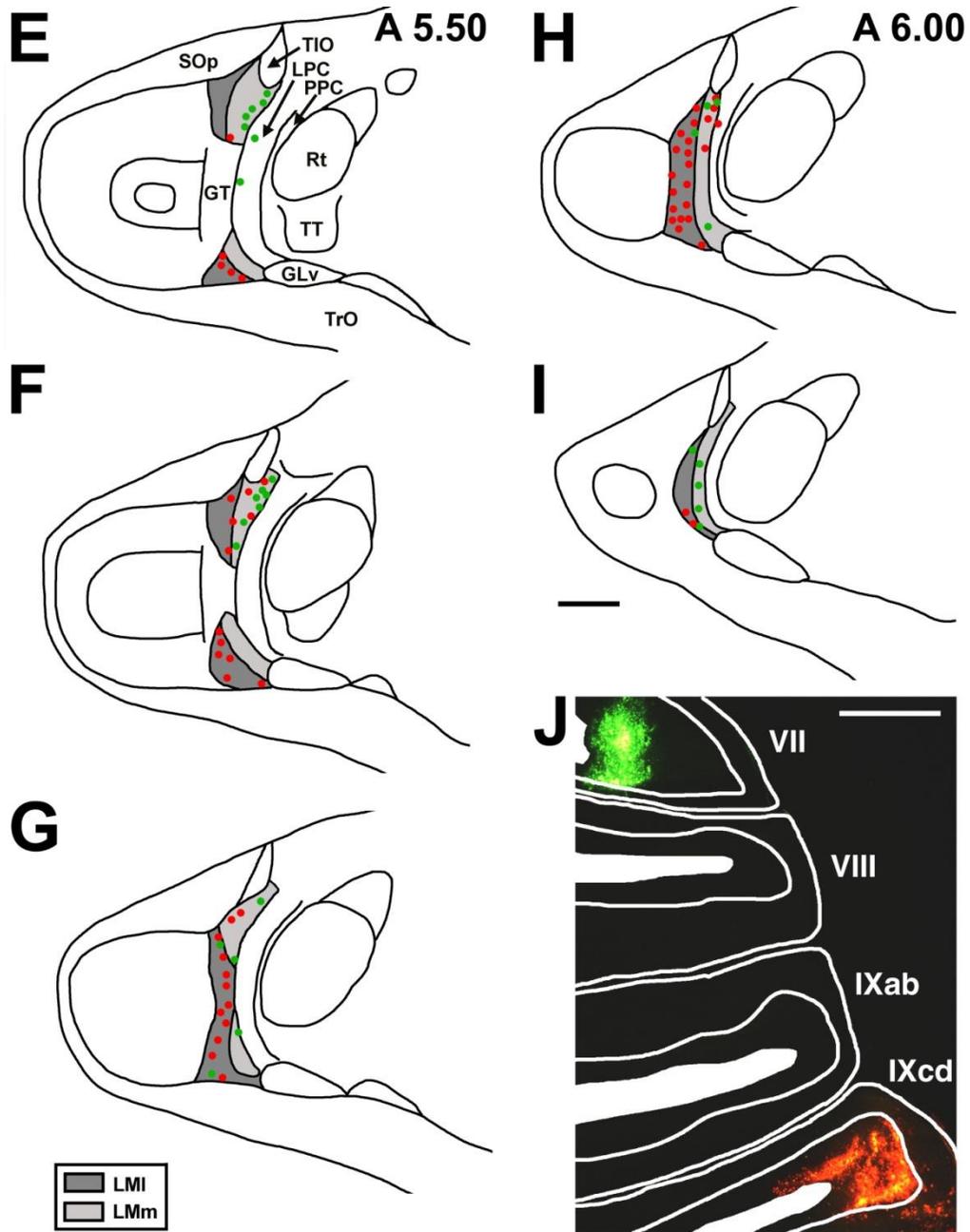


**Figure 2.2** Location and extent of injection sites in the cerebellum. A-E show representations of half coronal sections through the pigeon cerebellum illustrating each injection site (grey shading) according to case number. Vertical lines through the folia represent climbing fibre projection zones, which are labeled in A (see also Fig. 2.1). Each case had two injection sites; one in folium IXcd and one in either VI, VII, or VIII. Au = auricle. Scale bar = 1mm.

### ***2.2.2 Retrograde Labeling in LM, nBOR and Other Brain Regions***

Retrograde labeling was analyzed throughout the brain, focusing on several areas that project to folia VI-IXcd. Figure 2.3 shows selected tracings of coronal sections from case #3 which illustrate retrograde labeling from injections (Fig. 2.3J) in VII (green) and IXcd (red). From caudal to rostral, the main areas of interest include: the inferior olive (Fig. 2.3A), the medial and lateral pontine nuclei (Fig. 2.3B), the nucleus of the basal optic root (nBOR; Fig. 2.3C), the medial spiriform nucleus (Fig 2.3C) and LM (Fig. 2.3E-I). Surprisingly little labeling was seen in the ventral leaflet of the lateral geniculate nucleus even though we have observed labeling in previous studies from injections of retrograde tracer into folium IX (Pakan et al., 2006). In all cases only a few scattered cells were observed in caudolateral aspect of this nucleus. Retrogradely labeled cells were also found in the vestibular and cerebellar nuclei, particularly from the IXcd injections, but not analyzed in any detail. Labeled cells were observed in LM and nBOR from all injections (Fig. 2.4D-G and Figure 2.3C, E-I). Overall, labeling in the nBOR was equally abundant on the contra- and ipsilateral sides (48% vs. 52%). Labeling in the LM was greater on the contralateral side (60% vs. 40%). In Table 2.1 the number of cells found in the contralateral nBOR and LM are indicated for each injection. Figure 2.5A shows an histogram of the relative percentage of cells labeled in the contralateral nBOR vs. LM averaged across all injections in IXcd (left) vs. injections in VI-VIII (right). Note that there

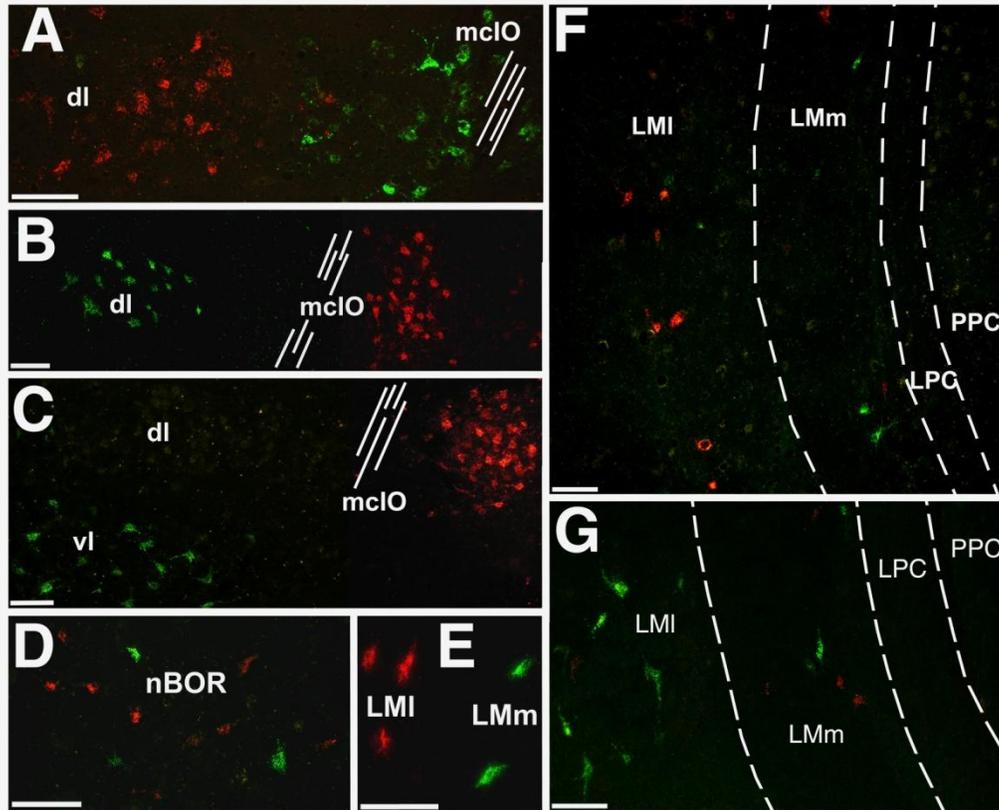




**Figure 2.3 Distribution of Retrograde labeling from injections into folium IXcd and folium VII (case#3).** **A-D** show tracings of coronal sections, caudal to rostral, through the inferior olive, the pontine nuclei, the nucleus of the basal optic root, and the spiriform nuclei, respectively. The approximate anterior-posterior locations according to the atlas of Karten and Hodos (1967) are listed on the right side. The red (folium IXcd projecting cells) and green (folium VII projecting cells) dots represent retrogradely labeled cells resulting from injections shown in **J**. **E-I** show tracings, caudal to rostral approximately 160 $\mu$ m apart, through the contralateral LM. The dark and light gray shading represents the lateral and medial subnuclei of LM, respectively. **J** shows a fluorescent image of the injection sites. The folia are outlined in white, and the white matter is shown as solid white. See list for abbreviations. The scale bar in **D** applies for **A-D**. The scale bar in **I** applies for **E-I**. All scale bars = 1mm.

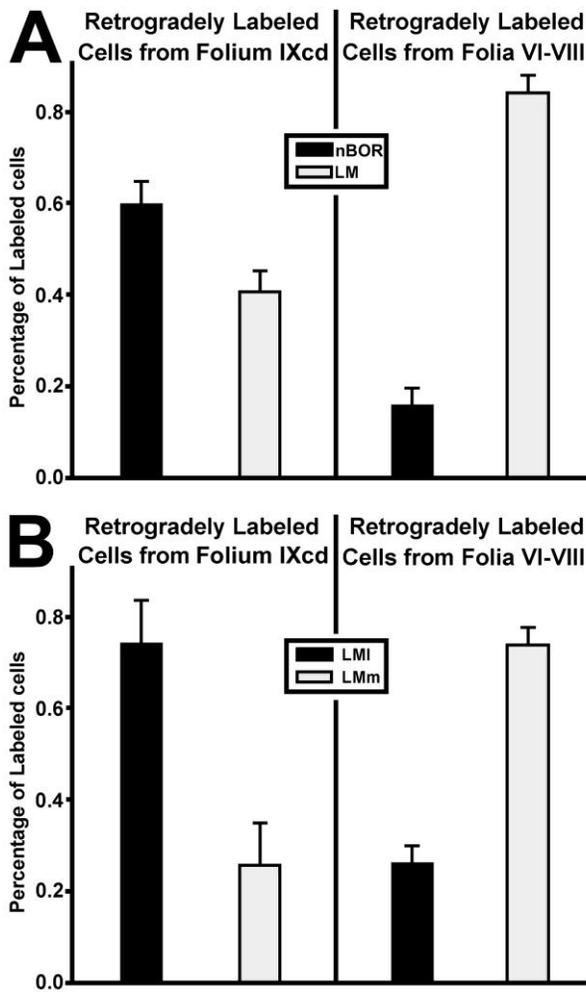
was relatively more input to IXcd from nBOR compared to LM (60:40; see also Fig. 2.4D). However, the input to VI-VIII was much heavier from LM compared to nBOR, as the projection from nBOR to VI-VIII was sparse (Table 2.1; see also Fig. 2.3C).

The drawings in Figure 2.3E-I show the distribution of retrograde labeling in the contralateral LM from case#3. Figure 2.4E-G show photomicrographs of retrograde labeling in the contralateral LM from case #2 (E and F) and case #5 (G). These images represent the typical pattern of labeling observed in LM: retrogradely labeled cells from folia VI-VIII injections were found mainly in LMm whereas the input to IXcd was largely from LMI. Averaged across all cases, 73.8% of retrogradely labeled neurons from injections into VI-VIII were in LMm, and the remaining 26.2% were in LMI. The opposite pattern of retrograde labeling was found for injections in folium IXcd: 74.3% of labeled cells were in LMI and the remaining 25.7% were in LMm (Fig. 2.5B). In addition, there was also a dorsal-ventral difference in the distributions such that, more cells from the injections in VI-VIII were labeled in the dorsal region of LM and more cells from the IXcd injections were labeled in the ventral region of LM. This differential aspect of the distribution is particularly evident in the caudal regions of LM (see Fig. 2.4 E,F), and was present in all cases. Nine double labeled LM cells were observed and all were from case #2. As mentioned above, it was possible that the injection in IXcd for this case may have spread into IXab. Thus, with few exceptions, individual LM neurons do not project to both IXcd and VI-VIII.



**Figure 2.4** Photomicrographs of retrograde labeling in the accessory optic system, pretectum, and inferior olive. **A-C**: Retrograde labeling in the inferior olive from cases #5, #3 and #2, respectively. The white lines highlight the genu of the hypoglossal nerve. Injections into folia VI-VIII labeled cells in the dorsal and ventral lamella of the inferior olive and injections into folium IXcd labeled cells in the medial column of the inferior olive (mcIO). **D** shows labeled cells in the nucleus of the basal optic root (nBOR) from a green injection in VII and a red injection in IXcd (case #3). The distribution within nBOR was not observed to be different between injection regions, however, more nBOR cells were labeled from injections in folium IXcd of the vestibulocerebellum compared

to folia VI-VIII injections. **E-G** show labeled cells in the nucleus lentiformis mesencephali (LM) from case #2 (**E** and **F**; a green injection in VII and a red injection in the flocculus of IXcd) and #5 (**G**; a green injection in the uvula of IXcd and a red injection in VII). The dashed white lines represent the borders of the subnuclei and surrounding areas. There was approximately 3 times more labeling observed in the lateral subnuclei of LM (LMI) compared to the medial subnuclei of LM (LMm) from injections into folium IXcd. Conversely, approximately 3 times more labeling was observed in LMm compared to LMI from injections into folia VI-VIII. LPC, nucleus laminaris precommisuralis; PPC, nucleus principalis precommisuralis. Scale bars = 100 $\mu$ m.



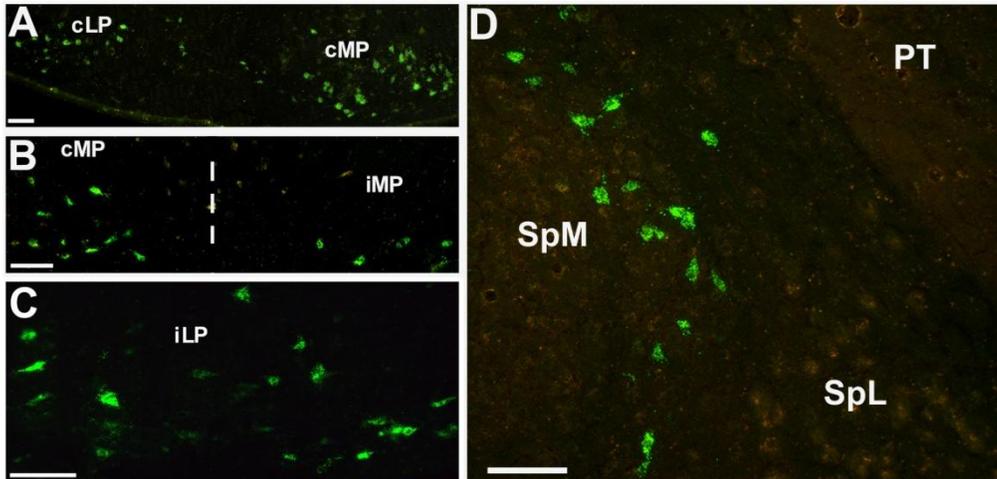
**Figure 2.5 Magnitude of labeling in the accessory optic system and pretectum from injections into folium IXcd vs. folia VI-VIII.** **A** shows a histogram of the relative percentage of cells labeled in the contralateral nucleus of the basal optic root (nBOR; black bars) compared to the contralateral nucleus lentiformis mesencephali (LM; grey bars) averaged across all injections in folium IXcd (left) vs. injections in folia VI-VIII (right). A higher percentage of cells were labeled

in nBOR from IXcd injections and a higher percentage of cells were labeled in LM from VI-VIII injections. **B** shows a histogram of the relative percentage of cells in the contralateral lateral subnucleus of lentiformis mesencephali (LMI; black bars) and medial subnucleus of lentiformis mesencephali (LMm; grey bars) averaged across all injections in IXcd (left) vs. injections in VI-VIII (right). Most of the labeled neurons from injections into VI-VIII were in LMm. Conversely, most of the labeled neurons from injections into IXcd were in LMI.

With respect to the retrograde labeling in nBOR (Figs. 2.3C, 2.4D), there was no apparent difference in the distribution of cells labeled from injections into IXcd vs. VI-VIII. Recall that comparatively few cells were labeled from the VI-VIII injections, but from all injections labeled cells were distributed throughout the nucleus including the dorsal and the lateral extensions. In total, 10 double labeled nBOR cells were found in 4 cases. Thus, few individual nBOR cells project to both IXcd and VI-VIII.

Figure 2.6A-C show photomicrographs of retrograde labeling in the pontine nuclei from injections into folia VII (cases #2 and #3). Heavy labeling was found throughout the medial and lateral pontine nuclei following injections into VI-VIII. This labeling was bilateral, but in general there was more labeling on the contralateral side. From the injections into VII, there was more labeling in the medial pontine nuclei compared to the lateral pontine nuclei. Retrograde labeling in the medial and lateral pontine nuclei were not observed from the flocculus injections, but a few cells were labeled from the uvula injection (see also Fig. 2.3B).

Figure 2.6D shows photomicrographs of retrogradely labeled cells in the medial spiriform nucleus from an injection into folia VII in case #3 (see also Fig. 2.3D). From all injections into VI-VIII a strip of cells was labeled along the dorsolateral border of the medial spiriform nucleus. Diffuse labeling of large multipolar cells was also found dorsomedial to the medial spiriform nucleus. It was difficult to ascribe these to a specific nucleus, but they appeared to reside



**Figure 2.6** Retrograde labeling in pontine nuclei and the medial spiriform nucleus. **A-C** show photomicrographs of retrograde labeling in the pontine nuclei from injections into folia VII (case #2 and #3). Labeled cells can be seen throughout the pontine nuclei on the ipsilateral and contralateral side. There was more labeling in the medial pontine nucleus (MP) compared to the lateral pontine nucleus (LP) from folia VII injections (**A**). In general, there was more labeling observed in the contralateral pontine nuclei (**B**; the broken line represents midline). **D** shows a photomicrograph of retrogradely labeled cells in the medial spiriform nucleus (SpM) from an injection into folium VII (case #3). From injections into folia VI-VIII a specific and reliable distribution of labeling was seen in SpM, namely, a strip of cells found on the dorsolateral boarder of the nucleus. There was no labeling found in SpM from folium IXcd injections in any of the cases. *i/cMP*, ipsilateral/contralateral medial pontine nuclei; *iMP*, *i/cLP* ipsilateral/contralateral lateral pontine nuclei; SpL, lateral spiriform nucleus; PT, pretectal nucleus. Scale bars = 100 $\mu$ m.

within the medial pretectal nucleus, the dorsolateral nucleus of the posterior thalamus and/or the area pretectalis. There was no labeling in the medial spiriform nucleus from any IXcd injections.

### **2.3 Discussion**

In pigeons, we have shown that there is a topographic projection from the pretectal nucleus LM to the cerebellum. The projection to folium IXcd of the vestibulocerebellum arises mainly from LMI, whereas that to folia VI-VIII arises mainly from LMm. Previous electrophysiological studies have not revealed a difference between LMI and LMm: both subnuclei process optic flow information (Winterson and Brauth, 1985; Crowder et al., 2003). Neurons in the avian LM have extremely large receptive fields (average = 60 deg) and exhibit direction-selectivity in response to moving large-field stimuli. Most neurons prefer temporal to nasal motion in the contralateral visual field (McKenna and Wallman, 1985; Winterson and Brauth, 1985; Wylie and Crowder, 2000). Neurons with essentially identical properties have been found in the NOT (Collewijn, 1975a; Hoffmann and Schoppmann, 1981; Winterson and Brauth, 1985; Mustari and Fuchs, 1990). Previous lesion and stimulation studies of LM and NOT have emphasized their role in the optokinetic response (Gioanni et al., 1983; Cohen et al., 1992): lesions to LM or NOT markedly impair or abolish the optokinetic response to stimuli moving in the temporal to nasal direction. Whereas the LM has only been linked to the generation of the optokinetic response, studies suggest

the NOT is involved in other behaviors including pupillary constriction and convergence ('near' response; Büttner-Ennever et al., 1996a), smooth pursuit (Mustari and Fuchs, 1990; Ilg et al., 1993; Büttner-Ennever et al., 1996a; Yakushin et al., 2000) and saccades (Ballas and Hoffmann, 1985; Sudkamp and Schmidt, 1995; Schmidt, 1996; Price and Ibbotson, 2001).

### 2.3.1 *Mossy Fibre Inputs to the Posterior Cerebellum*

Similar to pigeons, a direct mossy fibre pathway from LM and nBOR to the cerebellum has been reported in turtles and fish, but not frogs (fish: Finger and Karten, 1978; turtle: Reiner and Karten, 1978; frogs: Montgomery et al., 1981). In mammals, there has been no report of a mossy fibre pathway from the NOT to the cerebellum, but a mossy fibre projection from the medial terminal nucleus of the AOS to the vestibulocerebellum has been reported in some species (chinchilla: Winfield et al., 1978; tree shrew: Haines and Sowa, 1985), but not others (cats: Kawasaki and Sato, 1980; rats and rabbits: Giolli et al., 1984). There is evidence of several indirect pathways from NOT to the cerebellum through which optic flow information can be conveyed. Most of the mossy fibre input to the vestibulocerebellum arises in the vestibular nuclei and the prepositus hypoglossi (Voogd et al., 1996; Ruigrok, 2003), but there are also projections originating in the reticular formation, the raphe nuclei, and neurons located within and around the medial longitudinal fasciculus (Blanks et al., 1983; Sato et al., 1983; Gerrits et al., 1984; Langer et al., 1985; Voogd et al., 1996; Ruigrok, 2003). The NOT

projects to many of these structures, including the vestibular nuclei, the medial and dorsolateral nuclei of the basilar pontine complex, the mesencephalic reticular formation, the prepositus hypoglossi, and the nucleus reticularis tegmenti pontis (Itoh, 1977; Terasawa et al., 1979; Cazin et al., 1982; Holstege and Collewyn, 1982; Giolli et al., 1984; 1985; Torigoe et al., 1986b; a; Giolli et al., 1988; for review see Simpson et al., 1988). Information from the NOT also reaches folium VII of the oculomotor vermis via the dorsal, medial and dorsolateral pontine nuclei and the nucleus reticularis tegmenti pontis (Torigoe et al., 1986b; Yamada and Noda, 1987; Thielert and Thier, 1993; Voogd and Barmack, 2006).

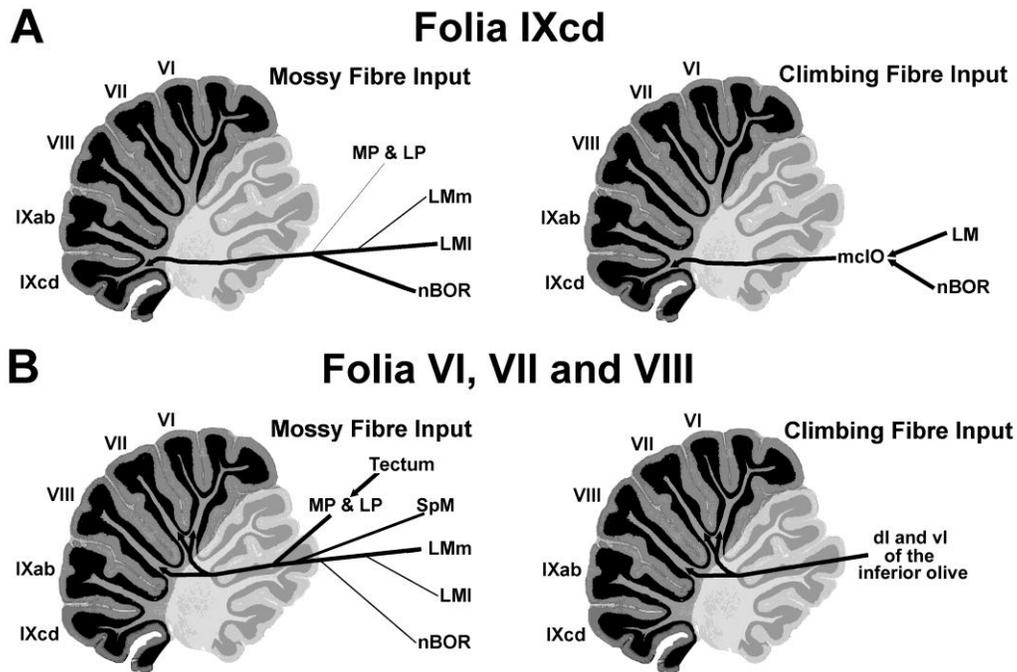
### ***2.3.2 Visual Projections to the Vestibulocerebellum and Folia VI-VIII***

Figure 2.7A shows a schematic of the connectivity from LM and nBOR to folium IXcd in pigeons. Included are mossy fibre connections (present study; Clarke, 1977; Brecha et al., 1980; Gamlin and Cohen, 1988a) and visual climbing fibre inputs (Arends and Voogd, 1989; Lau et al., 1998; Wylie et al., 1999; Crowder et al., 2000; Wylie, 2001). There is heavy mossy fibre input to folium IXcd from nBOR and LMI, but much less from LMm. Input from the pontine nuclei is minimal.

The climbing fibre pathway to the vestibulocerebellum arises from the mcIO, which receives topographic input from the LM and nBOR (Wylie, 2001). The connectivity of the optokinetic-olivo-vestibulocerebellum pathway is remarkably similar in mammals (Voogd et al., 1996; Voogd and Wylie, 2004).

Furthermore, the zonal organization of the flocculus is strikingly similar in mammals and birds (Voogd and Wylie, 2004). Research in many species has shown that the flocculus is critical for integrating optokinetic, vestibular and eye muscle proprioceptive information to control compensatory eye movements (Büttner-Ennever et al., 1996a; b; Büttner-Ennever and Horn, 1996; Büttner-Ennever, 2005). The optic flow input is critical for the modification of the VOR gain (e.g. Robinson, 1976; Zee et al., 1981; Ito et al., 1982; Nagao, 1983; Waespe and Cohen, 1983; Lisberger et al., 1984; Lisberger et al., 1994). Compared to the flocculus, the role of the ventral uvula and nodulus is not as clear. In a recent review, Voogd and Barmack (2006) concluded that the nodulus does not control eye movement per se, but is involved in refining movement and postural reflexes.

Figure 2.7B shows a schematic of the input to folia VI-VIII in pigeons. The mossy fibre input is largely from LMm, but weak from LMI and nBOR, and there is a heavy input from the pontine nuclei (medial and lateral) and the medial spiriform nucleus. In pigeons, the cerebellar projection from the pontine nuclei had been reported previously (Clarke, 1977). Karten and Finger (1976) reported that the medial spiriform nucleus projects to folia VIb-IXa of the cerebellum and that it receives afferents from the telencephalon. Given the fact that there is not a substantial cortico-ponto-cerebellar pathway in birds, these authors proposed that the medial spiriform nucleus was similar to a pontine nucleus in mammals insofar as it provides a pathway from the telencephalon to the cerebellum. However, little is known about the function of the medial spiriform nucleus.



**Figure 2.7 Visual mossy fibre and climbing fibre pathways to the cerebellum.** The thicker lines represent a stronger projection. On a mid-sagittal section of the pigeon cerebellum, **A** illustrates the mossy fibre (left) and climbing fibre (right) input to folium IXcd. Likewise, **B** illustrates the mossy fibre (left) and climbing fibre (right) input to folia VI-VIII. See text for details. LPC, nucleus laminaris precommisuralis; PPC, nucleus principalis precommisuralis; LMI, lateral subnucleus of the nucleus lentiformis mesencephali; LMm, medial subnucleus of the nucleus lentiformis mesencephali; SpM, medial spiriform nucleus; vl, ventral lamella of the inferior olive; dl, dorsal lamella of the inferior olive; mclO, medial column of the inferior olive; nBOR, nucleus of the basal optic root.

In pigeons, the climbing fibre input to folia VI-VIII is from the dorsal and ventral lamellae of the inferior olive, and is zonally organized (Arends and Zeigler, 1991). The information carried by these climbing fibre inputs to VI-VIII is unclear and studies are wanting. Larsell (1967) suggested that folium VII is responsible for “visual power”, noting that this folium was large in raptors. Clarke (1974) recorded visual units in VI-VIII and suggested they were of tectal origin. In the tectum, neurons respond to small moving stimuli or relative motion (Frost and Nakayama, 1983) and there is a heavy projection from the tectum to the medial and lateral pontine nuclei (Clarke, 1977). Albeit to a lesser degree, LM also projects to the pontine nuclei (Clarke, 1977; Gamlin and Cohen, 1988b). Thus, VI-VIII receives both optic flow information, from LMm, and local motion information, from a tecto-pontine system.

Larsell (1967) considered the folia of the avian cerebellum to be homologous to those in the vermis of mammals. However, since stem reptiles lack a highly foliated cerebellum, any explicit homology should be viewed with caution. Nonetheless there are striking similarities between folia VI-VIII in birds and mammals. In mammals VI-VIII is considered the oculomotor cerebellum. There are numerous tecto-pontine inputs and it has been implicated in the control and modification of saccades and smooth pursuit eye movements (for review see Voogd and Barmack, 2006). Thus, folia VI-VIII in birds could be involved in the control of saccades. However, this cannot be the sole role of folia VI-VIII. For the mammalian cerebellum, Voogd and Barmack (2006) have emphasized that the

term ‘oculomotor cerebellum’ is misleading insofar as the function of this region likely goes beyond oculomotor control. First, only two zones in VII are related to saccades, and the function of the other zones is unknown. Second, the input to folia VI-VIII is multimodal and includes visual, auditory, and trigeminal inputs. Similarly, in birds there is a heavy trigeminal input to folia VI-VIII (Arends et al., 1984; Arends and Zeigler, 1989) and electrophysiological studies have noted visual, auditory and somatosensory responses in these folia (Whitlock, 1952; Gross, 1970). These findings suggest that, in both mammals and birds, the function of folia VI-VIII extends beyond oculomotor control.

### ***2.3.3 Proposed Role of Folia VI-VIII in “Steering” Behavior***

As illustrated in Figure 2.7B, folia VI-VIII are receiving two types of visual information: optic flow information from LMm and local object motion from a tecto-pontine system. What is the function of this visual-visual integration? One possible explanation comes from recent work done on area MST of primate cortex. Like pretectal and AOS neurons, MST neurons respond best to largefield stimuli simulating patterns of optic flow (Duffy and Wurtz, 1991) but recent studies of MST have been exploring interactions between optic flow and local motion (Logan and Duffy, 2006). Electrophysiological, psychophysical and modeling studies concluded that such optic flow and local motion integration is important for ‘steering’ during locomotion through a complex environment

consisting of objects and surfaces (Sherk and Fowler, 2001; Elder et al., 2005; Page and Duffy, 2005; Sato et al., 2005).

Clearly, obstacle avoidance and precise steering would be critical for a variety of behaviors, including flight in birds, and there is some evidence to suggest that folia VI-VIII might be involved in steering. Iwaniuk et al. (2007) showed that folium VI and VII are hypertrophied in bird species that are classified as strong fliers. Hellmann and colleagues (2004) suggested that the tectopontine projection in pigeons is involved in object avoidance. Similarly, Glickstein et al., (1972) implicated ponto-cerebellar pathways in mammals in the visual control of movement. In general, the tectum is thought to be important for numerous visuomotor behaviors such as figure-ground segregation and the discrimination of the motion of an object from self-induced optical motion (Frost and Nakayama, 1983), orienting responses, and object avoidance behavior (Ewert, 1970; Ingle, 1970). Perhaps this tectal information is integrated with optic flow information from LMm in the cerebellum to facilitate obstacle avoidance during locomotion. For example, during translation, a radial optic flow pattern would result, which would be detected by neurons in LMm. In addition, stationary objects would move relative to the background creating the ideal stimulus to activate deep tectal cells (Frost and Nakayama, 1983), which project to the pontine nuclei (Hellmann et al., 2004). Thus, steering likely involves the integration of multiple visual cues and the anatomical data found in this study indicates that this integration could be accomplished in folia VI-VIII. Of course, the anatomical data itself does not

conclusively validate the function of these folia, however it does provide the basis for further investigation.

**Table 2.1 A summary of the injection site locations and retrograde labeling in the medial and lateral subnuclei of the pretectal nucleus lentiformis mesencephali (LMm, LMI) and nucleus of the basal optic root (nBOR) contralateral to the injection site.**

			Number of Cells Labeled From IXcd Injections			Number of Cells Labeled From VI-VIII Injections		
Case	IXcd Injection	VI-VIII Injection	nBOR	LMm	LMI	nBOR	LMm	LMI
1	Flocculus Zone 2	VI (red) Zone A <sub>2</sub>	35	6	20	69	173	86
2	Flocculus Zone 1, 2	VII (green) Zone C	111	63	45	35	344	53
3	Flocculus Zone 0-3	VII (green) Zone A <sub>1</sub>	133	21	96	12	41	22
4	Flocculus Zone 2	VIII (red) Zone A <sub>1</sub>	66	0	17	28	54	21
5	Uvula All Zones	VII (red) Zone A <sub>1</sub>	503	109	263	2	29	8
Total			848	215	518	146	641	190

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Chapter 3: **Purkinje Cell Compartmentation as Revealed by Zebrin II  
Expression in the Cerebellar Cortex of the Pigeon**

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The gross anatomy of the cerebellum varies from a single leaf in amphibians to the elaborately foliated structure of birds, mammals and some fish (e.g., Voogd and Glickstein, 1998). Although the cerebellum is divided into clearly defined lobes and lobules (generally referred to as “lobules” in mammals and “folia” in birds), there is substantial evidence to suggest that a more fundamental cerebellar architecture is built around arrays of parasagittal zones of Purkinje cells that cut across the folia. These parasagittal stripes can be defined by climbing and mossy fibre input, Purkinje cell projection patterns, Purkinje cell response properties and topography, and cerebellar interneurons (Voogd, 1967; Hawkes and Gravel, 1991; Hawkes, 1992; Voogd et al., 1996; Hawkes, 1997; Herrup and Kuemerle, 1997; Oberdick et al., 1998; Voogd and Glickstein, 1998; reviewed in, Armstrong and Hawkes, 2000). A parasagittal organization has also been revealed with molecular markers. The most thoroughly studied marker is zebrin (Brochu et al., 1990), which cloning studies demonstrate is the metabolic isoenzyme aldolase C (Ahn et al., 1994; Hawkes and Herrup, 1995). In mammals, zebrin is expressed strongly by subsets of Purkinje cells whereas other Purkinje cells, and other cell types, express zebrin either very weakly or not at all (e.g. Walther et al., 1998). In all mammals studied thus far, zebrin II-immunopositive (zebrin+) Purkinje cells are distributed as an array of immunoreactive parasagittal stripes - more than a dozen in some places - separated by intervening zebrin II-immunonegative (zebrin-) or only weakly immunopositive stripes (Brochu et al., 1990; Eisenman and Hawkes, 1993; Sillitoe and Hawkes, 2002). These stripes

occur in both the vermis and the cerebellar hemispheres and the basic organization of these stripes within the vermis is highly conserved among mammals (Sillitoe et al., 2005).

