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BRIEF COMMUNICATION

Expression of calcium-binding proteins in cerebellar- and inferior olivary-projecting neurons in the nucleus lentiformis mesencephali of pigeons

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Abstract

In the avian brain, the optokinetic response is controlled by two retinal-recipient nuclei: the nucleus of the basal optic root (nBOR) of the accessory optic system and the pretectal nucleus lentiformis mesencephali (LM). Although considered sister nuclei because of their similar response properties and function, there are both similarities and differences with respect to efferent projections and neurochemistry. Both nBOR and LM project to the cerebellum (Cb) directly as mossy fibers but also indirectly *via* the inferior olive (IO). In a previous report, we showed that the cerebellar- and inferior olivary-projecting neurons in nBOR of pigeons differentially express the calcium-binding proteins calretinin (CR) and parvalbumin (PV). Both CR and PV are expressed in the somata of LM neurons, although the latter is not as prevalent, and whether expression of CR and PV reflects cerebellar and IO projections is not known. In this report, by combining retrograde neuronal tracing from the Cb and IO with fluorescent immunohistochemistry, we examined the expression of these calcium-binding proteins in the pigeon LM. Half (52%) of the cerebellar-projecting neurons were CR+ve, but only 15% were PV+ve. Almost all (>95%) these PV+ve cells also expressed CR. In contrast, few of the IO-projecting neurons expressed CR or PV (≤5%). This is strikingly similar to what we observed in nBOR and reveals that calcium-binding protein expression is concordant with projection patterns in two nuclei that share similar functions.

Keywords: Calretinin, Parvalbumin, Pretectum, Cerebellum, Inferior olive, Optokinetic

Introduction

When an animal is presented with movement of the entire visual world (i.e., optic flow), an optomotor response is initiated in the direction of the flowfield, thereby minimizing any motion on the retina. This is known as the optokinetic response (OKR) and is one of several behaviors that help to stabilize the retinal image (Waespe & Henn, 1987). Without retinal image stabilization, both visual acuity and relative velocity discrimination suffer (Westheimer & McKee, 1975; Nakayama, 1985).

In all vertebrate classes, retinal-recipient nuclei in the pretectum and accessory optic system (AOS) are involved in the analysis of optic flow and the generation of OKR. In birds, these structures are the nucleus lentiformis mesencephali (LM) of the pretectum and the nucleus of the basal optic root (nBOR) of the AOS. The mammalian homologs of the LM and nBOR are, respectively, the nucleus of the optic tract (NOT) of the pretectum and the medial terminal nucleus (MTN) and lateral terminal nucleus of the AOS (Simpson, 1984; Fite, 1985; McKenna & Wallman, 1985*a*; Weber, 1985; Simpson et al., 1988; Gamlin, 2006; Giolli et al., 2006). In several reports, the LM and nBOR have been erroneously grouped together as the AOS. Despite their similarity in function, Giolli et al. (2006) have emphasized that the optokinetic nuclei in the pretectum and AOS differ with respect to their connectivity and neurochemistry.

The nBOR and LM have similar projection pathways to the cerebellum (Cb). From both nuclei, there are direct mossy fiber projections to the posterior Cb (folia VI–IXcd) as well as indirect projections to the vestibulocerebellum (folia IXcd and X) *via* the medial column of the inferior olive (IO). Thus, there is a convergence of information from these two pathways in folium IXcd (Clarke, 1977; Brecha et al., 1980; Gamlin & Cohen, 1988*b*; Arends & Voogd, 1989; Lau et al., 1998; Wylie et al., 1999; Crowder et al., 2000; Wylie, 2001; Pakan & Wylie, 2006). As shown in Fig. 1A, the IO-projecting LM cells are found caudally in the LM, localized in a thin strip along the border of the medial and lateral subnuclei (LMm and LMl, respectively; Gamlin & Cohen, 1988*b*; Wylie, 2001). These IO-projecting cells are fusiform

