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BRIEF COMMUNICATION

Expression of calcium-binding proteins in pathways from the nucleus of the basal optic root to the cerebellum in pigeons

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Abstract

Calcium-binding protein expression has proven useful in delineating neural pathways. For example, in birds, calbindin is strongly expressed in the tectofugal pathway, whereas parvalbumin (PV) is strongly expressed in the thalamofugal pathway. Whether neurons within other visual regions also differentially express calcium-binding proteins, however, has not been extensively studied. The nucleus of the basal optic root (nBOR) is a retinal-recipient nucleus that is critical for the generation of the optokinetic response. The nBOR projects to the cerebellum both directly and indirectly *via* the inferior olive (IO). The cerebellar and IO projections originate from different neurons within the nBOR, but whether they can also be differentiated based on calcium-binding protein expression is unknown. In this study, we combined retrograde neuronal tracing from the cerebellum and IO with fluorescent immunohistochemistry for PV and calretinin (CR) in the nBOR of pigeons. We found that about half (52.3%) of the cerebellar-projecting neurons were CR+ve, and about one-third (33.6%) were PV+ve. Most (90%) of these PV+ve cells were also labeled for CR. In contrast, very few of the IO-projecting neurons expressed CR or PV ($\leq$2%). Thus, the direct nBOR–cerebellar and indirect nBOR–olivocerebellar pathways to the cerebellum can be distinguished based on the differential expression of CR and PV.

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

Introduction

Although calcium-binding proteins have numerous cellular functions (Kohr et al., 1991; Yamaguchi et al., 1991; Baimbridge et al., 1992; Schwaller et al., 2002), their role from a system's perspective is not well understood. Across several species, the restricted expression of the calcium-binding proteins calretinin (CR), parvalbumin (PV), and calbindin (CB) has been described in distinct subpopulations of the central nervous system (e.g., Van Brederode et al., 1990; Resibois & Rogers, 1992; Pfeiffer & Britto, 1997; Pritz & Siadati, 1999). This differential expression of calcium-binding proteins has been proven useful in distinguishing individual neural pathways, particularly in sensory systems (Blümcke et al., 1990; Celio, 1990; Wild et al., 2005). In the avian visual system, for example, Heyers et al. (2008) examined the expression of calcium-binding proteins in the tectofugal and thalamofugal visual pathways of the zebra finch. PV was primarily expressed in structures of the thalamofugal pathway, whereas CB was expressed in the tectofugal pathway. Whether or not this differential expression applies to other nuclei of the visual system has not been extensively studied.

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The nucleus of the basal optic root (nBOR) of the accessory optic system is the homologue of the mammalian medial and lateral terminal nuclei (MTN/LTN; Simpson, 1984; Giolli et al., 2006). The nBOR resides at the base of the brain at the mesodiencephalic border (Fig. 1A) and receives direct retinal input from displaced ganglion cells (Karten et al., 1977; Reiner et al., 1979; Fite et al., 1981). The nBOR is involved in the analysis of optic flow that results from self-motion, and it is critical for the generation of the optokinetic response (Burns & Wallman, 1981; Morgan & Frost, 1981; Gioanni et al., 1983, 1984; Wylie & Frost, 1999*b*). Information from the nBOR reaches the vestibulocerebellum (VbC, folia IXcd and X) *via* an indirect and a direct pathway. Indirectly, the nBOR projects to the medial column of the inferior olive (IO), which provides a climbing fiber input to the VbC (Brecha et al., 1980; Arends & Voogd, 1989; Wylie et al., 1997, 1999*b*; Lau et al., 1998; Crowder et al., 2000; Wylie, 2001). The nBOR also projects directly to the VbC as mossy fibers, although this projection is restricted to IXcd (Brecha et al., 1980; Wylie & Linkenhoker, 1996; Wylie et al., 1997). Using double-retrograde labeling with fluorescent tracers, Wylie et al. (2007) emphasized that the IO- and VbC-projecting nBOR neurons differ with respect to size, morphology, and distribution in the nBOR. As shown in Fig. 1B, the VbC-projecting neurons are large (see also Fig. 2) multipolar neurons found throughout the