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

### **3.1 Methods**

Animal procedures conformed to institutional regulations and the *Guide to the Care and Use of Experimental Animals* from the Canadian Council for Animal Care. Adult pigeons (*Columba livia*) were obtained from a local supplier. Pigeons were deeply anaesthetized with sodium pentobarbital (100 mg/kg, i.p.) and

transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The brains were then removed and post-fixed by immersion at 4°C in the same fixative for several days.

### 3.1.1 *Immunohistochemistry*

#### 3.1.1.1 *Whole mount Immunohistochemistry*

A whole mount of the cerebellum was immunostained using a protocol slightly modified from one originally designed for the mouse cerebellum (Sillitoe and Hawkes, 2002). The cerebellum was dissected from the brain by cutting through the cerebellar peduncles. After incubating the pigeon cerebellum in fixative for 24-48 h, it was post-fixed overnight at 4°C in Dent's fixative (Dent et al., 1989). Next the cerebellum was incubated in Dent's bleach (Dent et al., 1989) for ~8 h and then dehydrated in 2 x 30 min each 100% methanol. The tissue was passed through 4-5 cycles of chilling to -80°C and thawing to room temperature in 100% methanol followed by overnight incubation in methanol at -80°C. Anti-zebrin II is a mouse monoclonal antibody (Brochu et al., 1990), subsequently shown to bind the respiratory isoenzyme aldolase C (Aldoc: Ahn et al., 1994: it was used directly from spent hybridoma culture medium diluted 1:200). For zebrin staining, the cerebellum was rehydrated for 90 min each through 50% methanol, 15% methanol, and phosphate-buffered saline (PBS), then enzymatically digested in 10 µg/ml proteinase K (>600 units/ml; Boehringer

Mannheim, Inc.) in PBS for 5 min at room temperature. After rinsing 3 x 10 min in PBS, the tissue was incubated in blocking buffer (Davis, 1993) for 6-8 hrs at room temperature. The tissue was then incubated for 48-96h in anti-zebrin II antibody (1:200), rinsed 3 x 2h at 4°C, and incubated overnight at 4°C in secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA). Finally, the cerebella were rinsed 4 x 3h each at 4°C in PBS followed by a final overnight rinse, incubated in 0.2% bovine serum albumin, 0.1% Triton X-100 in PBS for 2h at room temperature, and antibody binding sites was revealed with diaminobenzidine (DAB).

#### *3.1.1.2 Zebrin Immunohistochemistry for Serial Sections*

For section immunohistochemistry, the brain was post-fixed in 4% paraformaldehyde (in 0.1M PB) for several days. The tissue was equilibrated in sucrose (30% in 0.1M PB) and serial (40µm thick) coronal or sagittal sections were cut through the extent of the cerebellum using a cryostat. Tissue sections were rinsed thoroughly in 0.1M PBS and blocked with 10% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA) and 0.4% TritonX-100 in PBS for 1 hour. Tissue was then incubated in PBS containing 0.1% TritonX-100 and the primary antibody, mouse monoclonal anti-zebrin II (kindly provided by Richard Hawkes, University of Calgary; Brochu et al., 1990) for 60-75 hours at room temperature. Anti-zebrin II is a monoclonal antibody grown in mouse,

produced by immunization with a crude cerebellar homogenate from the weakly electric fish *Apteronotus* (Brochu et al., 1990) and recognizes in mouse a single polypeptide band with an apparent molecular weight 36 kDa, which cloning studies have shown to be the metabolic isoenzyme aldolase C (Ahn et al., 1994; Hawkes and Herrup, 1995). It was used directly from spent hybridoma culture medium diluted 1:200. Anti-zebrin 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).

Tissue was then rinsed in PBS and for the HRP- conjugated antibodies, the sections were incubated in HRP-conjugated goat anti-mouse antibodies (Jackson Immunoresearch Laboratories, West Grove, PA: each diluted 1:1000 in blocking solution) for 2 hours at room temperature, and antibody binding was revealed by using diaminobenzidine (DAB). Sections were dehydrated through an alcohol series, cleared in xylene, and cover-slipped with Entellan mounting medium (BDH Chemicals, Toronto, ON). In some cases an epitope retrieval protocol was employed (Namimatsu et al., 2005; Yamashita and Okada, 2005). The antigen distributions revealed by the different methods were not, however, different.

When fluorescent secondary antibodies were used, the initial processing was as described as above, except that the brain was embedded in gelatin prior to sectioning and was incubated in the primary for 72 hours at room temperature. Tissue was then rinsed in PBS and sections were incubated in either Cy3, Cy2 or

AMCA conjugated donkey anti-mouse antibody (Jackson Immunoresearch Laboratories, West Grove, PA: diluted 1:100 in PBS, 2.5% normal donkey serum, and 0.4% TritonX-100). The tissue was then rinsed in PBS and mounted onto gelatinized slides for viewing.

### *3.1.1.3 Double Labeling of Cerebellar Sections for Calbindin and Zebrin*

Cerebellar sections were processed using immunohistochemistry for double fluorescence. Briefly, tissue sections were washed, blocked in PBS containing 10% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, PA) and incubated with gentle agitation in blocking solution containing a combination of primary antibodies: mouse anti-zebrin II (spent culture medium diluted 1:200; Brochu et al., 1990) and rabbit anti-calbindin (1:1000; C7354; Sigma Immunochemicals, St. Louis, MO) overnight at 4°C. Anti-Calbindin D-28K (KD-15) is a synthetic peptide corresponding to the C-terminal region of rat calbindin-D-28K (amino acids 185-199) and is developed in rabbit. This antibody recognizes a band of 28 kDa on Western blots. The sequence is identical in the corresponding human, mouse and bovine calbindin-D-28K sequences and is highly conserved (single amino acid substitution) in chicken and frog calbindin-D-28K (manufacture's information). Following incubation in primary antibodies, sections were washed and then left in PBS containing Cy3-conjugated goat anti-rabbit secondary antibody and Cy2-conjugated goat anti-mouse secondary antibody (both diluted 1:1000, Jackson Immunoresearch Laboratories, West

Grove, PA) for 2 hours at 4°C. The sections were then washed in 0.1M PBS buffer, mounted onto chrome-alum and gelatin subbed slides, air-dried for 2 h and cover-slipped in non-fluorescing mounting medium (Fluorsave Reagent, Calbiochem, La Jolla, CA).

### 3.1.2 *Microscopy and Image Analysis*

For the whole mounts, the brains were examined, and images obtained, by using a Spot digital camera (Diagnostics Instruments, Sterling Heights, MI, USA) mounted on a Zeiss Stemi SV6 microscope. For the serial sections where zebrin expression was visualized using the DAB-peroxidase reaction product, the sections were examined by using a Spot CCD camera (Diagnostics Instruments, La Jolla, CA, USA) mounted on a Zeiss Axioplan II microscope. For microscopy and image capture methods for fluorescent immunochemistry see section 2.1.2

### 3.1.3 *Western blotting*

Western blot analysis of both pigeon and mouse tissue was carried out using a conventional protocol (Marzban et al., 2003a). Briefly, mice and pigeons were deeply anaesthetized with a lethal dose of sodium pentobarbital (see above), decapitated, and the cerebellum quickly removed from the skull, diced, rinsed in PBS and homogenized in RIPA (1× PBS, 1% Nonidet P-40 (Amresco, Cedarlane Laboratories, Hornby, ON, Canada), 0.5% sodium deoxycholate, 0.1% sodium

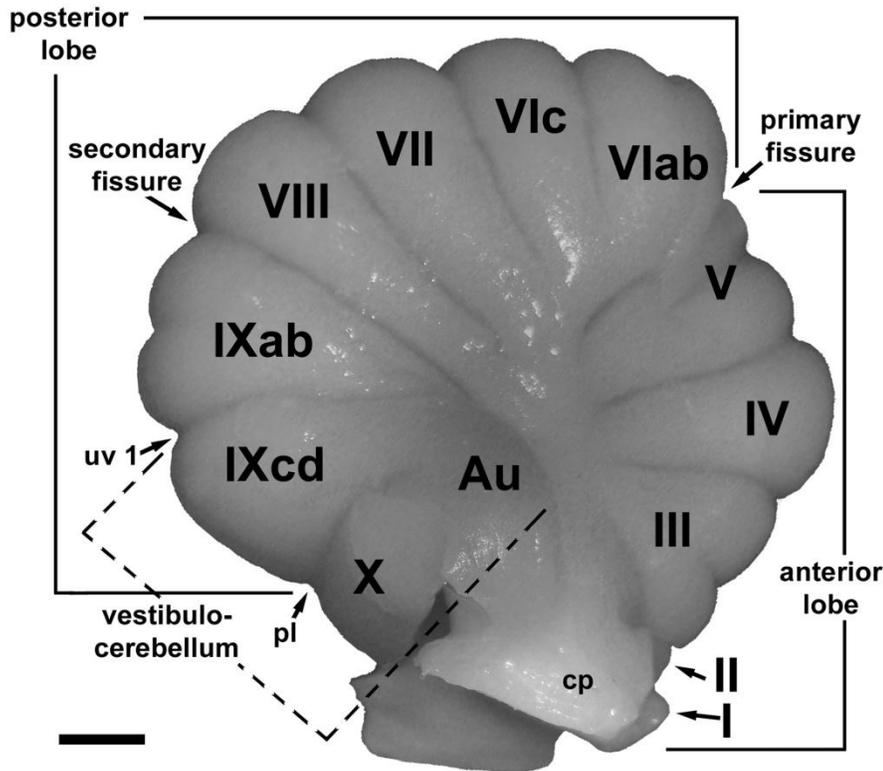
dodecyl sulfate) buffer containing the protease inhibitor pepstatin (1 µg/ml: Bioshop Canada, Burlington, ON, Canada). Cerebellar homogenates were separated by using polyacrylamide gel electrophoresis through a 10% gel (Gibco BRL, Burlington, ON, Canada) and transblotted onto a nitrocellulose membrane (Bio-Rad, Mississauga, ON, Canada) for 1 h by using a semi-dry blotting apparatus. Non-specific binding sites on the membrane were blocked for 2h in 5% skim milk powder / 1% bovine serum albumin in PBS. The membrane was incubated overnight at 4 °C in anti-zebrin II (1/1000 in 5% skim milk powder / 1% bovine serum albumin in PBS). After several rinses in 2% Tween 20 in PBS, the membrane was incubated for 1h in 1:5000 HRP-conjugated rabbit anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) in blocking solution. Several rinses in 2% Tween 20 in PBS and a final rinse in tris-buffered saline (10 mM Tris–HCl pH 7.6 and 150 mM NaCl) followed antibody incubation. The reaction was visualized directly on the nitrocellulose membrane by using 0.5 mg/ml DAB and 0.5 µl/ml 30% H<sub>2</sub>O<sub>2</sub> in PBS until the desired color intensity was achieved. Membranes were scanned using a flatbed scanner (UMAX Astra 1220s) operating under Vistascan (UMAX Data Systems) and thereafter images were transported into Adobe Photoshop.

#### 3.1.4 *Nomenclature*

The pigeon cerebellum consists of a vermis without evident hemispheres (Figure 3.1). Ten primary folia are recognized according to Larsell (1967). Larsell

(1967) suggested an explicit homology between folia I-X of birds and lobules I-X of the mammalian cerebellum. Homologies between avian cerebellar folia and the lobules of mammalian cerebella are conventionally drawn as follows: folium I in the pigeon corresponds to the lingula; folia II and III to the lobulus centralis; folia IV and V to the culmen; folium VI to the declive; folium VII to the folium plus tuber vermis; folium VIII to the pyramis; folium IX to the uvula; and folium X to the nodulus (Fox and Snider, 1967). There are no prominent cerebellar hemispheres in birds, but the unfoliated cortices covering the basis cerebelli may represent their rudimentary homologs (e.g., Larsell, 1948; see below).

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



**Figure 3.1** A lateral view of the pigeon cerebellum. The folia are numbered I-X (anterior to posterior) according to the nomenclature of Larsell (1967). Folia I and II are hidden behind the cerebellar peduncle (cp). Folia I-V comprise the anterior lobe which is separated from the posterior lobe (folia VI-IX) by the primary fissure. The posterior lobe is separated from folium X (nodulus) by the posterolateral fissure (pl). The vestibulocerebellum includes folia IXcd (ventral uvula) and X, which merge laterally and form the auricle (Au). uv 1, uvular sulcus 1. Scale bar = 1mm.

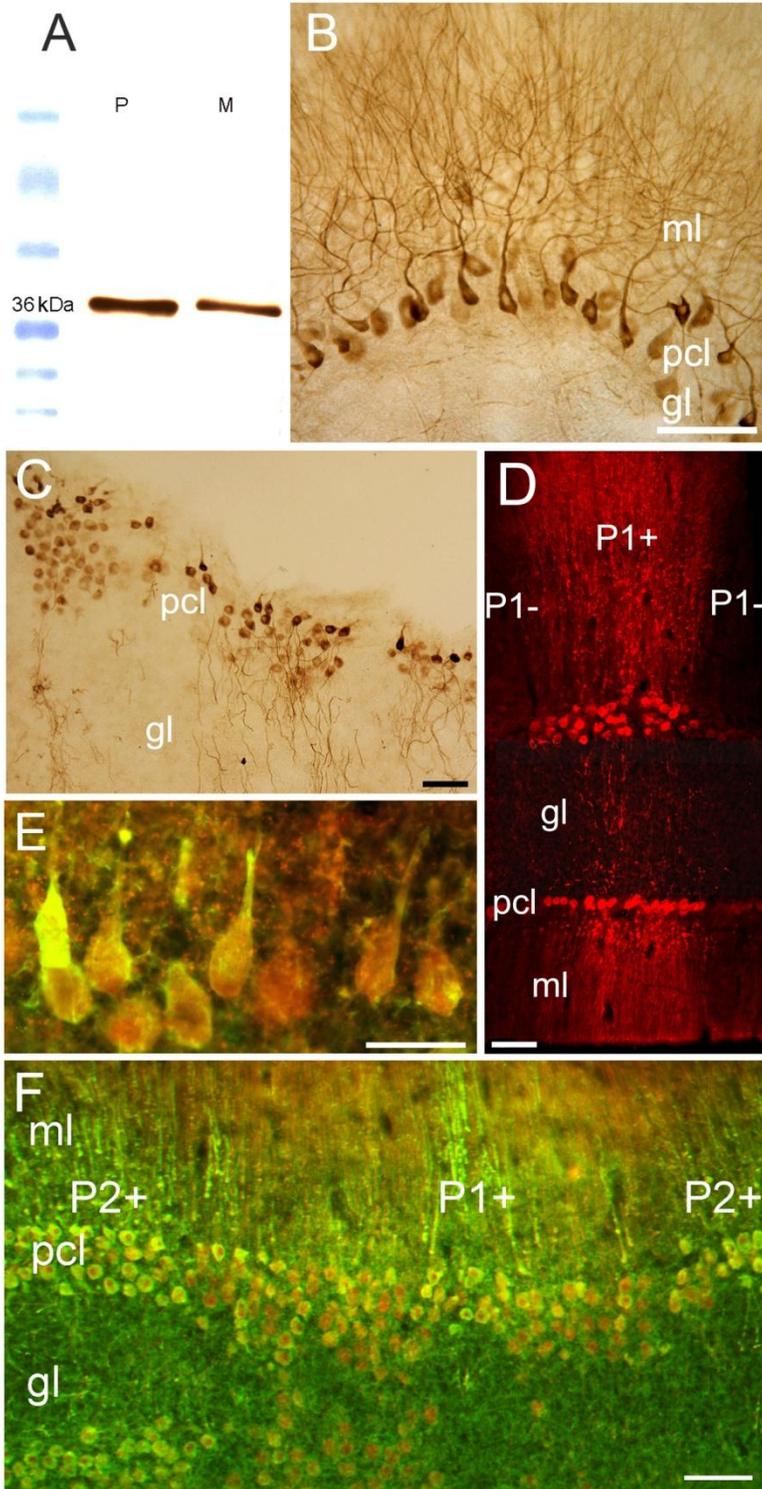
## 3.2 Results

### 3.2.1 *Western Blot Analysis*

Anti-zebrin II recognizes in mouse a single polypeptide band with an apparent molecular weight 36 kDa, which cloning studies have shown to be the metabolic isoenzyme aldolase C (Ahn et al., 1994; Hawkes and Herrup, 1995). Western blot analysis of pigeon cerebellum homogenate also detects a single immunoreactive polypeptide band, identical in size to the band detected in extracts from the adult mouse cerebellum (Fig. 3.2A).

### 3.2.2 *Zebrin Expression*

Purkinje cells were the only neurons that were immunoreactive for zebrin in the cerebellar cortex of the pigeon (Fig. 3.2B-D). Immunoreactivity was present in the dendritic arbors and in the somata of Purkinje cells. Purkinje cell axons in the white matter tracts and in the granular layer were strongly immunoreactive (Fig. 3.2C,D) but, as in mammals, there was no zebrin immunoreactivity in the Purkinje cell nuclei (Fig. 3.2B,E,F). Anti-zebrin II immunostaining was also detected in Purkinje cell axon terminals located within the cerebellar and vestibular nuclei. In some cases, weak zebrin immunoreactivity was also associated with astrocytes – for example, the somata of Bergmann glial cells and glial end feet on blood vessels. Although we cannot exclude background and/or non-specific staining, a similar low level of apparently specific, anti-zebrin



**Figure 3.2 Zebrin expression in the adult pigeon cerebellar cortex.** **A** shows a Western blot of pigeon (P) and mouse (M) cerebellar homogenates probed with anti-zebrin II. A single immunoreactive band is detected in both cases, apparent molecular weight 36 kDa. **B** and **C** show sections through the vermis, immunoperoxidase stained with anti-zebrin II. DAB reaction product is prominent in the Purkinje cell somata in the Purkinje cell layer (pcl) and the Purkinje cell dendrites in the molecular layer (ml) and in the axon fragments coursing through in the granular layer (gl) and the white matter. The tissue in **C** had been boiled, which removed most of the molecular layer. **D** shows a coronal section through the vermis, processed for zebrin using a fluorescent secondary antibody. Zebrin immunopositive (P1+) and immunonegative (P1-) Purkinje cells can be clearly distinguished. **E** and **F** show a section through pigeon cerebellum double immunofluorescence stained for calbindin (red) and zebrin (green). Zebrin immunoreactive Purkinje cells form a symmetrical array of stripes (P1+ at the midline: for stripe terminology, see Sillitoe and Hawkes, 2002). Purkinje cell somata are strongly zebrin immunoreactive in the “immunopositive stripes” and express immunoreactivity at lower levels or not at all in the intervening zebrin “immunonegative” Purkinje cells. Scale bars: B, C, D = 100µm; F = 250µm; E = 50µm.

immunoreactivity has also been reported in mouse glia (e.g., Walther et al., 1998). In Figure 3.2F, a section reacted for zebrin (green) and calbindin (red) is shown. Note the presence of calbindin labeled Purkinje cells in the P1- stripe, where zebrin reactivity was weak. That is, the zebrin- stripes were not simply devoid of Purkinje cells.

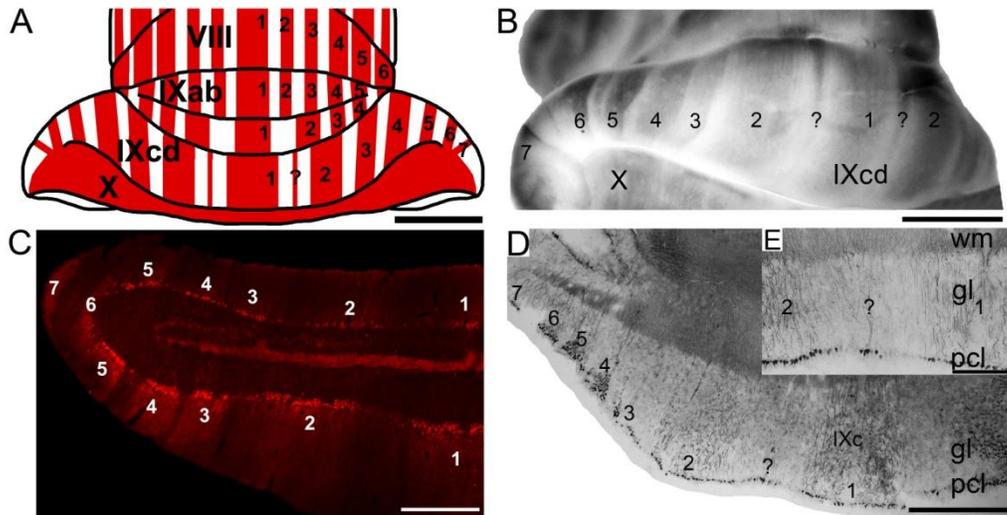
In the pigeon cerebellum, most Purkinje cell somata are zebrin immunoreactive but vary in the strength of their immunoreactivity, with alternating stripes of high and low immunoreactivity. The stripes are revealed most clearly through different levels of expression in the dendritic arbors. For example, Figures 3.2E and 3.2F show coronal sections double immunofluorescence-stained for zebrin (green) and calbindin (red: a marker of all Purkinje cells in both rodents (Baimbridge et al., 1982; Celio, 1990) and birds (Pasteels et al., 1987; de Talamoni et al., 1993; Sechman et al., 1994)). Clusters of strongly zebrin immunoreactive Purkinje cells alternate with clusters of Purkinje cells that only express zebrin immunoreactivity weakly. In what follows, the zebrin+/- terminology will be used to refer to the strong and weak subsets. The stripes themselves are numbered following the nomenclature used in mammals whereby the most medial positive stripe is designated P1+ and the number increases as the stripes move laterally to P7+ (Brochu et al., 1990; Eisenman and Hawkes, 1993; Ozol et al., 1999; Sillitoe and Hawkes, 2002; reviewed in Sillitoe et al., 2005), but no formal homology between individual stripes in birds and mammals should be inferred. Alternating zebrin+/- stripes were apparent in the

posterior (Fig. 3.3 and 3.4) and anterior lobes (Fig. 3.5). In the whole mounts, the contrast between the stripes was not as clear as in rodents, but resembled that seen in other mammals (e.g. cat: Sillitoe et al., 2003a; primate: Sillitoe et al., 2004; reviewed in: Sillitoe et al., 2005).

### *3.2.2.1 Posterior Lobe*

The most consistent and clear stripes were observed in folium IXcd (Figure 3.3). In both whole mounts (Fig. 3.3B) and serial sections (Fig. 3.3C,D), a zebrin+ stripe, numbered P1+, straddled the midline. Six other zebrin+ stripes, P2+ to P7+, were located laterally on either side of the cerebellar midline and extended into the auricle. The two medial stripe pairs (P1+/- and P2+/-) were wider than the five lateral pairs (P3+/- to P7+/-). In some cases, a narrow immunopositive stripe (indicated by “?” in Fig. 3.3) was present in folium IXcd, in the middle of P1-. This stripe was most often seen in the most caudal (and superficial) aspects of the folium.

In the ventral lamella of folium IXab the continuation of the P1+/- to P5+/- stripes from IXcd was apparent (Fig. 3.4A,B), however, in folium VII and VIII, six +/- stripes were observed in coronal sections (Fig. 3.4B). The dorsal lamella of folium IXab represented a transition between the two patterns. P1+ and P1- were narrower in folia VII-IXab than in folium IXcd, and P2+, which was wide in folium IXcd, became quite narrow, such that all stripes in folia VI were of approximately the same width. P3 and P4 of folium IXcd were aligned



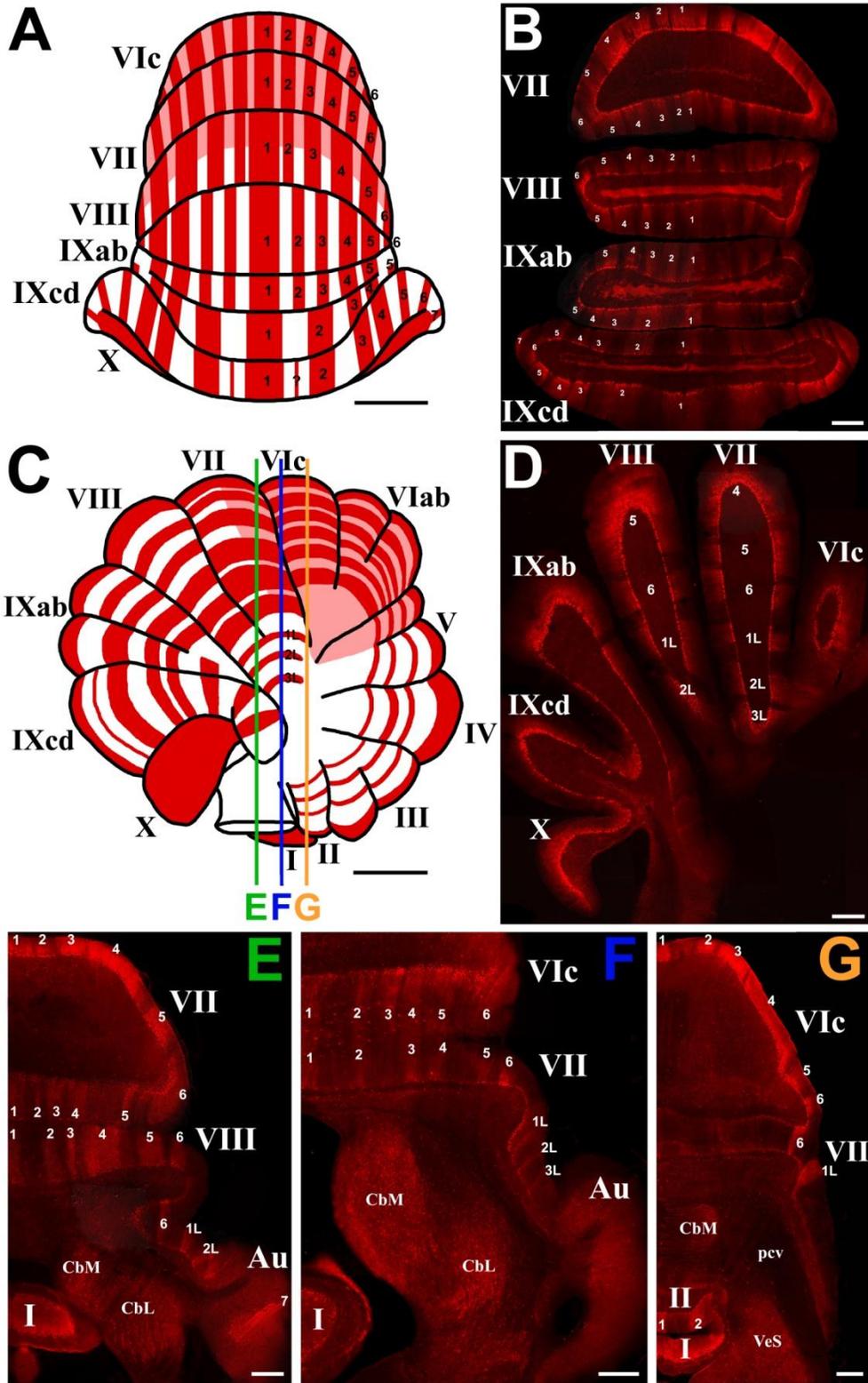
**Figure 3.3 Topography of zebrin expression in folium IXcd of the pigeon cerebellum.** **A** shows a schematic of the zebrin immunopositive (red) and zebrin immunonegative (white) bands in IXcd shown from a postero-ventral view. The immunopositive bands are numbered (1-7) in ascending order from the midline. The thin band (?) between 1 and 2 was observed in the dorsal lamella in some but not all cases. **B** shows a whole mount of the cerebellum from a posterolateral view, emphasizing the stripes in IXcd. **C** shows a coronal section through IXcd illustrating the stripes spanning the folium. Note the absence of the thin band between 1 and 2. **D** and **E** show adjacent horizontal sections through the dorsal lamella of IXcd. This tissue was boiled, which destroyed most of the molecular layer. gl, granular layer; pcl, Purkinje cell layer; wm, white matter. Scale bars: A, B, D = 2.5mm; C, E = 500 $\mu$ m.

reasonably well with P4 and P5 of dorsal IXab and VIII. In folium VI, the six zebrin<sup>±</sup> stripes were apparent in the molecular layer, but the contrast between the zebrin<sup>+</sup> and zebrin<sup>-</sup> stripes was reduced. This was because the overall expression of zebrin was greater in VI than in the other folia, and most Purkinje cells were zebrin<sup>+</sup>. The transition from the pattern in folium VII to that in VI began in the dorsal lamella of folium VII and was more gradual than indicated in Figure 3.4A. The pattern continued into the dorsal lamella of folium V (see Figure 3.5).

There were also three zebrin<sup>±</sup> stripe pairs that traversed the lateral surface of the cerebellum (Fig. 3.4C-G). This region of the cerebellum is formed by the lateral extensions of folia VI-VIII, and has been called the lateral unfoliated cortex (Larsell, 1967; Arends and Zeigler, 1991). These stripes, 1L-3L were clear in coronal sections that transected the middle of the cerebellum through the cerebellar nuclei (Fig. 3.4E-G), and sagittal sections through the lateral edge of the cerebellum (Fig. 3.4D).

### *3.2.2.2 Anterior Lobe*

In the anterior lobe, an array of zebrin immunoreactive parasagittal stripes spanning folia II-V was clearly seen (Fig. 3.5A) in both whole mount preparations (Fig. 3.5B) and coronal sections (Fig. 3.5C). There were four zebrin<sup>+</sup> (P1+ to P4+) alternating zebrin<sup>-</sup> stripes. The P1+ and P3+ zones were more distinct. Overall, the zebrin<sup>-</sup> stripes were slightly wider than the zebrin<sup>+</sup> stripes. In



**Figure 3.4 Topography of zebrin expression in the posterior lobe of the pigeon cerebellum.** **A** shows a schematic of the zebrin immunopositive (red), zebrin weakly positive (pink), and zebrin immunonegative (white) bands in folia VI-IXcd, shown from a posterior view. The pink in the immunonegative bands in folia VI is to represent that the overall zebrin expression was higher and the contrast between the immunopositive and immunonegative bands was less. **B** shows a photomicrograph of zebrin expression in a coronal section through folia VII-IXcd. **C** shows a schematic of zebrin expression from a lateral view. The lettered vertical lines through the cerebellum in **C** represent coronal sections shown in **E-G**, taken from their representative location in the anterior-posterior plane. **D** shows a photomicrograph of a parasagittal section taken about 3mm from midline, illustrating the lateral stripes in folia VII and VIII. Au, auricle; gl, granular layer; pcl, Purkinje cell layer; wm, white matter; CbM/CbL, medial/lateral cerebellar nucleus; pcv, cerebellovestibular process; VeS, superior vestibular nucleus. Scale bars: A, C = 2.5mm; B, D-G = 500 $\mu$ m.

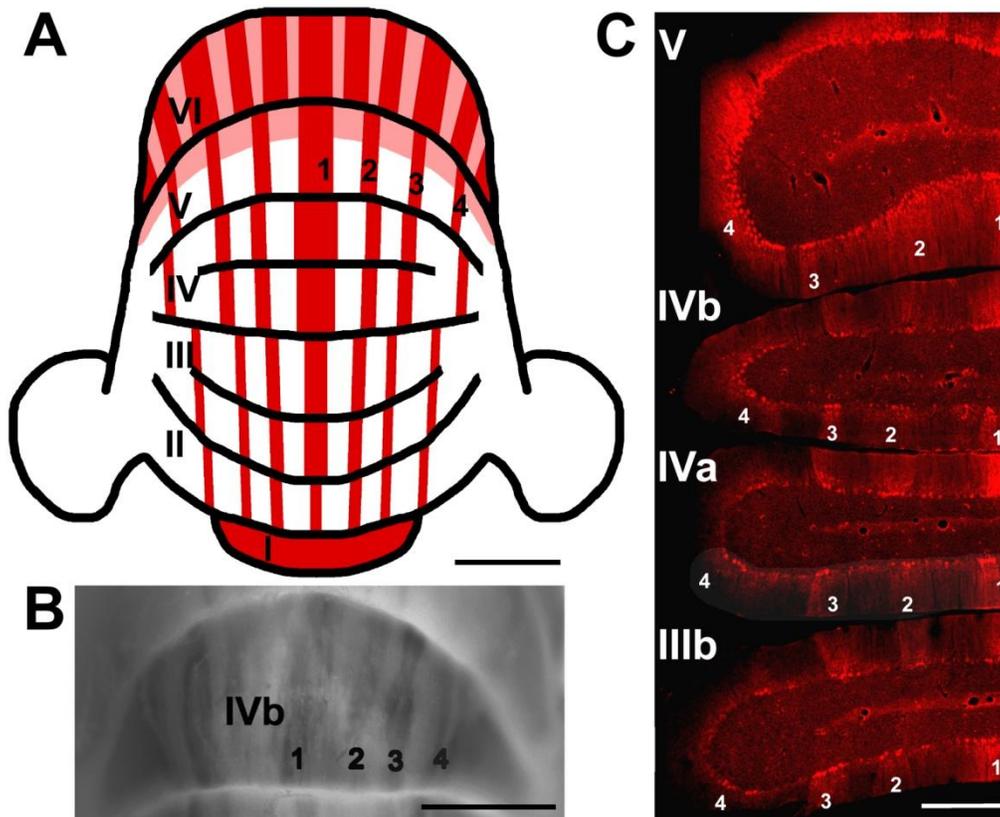
the dorsal lamella of folium V, the stripes appeared less distinct as in folium IV, because the overall zebrin immunoreactivity in the Purkinje cell somata was higher, but stripes were still apparent in the molecular layer.

### *3.2.2.3 Folia I and X*

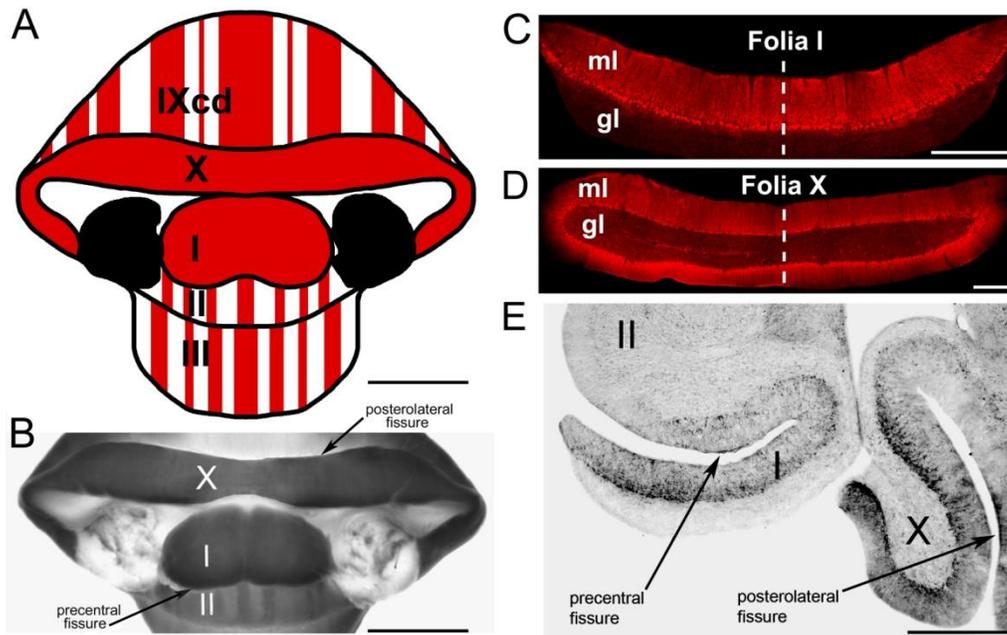
All Purkinje cells in folia I (lingula) and X (nodulus) were immunopositive. This is shown in Figure 3.6B in a ventral view of a whole mount preparation. The stripes can clearly be seen in folium II as opposed to the uniformly intense immunopositive labeling in folia I and X. This was also apparent in coronal sections (Fig. 3.6C,D). Figure 3.6E shows a sagittal section through folia I, II and X, through the P1- stripe. Note the immunopositive labeling in I and X opposed to the absence of labeling in folium II.