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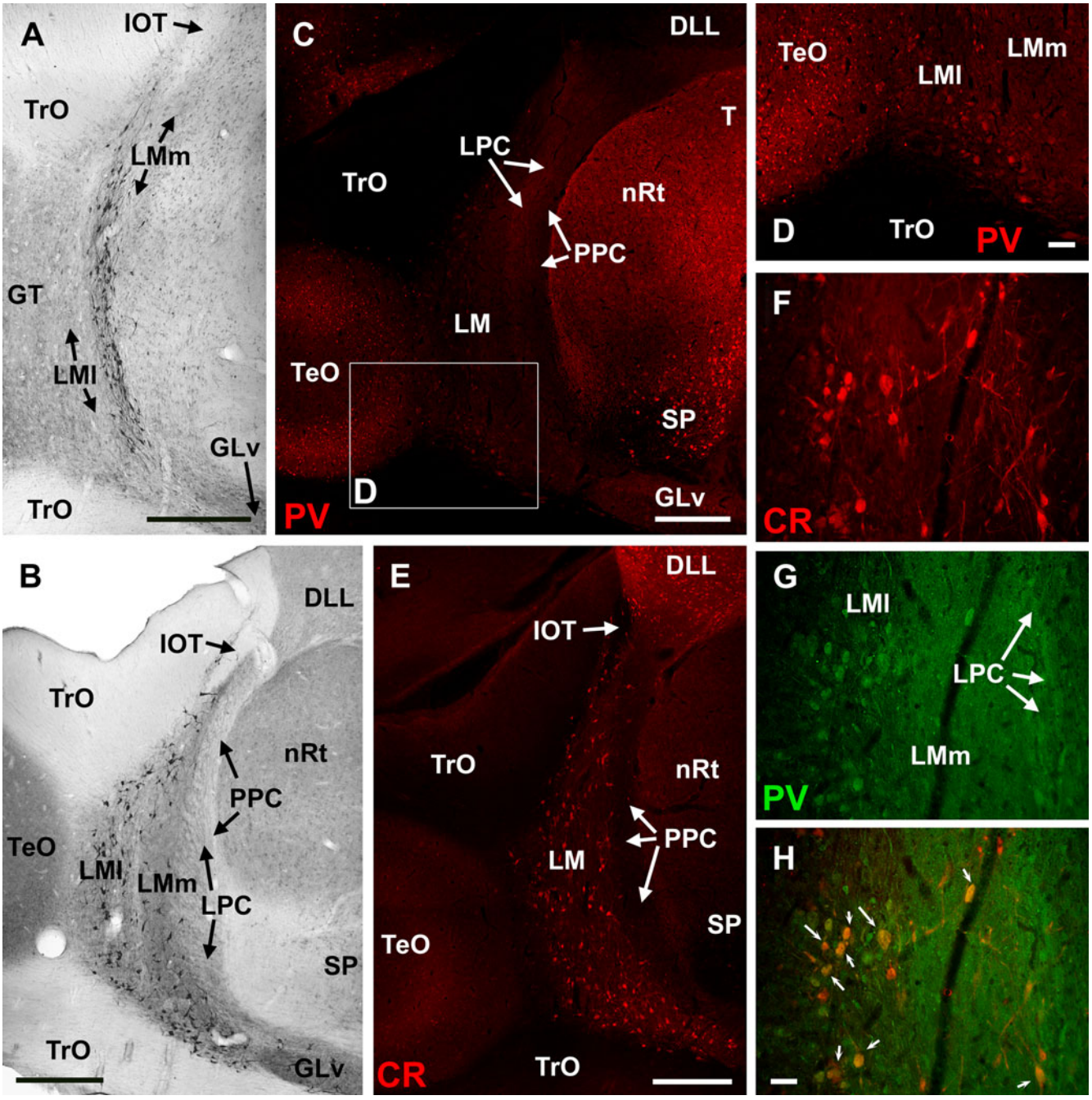

Fig. 1. Photomicrographs show cells in the retrogradely labeled cells in the nucleus LM after injections of cholera toxin subunit B in the IO (**A**) or vestibulocerebellum (VbC; **B**) (adapted from Pakan et al., 2006). The cells projecting to the VbC are large multipolar cells found throughout the medial and lateral subnuclei (LMm and LMl), whereas those projecting to the IO are localized to a strip located caudally, along the border of LMm and LMl. **C–H** show the expression of PV and CR in the LM and surrounding regions. PV was strongly expressed in nRt, triangularis (T), subpretectalis (SP), and the optic tectum (TeO) (**C**). Expression in the LM was not as intense; however, numerous PV-immunopositive (PV+ve) cell bodies were observed in LMl and the LPC (**D** and **G**). PV expression was apparent in the neuropil of LMm, but fewer neurons were PV+ve (**D** and **G**). CR was strongly expressed in the anterior dorsolateral thalamus, pars lateralis (DLL) and large multipolar cells throughout LM (**E** and **F**). **F–H** show a section reacted for both CR (**F**, red) and PV (**G**, green). The overlay (**H**) shows that many cells express both PV and CR, some of which are indicated by the white arrows. For all panels, left is lateral. GLv, lateral geniculate nucleus, pars ventralis; IOT, isthmo-optic nucleus; PPC, nucleus principalis precommissuralis; TrO, tractus opticus. Scale bars: 500 μm in **A–C** and **E**, 100 μm in **D**, and 50 μm in **H**.