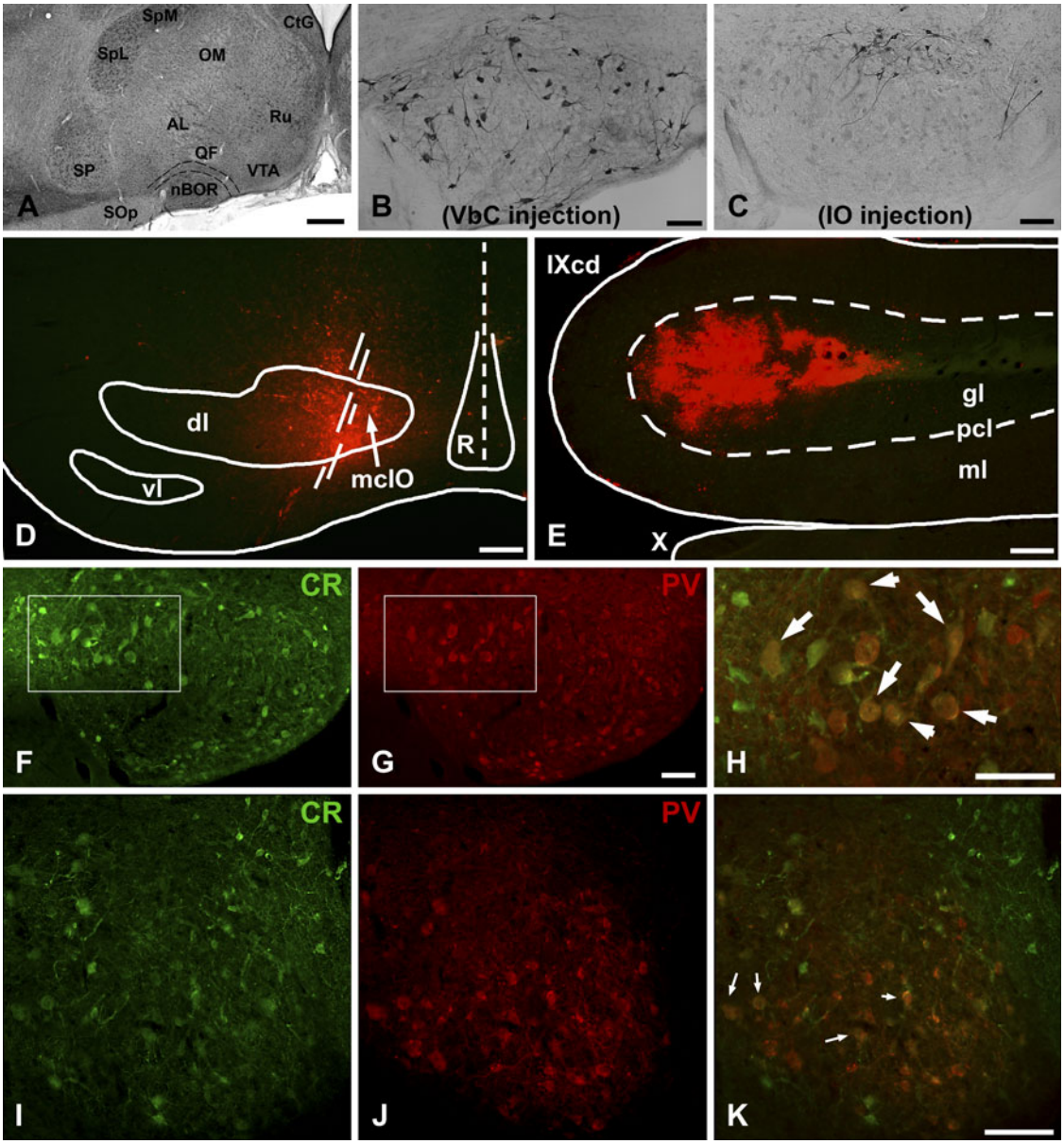

Fig. 1. A shows a coronal section through the pigeon brain at the mesodiencephalic border to illustrate the location of the nucleus of the basal optic root (nBOR) at the base of the brain. Photomicrographs show retrogradely labeled cells in the nBOR after injections of cholera toxin subunit B in the vestibulocerebellum (VbC; **B**) or inferior olive (IO; **C**) (adapted from Wylie et al., 2007). The cells projecting to the VbC are large multipolar cells found throughout the nBOR, whereas those projecting to the inferior olive (IO) are much smaller and localized to the dorsal nBOR. **D** and **E**, respectively, show typical injections of fluorescent retrograde tracer in the IO (biotinylated dextran amine) and folium IXcd of the VbC (LumaFluor). The dashed line in **D** represents midline. The dashed line in **E** represents the Purkinje cell layer (pcl). **F–K** show two sections through the nBOR immunoreacted for parvalbumin (PV; **G, J**, red) and calretinin (CR; **F, I**, green). Numerous cell bodies that are PV+ve and CR+ve can be seen. The overlays are shown in **H** and **K** on the right. For **H**, a restricted region is shown at higher magnification, which corresponds to the rectangular outline in **F** and **G**. The arrows in **H** and **K** indicate some of the cells that coexpress PV and CR. For all panels, left is lateral. AL, ansa lenticularis; CtG, central gray; OM, occipitomesencephalic tract; QF, tractus quintofrontalis; Ru, nucleus ruber; SOp, stratum opticum; SP, nucleus subpretectalis; SpL/SpM, lateral/medial spiriform nucleus; VTA, ventral tegmental area; dl, vl, dorsal and ventral lamella of the IO, respectively; mcIO, medial column of the IO; R, raphe; ml, molecular layer; gl, granule cell layer. Scale bars: 500 μm in **A**; 100 μm in **B, C, F–I**; 200 μm in **D** and **E**.