### *3.2.3 Other Molecular Markers*

Several other neurochemical markers reveal stripes in mammalian cerebella including motilin (Chan-Palay et al., 1981), acetylcholinesterase (Jaarsma et al., 1995), corticotropin-releasing factor (van den Dungen et al., 1988; Cummings, 1989; Cummings et al., 1989; King et al., 1997), heat shock protein 25 (Hsp25; Armstrong et al., 2000), human natural killer cell antigen (HNK)-1 (Eisenman and Hawkes, 1993; Marzban et al., 2004) and phospholipase c $\beta$ 4 (Sarna et al., 2006). In fact, over twenty such antigens can produce banding patterns in juvenile or adult mammals. In addition to zebrin, we also processed



**Figure 3.5 Topography of zebrin expression in the anterior lobe (folia I-V) of the pigeon cerebellum.** **A** shows a schematic of the zebrin immunopositive (red), zebrin weakly immunopositive (pink), and zebrin immunonegative (white) bands in folia I-VI shown from an anterior view. The pink in the immunonegative bands of the dorsal part of folium V and throughout folium IV (posterior lobe) is to represent that the overall zebrin expression was higher and the contrast between the immunopositive and immunonegative bands was less. **B** shows zebrin expression in folium IVb from a whole mount. **C** shows a coronal section through folia IIIb to V. Note the four (1-4) immunopositive stripes, but the greater overall expression in dorsal V (**C**). Scale bars: A, B = 2.5mm; C = 500 $\mu$ m.



**Figure 3.6 Zebrin expression in the folium I (lingula) and folium X (nodulus) of the pigeon cerebellum.** **A** shows a schematic of the zebrin immunopositive (red) and zebrin immunonegative (white) bands of the cerebellum shown from a ventral view. The cerebellar peduncles are shaded black. **B** shows a photograph of the same view of a whole mount cerebellum. The bands are apparent in folium II, but folium I and folium X are uniformly immunopositive. **C** and **D** show coronal sections through folium I and folium X, respectively, illustrating this uniform immunopositive expression. **E** shows a sagittal section through folia I, II and X. Purkinje cells are immunonegative through folium II, but immunopositive throughout folium I and folium X. Scale bars: A, B = 2.5mm; C, D, E = 500 $\mu$ m.

the pigeon cerebellum for acetylcholinesterase using a histochemical reaction and HSP25 and phospholipase c $\beta$ 4 using immunohistochemistry. None of these was effective in revealing a banding pattern in the pigeon cerebellum.

### 3.3 Discussion

In the present study we have shown that zebrin is expressed in alternating immunopositive and immunonegative stripes that are arranged parasagittally across the mediolateral extent of the pigeon cerebellum. Two lines of evidence suggest that zebrin II/aldolase C expression is evolutionarily conserved. First, Western blots of cerebellar tissue from numerous species ranging from fish to primates reveal a single immunoreactive polypeptide band of identical apparent molecular weight (36 kDa; rat: Brochu et al., 1990; opossum, *Monodelphis domestica*: Dore et al., 1990; squirrel monkey, *Saimiri sciureus*: Leclerc et al., 1990; weakly-electric fish: Lannoo et al., 1991a; rabbit: Sanchez et al., 2002; hamster, *Mesocricetus auratus*: Marzban et al., 2003b; cat: Sillitoe et al., 2003a; tenrec: Sillitoe et al., 2003b; primate: Sillitoe et al., 2004; reviewed in: Sillitoe et al., 2005). This is consistent with cloning studies that have revealed that in mice the zebrin antigen is the metabolic enzyme aldolase C (Ahn et al., 1994). Secondly, as in all other animals studied to date, except amphibians, in which no zebrin immunoreactivity has been detected (Sillitoe et al., 2005), the zebrin antigen is prominently expressed in pigeon Purkinje cells. Not all Purkinje cells in pigeons express zebrin immunoreactivity and this differential expression reveals

the elaborate underlying cerebellar topography. Zebrin immunocytochemistry has revealed a pattern of zones and stripes in all mammalian species examined thus far (>20), and the same is also true in chicken (unpublished data). In fish, two expression patterns have been reported – either all Purkinje cells are zebrin immunoreactive (e.g., zebrafish, *Danio rerio*: Lannoo et al., 1991b), or there are both zebrin<sup>+</sup>/<sub>-</sub> phenotypes, but not organized into stripes (e.g., *Eigenmannia*: Lannoo et al., 1991a; *Gnathonemu*: Meek et al., 1992).

### 3.3.1 *Comparison of Zebrin Stripes in Pigeons and Mammals*

In rodents, the fundamental cerebellar architecture consists of four transverse zones: the anterior zone (AZ: ~lobules I-V), the central zone (CZ: ~lobules VI-VII), the posterior zone (PZ: ~lobules VIII-IX), and nodular zone (NZ: ~lobules IX-X) (Ozol et al., 1999). Within the AZ and PZ, zebrin<sup>+</sup> Purkinje cells are distributed as an array of immunoreactive parasagittal stripes - more than a dozen in some places - separated by intervening zebrin<sup>-</sup> stripes (Brochu et al., 1990; Eisenman and Hawkes, 1993; Sillitoe and Hawkes, 2002). Based on the expression pattern of zebrin in the pigeon cerebellum we can tentatively identify four transverse zones reminiscent of those in mammals – two striped (folia II-V and folia VII- IX), and two essentially zebrin<sup>+</sup> (folium VI and folium X). We therefore suggest that folia II-V are homologous with the mammalian AZ, folium VI-VII with the CZ, folia VII- IX with the PZ and folium X with the NZ. In

addition, we identify a transverse expression domain associated with the lingula (folium I) that seems to have no mammalian homolog.

### *3.3.1.1 Anterior Zone*

The mammalian AZ is characterized by an array of thin stripes of Purkinje cells that express zebrin separated by broad stripes that either express no zebrin immunoreactivity (e.g., rat, mouse) or express it more weakly (e.g., cat, primate). Parasagittal stripes of zebrin+ Purkinje cells are also evident in the pigeon AZ. These stripes are not as narrow as those in the mammalian AZ, but the zebrin– stripes are slightly broader than the zebrin+ stripes. The homology between pigeon folia II-V and the mammalian AZ is supported by the similar distribution of afferent terminal fields: both zones are prominent targets of the spinocerebellar projection. The avian spinocerebellar tract has terminal fields in folia I-VIa,b. The projections from the neck, is primarily to folia II-IV, whereas the cervical enlargement, representing the wings, projects primarily to folia III-V and the lumbosacral enlargement, representing the legs, projects primarily to folia III-VIa,b (Fox and Snider, 1967; Schulte and Necker, 1998; Necker, 2001). In rats and mice, the spinocerebellar and cuneocerebellar tracts project strongly to lobules II-V of the anterior lobes (e.g. reviewed in: Voogd et al., 1996; Voogd and Ruigrok, 1997) where they segregate into parasagittal stripes in register with the

overlying Purkinje cell stripes (Gravel and Hawkes, 1990; e.g., Akintunde and Eisenman, 1994; Ji and Hawkes, 1994; 1995).

### *3.3.1.2 Central Zone*

The alternating zebrin<sup>±</sup> stripes that characterize the AZ in pigeons are replaced in folium VI by a more uniform pattern of zebrin expression, not unlike the CZ (lobules VIa,b,c) in rodents. Although zebrin does not reveal CZ heterogeneity, in rodents a parasagittally-striped organization has been shown in the afferent terminal field distributions (e.g., Serapide et al., 1994; Voogd and Ruigrok, 1997) and by the expression of the small heat shock protein Hsp 25 (Armstrong et al., 2000: no Hsp25 expression is seen in pigeon Purkinje cells – unpublished data). Other features lend support to the notion that folium VI in birds might be homologous with lobule VI in mammals. In both mammals and birds lobule/folium VII is considered part of the oculomotor vermis where there are both visual and trigeminal inputs (Gross, 1972; Clarke, 1977; Williams, 1995; for review see Voogd and Barmack, 2006). Moreover, folium/lobule VI is a site of heavy pontocerebellar inputs in both birds and mammals (Gerrits and Voogd, 1986; Yamada and Noda, 1987; Voogd and Barmack, 2006).

### 3.3.1.3 Posterior Zone

In the mouse, the transition from the CZ to the PZ lies in the prepyramidal fissure between lobules VII and VIII: on the dorsal aspect of lobule VII all Purkinje cells express zebrin and on the ventral aspect of lobule VIII a prominent array of stripes is apparent (e.g., Eisenman and Hawkes, 1993). In the pigeon, a similar transition, albeit less striking, is seen between folia VII and VIII (Fig. 3.4A,C). A pattern of zebrin expression in which stripes of high-expressing Purkinje cells are separated by equal-width stripes of anti-zebrin unreactive cells characterizes the PZ in many mammals, including mouse (e.g., Sillitoe and Hawkes, 2002), rat (e.g., Brochu et al., 1990), rabbit (Sanchez et al., 2002), guinea pig (*Cavia porcellus*: Larouche et al., 2003), hamster (Marzban et al., 2003b), cat (Sillitoe et al., 2003a) and primates (Sillitoe et al., 2004). In the pigeon cerebellum, stripes of zebrin expressing Purkinje neurons are separated by narrow immunonegative stripes in folia VII and VIII, and the dorsal lamella of IXab, and by wider stripes in folia IXcd. Zebrin compartmentation is clearly distinguished in lobule IXcd of the pigeon cerebellum as broad parasagittal P1+ to P3+ stripes of highly immunoreactive Purkinje cells alternating with Purkinje cells that are either zebrin- or express the antigen at low levels. In addition, within the vermis a pair of narrow zebrin+ stripes is seen in the pigeon on either side of P1+ (Fig. 3.3C-E); these are only seen irregularly in the rodent cerebellum (so called 'satellite bands', e.g., Hawkes and Leclerc, 1987). The P4+ to P7+ stripes in folia IXcd in pigeons are present on the lateral extension of folia IXcd into the

auricle (flocculus). Thus, based on the pattern of zebrin expression, it appears that the PZ of mammals (lobules VII and VIII) corresponds with folia VII-IXcd. A similar striped pattern of expression is also found in folium IX of the chick cerebellum, supporting the hypothesis that folium IX constitutes the PZ of the avian cerebellum (Hawkes et al., unpublished data). In mammals, lobule IX is part of the NZ, where there is uniform expression of zebrin (see below). Thus, folium IX in pigeons appears to correspond to lobule VIII in mammals. This postulation is consistent with data on mossy fibre afferent terminal field distributions. For example, spinocerebellar pathways to the posterior cerebellum predominantly terminate in lobule VIII (pyramis) in mammals, but folium IX (uvula) in the chicken (Vielvoye and Voogd, 1977) and pigeon (e.g., Necker, 1992).

Other data, however, argue against the postulated homology of folia IX in birds and lobule VIII in mammals. An extensive literature has examined the physiology and climbing fibre afferents to folia IXcd and X in birds (Wylie and Frost, 1991; 1993; Lau et al., 1998; Wylie et al., 1998; Wylie and Frost, 1999; Wylie et al., 1999b; Crowder et al., 2000; Winship and Wylie, 2001; Wylie, 2001; Winship and Wylie, 2003). The climbing fibres carry optokinetic information to folia IXcd and X, and form parasagittal stripes spanning the two folia. In the lateral half of folia IXcd and X, Purkinje cells respond to rotational optokinetic stimuli in precisely the same manner as the flocculus in mammals (Graf et al., 1988). Moreover, the zonal organization is remarkably similar to the flocculus in rats and rabbits (Voogd and Wylie, 2004). Thus, based on response properties and

the P4+/- to P7+/- stripes, folium IXcd in pigeons appears to be the homolog of the flocculus, but the flocculus in mammals is uniformly zebrin+ (e.g., Ozol et al., 1999; Sanchez et al., 2002; Sillitoe and Hawkes, 2002; Marzban et al., 2003a). In the medial parts of the ventral lamella of folium IXcd and in folium X of pigeons Purkinje cells respond to translational optokinetic stimulation (Wylie and Frost, 1999). These responses are reminiscent of those in the uvula (lobule IX) and nodulus (lobule X) of mammals, where Purkinje cells respond to either optokinetic stimuli or vestibular stimulation originating in the otolith organs (Barmack and Shojaku, 1995). Thus, despite the stripes, the medial parts of folium IXcd more resemble the uvula in mammals.

#### *3.3.1.4 Nodular Zone*

In mammals, the NZ is characterized by uniform zebrin expression in lobules IX and X. In mouse, the interdigitated boundary between the PZ and the NZ lies in the ventral face of lobule IX (Ozol et al., 1999; Armstrong and Hawkes, 2000). In the pigeon, an NZ in which all Purkinje cells uniformly express zebrin is restricted to folium X (the nodulus). Folium X is separated from the rest of the cerebellar cortex by the posterolateral fissure – the first to form during cerebellar development (Larsell, 1967). The lateral extensions of folium X, the flocculi, are uniformly zebrin+ in pigeon, as in mammals. Primary vestibular afferents are restricted to lobules IX and X in mammals (Fox and Snider, 1967;

Voogd and Wylie, 2004) and folium IXcd and X in birds (Schwarz and Schwarz, 1983), perhaps indicative in birds of the same interdigitation of transverse zones also identified in mammals (e.g., Ozol et al., 1999). There is also a prominent external granular layer boundary in this region for Tlx-3 expression in chick (Logan et al., 2002). Granule cell lineage boundaries between the PZ and NZ have been described in mice (e.g., Hawkes et al., 1999). Vestibular afferents also terminate in lobules I and II, perhaps hinting at a relationship between the nodular zone and a putative lingular zone (see below).

#### *3.3.1.5 Lingular Zone*

The most obvious discrepancy between the cerebellar ground plan in mammals and birds, as reflected in the present data, is the strong uniform expression of zebrin by all Purkinje cells in folium I. There is some reason to consider the lingula as a distinct transverse zone, with no equivalent in mammals. For example, the lingula is always separated from the more ventral lobulus centralis by a deep precentral fissure and for this reason, together with its large size in birds, the lingula was regarded by Larsell (1948) as a distinct primary folium. Functionally, the avian lingula has been associated with the control of tail feathers and the tail musculature (Larsell, 1948), although Necker (2001) reported a high concentration of neck afferents in I. It is possible that lobule I in mammals is a rudimentary homolog of the avian lingula, but given their very different patterns of zebrin expression, we favor the hypothesis that the avian lingula is a

unique transverse zone with no mammalian homolog, and that lobule I in mammals is simply the anterior tip of the AZ, continuous with lobules II-V. A second possibility is that the lingula in pigeon is derived embryologically from the NZ, with which it shares a zebrin expression profile. Although folium I is located at the extreme anterior of the cerebellum and X the posterior extreme, because of the way the cerebellum folds, they end up located adjacent to one another, but separated by the recess of the fourth ventricle. This is consistent with afferent terminal field maps showing that mossy fibre afferent fibres from the reticular nucleus of the pons terminate in all folia of the vermis, except I and X (Kawamura and Hashikawa, 1981; Gerrits and Voogd, 1986) and with the observations that both I and X are vestibulocerebellar receiving areas (Fox and Snider, 1967; Williams, 1995). In this context, it is pertinent that the flocculonodular lobe, which receives mostly vestibular inputs, and the lingula, which receives spinocerebellar and vestibulocerebellar inputs, together form the oldest part of the cerebellum, known classically as the archicerebellum (Williams and Warwick, 1980).

### **3.3.2 Are there hemispheres in the pigeon cerebellum?**

Many previous researchers have regarded the large central body of the avian cerebellum as the homolog of the mammalian cerebellar vermis (Fox and Snider, 1967). However, Larsell (1948) directed attention to the fact that on either

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

### **3.3.3 Evolutionary implications**

Our results clearly demonstrate the zebrin is expressed as a pattern of parasagittally oriented stripes in the cerebellar cortex of pigeons. As such, this is not only the first demonstration of zebrin immunoreactivity in a bird, but also the

first published study of zebrin expression in a vertebrate other than fish (Brochu et al., 1990; Lannoo et al., 1991a; Lannoo et al., 1991b; Meek et al., 1992) or mammals (Sillitoe et al., 2005). Although we suggest that the pattern of zebrin labeling is largely consistent, and possibly homologous, with that of mammals, the prominent differences in the flocculus and lingula between birds and mammals also suggests that this may not be true for all folia and lobules. Whether these regions truly are homologous and whether the lateral stripes actually represent the hemispheres requires the further examination of zebrin expression in other groups of vertebrates, especially in non-avian reptiles (i.e., snakes and lizards) and crocodylians. The cerebellum of non-avian reptiles consists of a single, leaf-shaped sheet in which the granule cell layer is inverted such that it is the dorsal most layer of the cerebellar cortex (Larsell, 1967). Crocodylians, on the other hand, possess an avian-like cerebellum with three folds that have been homologized with the avian cerebellar folia (Larsell, 1967) in the following combinations: I-V, VI-VIII and IX-X. If zebrin expressing zones are evolutionarily conserved among amniotes as they are within mammals (Sillitoe et al. 2005), then similar transitions in stripe patterns between the AZ, CZ, PZ and NZ should be apparent in both non-avian reptiles and crocodylians. If not, then the zonal organization of the cerebellum may be an example of convergent evolution between birds and mammals (i.e., homoplasy) and not necessarily a homologous trait.

### 3.4 References

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Chapter 4: **Congruence of Zebrin II Expression and Functional Zones  
defined by Climbing Fibre Topography and Purkinje Cell Response  
Properties in the Flocculus**

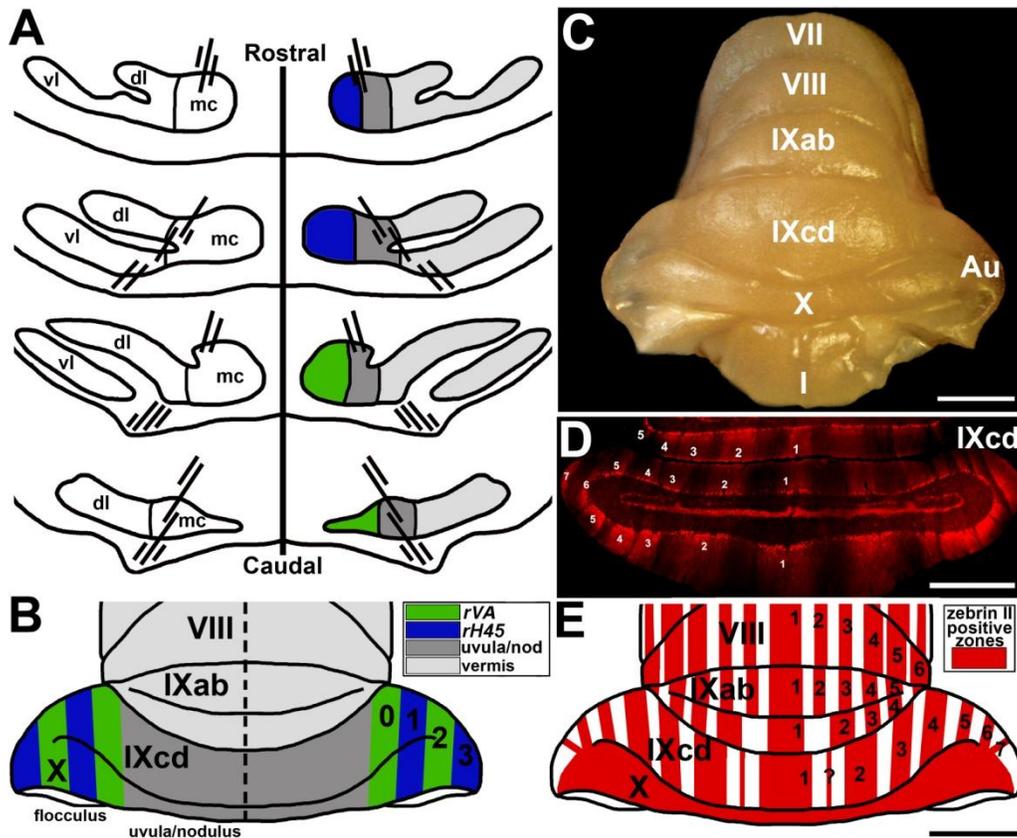
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The fundamental architecture of the cerebellum consists of parasagittal zones that are oriented perpendicular to the length of the folia (e.g. Voogd and Bigaré, 1980). These parasagittal zones can be defined by climbing fibre (CF) and mossy fibre (MF) input, Purkinje cell projection patterns, and Purkinje cell response properties (Llinas and Sasaki, 1989; De Zeeuw et al., 1994; Voogd and Glickstein, 1998; Wu et al., 1999; Ruigrok, 2003; Apps and Garwicz, 2005). A parasagittal organization has also been revealed with several molecular markers (for review see Herrup and Kuemerle, 1997), but the most thoroughly studied of these is zebrin II (aldolase C; Brochu et al., 1990; Ahn et al., 1994; Hawkes and Herrup, 1995), which is expressed by Purkinje cells. Zebrin immunopositive (zebrin+) Purkinje cells are distributed as a parasagittal array of stripes, separated by zebrin immunonegative (zebrin-) stripes (e.g. Larouche and Hawkes, 2006). Zebrin stripes have been shown in several mammalian species, and recently we have shown that zebrin is also expressed in the pigeon cerebellum with a pattern that is strikingly similar to that found in mammals (Fig. 4.1D,E; Pakan et al. 2007). Thus, the pattern of zebrin stripes is highly conserved, and is likely critical for fundamental cerebellar function. One difference between mammals and the pigeon is in the vestibulocerebellum (uvula, nodulus and flocculus; see Fig. 4.1B,C). In both pigeons and mammals, the nodulus (folium X) is uniformly zebrin+ (see Fig. 4.1E; Hawkes and Herrup, 1995; Pakan et al., 2007). Folium IXcd in the pigeon, which includes the uvula (medial half) and the flocculus (lateral half; Wylie and Frost, 1993; Wylie et al., 1993), consists of an array of

seven striking zebrin<sup>+</sup>/<sup>-</sup> stripes (P1<sup>+</sup>/<sup>-</sup> through P7<sup>+</sup>/<sup>-</sup>; see Fig. 4.1D,E; Pakan et al. 2007). In mammals, the uvula also consists of a series of zebrin<sup>+</sup>/<sup>-</sup> stripes, however, the flocculus is uniformly zebrin<sup>+</sup> (Brochu et al., 1990; Hawkes and Gravel, 1991; Hawkes et al., 1993; Hawkes and Herrup, 1995; Ozol et al., 1999; Armstrong and Hawkes, 2000; Sanchez et al., 2002; Marzban et al., 2003b; Sillitoe et al., 2003a). Although the specific function of zebrin expression in the cerebellum is largely unknown, zebrin expression is useful for, and often used as, a positional landmark in the cerebellar cortex (Hawkes and Gravel, 1991; Hawkes, 1992; Hawkes et al., 1993; Ozol et al., 1999).

The purpose of the present study was to examine the functional relationship between CF input, the electrophysiological response properties (Purkinje cell complex spike activity; CSA), and zebrin stripes in the flocculus of pigeons, for two main reasons. First, based on studies in rodents, it has been suggested that the CFs to the zebrin<sup>-</sup> stripes carry somatosensory information, whereas the CFs that project to the zebrin<sup>+</sup> stripes carry information from the visual and perhaps other sensory systems (Voogd et al., 2003; Sugihara and Shinoda, 2004; Sugihara and Quy, 2007; Sugihara and Shinoda, 2007). This scheme cannot apply to the pigeon flocculus. Folium IXcd of the pigeon flocculus clearly consists of zebrin<sup>+</sup> and – stripes (see Fig. 4.1D; Pakan et al., 2007), yet there is no somatosensory CF input. The pigeon inferior olive consists of three regions, the ventral and dorsal lamella, and the medial column (mcIO; Arends and Voogd, 1989). The flocculus receives CFs from medial subnuclei of the mcIO,



**Figure 4.1 Parasagittal organization of the pigeon flocculus.** Purkinje cell complex spike activity in the flocculus, the lateral portion of folium IXcd and X, responds best to rotational optic flow about either the vertical axes (rVA; green) or an horizontal axis oriented 45° to midline (rH45; blue; Graf et al., 1988; Wylie and Frost, 1993). **C** shows a photograph of the posterior pigeon cerebellum, indicating folia IXcd and X, as well as their lateral extension forming the auricle (Au). Shown in **B**, the rVA and rH45 cells are organized into four zones in the flocculus: two rVA zones (0 and 2, green) interdigitated with two rH45 zones (1 and 3, blue; Winship and Wylie, 2003). The rVA and rH45 zones receive climbing fibre input from the caudal and rostral medial column (mc) of the

inferior olive, respectively (A; Wylie et al., 1999c). **D** and **E** illustrate the pattern of zebrin expression in the pigeon posterior cerebellum (adapted from Pakan et al., 2007), shown with a coronal section through the posterior cerebellum (**D**) and a schematic of the pattern of zebrin positive stripes (**E**). The zebrin stripes are numbered from 1-7 through folium IXcd (**D,E**). dl, vl, = dorsal and ventral lamellae of the inferior olive. All scale bars = 1mm.

and it appears that this input conveys only visual optic flow information. This is supported by anatomical studies demonstrating that the mcIO receives visual input from retino-recipient nuclei in the pretectum and accessory optic system (Clarke, 1977; Brecha et al., 1980; Gamlin and Cohen, 1988a; Wylie et al., 1997; Wylie, 2001), and by electrophysiological studies detailing the responses of neurons in the mcIO and flocculus to optic flow stimuli (Wylie and Frost, 1993; Winship and Wylie, 2001). Also, somatosensory information, from both ascending (Wild, 1989a) and descending systems (Wild and Williams, 2000) reaches the ventral lamella of the inferior olive and not the mcIO. Second, there have been recent attempts to determine if the zebrin stripes correspond with functional zones of the cerebellum (Chockkan and Hawkes, 1994; Hallem et al., 1999; Voogd et al., 2003; Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004; Pijpers et al., 2006; Sugihara et al., 2007; Sugihara and Shinoda, 2007) and the pigeon flocculus offers a remarkable opportunity in this regard. The functional zonal organization of the flocculus has been extensively documented, and is essentially identical in mammals and birds (Voogd and Wylie, 2004). The CSA of floccular Purkinje cells, which represents CF activity (Eccles et al., 1966), responds best to patterns of optic flow that result from self-rotation about one of two axes: either the vertical axis (rVA, Fig. 4.2C), or a horizontal axis oriented at 45° azimuth (rH45; Fig. 4.2D; rabbits, Simpson et al., 1981; Graf et al., 1988; pigeon, Wylie and Frost, 1993). In several species, it has been shown that the rVA and rH45 cells are organized into parasagittal zones (Voogd and Wylie, 2004).

The pattern of the zones in the pigeon flocculus is shown in Figure 4.1B. There are two rVA zones (0 and 2) interdigitated with two rH45 zones (1 and 3). In caudal sections, the four zones can be seen in the coronal plane, but much of zone 3 resides rostromedially in the auricle, a distinct lateral protrusion of the avian cerebellum (Larsell, 1967). The CF inputs to the rVA and rH45 zones originate in the caudal and rostral regions of the mcIO, respectively (see Fig. 4.1A; Wylie et al., 1999c; Winship and Wylie, 2003). These subregions of the mcIO can be considered homologous with the mammalian dorsal cap of Kooy as the connectivity is strikingly similar. It has been shown in several mammalian species that the caudal and rostral regions of the dorsal cap project to the rVA and rH45 zones, respectively (for review see Voogd and Wylie 2004).

In the first part of this study, to determine the relationship of the zebrin stripes to the CF projections in the pigeon flocculus, we made small injections of the anterograde tracer biotinylated dextran amine (BDA) into either the caudal (rVA) or rostral (rH45) regions of the mcIO in pigeons and examined the resulting olivocerebellar CF labeling in relation to the zebrin expression pattern in the flocculus. In the second part of the study, using single-unit extracellular recording techniques, we mapped out the visually responsive Purkinje cells in the various zones of the flocculus and made small fluorescent tracer injections to mark the boundaries of the zones; we then visualized zebrin expression to determine the location of each marked recording site in relation to the zebrin stripes.

## 4.1 Methods

### 4.1.1 *Surgical Procedures*

The methods reported herein conformed to the guidelines established by the Canadian Council on Animal Care and were approved by the Biosciences Animal Care and Policy Committee at the University of Alberta. Silver King and Homing pigeons (*Columba livia*), obtained from a local supplier, were anesthetized by an intramuscular injection of a ketamine (65 mg/kg) /xylazine (8 mg/kg) cocktail. Supplemental doses were administered as necessary. Animals were placed in a stereotaxic device with pigeon ear bars and a beak bar adapter so that the orientation of the skull conformed to the atlas of Karten and Hodos (1967).

#### 4.1.1.1 *Inferior Olive Injections and Climbing Fibre Labeling*

For the first part of the study, the intent was to make localized injections into the subnuclei of the inferior olive that provide climbing fibre input to the flocculus. The pigeon inferior olive is divided into ventral and dorsal lamella, which are conjoined medially by the mcIO (Arends and Voogd, 1989).

Throughout the text we refer to the olivary input to the rVA and rH45 zones as originating in the caudal and rostral mcIO, respectively, as illustrated in Figure 4.1A. The rostro-caudal extent of the mcIO ranges from about 1.5-1.8mm in length. From our previous work (Wylie et al., 1999c) we showed that retrograde labeling from injections into the rVA zones is concentrated in the caudal 700-

800 $\mu$ m, whereas labeling from rH45 zones is concentrated in the rostral 700-800 $\mu$ m. The border between those areas projecting to the rVA and rH45 zones could be quite sharp (see Fig. 1 of Wylie et al., 1999), and double-labeling has shown that there is no overlap (Pakan et al., 2005). There is an essentially identical distinction between the caudal and rostral dorsal cap in rabbits and rats, which project to the rVA and rH45 zones in these species (Voogd and Wylie, 2004).

To access the inferior olive, bone and dura were removed from the dorsomedial surface of the cerebellum, lateral to the mid-sagittal sinus. To ensure that we were in the desired olivary subnuclei, single-unit extracellular recordings were used to confirm the location of the injection sites. To record the activity of optic flow units in the inferior olive, glass micropipettes filled with 2 M NaCl, with tip diameters of 4-5 $\mu$ m, were advanced through the cerebellum and into the brainstem using an hydraulic microdrive (Frederick Haer & Co.). Extracellular signals were amplified, filtered, and fed to a window discriminator. Inferior olivary units are easily identified based on their characteristically low firing rate (approximately 1 spike/sec) and proximity to the base of the brain. Upon isolation of a unit in the inferior olive, the optic flow preference of the unit was qualitatively determined. The direction-selectivity of the olivary neuron was determined by moving a large (90 X 90°) hand-held visual stimulus, consisting of a random pattern of dots and lines, in the receptive field of the unit. With such stimuli, rVA and rH45 units are easily identified (Winship and Wylie, 2001;

2003). Once the desired area was isolated, the recording electrode was replaced with a micropipette (tip diameter 20-30 $\mu$ m) containing fluorescent BDA; either mini-ruby (red; D-3312) or mini-emerald (green; D-7178; 10,000 molecular weight; Invitrogen, Carlsbad, CA). The tracers (0.01-0.05  $\mu$ l of 10% solution in 0.1M phosphate buffer) were pressure injected using a Picospritzer II (General Valve Corporation).

#### *4.1.1.2 Electrophysiological Recording of Visual Response Properties*

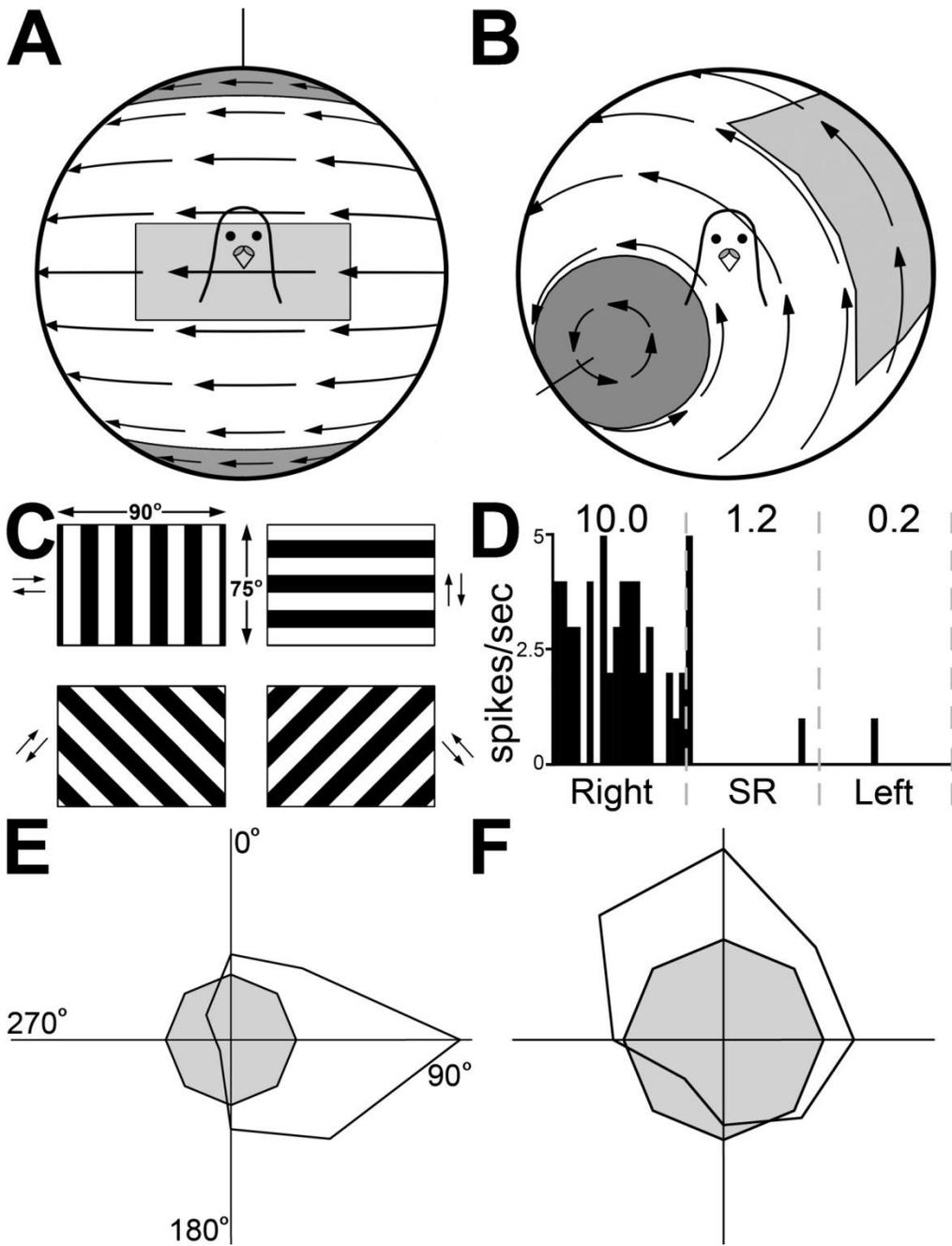
For the second part of the study, the flocculus was accessed by removing the bone surrounding the semicircular canals, as the dorsal surface of the flocculus (folium IXcd) lies within the radius of the anterior semicircular canal. This exposure allows easy access to the two rVA zones (zones 0 and 2) and the rH45 zones (zone 1 and 3; see Figure 4.8A for flocculus exposure example).