neurons that are oriented dorsoventrally (Pakan et al., 2006). The cerebellar-projecting neurons are large (Fig. 2) multipolar neurons found throughout LMl and LMm (Fig. 1B; Gamlin & Cohen, 1988*b*; Pakan et al., 2006). Likewise, in nBOR, the cerebellar-projecting neurons are large multipolar cells, whereas the IO-projecting neurons tend to be small fusiform neurons localized to the dorsal nBOR (Brecha et al., 1980; Wylie, 2001; Wylie et al., 2007).

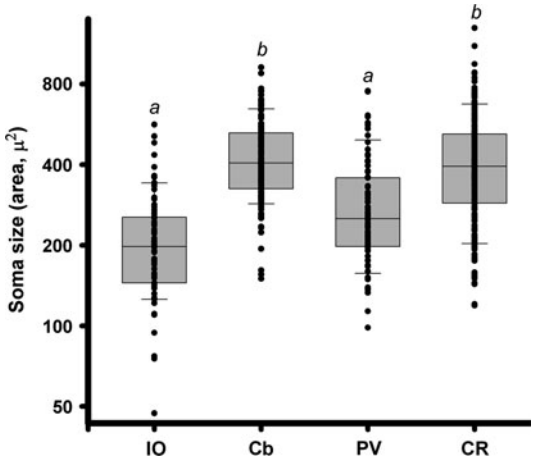

Fig. 2. A comparison of the sizes of neurons in nucleus LM. Box plots show the cross-sectional areas of PV- and CR-immunopositive neurons in LM, as well as LM neurons retrogradely labeled from injections into the vestibulocerebellum and IO. The measurements were subject to a log transformation to normalized the data sets. The boundaries of the box indicate the 25th and 75th percentiles, and the horizontal line marks the median. Bars mark the 90th and 10th percentiles. "*a*" indicates a significant difference from all other categories (Tukey Honestly Significant Difference (HSD)). "*b*" indicates significant difference from PV and IO groups (Tukey HSD).

In a recent report, we showed that a substantial proportion of the cerebellar-projecting nBOR neurons express the calcium-binding proteins parvalbumin (PV) and calretinin (CR), whereas very few of the IO-projecting neurons express either of these (Wylie et al., 2008). In this report, we investigate whether the equivalent pathways originating in LM also differ in their expression of calcium-binding proteins. Calcium-binding proteins are expressed in LM; CR immunoreactivity is intense in the perikarya and neuropil of LM, whereas PV immunoreactivity is moderate in the perikarya and neuropil (Pfeiffer & Britto, 1997; De Castro et al., 1998). In contrast, calbindin is not expressed in perikarya, but immunoreactivity is moderate in the neuropil (Pfeiffer & Britto, 1997). De Castro et al. (1998) reported that many of the cerebellar-projecting neurons in LM (which they refer to as the external pretectal nucleus) express CR, but other calcium-binding proteins and the IO-projecting neurons have not been investigated.