nBOR (Brecha et al., 1980; Wylie et al., 2007). In contrast, the IO-projecting cells are much smaller in size, fusiform in shape, and localized to the dorsal margin of the nBOR and the adjacent ventral tegmental area (Fig. 1C, 3; Brecha et al., 1980; Wylie, 2001; Wylie et al., 2007).

In the present study, we investigated if these projection neurons could also be distinguished with respect to the expression of calcium-binding proteins, by combining fluorescent retrograde tracing with immunochemistry in the pigeon (*Columba livia*). Calcium-binding proteins are expressed in the nBOR; perikarya

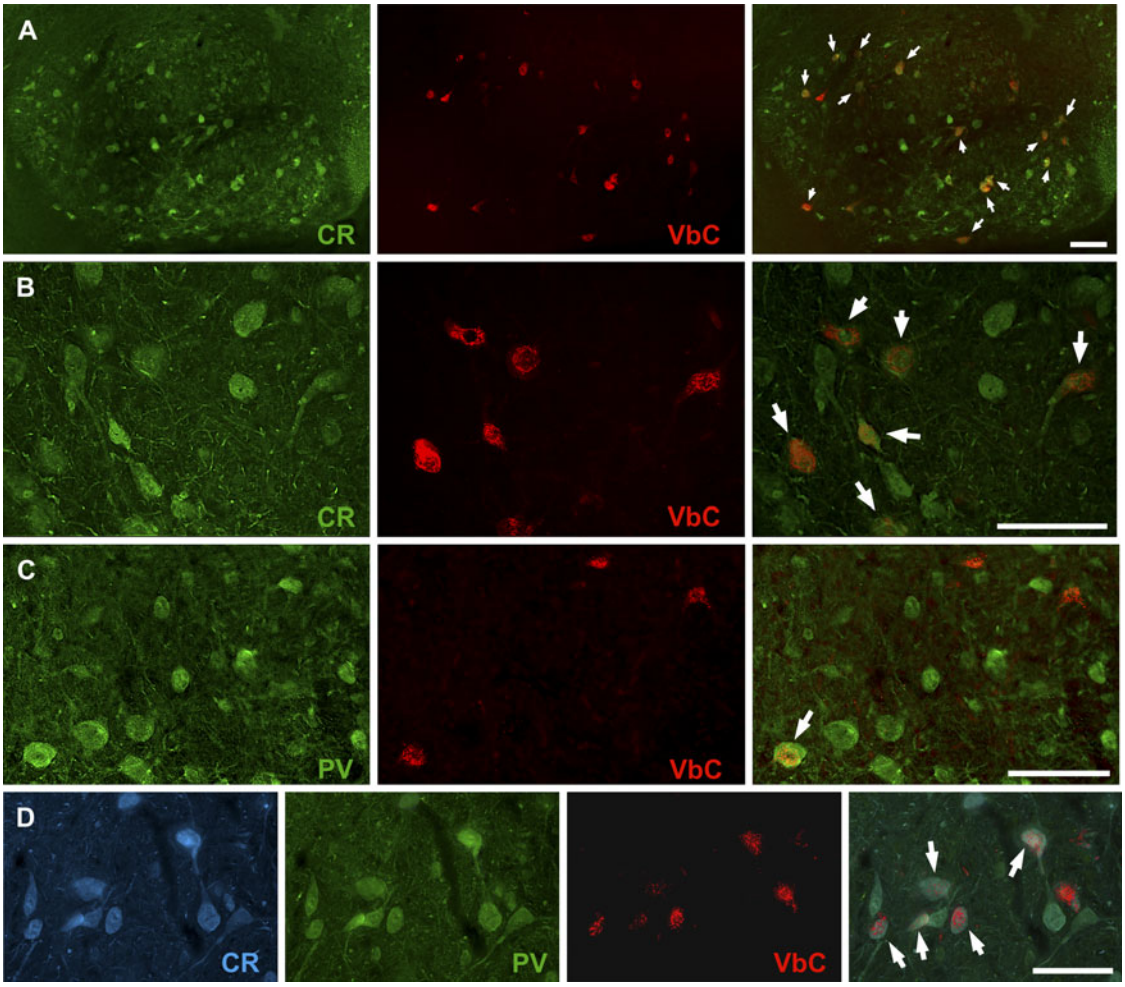

Fig. 2. Expression of calcium-binding proteins in vestibulocerebellar (VbC)-projecting neurons in the nucleus of the basal optic root (nBOR). The nBOR cells were retrogradely labeled with red LumaFluor from injections into the VbC, and the sections were immunoreacted for calretinin (CR; green in **A** and **B**), parvalbumin (PV; green in **C**), or both PV and CR (CR blue; PV green in **D**). The overlays are shown on the right. The arrows indicate retrogradely labeled cells that were also immunopositive for CR and/or PV. All scale bars = 100 μm.