Extracellular single unit recordings were then made using glass micropipettes filled with 2M NaCl (tip diameters of 3-5 $\mu$ m). Electrodes were advanced using a hydraulic microdrive (Frederick Haer & Co.) and raw signals were amplified, filtered and fed to a data analysis system (Cambridge Electronic Designs (CED) *1401plus*). The raw trace of the extracellular recording was spike-sorted to ensure isolation of a single unit using *Spike2* software (CED). Peri-stimulus time histograms (PSTHs) were constructed using *Spike2* (See Fig. 4.2D for example).

The CSA of Purkinje cells was identified and isolated based on their characteristic spike shape and spontaneous firing rate of about 1 spike/s. Isolated

units were first stimulated with the handheld stimulus mentioned above to determine if the cell was sensitive to visual stimulation. By moving this stimulus in different areas of the panoramic binocular visual field, the optic flow preference of each unit was qualitatively determined. The visual test stimuli were then back-projected onto a screen measuring  $90^\circ \times 75^\circ$  (width  $\times$  height) that was positioned in the frontal visual field (from  $45^\circ$  ipsilateral to  $45^\circ$  contralateral azimuth). The stimuli consisted of drifting square wave gratings (in one of four orientations; illustrated in Fig. 4.2C) of an effective spatial and temporal frequency (spatial frequency = 0.5 cycles per degree, temporal frequency = 0.5 Hz), generated by a *VSGThree* (Cambridge Research Services) and back-projected (*InFocus* LP750) onto the screen. Direction tuning curves were obtained by moving the gratings in 8 different directions (see Fig. 4.2C). Responses were averaged over at least 3 sweeps, where each sweep consisted of 5 seconds of motion in one direction, a 5 second pause, and 5 seconds of motion in the opposite direction, followed by a 5 second pause.

Several electrode penetrations were made to map out the locations of zones (e.g. see Fig 4.8A for marked recording electrode tracks). Upon completion of recording, in some cases the recording electrode was replaced with a micropipette (tip diameter 20-30 $\mu$ m) containing fluorescent BDA (see above) or fluorescent retrograde microspheres (Lumafuor Corp, Naples, FL). The tracers (0.01-0.05  $\mu$ l of 10% solution in 0.1M phosphate buffer) were pressure injected using a Picospritzer II (General Valve Corporation) at the site of recording.



**Figure 4.2 Electrophysiological recording of rotation sensitive neurons in the Pigeon VbC.** **A** and **B** show optic flow-fields generated by self-rotation with arrows indicating the motion vectors in the optic flow-field. The flow-field in **A** consists of a circular flow-field rotating about a vertical axis (rVA). The flow-field motion is opposite to the direction of head rotation. **B** illustrates the optic flow-field resulting from clockwise head rotation. The arrows, as projected onto a sphere, illustrate the counter clockwise rotation of local motion in the flow-field, which collectively constitute the whole-field rotational optic flow. The “pole” is shown with dark grey shading and represents the axis of rotation; at the “equators” (light grey shading) of the sphere, the flow-field is laminar, with all vectors pointing in approximately the same direction. **C** shows the four grating orientations used to determine preferred direction of motion and tuning curves. Each grating moved in both directions, perpendicular to the orientation of the grating, to produce a tuning curve with a total of 8 directions (45° increments). **D** shows an example of the modulation of an rVA unit, during one sweep consisting of 5 seconds upward motion (avg of 10.0 spikes/sec), followed by a 5 second pause (spontaneous rate – SR - avg of 1.2 spikes/sec) and then 5 seconds of downward motion (avg of 0.2 spikes/sec). **E** and **F** are local motion tuning curves for representative rotation units. In **E**, an rVA unit is shown which was recorded at injection site #3 in Figure 4.8 and **F** shows an rH45 unit which was recorded at injection site #1 in Figure 4.8. The polar plots show firing rate (black line) minus spontaneous rate, with grey shading representing negative values (i.e. inhibition).

After all surgeries the craniotomy was filled with bone wax and the wound was sutured. Birds were given an intramuscular injection of buprenorphine (0.012mg/kg) as an analgesic. After a recovery period of 3-5 days, the animals were deeply anesthetized with sodium pentobarbital (100mg/kg) and immediately transcardially perfused with phosphate buffered saline (PBS; 0.9% NaCl, 0.1M phosphate buffer) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brain was extracted from the skull and immersed in paraformaldehyde for 7 days at 4°C. The brain was then embedded in gelatin and cryoprotected in 30% sucrose in 0.1M PBS overnight. Using a microtome, frozen serial sections in the coronal plane (40µm thick) were collected throughout the rostro-caudal extent of the cerebellum.

#### 4.1.2 *Zebrin Immunohistochemistry*

Zebrin immunohistochemical procedures are described in section 3.1.1.2. The primary antibody, mouse monoclonal anti-zebrin II (Brochu et al., 1990) was kindly provided by Richard Hawkes, University of Calgary.

#### 4.1.3 *Microscopy*

For details on microscopy as well as image capture and analysis see section 2.1.2.

#### 4.1.4 *Nomenclature of the Pigeon Flocculus*

For the nomenclature of the pigeon flocculus see section 2.1.3.

## 4.2 Results

In all animals we observed the expected pattern of zebrin immunoreactivity in folium IXcd (Figs. 4.1D,E, 4.5-4.8) consisting of seven zebrin<sup>±</sup> stripes. The stripes themselves are numbered following the nomenclature used in Pakan et al. (2007) which is the same as that in mammals, whereby the most medial positive stripe is designated P1<sup>+</sup> and the number increases as the stripes move laterally to P7<sup>+</sup> (see Fig 4.1D,E; Brochu et al., 1990; Eisenman and Hawkes, 1993; Ozol et al., 1999; Sillitoe and Hawkes, 2002; reviewed in Sillitoe et al., 2005). The seven stripe pairs were consistently seen in all specimens; the width of individual stripes can vary both between animals, as well as along the rostro-caudal dimension within animals. Therefore, in designating the band numbers it is important to complete an examination of all sections throughout the rostro-caudal extent of the vestibulocerebellum (see Figure 4.7 for a complete reconstruction in this regard). Folium X is generally immunopositive, with the exception of the dorsal lamella at rostral levels, where IXcd and X merge to form the auricle (see below, Figs. 4.6, 4.7).

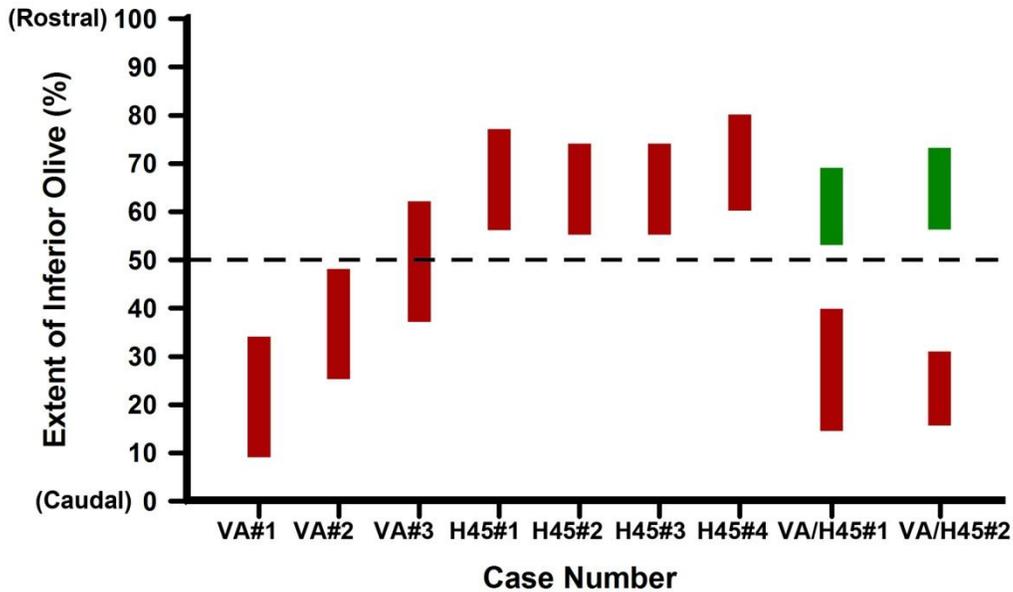
#### 4.2.1 *Inferior Olive Injections and Climbing Fibre Labeling*

For the first part of the study, the results are based on observations in nine animals, where injections of red and/or green fluorescent BDA were made into the mcIO. In seven animals, a single injection of red BDA was made in the mcIO. In 3 of these cases, the injections were aimed at the caudal mcIO and rVA cells were recorded at the injection sites (cases VA#1-3). In the other four cases the injections were aimed at the rostral mcIO and rH45 cells were recorded at the injection sites (cases H45#1-4). The remaining two animals received two injections each: a red injection was made into the caudal mcIO (rVA) and a green injection was made into the rostral mcIO (rH45) (cases VA/H45#1 and 2). The extent of the injections is shown in Figure 4.3. The injections were small, covering from 200 to 360 $\mu$ m of the rostro-caudal extent of the mcIO (average= 270 $\mu$ m). All injections at sites where H45 cells were recorded were confined to the rostral mcIO. All but one of the injections at sites where rVA cells were recorded, were confined to the caudal half of the mcIO. In the exception (case VA#3), the injection was largely in the caudal mcIO, but spread to the rostral half. Critically, in the cases involving two injections (VA/H45 #1 and 2), the red and green injections were non-overlapping. Figure 4.4A,B show photomicrographs of representative injection sites (case VA/H45#2), illustrating a red BDA injection in the caudal (rVA) mcIO (A) and a green BDA injection in the rostral (H45) mcIO (B).

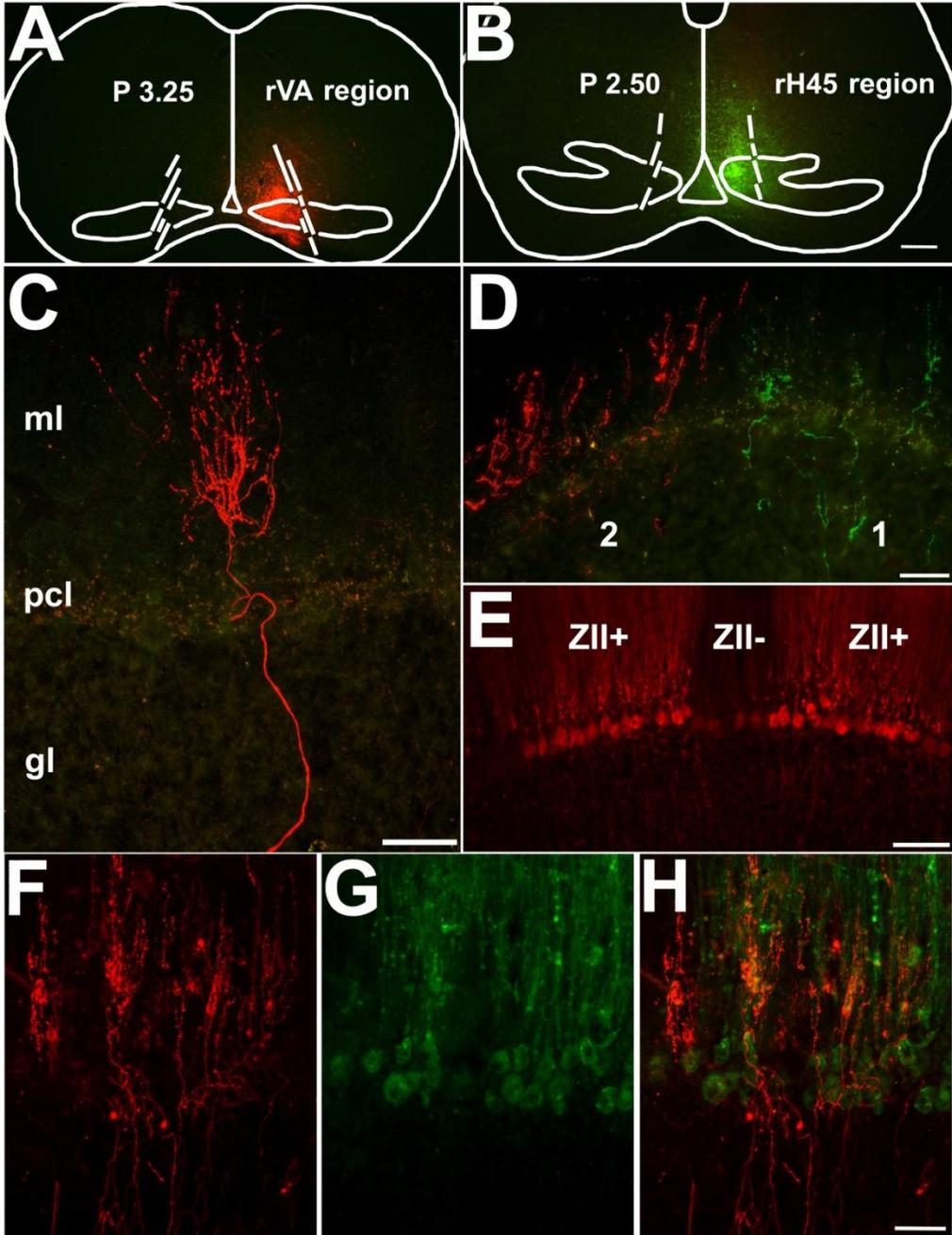
The resulting CF labeling from both the red and green BDA was entirely contralateral in all cases, robust, and easily distinguishable in the molecular layer (Figure 4.4C,D,F and H). From our injections in the caudal (rVA) mcIO resulting CF labeling consisted of a caudomedially located zone (zone 0) and a more rostromedially located zone (zone 2) in both folia IXcd and X. From our injections in the rostral (H45) mcIO we observed CF labeling clearly organized into two zones (zones 1 and 3). In cases VA/H45#1 and 2, the CF labeling from the caudal (red) and rostral (green) mcIO were clearly interdigitated and non-overlapping (see Figs. 4.4D, 4.5-4.7).

#### *4.2.1.1 Correlation of Climbing Fibres to the Zebrin Expression Pattern*

From the injections in the caudal and rostral mcIO (rVA, rH45), there was a clear correspondence with the zebrin stripes, which is illustrated in Figures 4.5-4.7. The injections in the caudal mcIO resulted in CF labeling in zebrin zones P4+/-, and P6+/-, whereas the injections in the rostral mcIO resulted in CF labeling in zebrin zones P5+/-, and P7+/- (see Table 4.1). Figures 4.5 and 4.6A-C show data from case VA/H45#1, where red- and green-BDA injected in the caudal and rostral mcIO, respectively. From the green-BDA injection labeling was seen in two zones (1 and 3; Figs. 4.5A, 4.6A) interdigitated with labeling in two zones (0 and 2; Figs. 4.5B, 4.6A) from the red-BDA injection. This labeling spanned the P4+/- to P7+/- zebrin stripes in IXcd (Figs. 4.5C, 4.6B). From the overlay of the CF and zebrin labeling (Figs. 4.5D and 4.6B), the correspondence



**Figure 4.3** Location and extent of the injection sites in the inferior olive. A bar graph shows the location and size of each injection, expressed as a proportion of the rostro-caudal extent of the medial column of the inferior olive (mcIO). The caudal and rostral borders of mcIO olive are designated 0% and 100%, respectively, and the dashed line represents the midpoint. It is known from previous research that the caudal half of the mcIO contains rVA cells and projects to zones 0 and 2 in the flocculus. Likewise, the rostral half of the mcIO contains rH45 cells and projects to floccular zones 1 and 3 (Wylie et al., 1999c; Pakan et al., 2005). Single injections of red-BDA were made in cases VA#1-3 and H45#1-4. In cases VA/H45#1 and 2, red- and green-BDA were injected in the caudal and rostral mcIO, respectively.

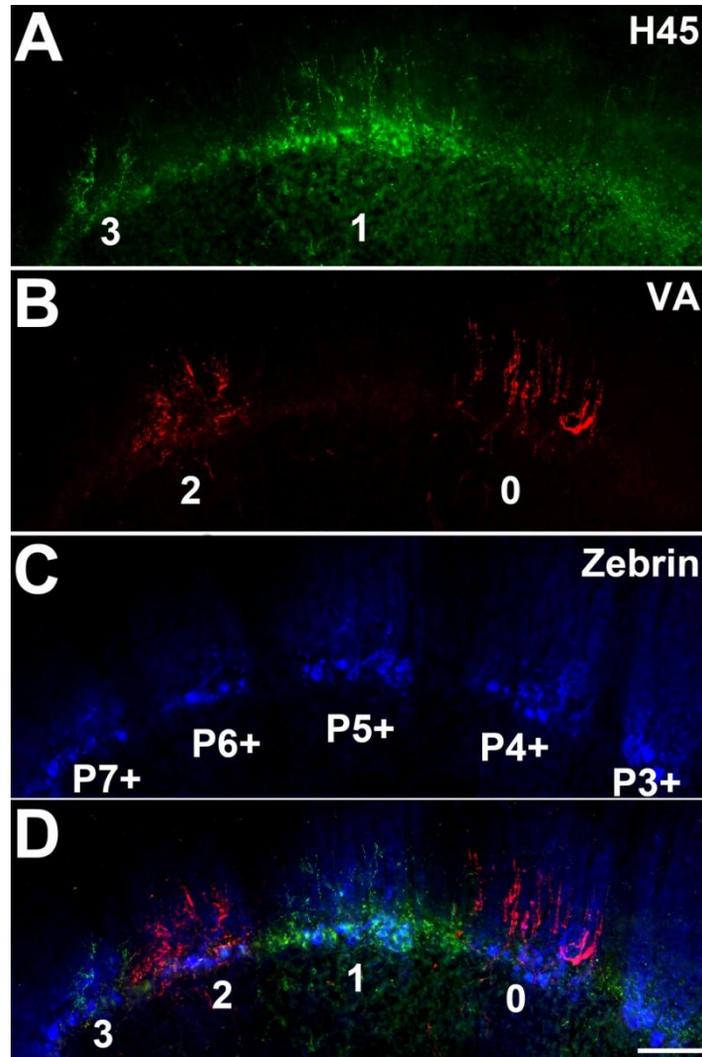


**Figure 4.4 Olivary injections sites, climbing fibre labeling, and zebrin immunohistochemistry in the flocculus.** **A** and **B**, respectively, show photomicrographs of red- and green-BDA injections in the caudal (rVA) and rostral (rH45) regions of the medial column of the inferior olive (mcIO) from case VA/H45#2. P3.25 and P2.50 refer to the rostrocaudal location of the coronal section in the pigeon atlas of Karten and Hodos (1967). In **C-H**, the molecular layer (ml) is represented dorsally, followed by the Purkinje cell layer (pcl), and the granule layer (gl) ventrally. **C** and **D** show photomicrographs of typical BDA labeled climbing fibres from case H45#2 and VA/H45#1, respectively. In **D**, the red and green labeling are easily distinguishable, as illustrated with the labeling in zones 1 (rH45) and 2 (rVA). **E** shows an example of zebrin immunohistochemistry from case H45#1, illustrating zebrin immunopositive (zebrin+) and immunonegative (zebrin-) stripes. The zebrin expression is apparent in the Purkinje cell bodies, axons and dendrites. **F-H** show red BDA labeled climbing fibres (**F**) in a zebrin+ stripe (green, **G**) from case VA#1. The overlay is shown in **H**. Scale bars: **B**= 250µm (also applies to **A**); **C-E**= 100µm; **H**=50µm (also applies to **F** and **G**).

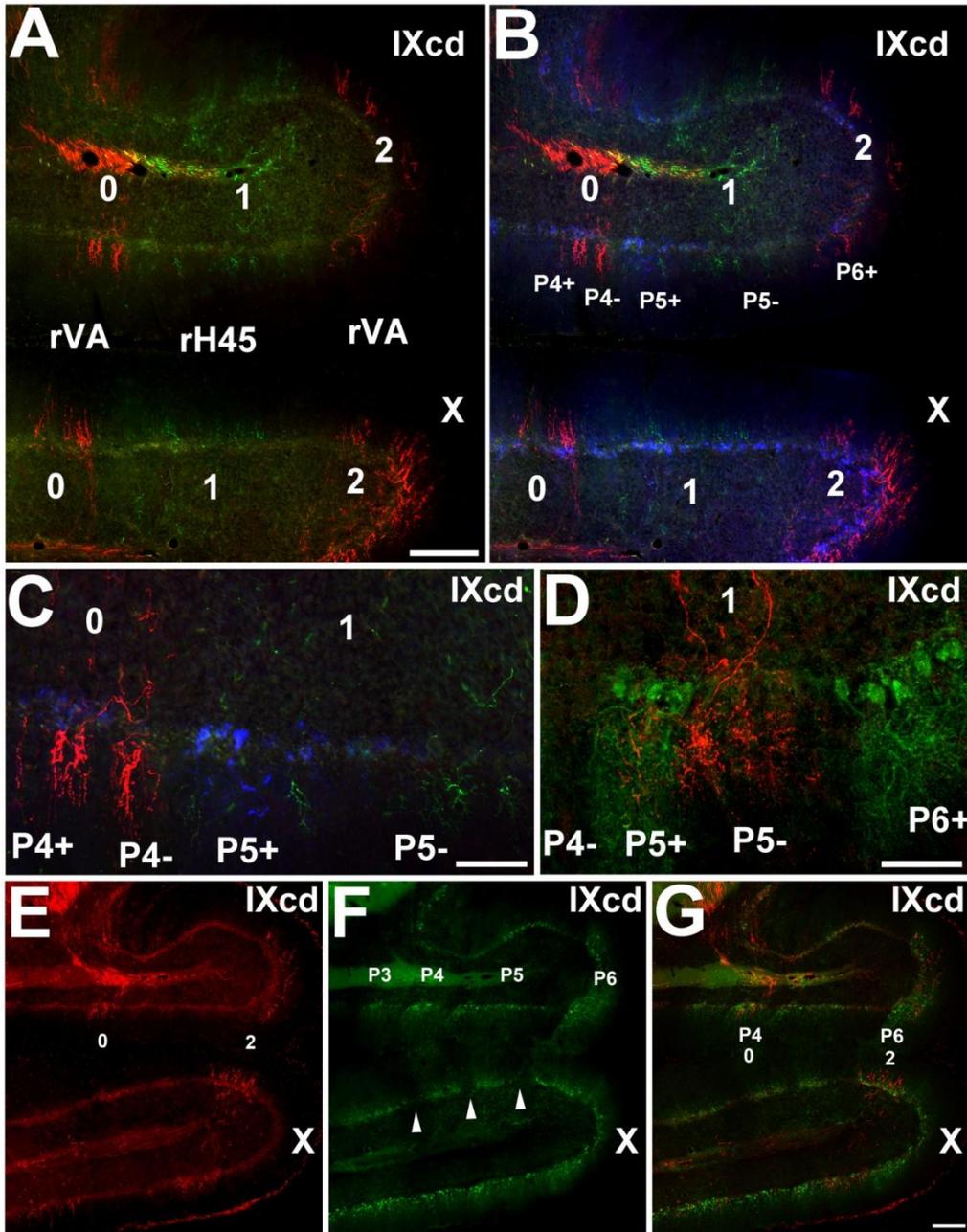
was clear: CF zones 0 and 2 (rVA) correspond to zebrin stripes P4+/-, and P6+/-, whereas CF zones 1 and 3 (rH45) correspond to zebrin stripes P5+/-, and P7+/- (see also Fig. 4.7). As indicated in Table 4.1, there was strong support for this scheme from all cases. The only exceptions were that from the injections in the caudal mcIO in cases VA/H45#1 and 2, a very small amount of CF labeling was observed in the P7- stripe (see Fig 4.7). In both cases, this labeling was found on the ventral-lateral border of the auricle, where X joins IXcd. We are unsure if this area actually represents ventral lamella of X as opposed to IXcd. The injection from case VA#3, which was centered in the caudal rVA region but spread to the rostral half of the mcIO, resulted in CF labeling which was largely in P4+/- and P6+/-, but also included moderate labeling in P7- and a small amount in P7+.

We wish to emphasize, that a CF zone (i.e. zone 0, 1, 2, or 3) spanned a single zebrin stripe including the positive and negative portion. This is illustrated in Figure 4.6D where a red BDA injection was made in the rH45 region of the mcIO (from case H45#1) and zebrin expression was visualized in green. This photomicrograph shows CF's terminating in zone 1 which is concentrated in P5-, but clearly spans into the adjacent P5+ as well. The presence of CF's in both positive and negative portions of a single zebrin stripe can also be seen in Figures 4.5D, 4.6B,C,G, and 4.7.

From injections in the caudal and rostral mcIO, the four CF zones (0-3) were clearly visible in folium X, and contiguous with the zones in IXcd (Figs. 4.6A,E,F and 4.7A). Folium X is generally uniformly immunopositive for zebrin,



**Figure 4.5** Correspondence of climbing fibre zones and zebrin stripes in folium IXcd of the flocculus. **A** and **B** show climbing fibre labeling in IXcd after an injection of green BDA into the rostral medial column of the inferior olive (mcIO; rH45 region) and a red BDA injection into the caudal mcIO (rVA region), respectively. The two rH45 zones (1 and 3), and two rVA zones (0 and 2) were clearly labeled. **C** shows zebrin labeling in blue, with the positive zebrin stripes labeled P3+ to P7+. **D** is the overlay of all three colors and illustrates the concordance between zone 0 and zebrin stripe P4, zone 1 and zebrin stripe P5, zone 2 and zebrin stripe P6 and zone 3 and zebrin stripe P7. Scale bar = 100 $\mu$ m.



**Figure 4.6 Climbing fibre zones and zebrin stripes in the flocculus of folia IXcd and X.** **A** and **B** show photomicrographs of a coronal section of IXcd and X through the flocculus from case VA/H45#1. In **A**, red climbing fibres can be seen from a BDA injection in the caudal medial column of the inferior olive (mcIO; rVA region), in two parasagittal clusters: a medial zone 0 and a lateral zone 2. Green climbing fibres can be seen in zone 1 from a BDA injection in the rostral mcIO (rH45 region). This injection also resulted in climbing fibre labeling in zone 3 (not shown). The climbing fibres zones clearly extend through folium X as well. In **B**, zebrin expression (blue) is superimposed with the climbing fibre zones to reveal the concordance in IXcd of the P4, P5 and P6 zebrin stripes with climbing fibre zones 0, 1 and 2 respectively. In folium X, the zebrin expression pattern is uniformly positive throughout the mediolateral extent of the flocculus. **C** shows a magnified version of zone 0 and 1 in the ventral lamella of folium IXcd. This panel illustrates both the concordance of the boundaries of the zebrin and climbing fibre zones, as well as the presence of climbing fibres in both the positive and negative regions of a particular zebrin stripe (eg. zone 0 climbing fibres (red) in both P4+ and P4- zebrin regions, and zone 1 climbing fibres (green) in both P5+ and P5- zebrin regions, with no spread from either colour into the adjacent zebrin stripe). **D** shows a photomicrograph of IXcd from case H45#1 where red labeled climbing fibres resulting from an injection in the rostral mcIO (rH45 region) extend into both the P5+ and P5- zebrin regions but not into the P4

or P6 stripes. **E-G** shows a photomicrograph of a coronal section of the flocculus through folia IXcd and X, at the region where these two folia are joining to eventually form the auricle (case VA#2). **E** shows climbing fibre labeling in zones 0 and 2 from an injection of red-BDA in the caudal region of the medial column of the inferior olive (rVA region). **F** shows zebrin expression in green, and **G** shows the overlay, illustrating the concordance of the climbing fibre zones 0 and 2 in IXcd with zebrin stripes P4 and P6, respectively. In folium X, the climbing fibres are contiguous with those in IXcd. Although the majority of folium X shows uniform positive zebrin expression, three areas of weak/negative zebrin expression can be seen in the dorsal lamella (indicated by arrowheads; see also reconstruction in Fig. 4.7A). These zebrin immunonegative regions in folium X can only be seen in rostral sections, immediately before folia IXcd and X join to become the auricle, and are prominent only in the dorsal lamella and the lateral regions. These three zebrin negative stripes in folium X correspond to P3-, P4- and P5- from medial to lateral. Scale bars: **A** = 250 $\mu$ m (also applies to **B,E-G**); **C,D**= 100 $\mu$ m.

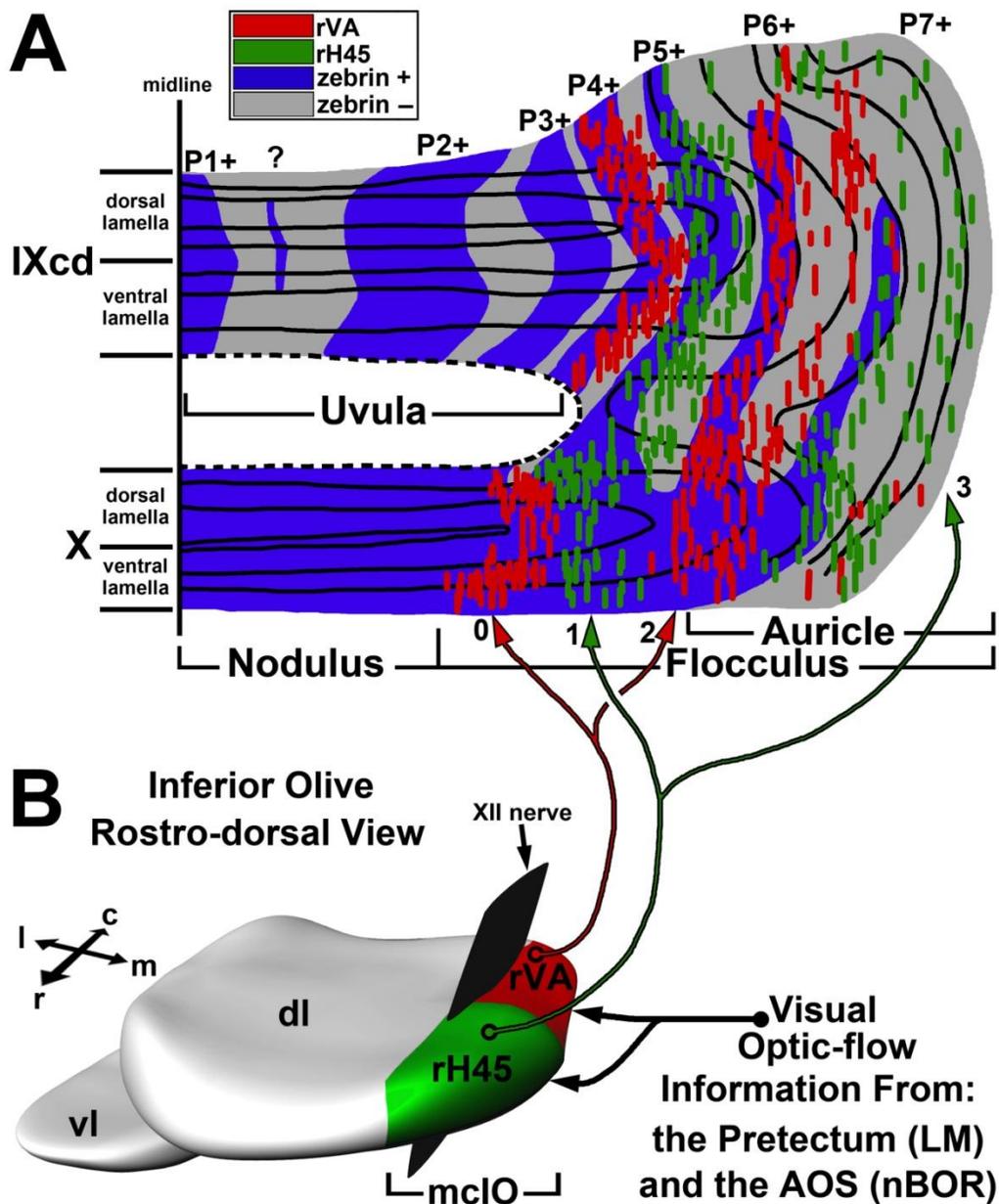
especially in caudal regions of the folium (Fig. 4.6B). In the rostral part of the dorsal lamella of X, where it joins with IXcd, there appears to be a region of transition, where the zebrin stripes in IXcd persist into the lateral margin of X. This is shown in the zebrin expression pattern in Figure 4.6F (green); arrowheads indicate where the immunonegative regions of P3-, P4- and P5- of IXcd are extending into dorsal X (see also Fig.4.7). With the exception of this transition region in the lateral portion, folium X is uniformly zebrin positive, without stripes. The CF zones however persist, with the same pattern observed in IXcd.

Figure 4.7 shows a reconstruction of the pattern of CF labeling as related to the zebrin stripes from case VA/H45#2. The Purkinje cell layer from 27 serial sections has been unfolded and flattened onto the surface of the page, illustrating the CF labeling from injections in the rostral (rH45; green) and caudal (rVA; red) mIO along with the zebrin+ (blue) and zebrin- (grey) stripes. The congruence of zones 0 and 2 with the P4+/- and P6+/- stripes and zones 1 and 3 with the P5+/- and P7+/- stripes is evident.

#### ***4.2.2 Electrophysiological Recording of Visual Response Properties***

The activity of 31 neurons sensitive to optic flow stimuli was recorded from the left flocculus in 4 pigeons. The average spontaneous firing was  $1.26 \pm 0.08$  spikes/s (mean  $\pm$  s.e.m.). These cells responded to large moving visual stimuli and preferred particular patterns of optic flow resulting from self-rotation. about

either a vertical (rVA), or horizontal axis oriented 45° contralateral azimuth (rH45). The identification of these cells was made by examining the responses to drifting large-field sine wave gratings presented to the frontal visual fields (see Fig. 4.2). When recording from the left flocculus, rVA neurons prefer large-field stimuli moving forward (temporal-to-nasal; T-N) and backward (N-T) in the ipsi- and contralateral visual fields, respectively, and rightward motion in the frontal visual field (see Fig 4.2A); in the natural environment, this type of visual stimulation would occur in response to a leftward rotation of the head about the vertical axis. In the left flocculus, rH45 neurons prefer large-field stimuli moving upward in the frontal and ipsilateral visual fields, and downward motion in the contralateral visual field (clockwise optic flow about an horizontal axis oriented at 45° azimuth; see Fig 4.2B); in the natural environment, this type of visual stimulation would occur in response to counter-clockwise rotation of the head about an horizontal axis oriented at 45° contralateral azimuth. In other words, the direction of the optic flow (the visual stimulus) is opposite to the direction of head rotation. Little or no modulation occurred in response to optic flow along/about axes orthogonal to the best axis. A modulation index (MI) was calculated for each neuron by taking the ratio of the firing rates in response to the two directions of optic flow about/along the preferred axis (max/min). A neuron was deemed optic flow sensitive if the MI was  $> 1.5$ . The average MI for the 31 neurons was 3.16 (range = 1.5 to 10.6; for example see Fig 4.2.D).