Materials and methods

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. We used the similar animals as those of our previous paper that examined the expression of calcium-binding proteins in nBOR neurons projecting to the IO and Cb (Wylie et al., 2008). The details of the methods can be found in this paper. Briefly, Silver King and homing pigeons were anesthetized with an intramuscular injection of ketamine (65 mg/kg)/xylazine (8 mg/kg), and animals were placed in a stereotaxic device with pigeon ear bars and beak adapter so that the orientation of the skull conformed to the atlas of Karten and Hodos (1967). Sufficient skull and dura were removed to expose the brain surface and allow access to either the IO or the Cb. For the IO injections, we targeted the medial column, which is the region that responds to optokinetic stimuli and receives input from LM and nBOR (Clarke, 1977; Gamlin & Cohen, 1988*b*; Wylie et al., 1997; Winship & Wylie, 2001, 2003; Wylie, 2001). For the cerebellar injections, we targeted folium IXcd of the vestibulocerebellum. Previous studies using anterograde techniques have shown that the LM projection to the Cb is a bilateral mossy fiber projection, which terminates in the granule layer as mossy fiber rosettes. This projection is primarily directed to IXcd but also includes folia VI–IXab (Clarke, 1977; Gamlin & Cohen, 1988*b*). The projection to IXcd originates mainly in LMl, whereas the projection to folia VI–IXab is mainly from LMm (Pakan & Wylie, 2006). All target sites were localized using stereotaxic coordinates (Karten & Hodos, 1967), and single-unit recordings made with glass micropipettes (tip diameters 4–5 μm) confirmed that neurons were responsive to optokinetic stimuli. Once responsive neurons were located, we replaced the recording electrode with a micropipette (tip diameter 20 μm) containing a fluorescent retrograde tracer (red Lumafluor, fluorescent latex microspheres; LumaFluor Corporation, Naples, FL), 1% cholera toxin subunit B–AlexaFluor 594 conjugate (Molecular Probes, Eugene, OR), or 10% biotinylated dextran amine (BDA) (mini-ruby D-3312; Invitrogen, Carlsbad, CA). These were pressure injected using a Picospritzer II (General Valve Corporation, Fairfield, NJ). For the cerebellar injections, there is no issue of potential leakage into other structures that receive input from LM: As long as the injection is confined to the Cb, only the large multipolar LM neurons will be labeled (Gamlin & Cohen, 1988*b*). These neurons project only to the Cb (Pakan et al., 2006). The LM does not project to any other brain stem structure in the vicinity of, or caudal to, the IO. Thus, for the injections targeting IO, we are confident that the retrogradely labeled cells would only represent those projecting to the IO.

Postsurgically, the birds were given an intramuscular injection of buprenorphine (0.012 mg/kg) as an analgesic. After a recovery period of 2–5 days, the animals were deeply anesthetized with sodium pentobarbital (100 mg/kg) and immediately perfused with heparinized phosphate-buffered saline (0.9% NaCl, 1 ml/100 ml heparin, and 0.1 M phosphate buffer). As outlined in Wylie et al. (2008), serial sections in the coronal plane (40 μm thick) were collected throughout the rostrocaudal extent of the Cb, pretectum, and IO and were immunohistochemically stained for PV and CR. Sections were viewed with a fluorescence microscope (Leica DMRE, Richmond Hill, ON, Canada), and images were acquired using a Retiga EXi *FAST* Cooled mono 12-bit camera (Qimaging, Burnaby, BC, Canada) and analyzed with Openlab imaging software (Improvision, Lexington, MA).

Nomenclature of LM

For the nomenclature of LM, we relied on Gamlin and Cohen (1988*a*,*b*) who divided the LM into medial and lateral subdivisions (LMm and LMl), both of which contain large and small cells (Gottlieb & McKenna, 1986; Zayats et al., 2003). Continuous with the LMl at its lateral and caudal aspects is the tectal gray (GT), which contains mainly small cells. The LMm, LMl, and the rostral part of GT all receive retinal input (Gamlin & Cohen, 1988*a*). Previously, the LMm was known as the LM magnocellularis, and the LMl and GT were included as the LM parvocellularis (e.g., Karten & Hodos, 1967). The LMm is bordered medially by the nucleus laminaris precommissuralis (LPC), a thin strip of cells that does not receive input from the retina. Medial to LPC is the nucleus principalis precommissuralis, which is lateral to the nucleus rotundus (nRt). In Nissl-stained sections, the layers of the pretectum are relatively easy to distinguish, although the border between GT and LMl can be difficult to localize.

Results

The results presented are based on experiments performed on 12 pigeons. Five pigeons received injection of retrograde tracer in the Cb and five received injection in the medial column of the IO. The remaining two did not receive injections, but sections through the pretectum were processed for PV and CR immunoreactivity to examine their respective distributions. Examples of injection sites in the IO and Cb can be found in Wylie et al. (2008).

Fig. 1C shows a section through the LM immunoreacted for PV. Consistent with previous reports (Pfeiffer & Britto, 1997; Heyers et al., 2008), PV was strongly expressed in neurons in nRt, triangularis (T), subpretectalis, and the optic tectum. Expression in the LM was not as intense, but numerous PV+ve cell bodies were present in LMl, as shown in the inset (Fig. 1D). PV expression was apparent in the neuropil of LMm, but fewer neurons were PV+ve (Fig. 1G).

Fig. 1E shows a section through the LM immunoreacted for CR. Consistent with previous studies (Pfeiffer & Britto, 1997; Heyers et al., 2008), CR was strongly expressed in the anterior dorsolateral thalamus, pars lateralis. In LM, large multipolar cells throughout LM were strongly CR+ve (Fig. 1E and 1F). These cells clearly resembled those labeled from injections of retrograde tracer in the Cb, with respect to size, morphology, and distribution (Fig. 1B). Fig. 1F–1H shows a section reacted for both CR (panel F, red) and PV (panel G, green). The overlay (panel H) shows that many cells express both PV and CR and are therefore double labeled (white arrows). Overall, CR+ve cells were more abundant in LM compared to PV+ve cells. In a survey through the rostrocaudal extent of LM in two cases, we counted 1291 CR+ve and 558 PV+ve LM neurons, 173 of which were double labeled. That is, 13.4% of CR+ve neurons were also PV+ve and 31.0% of PV+ve neurons were also CR+ve.

Fig. 2 shows a plot of the sizes of CR+ve and PV+ve LM neurons, as well as the sizes of LM neurons that project to the Cb and IO and other structures. The cells that project to the Cb are larger than those that project to the IO (mean cross-sectional area, Cb = 416 μ^2 and IO = 216 μ^2), although there is overlap in their distributions. The CR+ve cells were large (422 μ^2), with a distribution very similar to that of the cerebellar-projecting LM cells. The PV+ve neurons were slightly smaller (291 μ^2), with the distribution overlapping both the cerebellar- and the IO-projecting LM neurons. An analysis of variance of cell size revealed a significant difference among the four categories in Fig. 2 ($F = 689.87$, $df = 4, 868$, $P < 0.01$). The CR+ve and cerebellar-projecting cells were not significantly different from each other but were significantly larger than all other groups (*post hoc* Tukey Honestly Significant Difference (HSD), $P < 0.05$). The PV+ve neurons were significantly different from all other groups.

Fig. 3 shows several examples of retrogradely labeled LM neurons, from injections of red LumaFluor in the Cb, in sections immunoreacted for CR (panels A–C and D–F), PV (panels G–I), and both PV and CR (panels J–M and N–Q). Many of the cerebellar-projecting neurons were CR+ve (Fig. 3C, 3F, 3M, and 3Q). Across all five cases, of 588 retrogradely labeled cells, 306 (52%) were CR+ve. In contrast, far fewer cells were PV+ve (Fig. 3I, 3M, and 3Q). Across all five cases, of 897 retrogradely labeled cells, 135 (15%) were PV+ve. In sections that were immunoreacted for both PV and CR, it was clear that almost all those retrogradely labeled cells that were PV+ve were also CR+ve (Fig. 3M and 3Q). From these sections, of 49 retrogradely labeled cells that were PV+ve, 48 were also CR+ve.

Fig. 4 shows examples of retrogradely labeled LM neurons from injections of BDA (red) in the IO in sections immunoreacted for PV (panels A–C) and CR (panels D–F). Although the retrogradely labeled cells were often surrounded by CR+ve and PV+ve neurons, very few of the IO-projecting neurons were immunopositive. Only 4.5% of the IO-projecting neurons were CR+ve (9 of 202 retrogradely labeled cells) and 5.8% were PV+ve (12 of 207 retrogradely labeled cells). In sections that were reacted for both PV and CR, none of the retrogradely labeled cells were positive for both CR and PV.

Discussion

The restricted expression of the calcium-binding proteins in neuronal populations in the central nervous system has been described in several species (e.g., Celio, 1990; Van Brederode et al., 1990; Resibois & Rogers, 1992; Pfeiffer & Britto, 1997; Pritz & Siadati, 1999). Calcium-binding proteins have numerous cellular functions (e.g., Kohr et al., 1991; Yamaguchi et al., 1991; Baimbridge et al., 1992; Schwaller et al., 2002; Schwaller, 2007, 2009), although their role from a systems perspective is not well understood. Nonetheless, an analysis of the differential expression of calcium-binding proteins has been very useful in elucidating neural circuitry, particularly in sensory systems (Blümcke et al., 1990; Van Brederode et al., 1990; Wild et al., 2005). For example, Heyers et al. (2008) examined the expression of calcium-binding proteins in the tectofugal and thalamofugal visual pathways of the zebra finch (*Taeniopygia guttata*). In both the diencephalon and the telencephalon, PV was primarily expressed in structures of the thalamofugal pathway, whereas calbindin was expressed in the tectofugal pathway.