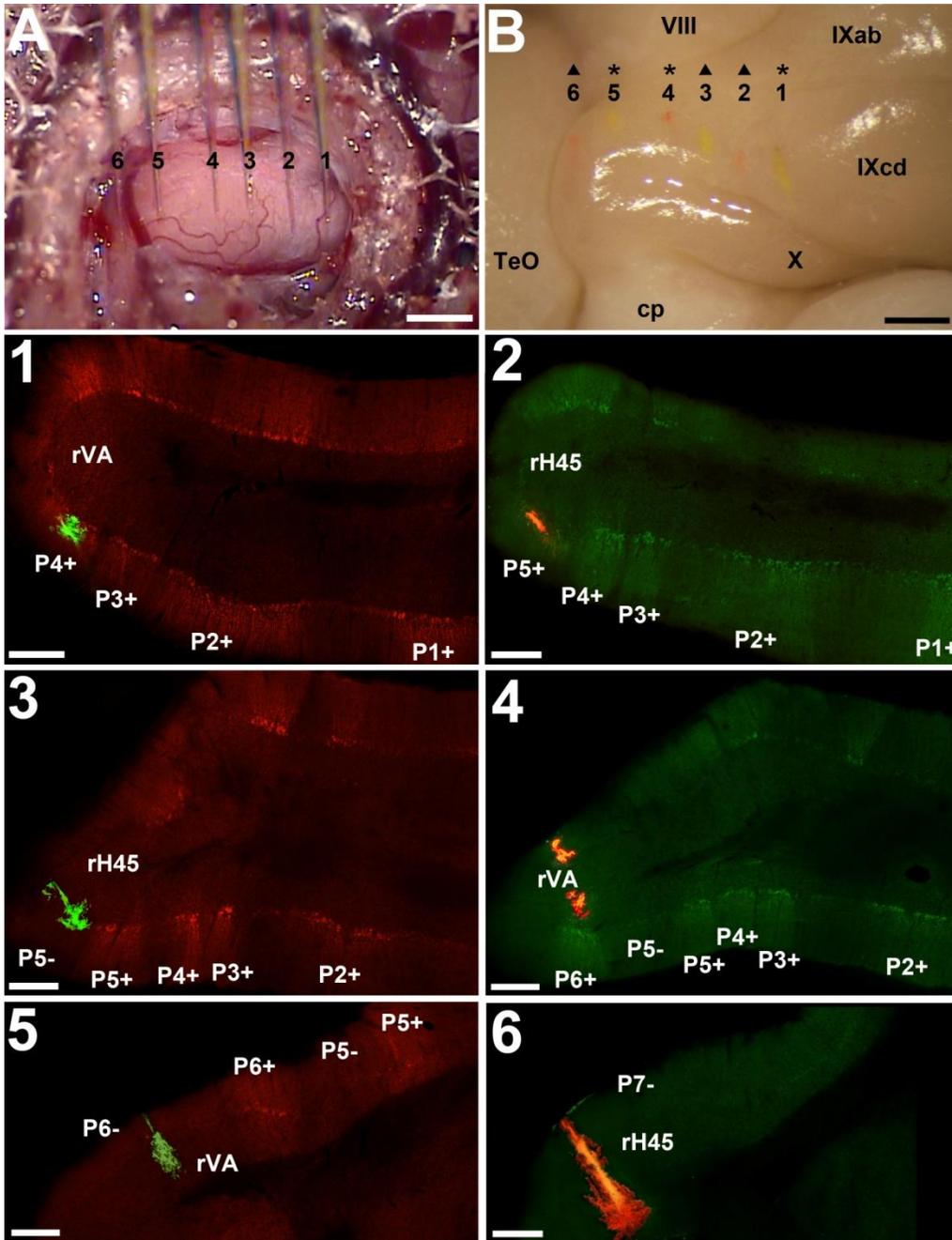
**Figure 4.7** A reconstruction of climbing fibre projections and zebrin expression in the flocculus and the origins of the climbing fibre projections from the inferior olive. A shows an “unfolded” reconstruction of labeled climbing fibres as well as zebrin expression in the right half of folia IXcd and X, including the lateral extension that forms the auricle, from case VA/H45#2. In

this case, an injection of red biotinylated dextran amine (BDA) was made in the caudal medial column of the inferior olive (mcIO; rVA region) and an injection of green- BDA was made in the rostral mcIO (rH45 region). The reconstruction was from 27 coronal sections (40 $\mu$ m thick and separated by 40 $\mu$ m) through the extent of the flocculus. The black lines represent the outline of the Purkinje cell layer of selected sections (approx every fourth) and indicate the shape of the folia at caudal (inside lines) and progressively more rostral (outside lines) extents. The dashed line indicates that IXcd has been cut away from X in the unfolding process, but in fact they are contiguous at this point. Each climbing fibre was marked with a line of the corresponding colour and the positive zebrin expression marked with blue (the zebrin immunonegative stripes are grey). The climbing fibre projection zones are labeled 0-3 and the zebrin stripes are labeled P1+ to P7+. **B** shows a 3-D schematic of the left inferior olive viewed from the rostradorsal aspect; the regions of the inferior olive which are responsive to rotational optic flow (the mcIO) are indicated in red (caudal rVA region) and green (rostral rH45 region). The climbing fibre projections from these two regions of the inferior olive to folium IXcd and X are indicated by arrows. The afferent information that the mcIO receives is also indicated and includes visual optic-flow information from two retinal-recipient nuclei, lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR; Wylie, 2001). dl, vl, = dorsal and ventral lamellae of the inferior olive, respectively. Scale bar in **A** = 1mm.

Recordings were made from 9 rVA neurons and 12 rH45 neurons. Tuning curves for these, representing the firing rate (spikes/sec relative to the spontaneous rate), were plotted as a function of the direction of motion in polar coordinates (see Fig. 4.2E,F for examples). The direction tuning curve for a representative rVA neuron is shown in Fig. 4.2A. The axes in the polar plots are equivalent to 0° (upward motion of the visual stimulus), 90° (rightward motion of the visual stimulus), 180° (downward motion of the visual stimulus) and 270° (leftward motion of the visual stimulus). For example, the response to the leftward/rightward *head rotation* about the vertical axis is represented by the positive/negative direction along the polar plots horizontal axis (rVA cell). Likewise, in the positive direction along the polar plots vertical axis, the response to a counter-clockwise roll of the *head* is represented; that is, the response to clockwise optic flow is plotted (rH45 cell). Because all recordings were made from the left flocculus in the frontal visual field, for rVA cells, maximal excitation should occur in response to rightward motion of the visual stimulus (90° on polar plots) and maximal inhibition to leftward motion of the visual stimulus (270° on polar plots; Fig. 4.2E,F). For rH45 cells, maximal excitation should occur in response to upward motion of the visual stimulus (0° on polar plots) and maximal inhibition to downward motion of the visual stimulus (180° on polar plots; Fig. 4.2F).

#### *4.2.2.1 Correlation of Recording Sites to the Zebrin Expression Pattern*

Eighteen injections were made at known recording sites in 4 birds such that the functional organization of Purkinje cell CSA could be assessed in relation to the pattern of zebrin expression in the flocculus. Nine injections were made with associated recording data collected (2 birds) and nine injections in which the optic flow preference was determined with the hand-held stimulus (2 birds). Table 4.2 shows each injection by case and response type. Figure 4.8 shows recording sites, injection sites and the results of Case #1. In Figure 4.8A, the flocculus exposure is shown within the radius of the anterior semicircular canal. In this case, 15 neurons were recorded from in order to map out the boundaries of the rotation sensitive zones in the flocculus, and six injections were made in appropriate regions with clearly identifiable response properties (i.e. either rVA or rH45 cells). In Figure 4.8, the six injection sites are shown in (A), which are photomicrographs taken during surgery at the time of the injection, and in (B), which were taken after perfusion and extraction of the brain. The brain was then sectioned and zebrin expression was visualized in serial coronal sections. In the remainder of Figure 4.8, the six injection sites are shown with the zebrin expression pattern. In all cases, injection/recording sites could be clearly seen in relation to the zebrin immunoreactivity, and the location of each injection/recording site was assigned to the appropriate zebrin stripe; this is summarized in Table 4.2. Because the P7+ zebrin zone is quite small (only 2-3 Purkinje cells wide), it would be very difficult to get a recording specifically from



**Figure 4.8 Correlation of electrophysiological zones in the flocculus and zebrin expression.** **A** is a photomicrograph of the exposure site, with the flocculus exposed within the radius of the anterior semicircular canal. **A** composite of the six injection sites (of the anterograde tracer, fluorescent biotinylated dextran amine) from this case (Case #1) and their associated recording tracks are shown from images taken immediately prior to injection. **B** shows a photograph of the flocculus after the brain had been perfused and extracted; the six injection sites can clearly be seen on the surface of the brain. The associated cell type from the recordings at each site is indicated above the numbers: an asterisk represents an rVA cell and a triangle represents an rH45 cell. The remaining panels (1-6) are photomicrographs of coronal sections through the corresponding injection sites. These coronal sections were also processed for zebrin immunoreactivity in an alternating colour to the injection. The association between a recording/injection sites and a particular zebrin stripe/interstripe can clearly be made (e.g. injection #3 is in the P5- zebrin interstripe, which is an rH45 responsive zone). TeO, optic tectum; cp, cerebellar peduncle; r. rostral; c, caudal; m, medial; l, lateral. Scale bars in **A**, **B** = 1mm; panels #1-6 = 300µm.

these neurons, therefore, this was the only zebrin zone that is not represented in this data.

A clear relationship can be seen between the climbing fibre zones found in the first part of this experiment, the Purkinje cell CSA, and the zebrin expression pattern. All injections where the Purkinje cell CSA was responsive to rotation about the horizontal axis (rH45 neurons; 10 injections) were located in zebrin P5+/- or P7+/- stripes and all injections where the Purkinje cell CSA was responsive to rotation about the vertical axis (rVA neurons; 8 injections) were located in the P4+/- or P6- stripes. Importantly, in a single animal, rVA cells were found in both the positive and negative zebrin regions of a single zebrin stripe and inter-stripe (i.e. in P6+ and P6-), and the same applied for rH45 cells (i.e. in P5+ and P5-; see Fig 4.8). This confirms the climbing fibre projection pattern we observed from visually responsive regions of the mcIO.

### **4.3 Discussion**

For several decades it has been known that the climbing fibre inputs to the cerebellum are organized into parasagittal zones (Voogd and Bigaré, 1980). This is especially apparent in the flocculus, where there are several rVA zones interdigitated with rH45 zones. Although the absolute number of zones varies between species, a similar pattern is observed in both mammals and aves, indicating that the system is highly conserved. Current research suggests that

there are five visual zones in rats, three rVA zones (zones 0, 2 and 4) interdigitated with two H45 zones (zones 1 and 3; Sugihara et al., 2004), four zones in pigeons (zones 0-3; Winship and Wylie, 2003) and four zones (zones 1-4) in rabbits (De Zeeuw et al., 1994; Tan et al., 1995a) and mice (for review see Voogd and Wylie, 2004; Schonewille et al., 2006).

Zebrin and other molecular markers are also expressed as a series of parasagittal stripes in the cerebellum (Hawkes and Gravel, 1991; Herrup and Kuemerle, 1997). Again, this principle is highly conserved as a similar pattern of zebrin stripes is apparent in avian and mammalian species, although there are some differences (Pakan et al., 2007). Perhaps the most striking difference is, whereas zebrin positive and negative stripes are quite distinct in IXcd of the avian flocculus, the mammalian flocculus is uniformly zebrin positive (Ozol et al., 1999; Sanchez et al., 2002; Sillitoe and Hawkes, 2002; Marzban et al., 2003b). This is quite surprising in view of physiological and anatomical studies that have underscored that in terms of function, response properties and connectivity, the flocculus is virtually identical in birds and mammals (Wylie, 2001; Voogd and Wylie, 2004). Nonetheless, the appearance of zebrin stripes in the avian flocculus, combined with an extensive literature that has examined the physiology and climbing fibre afferents to the flocculus in birds (Wylie and Frost, 1991; 1993; Lau et al., 1998; Wylie et al., 1998b; Wylie and Frost, 1999a; Wylie et al., 1999c; Crowder et al., 2000; Winship and Wylie, 2001; Wylie, 2001; Winship and Wylie,

2003), affords a unique opportunity to examine the relationship between zebrin expression, climbing fibre zones, and physiology in the flocculus.

#### 4.3.1 *The Relationship between Climbing Fibre Zones and Zebrin Stripes*

In this study, by injecting anterograde tracers into the mcIO and observing CF labeling as well as zebrin expression, we have shown that there is a strict concordance between CF zones and zebrin stripes in folium IXcd of the flocculus in pigeons. Injection of anterograde tracer in the caudal mcIO resulted in CFs in zebrin bands P4+/- and P6+/-, whereas rostral mcIO injections resulted in CFs in zebrin bands P5+/- and P7+/- . Thus, zebrin stripes P4+/- and P6+/- correspond to the rVA zones 0 and 2, whereas P5+/- and P7+/- correspond to the rH45 zones 1 and 3, respectively. Figure 4.7 summarizes these findings with a schematic of the pigeon inferior olive and a reconstruction of the CF projections to the flocculus.

Note that the concordance between the zebrin stripes and the climbing fibre zones only applies to folium IXcd and the rostral-most parts of the dorsal lamella of X. The bulk of folium X does not contain zebrin stripes, but is uniformly zebrin positive (Figs. 4.6 and 4.7; Pakan et al. 2007), as is the case in mammals (Hawkes et al., 1993; Hawkes and Herrup, 1995; Ozol et al., 1999; Sanchez et al., 2002; Marzban et al., 2003b). However, the CF zones clearly extend through IXcd and X (Figs. 4.6 and 4.7). Perhaps the CF zones in X are related to the expression of some other molecular marker. For example, in the

mouse cerebellum, heat-shock protein-25 (Hsp 25) is expressed as parasagittal stripes of high and low immunoreactivity, but only in regions where zebrin immunoreactivity is uniformly positive, including the nodulus and flocculus (Armstrong et al., 2000). However, the equivalent to Hsp 25 in rats (Hsp 27) was not detected in the adult rat cerebellum by using immunocytochemistry (Wilkinson and Pollard, 1993; Plumier et al., 1997). Whether Hsp 25 is expressed as stripes anywhere in the pigeon cerebellum has yet to be thoroughly investigated.

The finding that a CF zone in the pigeon flocculus corresponds to a pair of positive/negative zebrin stripes, (i.e., zone 0 corresponds to zebrin stripes P4+ and P4-, etc.) is unique and perhaps contrary to previous investigations of the correspondence of CF afferents and zebrin stripes in mammals. Previous studies emphasized that an olivary subnucleus projects to either a positive or negative zebrin stripe, but not both (Gravel et al., 1987; Sugihara and Shinoda, 2004; Apps and Garwicz, 2005; Pijpers et al., 2006; Sugihara and Quy, 2007). Voogd and colleagues (2003) investigated the distribution of climbing fibres to the copula pyramidis and the paramedian lobule in relation to the pattern of zebrin expression in the rat. They found that, with few exceptions, olivocerebellar fibres originating from the rostral dorsal accessory olive innervate the zebrin negative stripes of the C1 and C3 zones, the rostral medial accessory olive and principle olive innervate the zebrin positive stripes of the C2 and D zones, respectively, and that the A2 zone corresponds to the region of the P4b+ and P5a+ bands in the

medial paramedian lobule and lobulus simplex. Voogd and Ruigrok (2004) investigated the zonal organization of the corticonuclear and the olivocerebellar climbing fibre projections to the vermis of the cerebellum in relation to zebrin stripes in rats. They found that small injections in various subnuclei of the inferior olive produced climbing fibre bands which were generally confined to either a positive or a negative zebrin stripe, but not both. In a comprehensive study of the entire cerebellum, Sugihara and Shinoda (2004) identified olivocerebellar projections to zebrin compartments by labeling climbing fibres with BDA injected into various small areas within the inferior olive in rats. They found that the principal olive (as well as neighboring areas) and several medial subnuclei innervated zebrin positive stripes, whereas the centrocaudal portion of the medial accessory olive innervated zebrin negative stripes in the vermis. The dorsal accessory olive and neighboring regions innervated zebrin negative and lightly positive stripes in the hemisphere and the rostral and caudal pars intermedia.

To reiterate, the correspondence between a given olivary region and zebrin stripes of a particular sign (positive or negative) was not found in the present study of the pigeon flocculus. Rather, an olivary region was associated with a particular positive/negative zebrin pair. Whether the type of zebrin-CF correspondence that we observed is peculiar to pigeons, or even just the flocculus of pigeons, remains to be seen. It is possible that within the cerebellum the nature of the concordance between zebrin stripes and CF zones is different for different olivocerebellar systems. This idea is supported by the fact that the zebrin-CF

concordance that we observed in the flocculus applies to folium IXcd but not X, despite the fact that the CF zones are identical in IXcd and X.

#### 4.3.2 *Linking Functional Cerebellar Zones with Zebrin Stripes*

In recent years there has been an attempt to reveal an underlying unit of function associated with zebrin stripes. Sugihara et al. (2004) concluded that the zebrin stripes are related to function insofar as inferior olive (IO) subnuclei project to either zebrin+ or zebrin- bands, and the subnuclei of the IO receive input from particular sensory systems. Furthermore, Sugihara et al. (2004) suggested that the zebrin- stripes receive input from CFs conveying somatosensory information whereas zebrin+ stripes receive input from CFs conveying information from visual, auditory and other sensory systems (see also Voogd et al., 2003; Voogd and Ruigrok, 2004; Sugihara and Quy, 2007; Sugihara and Shinoda, 2007). For example, in mammals, the ventral lateral outgrowth, which processes visual-optokinetic information, projects to zebrin+ regions in lobule X and ventral IXcd. In the dorsal margin of IXcd, thin zebrin- stripes are innervated by somatosensory olivary subnuclei (Voogd et al., 1996). Clearly, this scheme does not apply to the pigeon flocculus as the CF inputs convey only visual information (see introduction). Furthermore, there are several exceptions to this scheme in mammalian studies. For example, vestibular information appears to reach zebrin+ stripes in lobules VIII-X via the group beta and the dorsomedial cell

column, but also a zebrin- stripe in the lateral A subzone of the anterior vermis via the subnucleus B of the caudal medial accessory olive (Gerrits et al., 1985a; Voogd and Ruigrok, 2004; Voogd and Barmack, 2006). Also, the majority of the CF afferents to zebrin+ stripes arise from nuclei at the midline of the mesodiencephalic junction from structures regarded as motor, rather than sensory (including the red nucleus and accessory oculomotor nuclei; Swenson and Castro, 1983; Onodera, 1984; Holstege and Tan, 1988; de Zeeuw et al., 1989), as well as the subnucleus a, which receives input from the spinal cord (Matsushita et al., 1991).

Recent optical imaging studies have demonstrated that subsets of inferior olivary neurons activate parasagittal stripes of Purkinje cells that correspond to those seen using zebrin immunohistochemistry (Chen et al., 1996; Hanson et al., 2000). Additionally, in mammals, functional somatotopic maps that reflect afferent topography have been found in the cerebellum (Welker, 1990), and when compared directly to zebrin expression, the boundaries have been found to align. For example, there is a close relationship between zebrin stripes and boundaries in the tactile receptive field map (Chockkan and Hawkes, 1994; Bower, 1997b; Hallem et al., 1999). However, the functional significance of cerebellar heterogeneity for the processing of afferent information remains unknown.

In the present study, because there is a great deal of research detailing the optic flow information conveyed by CFs to the flocculus, we suggest a functional link between the four optokinetic zones and the zebrin stripes. The caudal and

rostral mcIO provide CF afferents to floccular zones 0 and 2, and 1 and 3, respectively. In the present study, we showed that each zone corresponds to a particular zebrin positive/negative stripe pair (P4+/- to P7+/-; see Fig. 4.7). Previous studies from our lab have shown that the caudal mcIO and zones 0 and 2 respond best to rVA optic flow, whereas the rostral mcIO and zones 1 and 3 respond best to rH45 optic flow (Winship and Wylie, 2001; Pakan et al., 2005). Thus the P4+/- and P6+/- stripes are rVA zones, and the P5+/- and P7+/- are rH45 zones. Whether the zebrin+ and zebrin- regions of each of the rVA and rH45 zones have different roles in oculomotor function is unknown. Because zebrin+ and zebrin- cells may differ with respect to plasticity and excitability (Welsh et al., 2002; Wadiche and Jahr, 2005) this may be the case. We must acknowledge that it is possible that the zebrin- and zebrin+ stripes receive differential sensory information. Although our research over the past two decades has shown that the CSA of all Purkinje cells in the flocculus respond to optokinetic stimulation (e.g., Wylie et al. 1991, 1993, 1998; Wylie and Frost 1993), as do the olivary cells that provide CF input to the flocculus (e.g. Winship and Wylie 2001), whether some cells also respond to vestibular stimulation has not been tested. This is the case in the rabbit uvula and nodulus (Shojaku et al., 1991) as well as the flocculus (Simpson et al., 2002). Thus, it is possible that either the zebrin+ or zebrin- stripes (or both) also receive vestibular CF information. If this was the case, it would likely arise from a secondary vestibular input, as a primary vestibular input to the

mcIO has not been reported in pigeons (Schwarz and Schwarz, 1986; Dickman, 1996; Dickman and Fang, 1996).

The direct congruence between several functional CF zones with clearly defined response properties and a series of zebrin stripes that we show in the pigeon flocculus in the present study, has not been described in other cerebellar systems in any species (cf. Chockkan and Hawkes, 1994; Hallem et al., 1999). However, a few recent papers have suggested a direct relationship between zebrin stripes and other aspects of cerebellar physiology. Sugihara et al. (2007) showed that synchronous activity was higher among Purkinje cells within a zebrin stripe. Gao et al. (2006) demonstrated that the inhibition evoked by parallel fibre stimulation results in parasagittal bands of decreases in activity along a folium that correspond to the location of zebrin stripes.

Finally, although the Purkinje cells in the flocculus are uniformly zebrin+ in mammals (Leclerc et al., 1992; Eisenman and Hawkes, 1993), stripes in either the Purkinje cell axons, the afferent input, or the Purkinje cells themselves are revealed with several other molecular markers, such as acetylcholine esterase (De Zeeuw et al., 1994; Tan et al., 1995b), corticotropin-releasing factor (Sawada et al., 2008) and Hsp25 (Armstrong et al., 2000). Schonewille et al. (2006) investigated the correspondence of the floccular zones, defined by recording responses to optic flow stimuli, with Hsp25. The relationship they found between this molecular marker (Hsp25) and the physiological zones in the mouse flocculus was completely different to what we found in the present study using zebrin as a

molecular marker. An Hsp25 positive stripe encompassed zones 1 (rH45) and 2 (rVA), and a Hsp25 negative stripe encompassed zones 3 (rH45) and 4 (rVA). Therefore, it is evident that the relationship between various molecular markers and the physiological zones in the cerebellum is complex. Even though the function of zebrin is unclear (cf. Welsh et al., 2002), the unique parasagittal organization of both zebrin and other molecular markers would suggest that examining the relationship between these markers and other well known aspects of cerebellar organization, such as anatomy and electrophysiology, will lead to insights on the fundamental functional organization of the cerebellar cortex.

**Table 4.1 Summary of zebrin and climbing fibre correlation from each case.** Single, double and triple “+” signs indicate the relative amount of climbing fibre labeling in each of the zebrin stripes. Note that this is not an indication of the density of the labeling in the zones. The P4- stripe is thinner than the P4+ stripe (see Fig. 4.1D,E). That is, there is generally less labeling in the P4- stripe compared to P4+ stripe simply because it is thinner. In contrast, the P7- stripe includes the majority of the auricle, and is much wider than the P7+ stripe (see Fig. 4.1E).

<b>Caudal mcIO Injections</b>								
<b>Case</b>	<b>P4+</b>	<b>P4-</b>	<b>P5+</b>	<b>P5-</b>	<b>P6+</b>	<b>P6-</b>	<b>P7+</b>	<b>P7-</b>
<b>VA#1</b>	+++	+			+	++		
<b>VA/H45#1</b>	+++	+			+++	+		+*
<b>VA/H45#2</b>	+++	+			+++	++		+*
<b>VA#2</b>	++	+			+++	++	+	
<b>VA#3</b>	++	+			+++	+	+	+
<b>Rostral mcIO Injections</b>								
<b>Case</b>	<b>P4+</b>	<b>P4-</b>	<b>P5+</b>	<b>P5-</b>	<b>P6+</b>	<b>P6-</b>	<b>P7+</b>	<b>P7-</b>
<b>VA/H45#1</b>			++	+			++	+++
<b>VA/H45#2</b>			++	++			+	++
<b>H45#1</b>			+++	++			+	+++
<b>H45#2</b>			++	+			++	+++
<b>H45#3</b>			++	+			+	+++
<b>H45#4</b>			++	+				++

**\*Very few climbing fibres were found ventrally in the auricle.**

**Table 4.2 Injection sites by case and response type.** In all cases, injection/recording sites can be clearly seen in relation to the zebrin immunoreactivity (zebrin immunopositive zones- grey shading), and the location of each injection/recording site was assigned to the appropriate zebrin stripe

Case	Inj #	P4+	P4-	P5+	P5-	P6+	P6-	P7+*	P7-
1	1	rVA							
	2			rH45					
	3				rH45				
	4					rVA			
	5						rVA		
	6								rH45
2	1			rH45					
	2				rH45				
	3						rVA		
	4								rH45
3	1	rVA							
	2				rH45				
	3						rVA		
	4								rH45
4	1	rVA							
	2				rH45				
	3					rVA			
	4								rH45

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Chapter 5:     **Organization of Visual Mossy Fiber Projections and Zebrin  
Expression in the Vestibulocerebellum.**

**A version of this chapter has been published:**

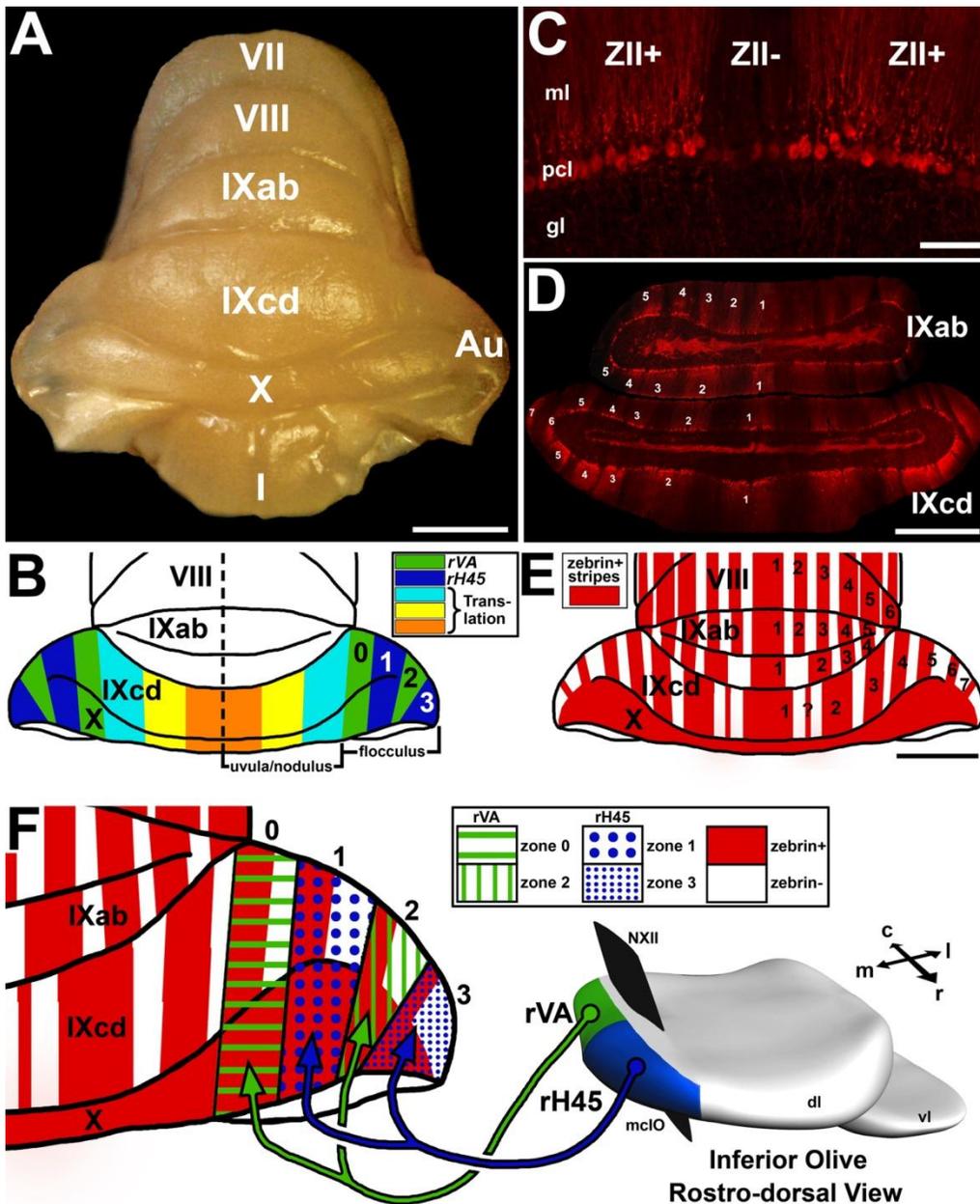
Pakan, J.M.P., Graham, D.J., and Wylie, D.R.W. (In Press). Organization of Visual Mossy Fibre Projections and the Zebrin Antigenic Map in the Vestibulocerebellum. *Journal of Comparative Neurology*. In Press.

The fundamental architecture of the cerebellar cortex consists of parasagittal compartments that have been revealed using anatomical, electrophysiological, and histological techniques (e.g. Voogd and Bigaré, 1980). Studies have shown that parasagittal zones can be defined by climbing fibre (CF) input, Purkinje cell efferents, and Purkinje cell response properties (Voogd, 1967; Oscarsson, 1969; Andersson and Oscarsson, 1978a; b; Voogd and Bigaré, 1980; Llinas and Sasaki, 1989; Sato and Kawasaki, 1991; De Zeeuw et al., 1994; Wylie et al., 1994; Wylie et al., 1995; Voogd and Glickstein, 1998; Ruigrok, 2003; Winship and Wylie, 2003; Wylie et al., 2003; Sugihara and Shinoda, 2004; Voogd and Wylie, 2004; Apps and Garwicz, 2005). Fewer anatomical studies have also investigated this parasagittal organization in relation to mossy fibre (MF) input, with particular focus on the topographical organization of somatosensory afferents to the cerebellar cortex from spinocerebellar and cuneocerebellar pathways (Voogd et al., 1969; 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).

Compartmentation of the cerebellar cortex has also been revealed immunohistochemically with several molecular markers (for review see Hawkes and Gravel, 1991; Herrup and Kuemerle, 1997), but the most thoroughly studied of these is zebrin II (aldolase C; Brochu et al., 1990; Ahn et al., 1994; Hawkes and Herrup, 1995), which is expressed by Purkinje cells. Zebrin immunopositive (zebrin+) Purkinje cells are distributed as a parasagittal array of stripes, separated

by zebrin immunonegative (zebrin-) stripes (e.g. Larouche and Hawkes, 2006). Zebrin parasagittal stripes have been shown in several mammalian species (for review see, Sillitoe et al., 2005), and recently we have shown that zebrin is also expressed in the avian cerebellum in a similar pattern (Fig. 5.1C-E; Pakan et al., 2007; Iwaniuk et al., 2009). Thus, the pattern of zebrin stripes is highly conserved among species, and likely contributes to underlying fundamental cerebellar function.