The extent to which this differential expression applied to other nuclei of the visual system is not, however, well understood. The present study and our study of the nBOR (Wylie et al., 2008) show that calcium-binding proteins are differentially expressed *within* a visual nucleus. For both nBOR and LM, few of the IO-projecting neurons expressed either CR or PV (nBOR <2%; LM <5%). In contrast, almost half of the cerebellar-projecting neurons in LM and nBOR express CR (52% for both LM and nBOR), and a smaller proportion express PV (15% in LM; 34% in nBOR), although most of these PV+ve neurons also expressed CR (90% in nBOR; 95% in LM). Why calcium-binding proteins are expressed differently in LM and nBOR is unknown.

As LM and nBOR neurons detect optic flow that results from self-motion (e.g., Burns & Wallman, 1981; Morgan & Frost, 1981; Winterson & Brauth, 1985; Wylie & Frost, 1990), one could imagine that they are active almost continuously and require substantial calcium buffering to prevent excitotoxicity. A recent report has suggested that CR, but not PV, protects neurons against glutamate excitotoxicity (D'Orlando et al., 2002). As retinal ganglion cell axons are glutamatergic (Cardozo et al., 1991), perhaps, the large multipolar neurons in LM and nBOR that project directly to the Cb receive direct retinal input, whereas the IO-projecting neurons do not receive glutamatergic input. If this is the case, enhanced CR expression might help prevent over-excitation of the cerebellar-projecting pathway. Related, $CR^{-/-}$ mice show an increased excitability in cerebellar granular cells (Gall et al., 2003).

On classifying the LM as an accessory optic nucleus

The nBOR is a nucleus of the AOS and, strictly speaking, homologous to the mammalian MTN (Simpson, 1984). The LM,

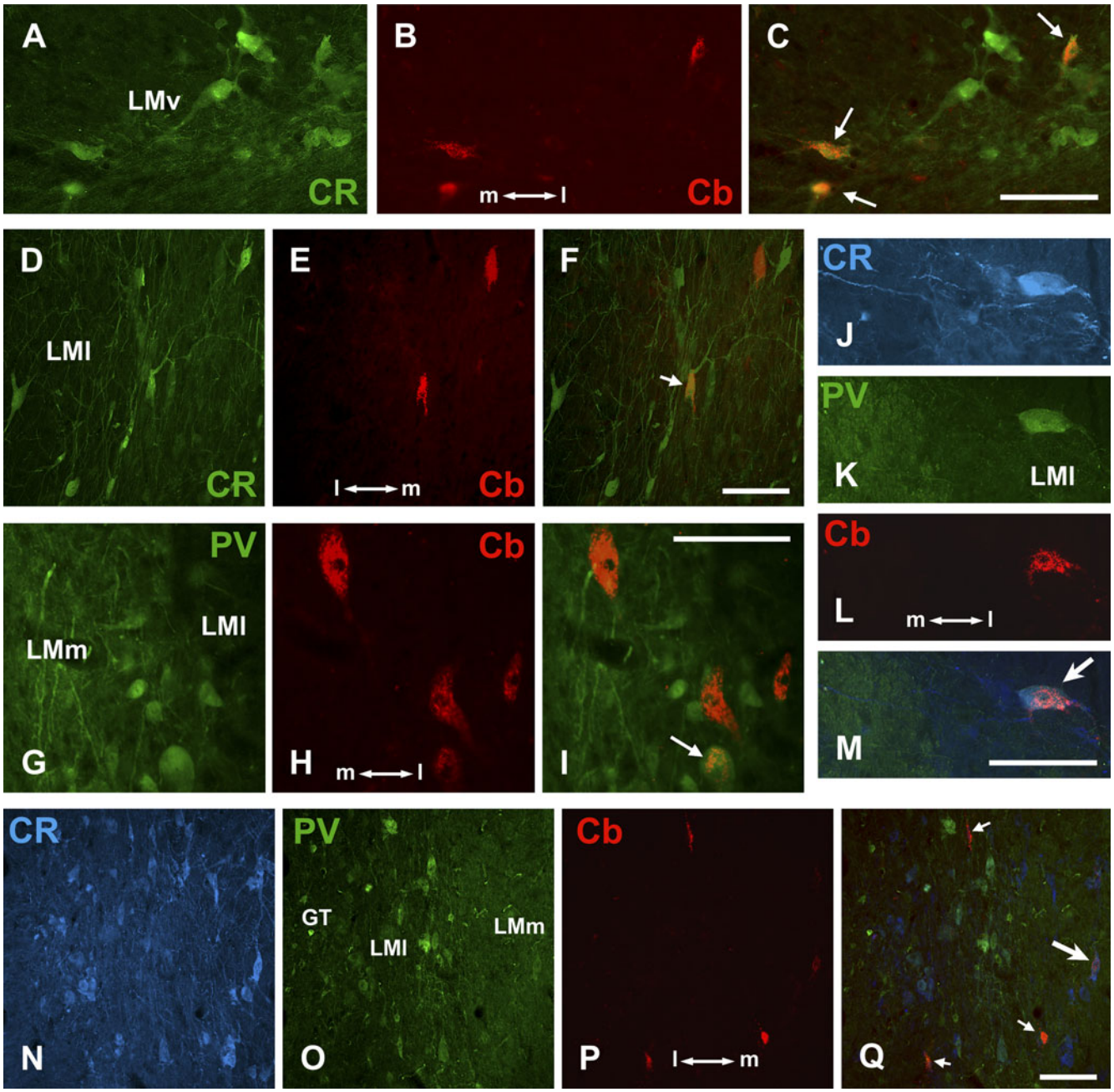

Fig. 3. Expression of calcium-binding proteins in cerebellar-projecting neurons in the nucleus LM. Cells were retrogradely labeled with red LumaFluor from injections into the vestibulocerebellum (**B**, **E**, **L,** and **P**), and the sections were reacted for CR (**A** and **D**), PV (**G**), or both PV and CR (**J–K** and **N–O**). The overlays are also shown (**C, F, I, M,** and **Q**). The arrows indicate retrogradely labeled cells that were also immunopositive for CR and/or PV. In **M** and **Q**, the larger stylized arrow indicates a retrogradely labeled cell that was PV+ve and CR+ve. In **Q**, the small arrows indicate three retrogradely labeled cells that were CR+ve but not PV+ve. All scale bars = 100 μm.

homologous to the mammalian NOT, resides within the pretectum (Simpson et al., 1988) but is often considered, erroneously, part of the AOS (Giolli et al., 2006). This is due to the fact that their response properties are quite similar: Neurons in LM and nBOR (and their mammalian homologs) show direction selectivity in response to large-field visual stimuli and are critical for mediating the OKR (e.g., Fite et al., 1979; Burns & Wallman, 1981; Morgan & Frost, 1981; Gioanni et al., 1983*a,b*; Simpson, 1984; McKenna & Wallman, 1985*b*; Winterson & Brauth, 1985; Simpson et al., 1988). Giolli et al. (2006) have noted that connections and neurochemistry of the pretectum and AOS are quite similar in mammals, although there are a few differences. The same can be said of the nBOR and LM. The LM and nBOR have reciprocal projections, and both project to the Cb, IO, pontine nuclei, ventral tegmental area, and dorsal thalamus (Clarke, 1977; Brecha et al., 1980; Gamlin & Cohen, 1988*b*; Wylie et al., 1997, 1998, 1999). Both nBOR and LM receive input from the visual Wulst, although from different regions (Wylie et al., 2005). The nBOR, unlike the LM, projects heavily to the interstitial nucleus of Cajal and other accessory oculomotor nuclei and the oculomotor complex (Brecha et al., 1980). The present