The pigeon vestibulocerebellum (VbC) is an ideal system for examining the correlation between functional, anatomical and molecular topographies of the cerebellar cortex for several reasons. First, the pigeon VbC is organized into easily identifiable zones that differ with respect to visual (i.e. optic flow) preference (Fig. 5.1B,CF; Wylie and Frost, 1999). Second, the climbing fibre input from the inferior olive (IO) has been extensively documented (Fig. 5.1F; Wylie and Frost, 1999; Crowder et al., 2000; Pakan et al., 2005) and the topography has been confirmed with single-unit recordings (Winship and Wylie, 2001). Third, the major source of optic flow MF afferents arises from two retinal recipient nuclei; the nucleus of the basal optic root (nBOR; Fig. 5.2A) of the accessory optic system (AOS), and the pretectal nucleus, lentiformis mesencephali (LM; Fig. 5.2B), which have both been extremely well characterized (Winterson and Brauth, 1985; Wylie and Frost, 1990; Wylie and Crowder, 2000). Finally, in pigeons, the most striking and reliable zebrin stripes

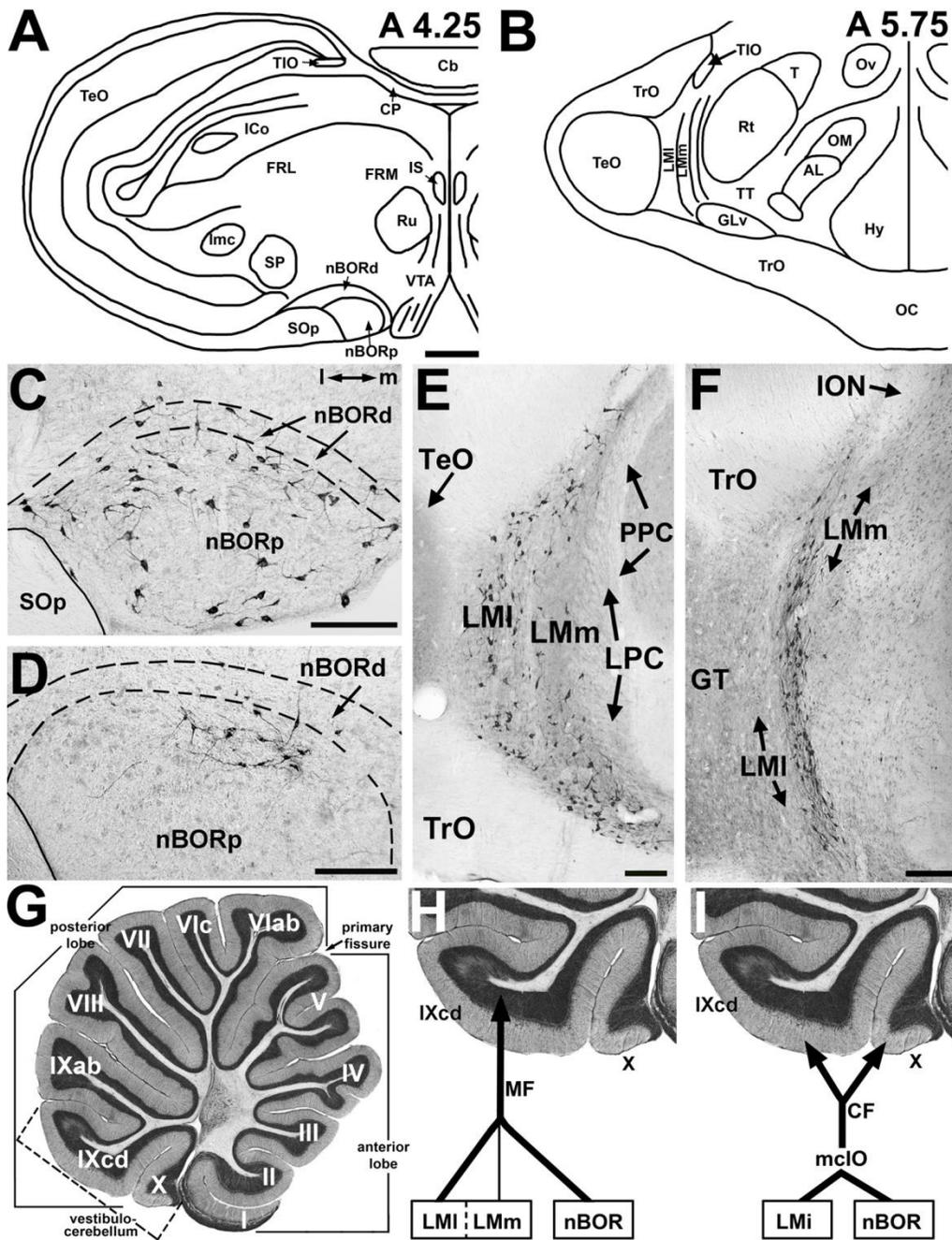


**Figure 5.1 Parasagittal organization of the pigeon flocculus.** **A** shows a photograph of the posterior pigeon cerebellum, indicating the vestibulocerebellum (VbC; folia IXcd and X), as well as their lateral extension forming the auricle (Au). **B** shows the Purkinje cell response properties in folium IXcd. The flocculus,

the lateral portion, responds best to rotational optic flow about either the vertical axis (rVA; green) or an horizontal axis oriented 45° to midline (rH45; blue; Graf et al., 1988; Wylie and Frost, 1993). Zones in the medial part of the ventral uvula and nodulus (teal, orange and yellow), responds best to translational optic flow stimuli (see Wylie and Frost, 1999). **C** shows a photomicrograph of zebrin expression in the pigeon cerebellum, illustrating subsets of Purkinje cells are immunopositive for zebrin (ZII+) separated by subsets of Purkinje cells that are immunonegative (ZII-). **D** and **E** illustrate the pattern of zebrin expression in the pigeon posterior cerebellum (adapted from Pakan et al., 2007), shown with a coronal section through the posterior cerebellum (**D**) and a schematic of the pattern of zebrin positive stripes (**E**). The zebrin stripes are numbered from 1-7 through folium IXcd. Shown in **F**, the rVA and rH45 cells are organized into four zones in the flocculus: two rVA zones (0 and 2, green) interdigitated with two rH45 zones (1 and 3, blue). The rVA and rH45 zones receive climbing fibre input from the caudal and rostral medial column of the inferior olive (mcIO), respectively. The caudal mcIO projects to the P4+/- (zone 0) and the P6+/- zebrin stripes (zone 2) and the rostral mcIO projects to the P5+/- (zone 1) and the P7+/- zebrin stripes (zone 3). dl, vl, dorsal and ventral lamellae of the inferior olive; gl, granular layer; ml, molecular layer; pcl, Purkinje cell layer. Scale bars **A,D,E** (applies also to **B**)= 1mm, **C** =100µm.

were seen in folium IXcd (Pakan et al., 2007), which, along with folium X, comprise the VbC (Fig. 5.1A,D,E; 5.2G). The lateral part of the VbC is the flocculus, which is responsive to visual stimuli resulting from rotational self-motion (Wylie and Frost, 1993; Voogd and Wylie, 2004), and the medial part of the VbC consists of the ventral uvula (folium IXcd) and the nodulus (folium X), which are responsive to visual stimuli resulting from translational self-motion (Wylie and Frost, 1991; Wylie et al., 1993; Wylie et al., 1998; Wylie and Frost, 1999). In pigeons, it has been shown that these optic flow responses are organized into parasagittal zones throughout the VbC (Fig. 5.1B; Wylie et al., 1993; Wylie and Frost, 1999; Winship and Wylie, 2003).

LM is homologous to the mammalian nucleus of the optic tract; (NOT; Collewijn, 1975; Hoffmann and Schoppmann, 1975; McKenna and Wallman, 1985)), and the nBOR is the homolog of the mammalian medial terminal nucleus (Simpson, 1984; Giolli et al., 2006). Neurons in the pretectum and AOS have large, contralateral receptive fields and exhibit direction-selectivity in response to large-field moving visual stimuli (Burns and Wallman, 1981; Simpson, 1984; Winterson and Brauth, 1985; Simpson et al., 1988; Grasse and Cynader, 1990; Wylie and Frost, 1990). The visual MF projections to the VbC target folium IXcd but not X, and originate from large multipolar cells in LM (mainly the lateral subnucleus) and nBOR (Fig. 5.2C,E,H). LM and nBOR also project indirectly to the VbC via the medial column of the IO (mcIO), and terminate as CFs in folia



**Figure 5.2** Nomenclature of the pigeon nucleus of the basal optic root (nBOR), lentiformis mesencephali (LM) and the vestibulocerebellum (VbC). A and B show tracings of coronal sections through nBOR and LM, respectively.

The approximate anterior-posterior locations according to the atlas of Karten and Hodos (1967) are listed in the top right. **C** and **D** are photomicrographs of coronal sections through the nucleus of the basal optic root (nBOR), showing retrogradely labeled neurons from injections of cholera toxin subunit B into the, VbC (**C**) and inferior olive (**D**; adapted from Wylie et al., 2007). Cells projecting to the VbC are very large multipolar cells found throughout nBOR (**C**). Cells projecting to the medial column of the inferior olive (mcIO) include a cluster of small cells dorsally in the nBOR (**D**). **E** and **F** are photomicrographs showing retrogradely labelled cells in the nucleus lentiformis mesencephali (LM) after injections of cholera toxin subunit B in the VbC (**E**) or inferior olive (**F**; adapted from Pakan et al. 2006). The cells projecting to the VbC are large multipolar cells found throughout LM, but mainly in the lateral subnucleus (LMI), whereas those projecting to the inferior olive are localized to a strip caudally, along the border of LMm and LMI. **G** shows a photomicrograph of a sagittal section through the pigeon cerebellum. The folia are numbered I-X (anterior to posterior) according to the nomenclature of Larsell (1967). The VbC includes folia IXcd and X. The granular layer of folium IXcd of the VbC receives direct mossy fibre projections from LM and nBOR (**H**). LM and nBOR also project indirectly to the VbC via the inferior olive, which then sends climbing fibres to the molecular layer of folia IXcd and X. For abbreviations see list. Scale bars: **A** = 1mm (applies to **A** and **B**); **C-F** = 200 $\mu$ m.

IXcd and X (Clarke, 1977; Brecha et al., 1980; Gamlin and Cohen, 1988; Arends and Voogd, 1989; Lau et al., 1998; Wylie et al., 1999a; Crowder et al., 2000; Winship and Wylie, 2003). The projections to these olivocerebellar pathways originate from small fusiform cells in the central region of LM and the dorsal regions of nBOR (Fig. 5.2D,F,I; Brecha et al., 1980; Gamlin and Cohen, 1988; Wylie and Linkenhoker, 1996; Winship and Wylie, 2003; Pakan et al., 2006; Pakan and Wylie, 2006; Wylie et al., 2007).

In a recent study (Chapter 4; Pakan and Wylie, 2008), we investigated the organization of this visual olivocerebellar pathway by making small injections of anterograde tracers into the IO in order to label CF projections to the flocculus. We then correlated the resulting CF labeling with zebrin expression and found that a single parasagittal zone spans an entire zebrin+ and zebrin- stripe (see Fig. 5.1F).

How the visual MF pathways from LM and nBOR are organized in folium IXcd is unknown. In the present study, we made small injections of the anterograde tracer biotinylated dextran amine (BDA) into LM and nBOR in pigeons toward three purposes: 1) to determine if there is a parasagittal organization of LM and nBOR MF terminals in folium IXcd; 2) to determine if LM and nBOR have differential MF projections to the VbC; and 3) to determine if the MF pathways to folium IXcd relate to the parasagittal organization of the zebrin stripes.

## 5.1 Methods

### 5.1.1 *Surgical Procedures*

The methods reported herein conformed to the guidelines established by the Canadian Council on Animal Care and were approved by the Biosciences Animal Care and Policy Committee at the University of Alberta. Silver King and Homing pigeons (*Columba livia*), obtained from a local supplier, were anesthetized by an intramuscular injection of a ketamine (65 mg/kg) /xylazine (8 mg/kg) cocktail and supplemental doses were administered as necessary. Animals were placed in a stereotaxic device with pigeon ear bars and a beak bar adapter so that the orientation of the skull conformed to the atlas of Karten and Hodos (1967). To access LM and nBOR, bone and dura were removed from the dorsal surface of the caudal telencephalon, lateral to the mid-sagittal sinus. To record the activity of optic flow units in the LM and nBOR, glass micropipettes filled with 2 M NaCl, with tip diameters of 4-5 $\mu$ m, were advanced through the telencephalon and into the midbrain using an hydraulic microdrive (Frederick Haer & Co, Bowdoin, ME.). Stereotaxic coordinates were used to approach LM and nBOR, but to ensure that our injections were in the desired nucleus, the localization was confirmed by recording the responses of neurons to a large (90°x 90°) moving hand-held stimulus in various areas of the visual field. Cells responsive to optic flow stimuli were found along the track at several depths so that the injection could be placed at a depth between the most dorsally and ventrally identified

cells. Once the desired area was isolated, the recording electrode was replaced with a micropipette (tip diameter 20-30 $\mu$ m) containing fluorescent BDA; either mini-ruby (red; D-3312) or mini-emerald (green; D-7178; 10,000 molecular weight; Invitrogen, Carlsbad, CA). The tracers (0.01-0.05  $\mu$ l of 10% solution in 0.1M phosphate buffer) were pressure injected using a Picospritzer II (General Valve Corporation, Fairfield, New Jersey). After surgery the craniotomy was filled with bone wax and the wound was sutured. Birds were given an intramuscular injection of buprenorphine (0.012mg/kg) as an analgesic.

After a recovery period of 3-5 days, the animals were deeply anesthetized with sodium pentobarbital (100mg/kg) and immediately transcardially perfused with phosphate buffered saline (PBS; 0.9% NaCl, 0.1M phosphate buffer) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brain was extracted from the skull and immersed in paraformaldehyde for 7 days at 4°C. The brain was then embedded in gelatin and cryoprotected in 30% sucrose in 0.1M PBS overnight. Using a microtome, frozen serial sections in the coronal plane (40 $\mu$ m thick) were collected throughout the rostro-caudal extent of the cerebellum.

### 5.1.2 *Zebrin Immunohistochemistry*

For zebrin immunohistochemical methods see section 3.1.1.2.

### 5.1.3 *Microscopy*

For details on microscopy as well as image capture and analysis see section 2.1.2.

### 5.1.4 *Nomenclature*

For detailed nomenclature of the pigeon flocculus see section 2.1.3.

Folia IXcd (uvula) and X (nodulus) comprise the VbC and merge rostromedially to form the auricle (Fig. 5.1A,B; 2G). In the lateral half of IXcd and X, CSA responds best to optic flow resulting from self-rotation about the vertical axis (rVA neurons; zones 0 and 2; Fig. 5.1B) or an horizontal axis oriented 45 degrees to the midline (rH45 neurons; zones 1 and 3; Fig. 5.1B; Wylie and Frost, 1993). These responses are essentially identical to those observed in the mammalian flocculus (Graf et al., 1988; Wylie and Frost, 1993). The numbering of the floccular zones, 0-3 as shown in Figure 5.1, follows that used for rats and rabbits (Voogd and Wylie, 2004).

In folium IXcd, it is usually impossible to tell on a coronal section precisely where the border is between the uvula and the flocculus, as this division can usually only be established by electrophysiological means; however, with the correlation of zebrin immunostaining and CF borders in Chapter 4, we (Pakan and Wylie, 2008) have determined that the medial border of the flocculus consistently corresponds to the medial edge of P4+. In what follows, we have

used this guideline to determine the mediolateral boundaries of the uvula and flocculus in folium IXcd.

For the nomenclature of LM, we relied on Gamlin and Cohen (1988) who divided the LM into a medial and a lateral subdivision (LMm, LMI respectively; see Fig. 5.2). Both subnuclei contain large multipolar cells, which project to the VbC (Fig. 5.2E; Gottlieb and McKenna, 1986; Gamlin and Cohen, 1988; Pakan et al., 2006). A strip of smaller fusiform cells located caudally along the boarder of LMm and LMI that project to the IO.

Brecha et al. (1980) divided the nBOR complex into three regions, the proper (nBORp), dorsal (nBORd), and lateral (nBORl). The large multipolar cells projecting to the VbC are found in nBORp and nBORl (Fig. 5.2C). The IO-projecting cells are found in the dorsal regions of nBORp and in nBORd (Fig. 5.2D; Wylie et al., 2007, 2001).

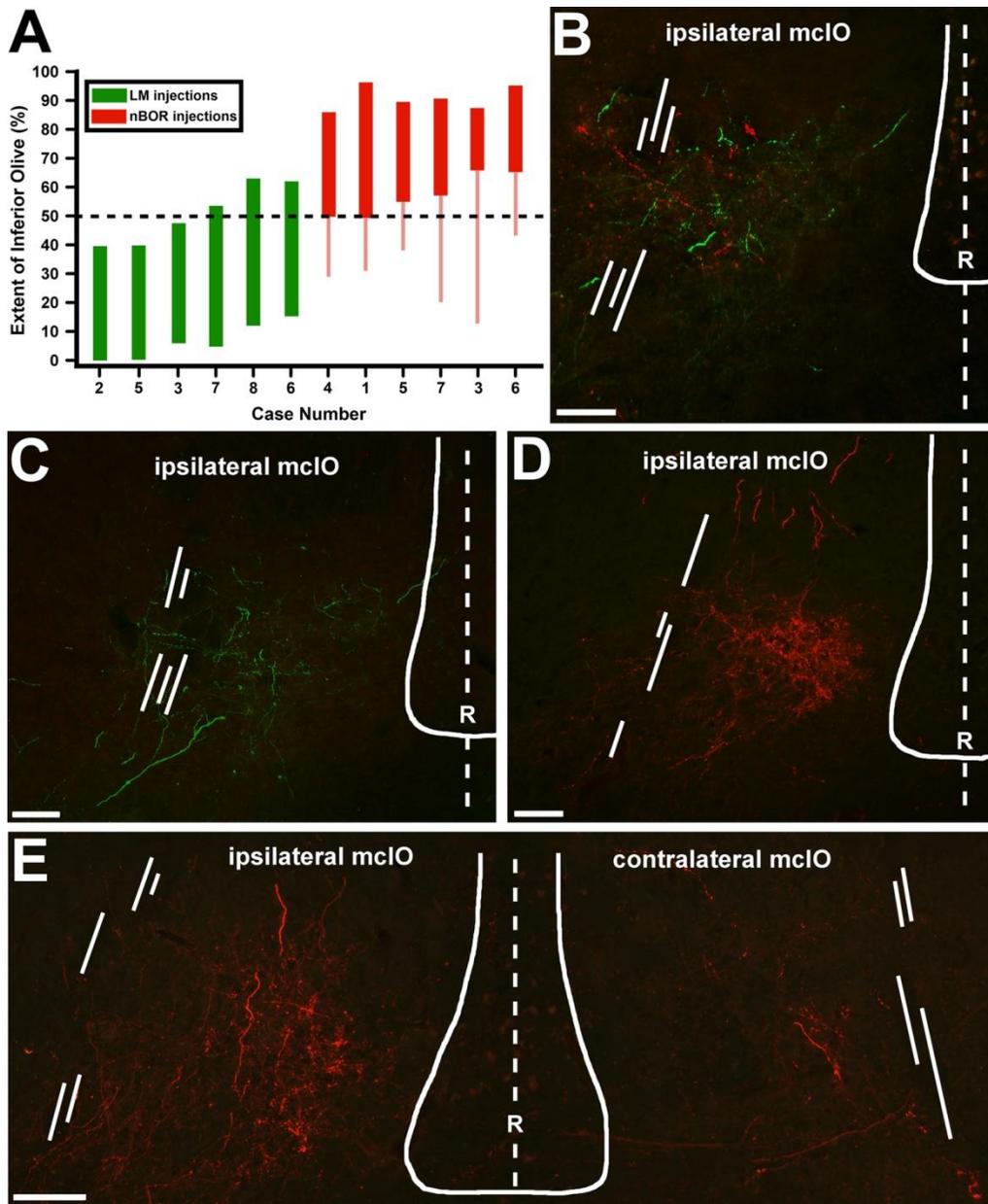
## **5.2 Results**

### ***5.2.1 Injections Sites and Inferior Olive labeling***

The results are based on twelve injections in eight animals. Four animals received single injections of red-BDA, two in LM (Case#2, Case#8), and two were in nBOR (Case#4, Case#1). The remaining animals received injections in both LM and the ipsilateral nBOR (Case#6, Case#3, Case#5, Case#7). Representative injection sites are shown in Figure 5.4A and B from Case#6 as

well as in Figure 5.6B and 5.7B from Case#5. All injections were largely confined to the target region, with the exception of the LM injection in Case#7, which spread ventrolaterally into the optic tectum, and Case#3, which spread dorsally and slightly medially to nBOR. Since there are no mossy fibre projections from these regions of spread to the cerebellum, this does not have any bearing on our results.

Terminal labeling in the inferior olive was observed from all injections. The graph in Figure 5.3A shows the extent of the labeling that was observed in the mcIO from each injection. From injections in nBOR, terminal labeling in the mcIO was heavy with the majority of terminals found in the rostral half of the ipsilateral mcIO (thick red bars in Fig. 5.3A; Fig. 5.4B,D,E). In these rostral regions, terminals here were heaviest in the most medial regions of the mcIO, where olivary cells respond to visual stimuli resulting from rotational self-motion (Winship and Wylie, 2001). There was also a comparatively small amount of terminal labeling more caudally in the mcIO (thin red bars in Fig. 5.3A), as well as in the contralateral mcIO (Fig. 5.3E). However, labeling in these regions tended to be more lateral in the mcIO (in regions surrounding the XII cranial nerve), where olivary cells respond to visual stimuli resulting from translational self-motion (Fig. 5.3B,E; Winship and Wylie, 2001). Fibres could be seen crossing the midline ventral to the raphe nucleus (Fig. 5.3E). The contralateral labeling from nBOR spanned the same rostral-caudal extent as the ipsilateral labeling (ipsilateral extent is shown in Fig. 5.3A). From injections in LM, labeling



**Figure 5.3** Pattern of terminal labeling in the inferior olive from injections in the nucleus lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR). **A** shows a graph of the rostro-caudal extent of terminal labeling observed in the medial column of the inferior olive (mcIO) resulting from

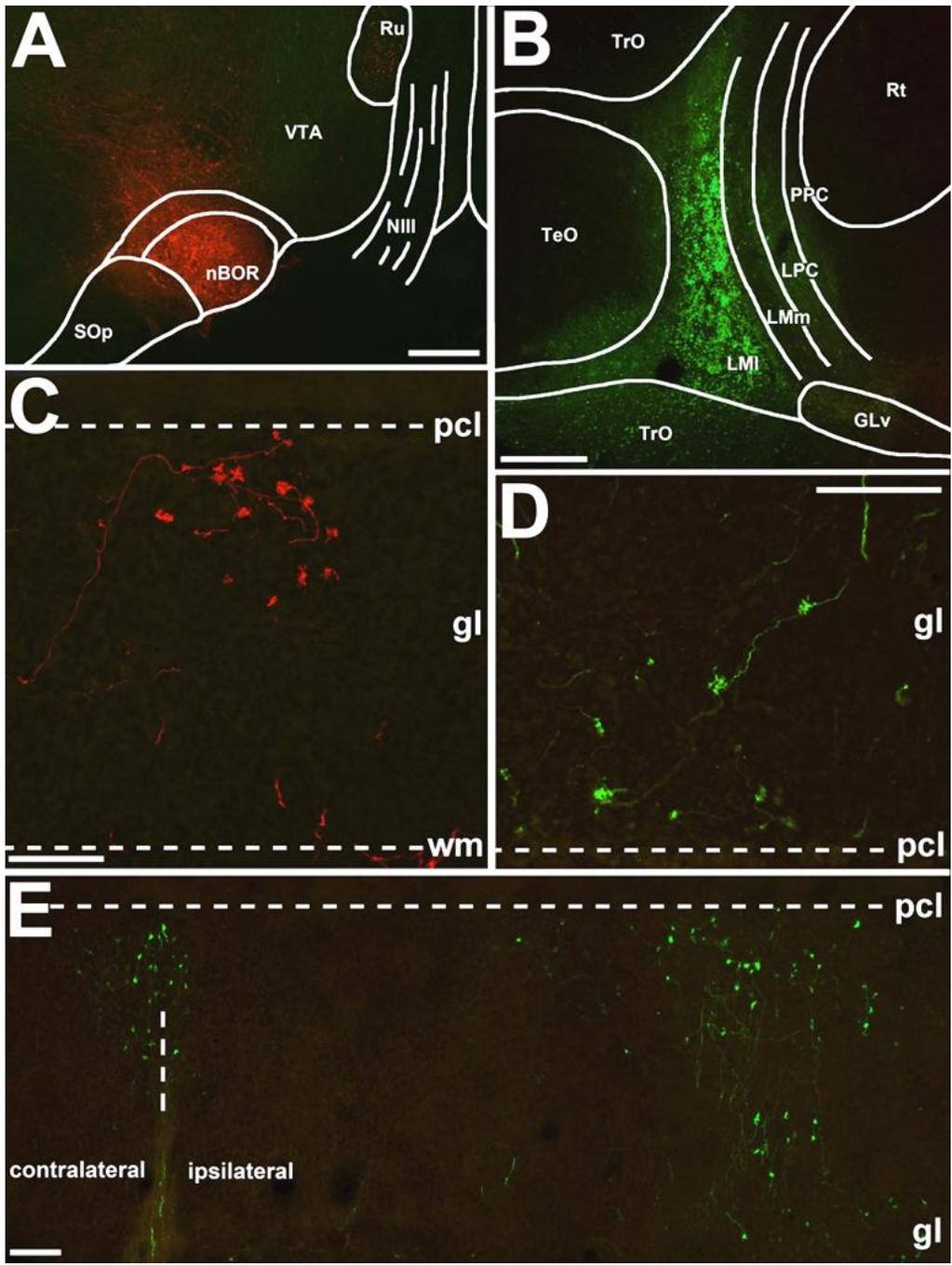
injections of anterograde tracers into the nucleus lentiformis mesencephali (LM; green bars) and the nucleus of the basal optic root (nBOR; red bars). The bars represent the presence of terminal labeling in the mcIO in the corresponding roostro-caudal region of the inferior olive, normalized and averaged over all cases. Thin red bars represent a comparatively small amount of labeling more caudal in the mcIO from nBOR injections. **B** is a photomicrograph of terminal labeling in the ipsilateral caudal mcIO from a green injection in LM and a red injection in nBOR. Note the amount of terminal labeling from nBOR is small and is located laterally in the mcIO. **C** is a photomicrograph of labeling from a green injection in LM illustrating terminal labeling in the medial and lateral regions of the mcIO. There was no terminal labeling observed in the contralateral mcIO from injections in LM. **D** and **E** are photomicrographs of terminal labeling in the rostral mcIO from a red injections in nBOR; note the heavy terminal labeling in the medial portions of the mcIO (**D** and **E**, left) and the presence of a small amount of terminal labeling in the contralateral mcIO in **E** (right), located slightly lateral compared to the ipsilateral labeling. **E** also shows fibres crossing the midline ventral to the raphe nucleus (R). Dotted lines represent midline; broken lines represent the position of the twelfth cranial nerve, which is used as a landmark for the lateral edge of the mcIO. Scale bars in B-E = 100 $\mu$ m

was observed in the caudal half of the ipsilateral IO, where terminals were found throughout the mediolateral extent of the mcIO (Fig. 5.3B,C). No labeling from LM injections was found in the contralateral mcIO or more rostral regions. Comparatively, the terminal labeling observed in the IO was much heavier from injections in nBOR than from LM injections.

### 5.2.2 *Mossy Fibre Input to Folium IXcd*

Axons from nBOR and LM travel from the injection sites and enter the brachium conjunctivum cerebellopetal where they then course caudally to the cerebellum, through the cerebellar white matter, and enter the granule cell layer as discrete fascicles which are organized in parasagittal bands (e.g. Fig. 5.4E). These fascicles pass through the internal regions of the granule cell layer and then spread horizontally to terminate as MFs, generally, in the external one-half of the granule cell layer, directly subjacent to the Purkinje cells (e.g. Fig. 5.4C,D). From all injections, MF terminal labeling (MF rosettes) could clearly be seen in the granule cell layer in folium IXcd of the VbC (e.g. Fig. 5.4,5.5).

As a representation of a typical double injection case, Figures 5.6-5.8 show photomicrographs of injections sites from Case#5 (Fig. 5.6B,5.7B) as well as the resulting MF terminal labeling in the form of reconstructions through the rostro-caudal extent of the dorsal and ventral lamellae of folium IXcd. The general pattern of terminal labeling was consistent between cases, although the

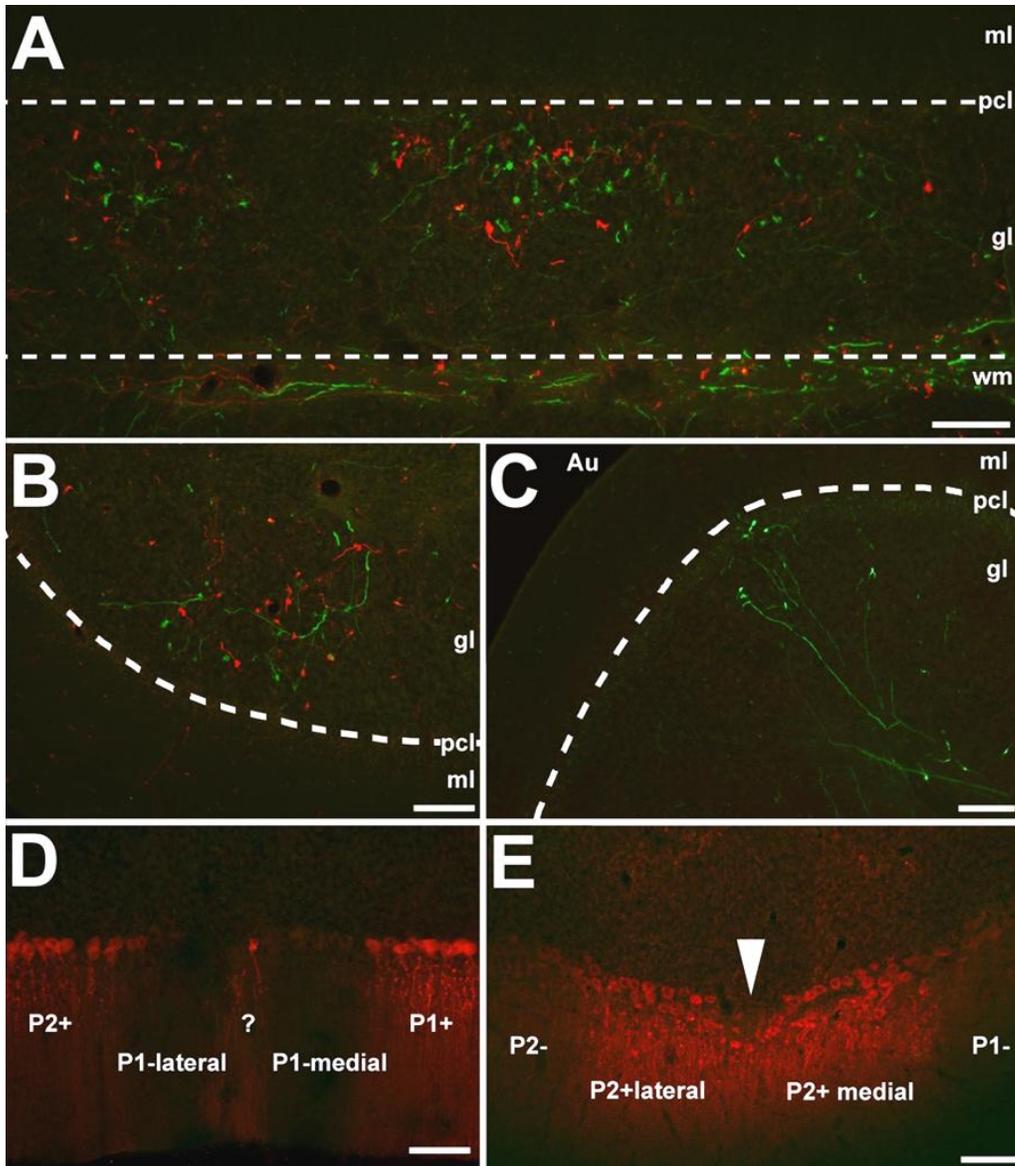


**Figure 5.4** Typical injection sites in nucleus lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR) and the resulting pattern of mossy fibre (MF) labeling in folium IXcd. **A** and **B** show photomicrographs of typical injection sites in nBOR (**A**; red) and LM (**B**;green). **C-E** show photomicrographs of typical resulting MF terminals (resettes) in folium IXcd from injections in nBOR (red) and LM (green). In **C** and **D**, note the proximity of the MF terminals in the superficial granular cell layer (gl), directly adjacent to the Purkinje cell layer (pcl) in both the dorsal lamella (**C**) and the ventral lamella (**D**). In **E**, it can be seen that the MF terminal pattern is organized in parasagittally oriented clusters in the granular layer. For abbreviations see list. Scale bars: **A,B** = 500 $\mu$ m; **C-E** = 100 $\mu$ m.

total number of MF rosettes differed according to the size of the injection. For quantification, MF rosettes were counted from serial coronal sections, and the numbers that follow are percentages averaged over all cases (see Table 5.1).

#### *5.2.2.1 Mossy Fibre Projections from nBOR*

Examining the MF terminal organization in Figure 5.6C and Table 5.1, the following observations can be made. MF projections from nBOR were distributed bilaterally throughout folia IXcd in parasagittal clusters of varying widths, with slightly heavier MF labeling on the contralateral side (ipsilateral labeling 45.6%, contralateral labeling 54.4%). More terminal labeling was seen in caudal regions of the folium, and labeling in rostral regions was sparse. There was also heavier terminal labeling in the dorsal lamella (55.6%) compared to the ventral lamella (44.4%), especially on the contralateral side and in rostral regions where the ventral lamella was nearly void of terminals (e.g. Fig. 5.8B). With respect to the parasagittal organization, the heaviest cluster of terminal labeling occurred surrounding the midline (20.5% directly surrounding midline, bilaterally), with the exception of the ipsilateral dorsal lamella, which had very few MF terminals (ipsilateral dorsal lamella – 1.1%, compared to the contralateral dorsal lamella – 6.4%; see green MFs in Fig. 5.8B for example). There were more apparent MF parasagittal clusters on the ipsilateral side, especially in the ventral lamella where a total of five clusters could be distinguished. In the dorsal lamella, there was a



**Figure 5.5** Pattern of mossy fibre (MF) labeling in folium IXcd from injections in nucleus lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR), and zebrin expression in folium IXcd. **A-C** show photomicrographs of the typical pattern of resulting MF terminals (rosettes) in folium IXcd from injections in nBOR (red) and LM (green). **A** shows an example of parasagittal clusters of labeled MF terminals in the dorsal lamella and **B** shows an example of parasagittal clusters of labeled MF terminals in the ventral flocculus from injections in nBOR (red) and LM (green). **C** shows a single parasagittal zone in the dorsal portions of the auricle (Au) from an injection in LM. **D** and **E** are examples of zebrin expression in folium IXcd shown in red. **D** shows the “?” zebrin immunopositive zone, dividing the P1- zone into medial and lateral regions, and **E** shows the natural paucity of Purkinje cells (arrowhead) in the mid region of the P2+ zebrin immunopositive zone, dividing the P2+ zone into medial and lateral regions. Dotted lines represent the boundaries of the granular cell layer. gl, granular layer; ml, molecular layer; Pcl, Purkinje cell layer; wm, cerebellar white matter. All scale bars are 100µm.

heavier MF projection to the uvula compared to the flocculus. The pattern of MF labeling in the dorsal flocculus was similar to the ventral flocculus with a total of three main parasagittal bands throughout its rostro-caudal extent. There was generally one parasagittal zone in the most lateral regions of the dorsal flocculus that received a small number of mossy fibre projections; this zone was seen consistently on the ipsilateral side and in the cases with large injection sites, few MF terminals were seen in the corresponding region on the contralateral side.

#### *5.2.2.2 Mossy Fibre Projections from LM*

The organization of MF projections from LM was similar to the distribution seen from nBOR injections, and MF terminals were generally in overlapping regions (e.g. Fig. 5.5A,B; Fig. 5.8A-C). Examining the MF terminal organization in Figure 5.7C and Table 5.1, the following observations can be made. From LM injections, MFs were distributed bilaterally and terminal labeling was heavier to the contralateral side (ipsilateral labeling 35.3%, contralateral labeling 64.7%). More terminal labeling was seen in caudal regions of the folium. Heavier MF terminal labeling was seen in the dorsal lamella (60.7%) compared to the ventral lamella (39.3%), especially in contralateral and rostral regions. With respect to the parasagittal organization, clusters were more apparent from the LM injections compared to nBOR injections. As with nBOR, LM injections also heavily labeled the midline region, with the exception of the

ipsilateral dorsal lamella (see Fig. 5.8B for example); this pattern was more pronounced from injections in LM (ipsilateral dorsal lamella – 1.2%, compared to contralateral dorsal lamella – 13.7%). In the ventral lamella, there were more apparent parasagittal clusters on the ipsilateral side where four clusters could be distinguished. In the dorsal lamella, there was a heavier MF projection to the uvula compared to the flocculus, and overall, the heaviest labeling was observed in the contralateral dorsal uvula. The pattern of MF terminals in the flocculus consisted of three main parasagittal bands throughout its rostro-caudal extent. From LM injections, there was also generally one parasagittal zone in the most lateral and dorsal flocculus, with more rosettes on the ipsilateral compared to the contralateral side (Fig. 5.5C).

#### *5.2.2.3 Comparison of Projections from LM and nBOR*

Because the labeling from LM and nBOR was by and large overlapping, the parasagittal bands are more obvious in Figure 5.8C. There were a few notable differences between LM and nBOR injections in the pattern of MF labeling. Figure 5.8C shows the reconstruction of the labeling from both LM and nBOR. The MF terminal labeling in the flocculus was similar from LM and nBOR injections, but, on average, the MF terminal labeling in the uvula was more horizontally dispersed from nBOR compared to LM.

#### *5.2.2.4 Projections Outside Folium IXcd*

The projections from LM and nBOR to other regions of the cerebellum were not the focus of the current study, but briefly, from injections in nBOR, we observed very few MF terminals in other folia of the posterior lobe (folia VI-IXab) and most of the terminals were found surrounding the midline of these folia. From injections in LM, substantially more MF terminals were observed in the posterior lobe, especially in folia VI-VIII, and the terminals were found in the highest density surrounding the midline, with additional clusters in the more lateral parts of the folia. From injections into LM and nBOR we also observed a small number of MF terminals in the lingula (folium I) in the anterior lobe. No mossy fibre terminals were observed in folium X of the VbC in any of the cases.

#### *5.2.3 Zebrin Expression in Folium IXcd*

Zebrin immunoreactivity is observed in the Purkinje cell dendritic arbors, somata, and axons, but not the nuclei (Fig. 5.1C). In the pigeon posterior lobe, clusters of strongly zebrin immunoreactive Purkinje cells alternate with clusters that are immunonegative or very weakly immunoreactive (Figure 5.1D,E; Pakan et al., 2007). Alternating zebrin<sup>+/-</sup> stripes observed in folium IXcd are the most consistent and clear stripes seen throughout the cerebellum. The stripes themselves are numbered following the nomenclature used in Pakan et al. (2007) which is the same as that in mammals (Brochu et al., 1990; Eisenman and

Hawkes, 1993; Ozol et al., 1999; Sillitoe and Hawkes, 2002; reviewed in Sillitoe et al., 2005), whereby the most medial positive stripe straddles the midline and is designated P1+ and the six other zebrin+ stripes, P2+ to P7+, are located laterally on either side of the cerebellar midline extending into the auricle.

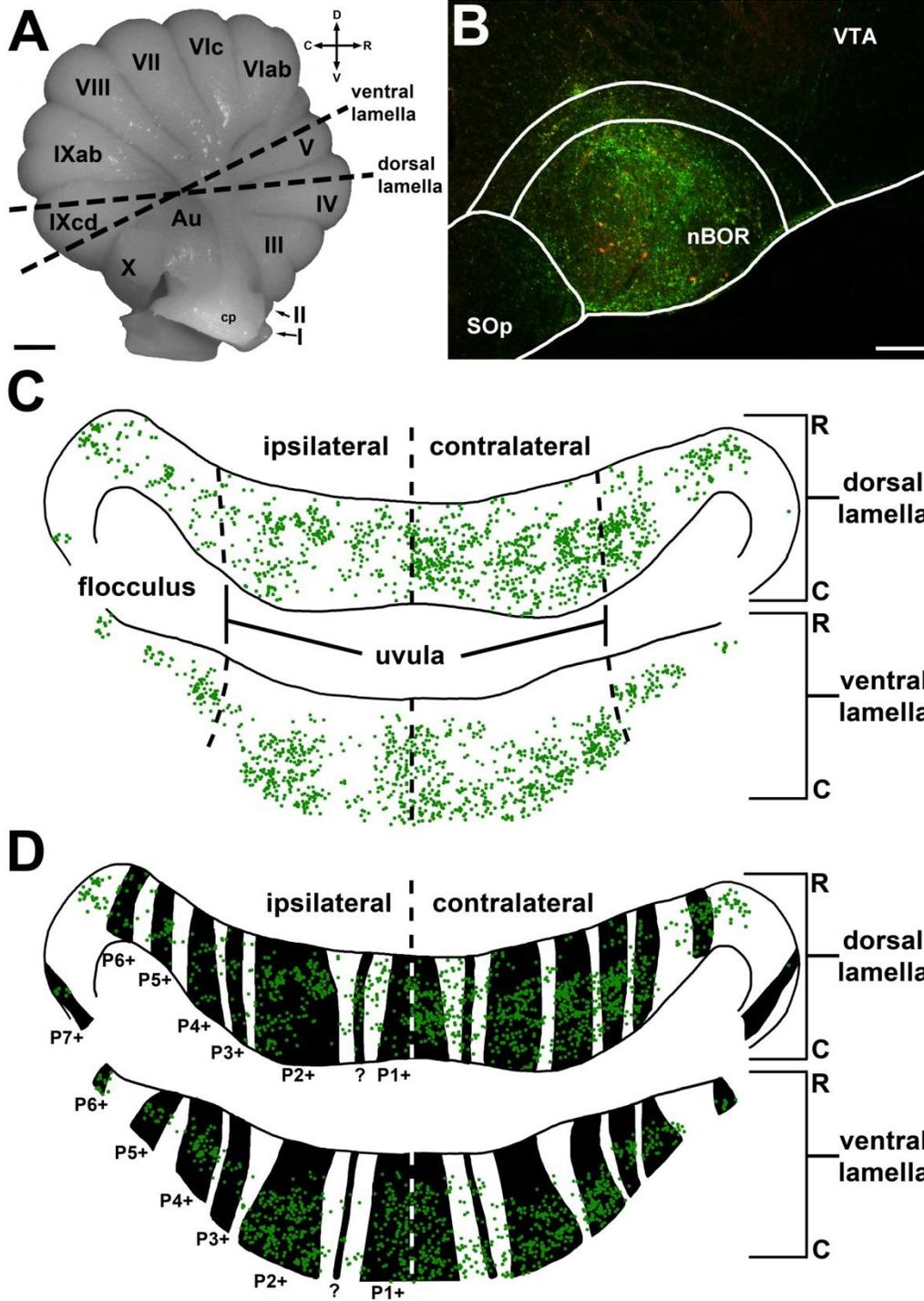
There are a few additional classifications that we have used in the current study to further delineate the various zebrin immunoreactive boundaries. In pigeons, there is often an additional, very small (perhaps 1-3 Purkinje cells wide), unclassified zebrin stripe which is between the large P1+ and P2+ stripes. We previously labeled this zone “?” in Pakan et al, (2007), because it was relatively inconsistently and usually only weakly immunopositive compared to the other surrounding positive stripes. However, in the current study, this small stripe was seen in all our cases (see Fig. 5.5D for example), especially in more caudal regions of folium IXcd, therefore we refer to P1-medial and P1-lateral as the zebrin negative regions surrounding the “?” zebrin+ zone. Secondly, the P2+ zebrin stripe is quite large and has a paucity of Purkinje cells in its mid-region, spanning about 2-4 Purkinje cells wide. Though this creates a pseudo-border, it is not a zebrin- stripe, and we refer to the portion medial to this division as P2+medial and the lateral portion as P2+lateral (see Fig. 5.5E for example).

#### *5.2.3.1 Mossy Fibre Labeling and Zebrin Expression in Folium IXcd*

In panel D of Figures 5.6-5.8, which show the reconstructions of MF terminal labeling from LM and nBOR as well as the zebrin expression pattern, the

correlation between the MF clusters and the zebrin stripes can be directly assessed. As is evident from these representative reconstructions, we found that, generally, MF terminals from LM and nBOR clustered within zebrin+ stripes. This can also be seen in Table 5.1, which shows percentages of MF terminal labeling in the various zebrin stripes, averaged across all cases. From nBOR injections, the concordance of the MF and the zebrin+ stripes is especially apparent in the ventral lamella, where 84% of the MF terminals were in zebrin+ zones. In the dorsal lamella 76% of terminals were in zebrin+ zones. Even within the P1- zebrin zone, much of the labeling fell within the very thin band of zebrin+ Purkinje cells, referred to as the “?” zebrin+ zone. From this perspective it seems that the nBOR MF projection is organized into several bands, each associated with a zebrin+ stripe.

From the LM injections - similar to that observed in nBOR - the concordance of the MF and the zebrin+ stripes is especially apparent in the ventral lamella, where 96% of the MF terminals were in zebrin+ zones. There were some general differences in the organization of zebrin stripes and MF terminals from injections in LM compared to nBOR. For instance, we did not observe a main cluster of MF rosettes in the “?” parasagittal stripe from injections in LM. Also, the parasagittal cluster corresponding to P3+ showed a striking difference between the contralateral and ipsilateral sides, with more MF terminal labeling on the contralateral side (7.3% on the contralateral compared to 1.3% on the ipsilateral side). There was also heavier terminal labeling in P4+ on the



**Figure 5.6 Reconstruction of mossy fibre (MF) terminals resulting from injections in the nucleus of the basal optic root (nBOR) from Case#5-Z5.**

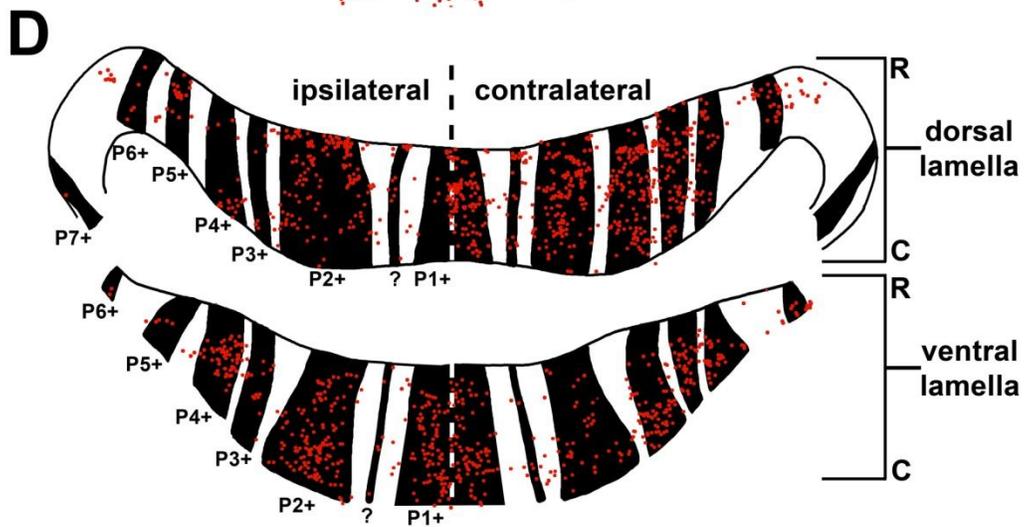
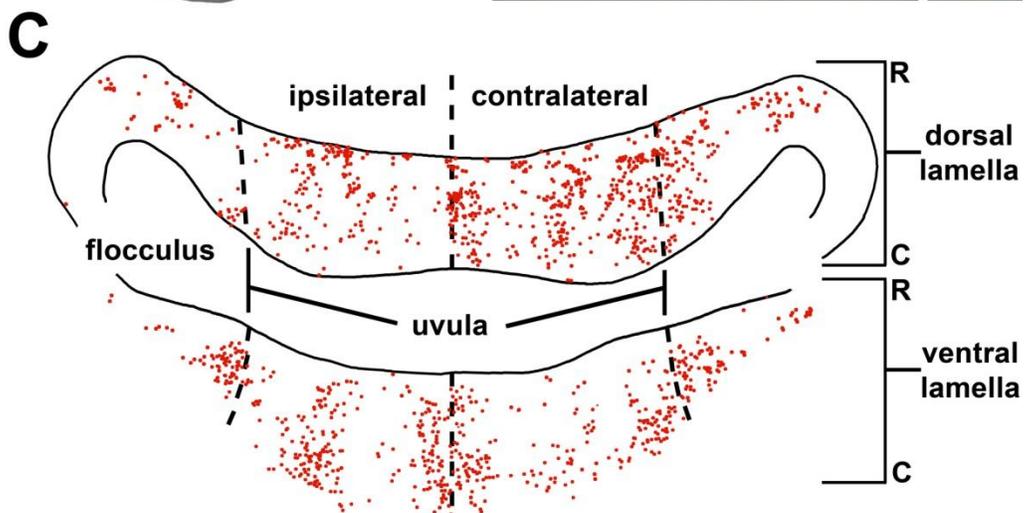
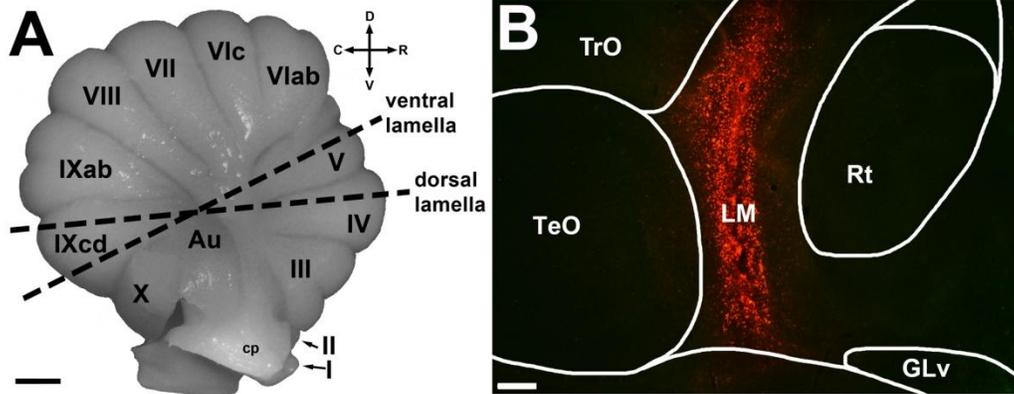
Figure 5.7 shows a reconstruction of MF terminals resulting from an injection in the nucleus lentiformis mesencephali (LM) from the same case. Figure 5.8 shows the overlay of the MF terminal labeling from these two injections. **A** shows the lateral view of the pigeon cerebellum and illustrates the plane of the reconstruction through the rostrocaudal extent of the folium. **B** shows the injection site in a photomicrograph of nBOR. **C** and **D** show reconstructions of folium IXcd through its rostrocaudal extent. Each dot on the reconstructions represent a MF terminal observed in serial coronal sections, but in order to present the overall pattern of labeling, the dorsal lamella and ventral lamella were separated and coronal sections were stacked from caudal to rostral. In other words, caudal coronal sections are represented more ventrally and rostral sections are represented more dorsally. **D** also shows the corresponding zebrin expression in folium IXcd. Au, auricle; R, rostral; C, caudal; D, dorsal, V, ventral; for other abbreviations see list. Scale bar in **A**= 1mm, **B** = 200µm.

contralateral side (6.7% contralateral compared to 2.1% ipsilateral), especially in the dorsal lamella (4.0% in contralateral dorsal lamella, 0.9% in ipsilateral dorsal lamella). As is apparent from Figure 5.6D (and Table 5.1) there was very little MF terminal labeling in the zebrin- zones from LM injections. On average, through the entire rostro-caudal extent of folium IXcd, thirteen of the sixteen zebrin- zones had less than 1% of MF terminal labeling associated with each of them.

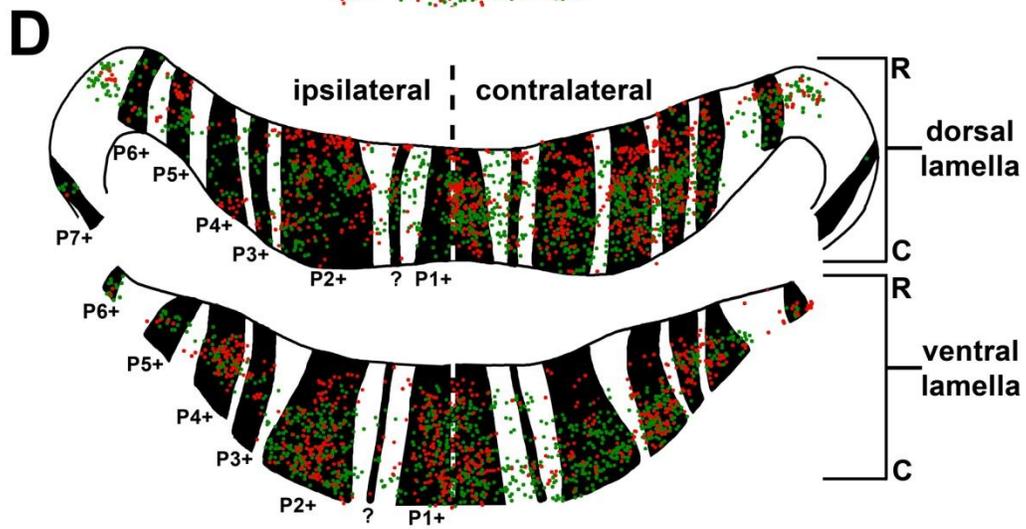
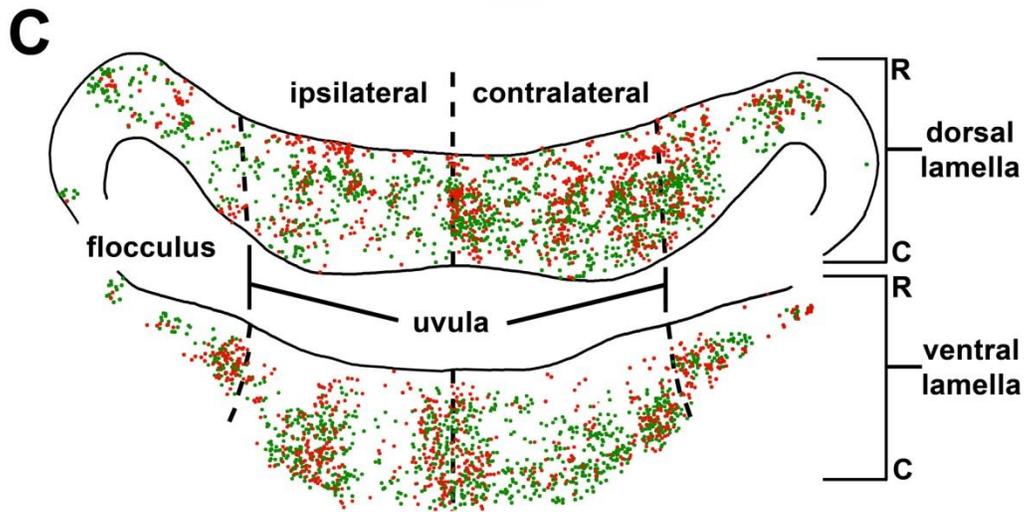
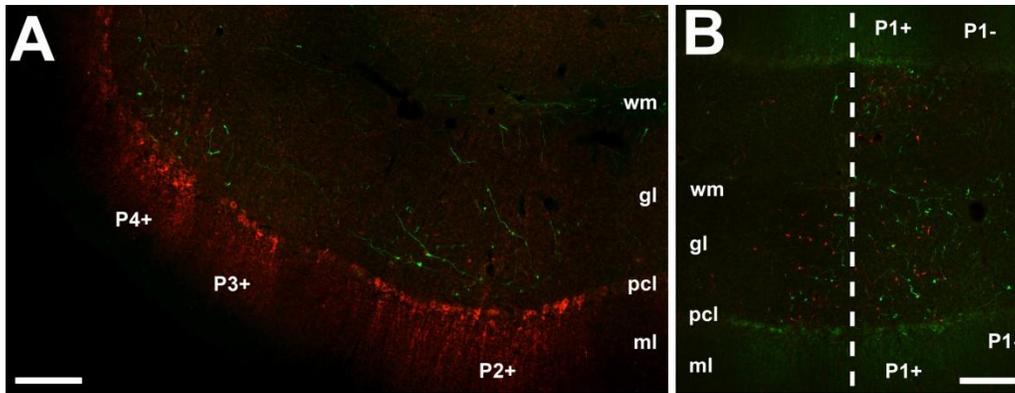
Although the abrupt zebrin+/- boundaries were not always strictly limiting boundaries for the MF clusters, generally, the zebrin+ Purkinje cells were directly superficial to the MF terminals. This is not an artifact of there simply being more zebrin positive Purkinje cells in folium IXcd. From our own measurements, we found that 57% of the Purkinje cells express zebrin (zebrin+ Purkinje cells) and 43% do not (zebrin- Purkinje cells). When we consider this along with the fact that 91% of MF terminals from LM injections and 80% of MF terminals from nBOR injections were correlated with zebrin+ stripes, it is clear that there was a strong bias for MF rosettes to cluster in zebrin+ stripes.

### **5.3 Discussion**

The parasagittal, zonal organization of climbing fibre projections from the IO has been well established in many cerebellar systems and in many different species (Voogd et al., 2003; Sugihara et al., 2004; Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004; Pijpers et al., 2005; Pijpers et al., 2006; Schonewille et al., 2006; Sugihara, 2006). The parasagittal organization of MF projections to the



**Figure 5.7 Reconstruction of mossy fibre (MF) terminals resulting from injections in the nucleus lentiformis mesencephali (LM) from Case#5-Z5. B** shows the injection site in a photomicrograph of LM. See Figure 5.6 caption for details of **A,C** and **D**. Scale bar in **A**= 1mm, **B** = 200 $\mu$ m.



**Figure 5.8 Reconstruction of mossy fibre (MF) terminals resulting from injections in the nucleus lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR) from Case#5-Z5. A and B show photomicrographs of the typical pattern of resulting MF terminals (rosettes) in folium IXcd from injections in nBOR (green) and LM (red) and the zebrin expression. In A, note the correspondence between the zebrin positive stripes (P2+ and P4+; red in Purkinje cell layer and molecular layer) and the MF terminals of LM and nBOR in the granular layer. B shows the region of folium IXcd directly surrounding the midline (dotted line) and the P1+ zebrin stripe (green in Purkinje cell layer and molecular layer); note the absence of MF terminal labeling in the ipsilateral dorsal lamella. gl, granular layer; ml, molecular layer; Pcl, Purkinje cell layer; wm, cerebellar white matter. See Figure 5.6 caption for details on C and D. Scale bars in A and B = 200µm.**

cerebellum has been less thoroughly studied, but the majority of research in mammals has found a zonal arrangement of terminals (for review see, Ozol and Hawkes, 1997).

In the present study, by injecting anterograde tracers into two retinal recipient nuclei in the pretectum and AOS, we have shown that these visual MF projections terminate in a zonal organization; this resulted in a number of parasagittal clusters of MF rosettes in the superficial granular layer of folium IXcd in the pigeon VbC. Generally the pattern of MF terminal labeling was very similar between LM and nBOR injections (Fig. 5.8), and consisted of 3-4 parasagittal clusters spanning the uvula and 4 clusters spanning the flocculus. The parasagittal demarcations were more defined in the ventral lamella of folium IXcd, especially compared to the dorsal uvula which showed more horizontal spread of MF terminal labeling from both LM and nBOR injections. Moreover, we showed that there was a clear organization of the MF terminals in relation to the zebrin stripes, to the extent that the bulk (80-91%) of the terminal labeling was contained within the zebrin+ stripes. We will consider these findings in light of what is known with regard to the visual information carried from LM and nBOR to the VbC through the MF and olivocerebellar pathways, and the functional organization of the olivocerebellar pathways with the zebrin stripes.

### 5.3.1 *Mossy Fibre Projections to the Vestibulocerebellum*

Previous investigations into the organization of MF terminals in the pigeon VbC using anterograde tracer ( $^3\text{H}$ -labeled amino acids) injections in LM (Clarke, 1977) and nBOR (Brecha et al., 1980) have described the resulting labeling in folium IXcd as “patchy” or “varied,” but the specific pattern of terminal labeling was not described. Gamlin and Cohen (1988), also used autoradiographic techniques to investigate the efferent projections from the pigeon LM, including MF pathways to the cerebellum. Similar to the current study, they found MF terminals bilaterally in folium IXcd, but more on the contralateral side, and in the external half of the granule cell layer. They did not provide a description of the organization of MF terminals, but from the samples of data provided in their Figures (Fig. 1 and 5 of Gamlin and Cohen, 1988) it is clear that the MF terminals are arranged in zonal clusters, although it is impossible to interpret the specific pattern from these examples. Schwartz and Schwartz (1983) investigated the organization of primary vestibular MF terminals in the pigeon VbC. They found that the labeled MF terminals were concentrated superficially in the granule cell layer (directly adjacent to the Purkinje cell layer) but did not note a parasagittal zonal arrangement.

The direct MF pathways from LM and nBOR to the VbC are not found in all vertebrates. Similar to pigeons, these direct MF pathways has been reported in turtles and fish, but not frogs (fish: Finger and Karten, 1978; turtle: Reiner and Karten, 1978; frogs: Montgomery et al., 1981; Weber et al., 2003). In mammals,

there has been no report of a mossy fibre pathway from the NOT to the cerebellum, however a mossy fibre projection from the medial terminal nuclei to the VbC has been reported in some species (chinchilla: Winfield et al., 1978; tree shrew: Haines and Sowa, 1985), but not others (cats: Kawasaki and Sato, 1980; rats and rabbits: Giolli et al., 1984). There is evidence of several indirect MF pathways from NOT and the AOS to the cerebellum through which optic flow information can be conveyed. Most of the mossy fibre input to the VbC arises in the vestibular nuclei and the prepositus hypoglossi (Voogd et al., 1996; Ruigrok, 2003), but there are also projections originating in the reticular formation, the raphe nuclei, a number of pontine regions, and neurons located within and around the medial longitudinal fasciculus (Blanks et al., 1983; Sato et al., 1983; Gerrits et al., 1984; Langer et al., 1985; Mustari et al., 1994; Voogd et al., 1996; Nagao et al., 1997; Ruigrok, 2003). The NOT and AOS project to many of these structures, including the vestibular nuclei, the medial and dorsolateral nuclei of the basilar pontine complex, the mesencephalic reticular formation, the prepositus hypoglossi, and the nucleus reticularis tegmenti pontis (NRTP; Itoh, 1977; Terasawa et al., 1979; Cazin et al., 1982; Holstege and Collewyn, 1982; Giolli et al., 1984; 1985; Torigoe et al., 1986b; a; Giolli et al., 1988; for review see Simpson et al., 1988; Gamlin, 2006; Giolli et al., 2006). In rats, Serapide and colleagues have observed that MF projections from the NRTP and the basal pontine nuclei terminate in parasagittal zones in the vermis of lobule IX, the flocculus and the paraflocculus (Serapide et al., 2001; Serapide et al., 2002).

### 5.3.2 *Visual Olivo-Vestibulocerebellar Pathways*

In this study, we also describe the topography of projections from LM and nBOR to the mcIO. We found a bilateral projection from nBOR, which was much heavier to the ipsilateral mcIO and a unilateral projection from the LM to the ipsilateral mcIO. This is in agreement with previous anterograde studies (Clarke, 1977; Brecha et al., 1980; Gamlin and Cohen, 1988; Wylie et al., 1997). We also observed a rosto-caudal difference, with nBOR projecting heavily to the rostral regions, and LM projecting exclusively to the caudal regions of the mcIO. This confirms the results from a retrograde study that noted more labeling of neurons in nBOR and LM from injections in the rostral and caudal mcIO, respectively (Wylie, 2001). The differences in the projections from LM and nBOR to the caudal and rostral mcIO, respectively, are similar to those seen in homologous pathways from the pretectum and AOS to the dorsal cap of Kooy, the homolog of the mcIO in mammals (Mizuno et al., 1973; Maekawa and Takeda, 1976; Takeda and Maekawa, 1976; Holstege and Collewyn, 1982; Mustari et al., 1994).

### 5.3.3 *Physiology of the Visual Projections to the VbC*

Most LM and nBOR neurons have large receptive fields restricted to the contralateral eye, and respond best to large-field stimuli such as checkerboards, gratings, and random dot patterns moving in a particular direction. Generally speaking, LM and nBOR are complimentary with respect to direction preference.

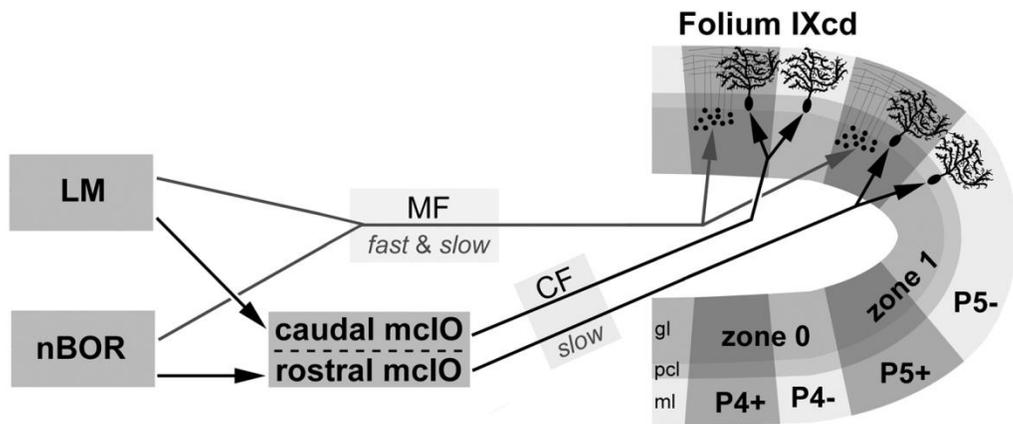
About half of LM neurons prefer temporal-to-nasal (T-N) motion. T-N neurons are rare in nBOR, where neurons that prefer upward, downward and N-T (nasal-to-temporal) motion are equally represented (Burns and Wallman, 1981; Morgan and Frost, 1981; Gioanni et al., 1984; Winterson and Brauth, 1985; Wylie and Frost, 1990; 1996). LM and nBOR neurons are also sensitive to speed (i.e. temporal frequency of moving gratings) and there are two response groups in both nBOR and LM: fast cells and slow cells (Wylie and Crowder, 2000; Crowder and Wylie, 2001; Crowder et al., 2003; Winship et al., 2006). Recording from the VbC, Winship et al. (2005) showed that the olivocerebellar system receives input from only slow cells in LM and nBOR, whereas the MF projections to the VbC arise from both slow and fast cells in LM and nBOR (see Fig. 5.9). In LM, the slow cells prefer T-N motion, whereas in nBOR the slow cells prefer upward, downward or N-T motion, but not T-N motion (Wylie and Crowder, 2000; Crowder and Wylie, 2001). Thus, the visual information to the mcIO from nBOR and LM differs with respect to direction preference and it is not surprising that these projections are topographic. The topography of these terminals in the mcIO is important in establishing the panoramic receptive fields of both the mcIO and Purkinje cells that are responsive to particular patterns of optic flow. For example, the caudal mcIO contains medially located rVA neurons, and the lateral regions of the caudal mcIO contain neurons that are responsive to visual stimuli resulting from self-translation in the backward direction. The preferred optic flow-field resulting from both of these response types consists of T-N motion in the

contralateral hemifield. Therefore, as we have shown in the present study, one would expect this caudal region to receive a large portion of its input from LM, which transmits T-N motion to the mcIO (see also, Wylie et al., 1999b; Crowder et al., 2000; Winship and Wylie, 2001; Wylie, 2001).

The fast units in nBOR and LM respond to all cardinal directions of motion; upward, downward, N-T and T-N (Wylie and Crowder, 2000; Crowder and Wylie, 2001). As the MFs to the VbC originate from both fast and slow cells in nBOR and LM (Winship et al., 2005), these nuclei are transmitting similar information to the VbC; this is not the case for the olivary inputs. Perhaps this is why the MF input from nBOR and LM to the VbC is overlapping and not topographically separated as they are in the mcIO.

#### ***5.3.4 Visual Inputs to IXcd In Relation to Zebrin Stripes***

In the present study, we are able to use the antigen zebrin II as a molecular marker in order to relate the various patterns of organization in the cerebellum. Although the specific function of zebrin II (or aldolase C) in the cerebellum is largely unknown (cf. Welsh et al., 2002; Wadiche and Jahr, 2005), zebrin expression is useful for, and often used as, a positional landmark in the cerebellar cortex (Hawkes and Gravel, 1991; Hawkes, 1992; Hawkes et al., 1993; For review see, Herrup and Kuemerle, 1997; Ozol et al., 1999). We have previously (Pakan and Wylie, 2008) investigated the correlation between CF zones and the zebrin expression pattern in the flocculus by making small anterograde tracer



**Figure 5.9** Optic flow input from the the nucleus lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR) to the vestibulocerebellum (VbC) in pigeons. This schematic illustrates the visual afferents projecting to the flocculus (of folium IXcd) from LM and nBOR. For simplicity, only floccular zones 0 and 1 are shown in this schematic. The mossy fibre (MF) inputs originate from both *fast* and *slow* cells in LM and nBOR, whereas the climbing fibre (CF) inputs via the medial column of the inferior olive (mcIO) originate primarily from *slow* cells in LM and nBOR (see Winship et al., 2005). The MF pathway terminates in the granular cell layer in zebrin immunopositive stripes (P4+ and P5+). The CF pathways terminate in parasagittal zones of a zebrin immunopositive and immunonegative pair (P4+/- and P5+/-), with the caudal mcIO projecting to the rVA responsive zones in the flocculus (zones 0) and the rostral mcIO projecting to the rH45 zones in the flocculus (zone 1). gl, granular layer; ml, molecular layer; Pcl, Purkinje cell layer; wm, cerebellar white matter.

injections into either the rostral or caudal mcIO and visualizing the resulting CF labeling with the zebrin stripes. We found that there was a strict concordance between CF organization and zebrin labeling such that a specific CF zone corresponded to a zebrin+/- pair in the flocculus (see Fig. 5.1E). For instance, the most caudomedial CF zone, an rVA zone, spanned the P4+ and P4- zebrin stripes, the adjacent rH45 zone spanned the P5+ and P5- stripes, the second rVA zone spanned the P6+ and P6- stripes, and the most rostrolateral rH45 zone spanned the P7+ and P7- zebrin stripes. This relationship of zebrin stripes to parasagittal CF zones was contrary to findings in other species and other regions of the cerebellum where CF zones generally project to either zebrin+ stripes or zebrin- stripes, but not both (Voogd et al., 2003; Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004; Pijpers et al., 2006; Sugihara and Quy, 2007; Sugihara and Shinoda, 2007; Pakan and Wylie, 2008). For instance, in a comprehensive study of the entire cerebellum, Sugihara and Shinoda (2004) identified olivocerebellar projections to zebrin compartments by labeling climbing fibres with BDA injected into various small areas within the inferior olive in rats. They found that there was a correspondence between a given olivary region and zebrin stripes of a particular sign, either positive or negative, but not both.

In this study, to determine the relationship of visual MF projections to the zebrin pattern, we examined the organization of the MF terminals from LM and nBOR to the granular layer and the parasagittal zebrin antigenic stripes in the

Purkinje cells of folium IXcd in the pigeon. Consistent and well demarcated zebrin antigenic stripes and parasagittal clusters of MF terminals from both LM and nBOR were visualized simultaneously, enabling a direct comparison of the zonal relationship. We found that the MF terminations were more pronounced in regions where Purkinje cells were zebrin+. This was a strong relationship with a clear relationship between MF parasagittal clusters and zebrin+ stripes, but the zebrin immunoreactive borders were not explicit boundaries for the MF terminals, and there were some MF rosettes observed in zebrin- regions as well.

There have been few studies investigating the relationship between MF terminal organization and zebrin expression; the results of these studies seem to implicate a consistent yet complicated relationship between MF zones and zebrin stripes (Gravel and Hawkes, 1990; Akintunde and Eisenman, 1994; Ji and Hawkes, 1994). However, a study by Matsushita et al., (1991) examined the topographic relationship between zebrin stripes and the distribution of spinocerebellar fibres originating from the central cervical nucleus in the rat. They found that in lobules 1-V, VIII and the copula pyramidis, the labeled MF terminals were seen clustered beneath zebrin positive bands. Similar to the results of this study, they also found that the borders of MF terminal distribution were not well-delineated, and were not strictly bounded by the borders of zebrin positive bands.

In general, the organization of the MF projections to the cerebellum appears less spatially restricted and seems more suited for carrying a wide

divergence of information; not only because MF information originates from many different regions in the central nervous system, but also due to the indirect nature of the MF projection to Purkinje cells through granule cells and parallel fibres. On the other hand, the CF system, originating solely in the IO, sends its projections directly to the Purkinje cell dendrites in narrow, parasagittally arranged stripes. The current study, along with our previous study on the CF correlation with zebrin (Pakan and Wylie, 2008), illustrate these differences between visual CF and MF projections from the pretectum and the AOS to the flocculus of the pigeon VbC. Along with the function of the Purkinje cells themselves, the organizational differences in these two afferent systems have been central to all major theories of cerebellar function (Marr, 1969; Albus, 1971; Ito, 1984); however, major portions of these theories remain largely unevaluated because there has been a lack of sufficient investigation into the anatomical interrelationship between these MF and CF systems, as well as the intrinsic biochemical properties of the Purkinje cells (cf. Pijpers et al., 2006).