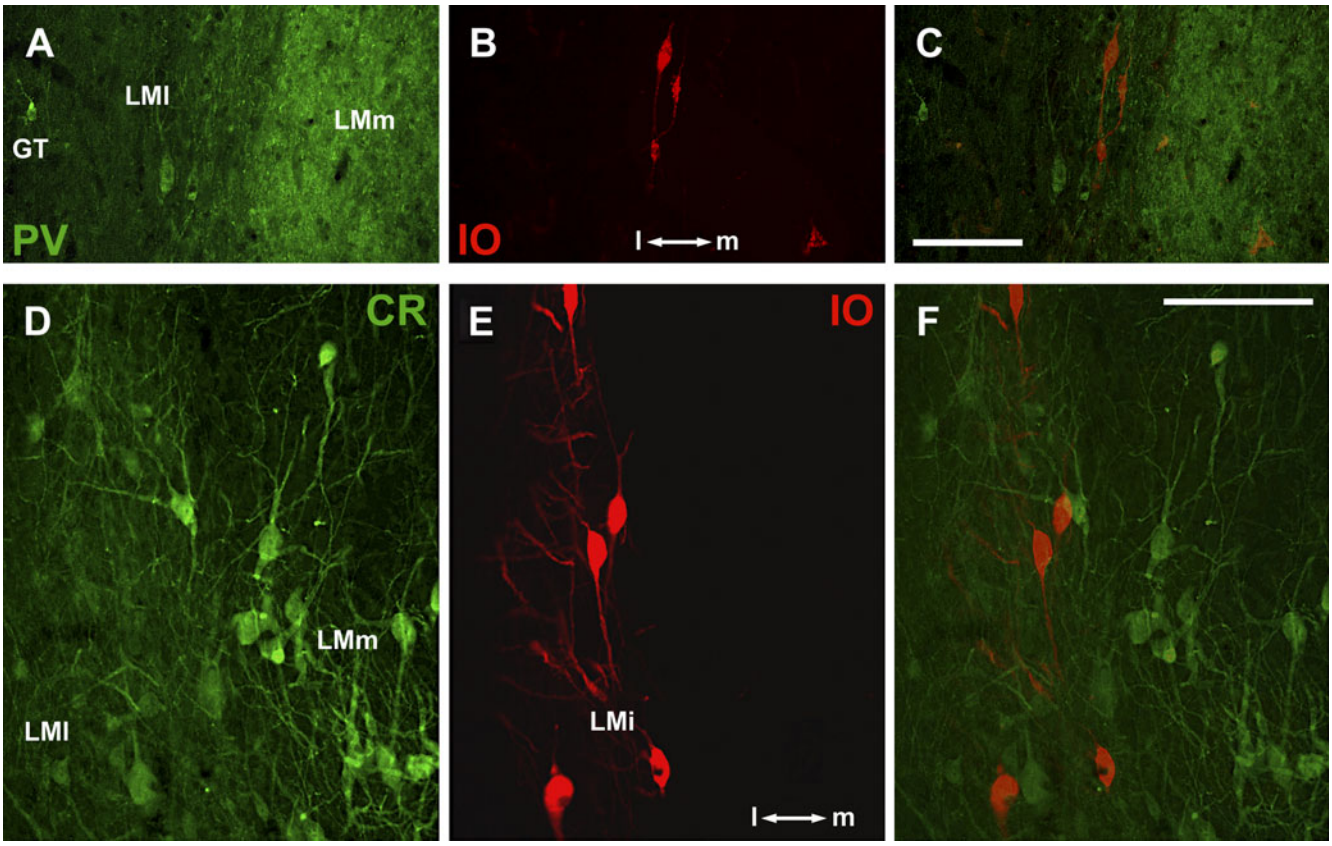

Fig. 4. Expression of calcium-binding proteins in IO-projecting neurons in the nucleus LM. Cells were retrogradely labeled with red BDA from injections into the IO (**B** and **E**), and the sections were reacted for either CR (**D**) or PV (**A**). The overlays are also shown (**C** and **F**). None of the retrogradely labeled cells was immunopositive for the calcium-binding proteins. All scale bars = 100 μm.

study speaks to more similarities with respect to the connectivity and neurochemistry of the pretectum and AOS in birds. For both LM and nBOR, the projection to the IO is from neurons that generally do not express CR or PV and the projection to the Cb is from large multipolar neurons, many of which express CR (Pakan et al., 2006; Wylie et al., 2007, 2008). However, the projection from the LM to the Cb targets folia VI–IXcd, whereas that from the nBOR is almost exclusive to IXcd (Clarke, 1977; Brecha et al., 1980; Gamlin & Cohen, 1988*b*; Pakan & Wylie, 2006).

Acknowledgments

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