Recent investigations into the collateralization of CF and MF terminals in the cerebellum in relation to zebrin expression, in rats, have made big strides in determining the anatomical relationship between these systems (Ruigrok, 2003; Voogd et al., 2003; Pijpers et al., 2006). For example, Pijpers and colleagues (2006) investigated the collateral terminations of MFs and CFs from small retrograde tracer injections and correlated the resulting terminal pattern with zebrin stripes. They found that labeled MF collaterals generally distribute to the

same lobules as CF collaterals and were always present in the granular layer directly subjacent to labeled CF collaterals. They also found that additional parasagittal clusters of MF terminals were labeled in other cerebellar regions, and these MF terminal zones often had the same zebrin signature as the source of the collateralization. They suggest that the various MF collateral zones, with the same zebrin expression characteristic, may be functionally linked. They also conclude that the MF and CF systems are closely aligned and that this is a consistent and widespread feature of cerebellar cortical organization.

In the pigeon VbC, the lack of sharp MF terminal boundaries and the fact that the relationship between visual MF afferents and zebrin expression seems to be more of a general pattern might suggest that, while the zebrin+ regions may share some underlying similarity in functional architecture with the visual MF terminal regions from LM and nBOR, it is unlikely that the zebrin boundaries themselves are driving the organization of the MF projection pattern. Taken together with the zebrin correlation to the CF projection pattern observed in the pigeon flocculus (Pakan and Wylie, 2008), our results suggests a complicated organizational relationship between visual afferents and the intrinsic zebrin antigenic map in the VbC.

### ***5.3.5 Functional Implications***

It has been well established that the VbC is important for integrating visual, vestibular and other sensori-motor information in producing compensatory

head and eye movements (for reviews see Simpson, 1984; Waespe and Henn, 1987; Voogd and Barmack, 2006). The functional implications of the findings of the present study, (the organization of visual MF inputs to the zebrin stripes), are dependent upon the actions of the granule-cell/parallel fibres on nearby Purkinje cells. A principle excitatory drive to a Purkinje cells arises from the synaptic contacts of ascending axons of granule cells that are directly subjacent to that Purkinje cell (Eccles et al., 1968; Brown and Bower, 2001; Lu et al., 2005). In this vein, the zebrin+ Purkinje cells in folium IXcd of the VbC would receive more excitatory visual MF input than the zebrin- Purkinje cells. In terms of the visual-vestibular interactions in mediating the vestibulo-ocular response, it is known that visual signals are especially useful at low head velocities where the vestibular response is quite sluggish (e.g. Wilson and Melvill Jones, 1979). That is, the zebrin+ Purkinje cells would be more active during low frequency head movements. Also, at the onset of a visual stimulus there is a very brief period (50-100 ms) prior to any compensatory eye movement when retinal slip velocity is high (Collewijn, 1972), and again one might expect that the zebrin+ Purkinje cells would be preferentially active. However, the actions of granule cells on Purkinje cells via parallel fibre synapses and inhibitory interneurons cannot be discounted (e.g. Lu et al., 2005). Indeed for the forelimb C3 region of the anterior lobe, Ekerot and Jorntell (2003) suggest that granule cells actually have a profound inhibitory influence through the actions of interneurons on directly adjacent Purkinje cells, and an excitatory influence of Purkinje cells in neighboring

microzones (see also Garwicz et al., 1998; Ekerot and Jorntell, 2001; Jorntell and Ekerot, 2006; Ekerot and Jorntell, 2008). Therefore, the functional implication of these zonal MF patterns remains to be resolved.

### 5.3.6 *Other Mossy Fibre Inputs to Folium IXcd*

As the visual MFs project mainly to the zebrin+ regions in folium IXcd, the question arises as to which afferent MF inputs are projecting to the zebrin- regions. There are several other inputs to folium IXcd in birds, including: primary vestibular inputs (although these are mainly to folium X; Schwarz and Schwarz, 1983), secondary vestibular inputs (Brecha et al., 1980; Arends and Zeigler, 1991; Diaz and Puelles, 2003; Pakan et al., 2008), pontocerebellar inputs (Freedman et al., 1975), and spinocerebellar inputs (Vielvoye and Voogd, 1977; Okado et al., 1987; Necker, 1994). Using anterograde techniques, the topographic distribution of mossy fibres has been described only with respect to the spinocerebellar and pontocerebellar projections. Vielvoye (1977) described in detail the topography of the spinocerebellar projections in white leghorn chickens (*Gallus Domesticus*). The spinocerebellar mossy fibres (and pontocerebellar mossy fibres (Freedman et al. 1975)) tend to project to deeper portions of the granular layer, rather than superficially as is the case for the LM and nBOR mossy fibre inputs. More importantly there is a clear parasagittal distribution of mossy fibre terminals in IXcd, where there are several clusters (Vielvoye, 1977; Okado et al., 1987). How

these clusters relate to the visual mossy fibre inputs, and the zebrin stripes is yet to be determined.

### **5.3.7 Conclusion**

From the results of the current study and our previous findings (Pakan and Wylie, 2008), a comprehensive picture of the visual MF and olivocerebellar inputs in relation to the zebrin stripes in the flocculus has emerged and is shown in schematic form in Figure 5.9. In folium IXcd of the pigeon flocculus, the functional units consist of two rVA zones (zones 0 and 2) and two rH45 zones (1 and 3), which receive input from the caudal and rostral mcIO, respectively. Each of these zones includes a zebrin + and zebrin – stripe. The visual MF projections terminate in a parasagittal organization and generally cluster in the zebrin+ regions of each of these larger CF zones.

**Table 5.1 Quantification of mossy fibre (MF) rosettes labeled in folium**

**IXcd from anterograde tracer injections into the nucleus lentiformis mesencephali (LM) and the nucleus of the basal optic root (nBOR).**

MF rosettes corresponding to zebrin stripes (labeled P1<sup>+/-</sup> through P7<sup>+/-</sup>) on each side of the folium were counted from serial coronal sections and expressed as percentages of the total number of labeled rosettes per case.

The numbers presented are averaged over all cases. I, ipsilateral; C, contralateral; Bi, bilateral.

	P1+	P1-med	?	P1-lat	P2+ med	P2+ lat	P2 -	P3 +	P3 -	P4 +	P4 -	P5 +	P5 -	P6 +	P6 -	P7 +	P7 -	Total all	Total +
<b>nBOR – I</b>	7.0	2.1	2.4	3.2	9.2	6.1	2.2	3.7	0.3	4.3	0.3	1.8	0.6	1.2	0.2	0.3	0.7	45.5	36.0
<b>nBOR – C</b>	13.5	2.7	2.3	3.5	7.5	5.0	1.3	5.9	0.8	5.4	0.9	3.1	0.7	1.1	0.3	0.1	0.4	54.5	43.9
<b>nBOR - Bi</b>	20.5	4.8	4.7	6.7	16.7	11.1	3.5	9.6	1.1	9.7	1.2	4.9	1.3	2.3	0.5	0.4	1.1	100	79.9
<b>LM – I</b>	10.5	0.9	1.0	0.4	7.6	7.3	0.6	1.3	0.3	2.1	0.0	2.0	0.2	0.4	0.2	0.0	0.7	35.3	32.2
<b>LM – C</b>	21.6	1.9	1.4	1.0	7.2	9.9	0.6	7.3	0.4	6.7	0.7	3.8	0.9	1.0	0.2	0.0	0.1	64.7	58.9
<b>LM - Bi</b>	32.1	2.8	2.4	1.4	14.8	17.2	1.2	8.6	0.7	8.8	0.7	5.8	1.1	1.4	0.4	0.0	0.8	100	91.1

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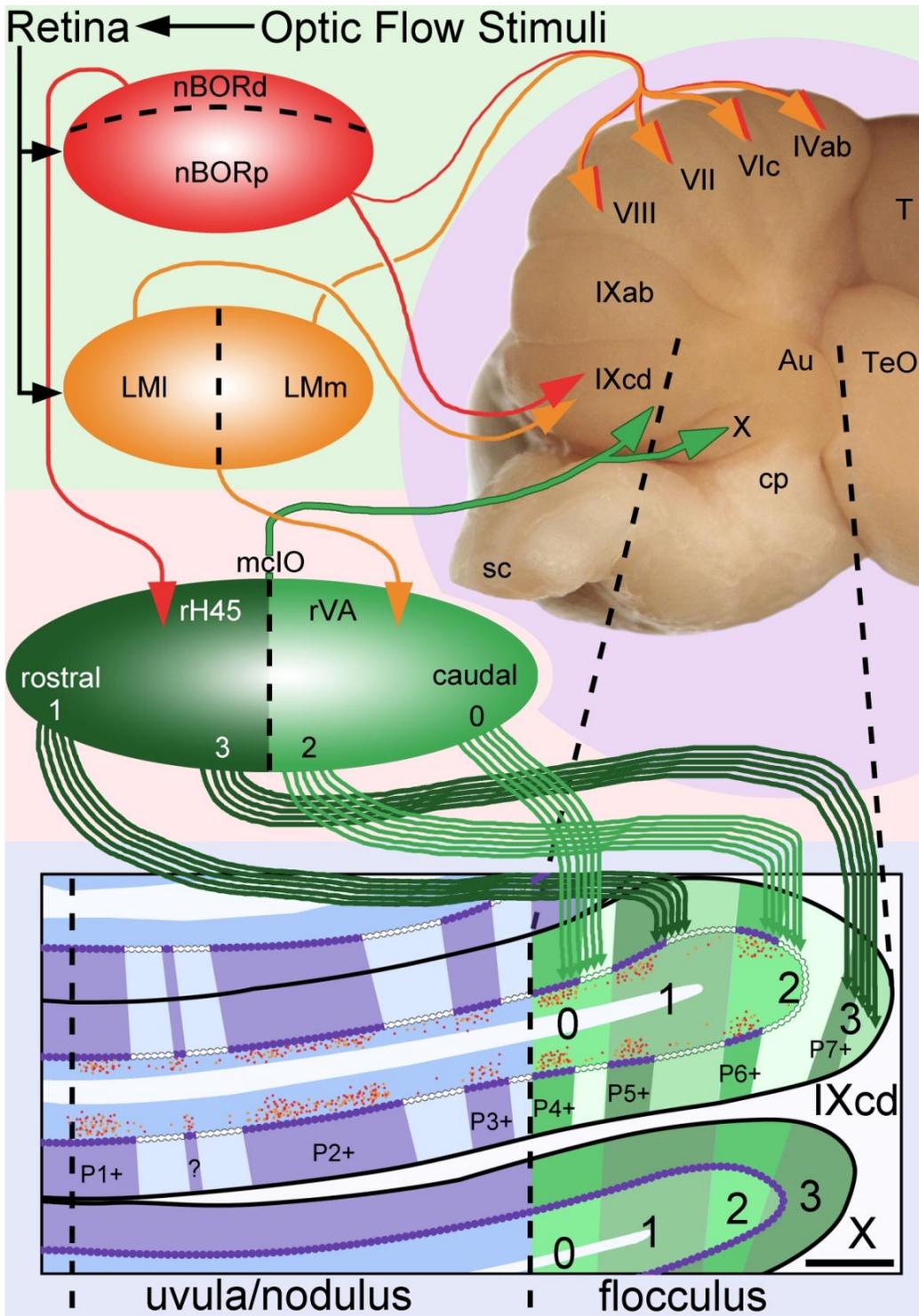
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## Chapter 6: **Summary and Future Directions**

The performance of various tasks, even ones that appear to be relatively simple in nature, involves rather complex patterns of parallel and sequential processing that could be accomplished only by a massively parallel and modularly organized system. Some of the best examples of this functional organization come from the visual system; traditionally, the geniculostriate visual pathway is discussed in this respect (e.g. Hubel and Wiesel, 1968; Berman et al., 1982; Bauer and Dow, 1991; Goodale and Milner, 1992; Milner and Goodale, 1993; Goodale, 1996; Lennie, 1998), but the AOS and associated pretectal visual pathway also serves as an excellent model to investigate these organizational principles. This is especially evident when we examine the complex structure and function of the projections of this visual pathway to the cerebellum (for summary see Figure 6.1).

The functional units throughout the cerebellum are now known to be a series of zones that lie in the sagittal plane, cutting transversely across the folia. This modular organization is revealed in several aspects: climbing fibre afferents to the cerebellar cortex terminate in parasagittal bands (Voogd & Bigaré 1980), there is growing evidence that mossy fibres also terminate in a parasagittal zonal pattern (Wu et al. 1999; Ruigrok 2003), Purkinje cells within a band show similar response properties (Apps & Garwicz 2005), and they tend to fire synchronously (Llinás & Sasaki 1989). Finally, this parasagittal organization is also revealed in the patterns of expression of numerous molecules (Herrup & Kuemerle, 1997).



**Figure 6.1 Summary of the organization of visual projections from the Accessory Optic System and associated pretectal area to the cerebellum.** The lentiformis mesencephali (LM), and the nucleus of the basal optic root (nBOR) receive optic flow information via projections directly from the retina. These two nuclei then project directly to folia VI-VIII and folia IXcd of the cerebellum as mossy fibres (LM: orange; nBOR: red), and indirectly to folia IXcd and X via the medial column of the inferior olive (mcIO; green). The caudo-lateral view of a whole cerebellum is shown in the top right. In the bottom panel a schematic of a coronal section through the vestibulocerebellum (VbC) is shown, indicating the pattern of mossy fibre terminals from LM and nBOR (red and orange dots), the climbing fibre projections (shades of green) and the zebrin expression pattern (purple = zebrin immunopositive regions/cells). Numbers 0-3 depict the rotation-sensitive electrophysiologically responsive zones in the flocculus. Au, auricle; cp, cerebellar peduncle; sc, spinal cord; T, telencephalon; TeO, optic tectum; LMI, LMm, lentiformis mesencephali, lateral and medial subnuclei, respectively; nBORp, nBORd, nucleus of the basal optic root, dorsal and proper regions, respectively. rVA, neurons responsive to optic flow stimuli resulting from rotation about a vertical axis; rH45, neurons responsive to optic flow stimuli resulting from rotation about a horizontal axis oriented 45° from midline.

The anatomy, physiology and biochemistry of the cerebellar cortex thus lead us to an important concept: the cerebellum can be subdivided into modules that perform specialized functions. This concept of cerebellar function may be gratifying from a researcher's perspective, since, historically, the insight that a complex brain region can be simplified by viewing it as a series of smaller repetitive units has advanced our understanding of neuroscience in many respects.

## **6.1 Summary of Chapters**

This dissertation consisted of four studies that investigated the organization of the visual optic flow pathways from the AOS and associated pretectum to the cerebellum, and the molecular, topographic, and functional modular organization of the cerebellar region processing this visual information, the vestibulocerebellum.

In Chapter 2 retrograde tracing techniques were used to investigate if the direct mossy fibre input from LM to IXcd differs from that to VI-VIII. Previous research has described a mossy fibre projection from LM to folia VI-VIII of the posterior cerebellum, and IXcd of the vestibulocerebellum, but the specific organization of these pathways had not been investigated (Clarke, 1977; Gamlin and Cohen, 1988; Pakan et al., 2006). In order to examine the organization of these pretectal-cerebellar projections, fluorescent retrograde tracers were injected into folia VI-VIII as well as the VbC, and the pattern of labeling in LM was observed. We found that large multi-polar neurons were labeled throughout LM

and that there is a topographic projection from the pretectum to the cerebellum. The projection to folium IXcd of the vestibulocerebellum arises mainly from LMI, whereas that to folia VI-VIII arises mainly from LMm. It is known that the vestibulocerebellum is involved in visual-vestibular integration, supporting gaze stabilization (Simpson et al., 1979; Waespe and Henn, 1987), but the function of folia VI-VIII in pigeons is not well understood to date. Previous research has shown that folia VI-VIII receive input from a tecto-pontine system (Clarke, 1977), which is likely involved with analyzing local motion as opposed to optic flow (Frost and DiFranco, 1976; Frost and Nakayama, 1983; Hellmann et al., 2004). The results of this study suggested that the subnuclei of LM and the subsequent visual pathways have differing roles in optic flow processing. In particular, the LMI-IXcd pathway is involved in generating the optokinetic response and we suggested that the pathway from LMm to VI-VIII is integrating optic flow and local motion to support various oculomotor and visuomotor behaviors including obstacle avoidance during locomotion. This work represents the first finding of a functional difference between the subnuclei in LM as well as the first suggestion of parallel visuomotor processing pathways from the pretectum to the cerebellum. An investigation into the precise organization of these pathways in the cerebellum, as well as electrophysiological experiments to establish the specific function of the cerebellar folia in birds are needed to further our understanding of this visuomotor processing.

Since the pigeon VbC is so well characterized with regard to the anatomical and functional parasagittal organization, we then wanted to investigate the biochemical modular organization, which has more recently been described in mammals. In Chapter 3 we used immunohistochemical techniques to investigate this modular organization in the avian cerebellum with the molecular marker zebrin. It has been shown that Purkinje cells in the cerebellum express the antigen zebrin II (aldolase C; Brochu et al., 1990; Ahn et al., 1994; Hawkes and Herrup, 1995) in many vertebrates (Hawkes et al., 1988; Brochu et al., 1990; Dore et al., 1990; Leclerc et al., 1990; Lannoo et al., 1991a; Lannoo et al., 1991b; Hawkes, 1992; Lannoo et al., 1992; Meek et al., 1992; Hawkes and Herrup, 1995; Sanchez et al., 2002; Marzban et al., 2003; Sillitoe et al., 2003; Sillitoe et al., 2005). In mammals, zebrin is expressed in a parasagittal fashion with alternating immunopositive and immunonegative stripes (for review see Hawkes and Herrup, 1995; Sillitoe et al., 2005). In this study we provided the first investigation into zebrin expression in an avian species. We found that, similar to mammals, zebrin expression in the pigeon cerebellum is prominent in Purkinje cells, including their dendrites, somata, axons and axon terminals. Parasagittal stripes were apparent with bands of Purkinje cells that strongly expressed zebrin (zebrin+) alternating with bands that expressed zebrin weakly or not at all (zebrin-). We found a pattern of zebrin expression in the pigeon cerebellum that was similar to the pattern seen in mammals. Interestingly, the zebrin stripes were most prominent in folium IXcd where there were seven zebrin+/- stripes, bilaterally. Therefore, this study

provided evidence of a biochemical modular organization in the avian cerebellum, and most importantly to this dissertation, in the pigeon VbC. The potential relationship of this biochemical parasagittal organization with the anatomical and physiological modular organization in the pigeon VbC can now be examined in an attempt to elucidate a functional underlying architecture that may be inherent in these parasagittal patterns.

In chapter 4, using both anterograde tracing and electrophysiological techniques in combination with immunohistochemistry, we investigated the specific functional organization of visually responsive climbing fibre projections from the mcIO to the VbC, and correlated this modular organization with the pattern of zebrin expression. Previous research has shown that floccular Purkinje cells respond to patterns of optic flow resulting from self-rotation about either the vertical axis (zones 0 and 2), or a horizontal axis (zones 1 and 3; Winship and Wylie, 2003; Wylie et al., 2003; Voogd and Wylie, 2004). Visual climbing fibre afferents projecting to the flocculus arise from the medial mcIO, zones 0 and 2 receive input from the caudal mcIO, whereas zones 1 and 3 receive input from the rostral mcIO (see Figure 6.1; Wylie et al., 1999b; Winship and Wylie, 2003). To investigate the relationship between these climbing fibre zones and the zebrin stripes, we made small injections of a fluorescent anterograde tracer into the rostral and/or caudal mcIO and visualized zebrin expression in the VbC. We also made single unit recordings of Purkinje cell CSA in response to optic flow stimuli, mapping out the responsive zones and then visualized zebrin expression.

We found a strict concordance between climbing fibre organization, Purkinje cell function and zebrin labeling: caudal mcIO injections resulted in climbing fibres in zebrin bands P4+/- and P6+/-, whereas rostral mcIO injections resulted in climbing fibres in zebrin bands P5+/- and P7+/- . Zebrin stripes P4+/- and P6+/- corresponded to the vertical axis zones 0 and 2, whereas P5+/- and P7+/- corresponded to the horizontal axis zones 1 and 3. Therefore, in this study we demonstrated that a series of zebrin stripes corresponds with functional zones in the cerebellum; this finding was one of the first studies in any species to correlate the biochemical, anatomical and functional organization in a cerebellar system.

Finally, In Chapter 5 we investigated the organization of the direct visual mossy fibre projections from the AOS and pretectum to the VbC and examined the relationship of these projections to the zebrin expression pattern. Compared to the well established parasagittal organization of climbing fibre afferents (for review see Brodal and Kawamura, 1980; Voogd and Ruigrok, 1997), less is known about the organization of mossy fibre afferents in general, and more specifically in relation to molecular markers such as zebrin. Visual afferents from the two retinal recipient nuclei, LM and nBOR, project directly to folium IXcd as mossy fibres and indirectly as climbing fibres via the inferior olive (Clarke, 1977; Brecha et al., 1980; Gamlin and Cohen, 1988; Arends and Voogd, 1989; Lau et al., 1998; Wylie et al., 1999a; Crowder et al., 2000; Winship and Wylie, 2003). Although it has been shown that these two nuclei project directly to folium IXcd the detailed organization of these projections has not been reported. In this study,

anterograde tracers were injected into LM and nBOR to investigate the organization of mossy fibre terminals and subsequently relate this organization to the zebrin expression pattern. We found a parasagittal organization of mossy fibre terminals in folium IXcd and observed a consistent relationship between mossy fibre organization and zebrin stripes: parasagittal clusters of mossy fibre terminals were concentrated in zebrin immunopositive regions. We also expand on previous studies with respect to the organization of the indirect visual pathways from LM and nBOR to the VbC by describing the detailed topography of these pathways from LM and nBOR to the inferior olive. The observations of this study underscore the functional differentiation of zebrin+ versus zebrin- Purkinje cells and suggest that the organization of the granular layer correlates to this patterning.

## **6.2 Future Directions**

There are many interesting questions that can be addressed as an extension of the work presented in this dissertation. A natural progression from Chapter 2 is the investigation into the visual response properties of neurons in, and the organization of visual afferents to, folium VI-VIII of the avian posterior cerebellum. Clearly, obstacle avoidance and visually guided steering are key functional behaviours in animals that fly. If this is indeed the function of the pretectal pathway to these folia in the cerebellum it would be very interesting to investigate not only the specific response properties of the Purkinje cells in this region to various visual stimuli, but also how the visual world is represented,

physiologically, in this region of the cerebellar cortex. If related to obstacle avoidance, a behaviour that would involve not only visual optic flow input from the AOS and pretectum, but tectal input, proprioceptive inputs, and perhaps other sensory inputs from the auditory system for example, this region of cortex could potentially contain very complicated overlapping sensory and motor representations of both the environment and the organism itself.

It would also be interesting to examine folia VI-VIII in relation to the pattern of zebrin expression, and the organization of the various afferents and functional properties. In fact, the investigation of zebrin expression is potentially interesting when applied to any region of the cerebellar cortex, especially in relation to the anatomical and functional patterns that also exist there. However, an equally interesting future direction of research into the biochemical parasagittal organization of the cerebellar cortex asks the question: what are the specific functions of these molecular markers? Zebrin, being the most thoroughly studied of these antigens, perhaps begs this question the most. To date the function of zebrin is largely unknown. The distribution of zebrin (aldolase C) in the cerebellum and the little we do know about the function of this metabolic enzyme would seem to present two main questions. First, what are the molecular details of how aldolase C expression is regulated and what are its cellular functions in the cerebellar cortex? Is aldolase C simply functioning in glycolysis in a subset of Purkinje cells, or is there some *x-factor* function of this enzyme that is not apparent to us yet? This is obviously an appealing possibility; perhaps, the

function of aldolase C in vitro (the cleavage of fructose-1,6-biphosphate in glycolosis) and its actual function in vivo may be quite different. There is apparently nothing obvious in the biochemistry of the aldolase C enzyme that might explain its remarkable distribution in the cerebellum (Ahn et al., 1994; Hawkes and Herrup, 1995). There are no known functional specializations of the zebrin+ Purkinje cell subsets that would require a particular set of aldolase properties and the enzyme characteristics of aldolase C in vitro do not differ significantly from that of the other brain isoform, aldolase A (Funari et al., 2007). However, if it does function to cleave a different substrate or have a different preferred substrate in vivo, this *x-factor* substrate may play a crucial role either in Purkinje cell function or in the maintenance of the parasagittal organizational patterns. To date, however, no alternative aldolase C substrate has been reported.

Some interesting and recent research that has provided insights into possible functional distinctions of the zebrin+ zones comes from studies of neurodegeneration and diseases which lead to Purkinje cell loss. Caution must be taken when interpreting the results of these studies and extrapolating to the normal function of the cerebellar cortex simply because these models represent abnormal conditions in vivo (i.e. disease states, damage, and mutant models). However, the results of these investigations can obviously still provide vital clues to the role of these molecular markers in normal situations. For instance, it has been suggested that zebrin+ Purkinje cells may have some means of neuroprotection above and beyond that of the zebrin- Purkinje cell subsets. This

suggestion comes from studies which found that the zebrin+ Purkinje cells are resistant to cell death in models of cerebellar degeneration and damage (Welsh et al., 2002; Slemmer et al., 2007; Williams et al., 2007; Heitz et al., 2008). For example, Williams et al. (2007) used a rat model of neonatal viral infection and found that Purkinje cells in the zebrin- stripes showed preferential cell death. Also, Slemmer et al. (2007) showed that zebrin- Purkinje cells were more likely to die after cerebral trauma in vivo and that this death was mediated by excitotoxic means in vitro. In addition, in many diseases involving generalized atrophy of the cerebellum, Purkinje cell degeneration is much more predominant in the anterior lobe (e.g. chronic alcoholism: Phillips et al., 1987; Wernicke encephalopathy: Baker et al., 1999; perinatal hypoxia: Connolly et al., 2007); this becomes pertinent when one notes that the anterior lobe consists of predominantly zebrin- Purkinje cells (Sillitoe et al., 2005).

Whether this functional aspect of the zebrin+ Purkinje cells is attributable in part or in whole (which is unlikely), to the function of the aldolase C enzyme in these Purkinje cells is unknown. However, in all probability, these neuroprotective factors stem from an interaction between many molecular markers that are also expressed exclusively in the subset of zebrin+ Purkinje cells. For instance, the excitatory amino acid transporter EAAT4 is expressed in a parasagittal pattern that is coincident with the zebrin+ Purkinje cells (Welsh et al., 2002). Interestingly, recent evidence indicates that the parasagittally restricted expression of the glutamate transporter EAAT4 plays a direct role in synaptic

plasticity in Purkinje cells and, furthermore, protects Purkinje cells against excitotoxic cell death during ischemia (Welsh et al., 2002; Wadiche and Jahr, 2005; Yamashita et al., 2006).

This preferential degeneration of a subset of Purkinje cells proves to be very interesting from a functional perspective, and has many applications towards future research. As just one example, prolonged thiamine deficiency produces extensive neuronal cell loss in select brain regions including the cerebellum, inferior olive, thalamus, and mammillary bodies. The initial degeneration is seen in the cerebellum, where there is a high rate of thiamine turnover, and the Purkinje cells are particularly vulnerable to thiamine deficiency; however the specific pattern of this degeneration and the mechanisms underlying it are unknown (Todd and Butterworth, 1999; Mulholland, 2006). Using models of thiamine deficiency encephalopathy that result in selective neuronal cell death in the cerebellum, and relating the pattern of degeneration to the parasagittal expression pattern of molecular markers, would be an excellent means of investigating if the presence of zebrin (and/or other molecular markers) provides neuroprotection to Purkinje cells. This model would also provide an interesting opportunity to examine the specific mechanisms that underlie the degeneration resulting from thiamine deficiency in the cerebellum.

The second key question about the function of zebrin relates to the functional consequences of the parasagittal expression pattern of this apparently unremarkable metabolic enzyme in the cerebellar cortex, especially in relation to

the modular anatomical and physiological organization of the cerebellar cortex. This problem so far has proven to be challenging to answer. By virtue of the developmental time at which these intrinsic molecular regional variations can be detected, it appears that the organization of the afferents is independent of zebrin expression. In rodents, olivocerebellar climbing fibres are already restricted to different parasagittal regions in the cerebellum by embryonic day 18 (Paradies and Eisenman, 1993; Paradies et al., 1996). At embryonic day 13-14, mossy fibres first enter the cerebellar cortex (even before the presence of granule cells; Altman and Bayer, 1987b; Altman and Bayer, 1987a; Grishkat and Eisenman, 1995) and transiently associate with Purkinje cells (Mason and Gregory, 1984; Ji and Hawkes, 1995); this association is essential for the mossy fibre to become parasagittally organized later in development (postnatal day 3-5; Arsenio Nunes and Sotelo, 1985; Arsenio Nunes et al., 1988; Ji and Hawkes, 1995). Because zebrin is not expressed in Purkinje cells until postnatal day 5 (Leclerc et al., 1988; Tano et al., 1992), this antigen can clearly have no causal effect on the parasagittal organization of afferents, which is established much earlier in development. So, even though the borders are generally correlated, zebrin does not function to establish or guide the parasagittal organization of the afferent input in development; the possibility remains that the afferent organization is somehow necessary for the parasagittal expression pattern of zebrin to develop. However, the results of both in vivo and in vitro experiments suggest that zebrin expression is not influenced by interactions with cerebellar afferents, cerebellar neurons, or

glia (Leclerc et al., 1988; Wassef et al., 1990; Seil et al., 1995). Leclerc et al. (1988) lesioned dorsal and ventral spinocerebellar mossy fibre afferents and olivocerebellar climbing fibre afferents in the neonatal or adult rat and found that the pattern of zebrin expression was unaffected; this suggested that sustained afferent input is not necessary for the development or maintenance of zebrin-positive and zebrin-negative Purkinje cells. Additionally, to determine if initial afferent contact during embryonic development is required to set up the pattern of zebrin expression Wassef et al. (1990) dissected cerebellar anlagen from embryos at embryonic day 12 (prior to any contact with afferents) and then transplanted the tissue ectopically into either the anterior chamber of the eye or the neocortex of adult hosts. They found that both zebrin<sup>+</sup> and zebrin<sup>-</sup> Purkinje cells were still seen in mature grafts. Taken together, the results of these studies clearly show that normal afferent input does not play a role in the determination of the zebrin phenotype in Purkinje cells.

To date, most research investigating the biochemical parasagittal organization in the cerebellum has used zebrin as a tool, and not specifically addressed the cellular function. The localization of the zebrin antigen can be taken as the archetype of the parasagittal pattern inherent in Purkinje cells, and we can utilize the zebrin expression pattern to represent regions of differential gene expression whose borders are consistent. The borders thus defined have functional significance to the cerebellum because they are respected by a number of factors: the response properties of Purkinje cells to physiological stimuli, Purkinje cell

climbing fibre afferents and, to a lesser extent, by the incoming granule cell afferents (for review see Herrup and Kuemerle, 1997). However, it is important to remember that the zebrin borders are not caused by the afferents, or vice versa. Therefore, the issue remains: if the intrinsic biochemical properties and the afferent terminal organizations are not causally related during development, or necessary for the maintenance of the pattern in adulthood – what is the functional relationship between these patterns, and why are they correlated?

### **6.3 Conclusions**

The visual projections of the AOS and associated pretectum are highly organized and the functional response properties of this pathway are highly conserved; this is evidenced by the similarity in the response properties and parasagittal zonal organization of the rotation-sensitive neurons in the inferior olive and flocculus between mammals and birds (Voogd and Wylie, 2004). Despite being only one synapse from the retina, neurons in the AOS and pretectum have complex responses to large moving visual stimuli (Winterson and Brauth, 1985; Soodak and Simpson, 1988). Only two synapses from the retina, binocular panoramic receptive fields are constructed in the inferior olive and VbC, where neurons respond to precise patterns of optic flow from self-motion, and share a common reference frame with the eye muscles and semi-circular canals of the vestibular system (Wylie and Frost, 1993; 1996; Wylie et al., 1998; Frost and Wylie, 2000). While the AOS and associated pretectal structures play an

essential role in compensatory responses to the visual consequences of self-motion, they are not restricted to this role and research into the various structures along this pathways have implicated these projections in the estimation of direction and speed of self-motion, spatial cognition, postural control, and visual obstacle avoidance (for review see Gamlin, 2006; Giolli et al., 2006). By examining the specific organizational principles and functional properties of this visual pathway, including the cerebellar cortex, a better understanding of how all visual systems cope with the challenges of representing the visual world may be attained.

In fact, the cerebellar cortex is increasingly being recognized as a powerful model system in which to study the organizational principles of the nervous system in general. Traditionally, model systems for parallel processing and modular organization have focused on the cerebral cortex and cortical pathways, especially the geniculostriate visual pathway (e.g. Hubel and Wiesel, 1968; Goodale, 1996; Milner and Goodale, 2008). However, the more researchers learn about the cerebellum, and the various sensory and motor systems functioning within this brain region, the more we realize the significant advantages this model has. For instance, the cerebellar cortex exhibits a uniform cytoarchitecture throughout the entire cerebellum (see section 1.2.1); this is not necessarily seen in various regions of the cerebral cortex (i.e. there are significant cytoarchitectural differences between the primary motor and the primary visual cortices in most species). Because of this uniformity, principles established in one region of the

cerebellar cortex can generally apply to other regions and are highly reproducible throughout the entire structure. Additionally, research to date suggests that the modular organization of the cerebellar cortex is affected by environmental factors to a lesser degree than cortical brain regions (e.g. the modular organization of the ocular dominance columns in the primary visual cortex, which can be altered substantially with development and experience; e.g. Hubel et al., 1976; 1977; LeVay et al., 1980). Factors such as these make the cerebellum a scientifically desirable model to establish and evaluate general principles of brain function and organization. Indeed, although for the last hundred years the cerebellar cortex may have appeared monotonous because of its nearly uniform cytoarchitecture, many functional and anatomical studies have already revealed an interesting functional architecture of modular, parasagittal organization hidden within its circuits (see section 1.2.2).

While these functional and anatomical studies have made great strides in unravelling the intricacies of cerebellar organization, by themselves, they leave open the question of where the information is stored that establishes these organizational patterns. In other words, the etiology of this afferent modular organization may arise from properties directly attributable to the intrinsic characteristics of the cells of the cerebellar cortex themselves, or the source of this organization could reflect patterns that are present in the afferent populations (i.e. the cerebellar cortex might be a passive recipient of these ordered projections). It is here that the discovery and investigation of molecular markers, and the

correlation with anatomical and functional organizational patterns, has proven, and will continue to prove, invaluable in our understanding of the functional of the cerebellar cortex (see section 1.2.2). Through the study of these markers we now know that the cells of the cerebellar cortex themselves (i.e. the Purkinje cells) participate in a pattern of parasagittally organized regions (for review see Hawkes and Gravel, 1991). Interestingly, many of these molecular markers share the same expression pattern in subsets of Purkinje cells and these patterns are seen in many different species (for review see: Hawkes and Eisenman, 1997; Armstrong and Hawkes, 2000; Sillitoe et al., 2005). So while we have made many significant discoveries in the past 20 years regarding the molecular, anatomical and functional principles governing the organization of the cerebellar cortex, there are still many questions left to be addressed; in fact, we have hardly scratched the surface in uncovering these organizational principles in the cerebellum.

## 6.4 References

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