within the nBOR and neuropil are immunoreactive for both CR and PV, but CB was not detected (Pfeiffer & Britto, 1997). De Castro et al. (1998) reported that about half of the VbC projection neurons in the nBOR express CR, but other calcium-binding proteins and the IO-projecting nBOR neurons have not been investigated. Here, we examine both direct and indirect projections from the nBOR to the VbC in relation to PV and CR expressions.

Materials and methods

The methods reported herein were approved by the Biosciences Animal Care and Policy Committee at the University of Alberta and conformed to the guidelines established by the Canadian Council on Animal Care. Silver King and homing pigeons, obtained from various suppliers, were anesthetized with an intramuscular injection of a ketamine (65 mg/kg)/xylazine (8 mg/kg) cocktail and were given supplemental doses as needed to maintain anesthesia. The 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 VbC. For the IO injections, we targeted the medial column, which is the region that receives input from the nBOR (Clarke, 1977; Gamlin & Cohen, 1988; Wylie et al., 1997; Wylie, 2001). For the VbC injections, we gained access to folium IXcd through the anterior vestibular canal. All target sites were localized using stereotaxic coordinates (Karten & Hodos, 1967) and single-unit recordings made with glass micropipettes (tip diameters 4–5 μm) filled with 2 M NaCl that were advanced using an hydraulic microdrive. The details of the stimuli and recording experiments can be found in previous papers (Wylie & Frost, 1990, 1993, 1999*b*; Wylie et al., 1993, 1998; Winship & Wylie, 2001, 2003; Winship et al., 2005). Briefly, neurons in the medial column of the IO and VbC respond to optic flow stimuli. Once responsive neurons were located, we replaced the recording electrode with a micropipette (tip diameter 20 μm) containing a fluorescent tracer. For retrograde tracers, we used 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, USA). Following injection, the electrode was undisturbed for 5 min. After surgery, the craniotomy was filled with bone wax, the wound was sutured, and 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 (PBS; 0.9% NaCl, 1 ml/100 ml heparin, 0.1 M phosphate buffer). The brains were extracted, embedded in gelatin, and cryoprotected in 30% sucrose. Using a microtome, frozen serial sections in the coronal plane (40 μm thick) were collected throughout the rostrocaudal extent of the VbC, nBOR, and IO.

Immunohistochemistry for PV and CR

Sections were rinsed thoroughly in 0.1 M PBS and blocked with 10% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA) and 0.4% TritonX-100 in PBS for 1 h. The sections were then incubated for 48 h at room temperature in PBS containing 0.1% TritonX-100 and primary antibodies to either PV (mouse anti-PV; P3088 Sigma, St. Louis, MI; 1:2000), CR, (rabbit anti-caltretinin; 7699/4 SWANT Bellinzona, Switzerland; 1:2000), or both PV and CR.

Tissue was then rinsed in PBS, and sections were incubated in Cy3, Cy2, or amino-methyl-coumarin-acetate conjugated donkey anti-mouse and/or anti-rabbit antibodies (as appropriate; Jackson Immunoresearch Laboratories: diluted 1:100 in PBS, 2.5% normal horse serum, and 0.4% TritonX-100) for 2 h at room temperature. The tissue was finally rinsed in PBS and mounted onto gelatinized slides for viewing.

Microscopy

Sections were viewed with a compound light microscope (Leica DMRE, Richmond Hill, ON, Canada) equipped with the appropriate fluorescence filters (rhodamine, fluorescein isothiocyanate, and 4-6-Diamidino-2-phenylindole). 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). Openlab was also used to measure soma size. Adobe Photoshop was used to adjust brightness and contrast.

Results

The results presented are based on experiments performed on 12 pigeons. Five pigeons received injection of retrograde tracer in the cerebellum 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.

Photomicrographs of typical injection sites in the VbC and IO are shown in Fig. 1D and E, respectively. For the IO injections, we targeted the medial column (mcIO), which, as shown from previous anterograde studies, is the region that receives bilateral input from the nBOR (Brecha et al., 1980; Wylie et al., 1997). Although the injections did spread outside the borders of the IO, they did not encroach upon other areas that receive input from the nBOR, such as the pontine or vestibular nuclei (Brecha et al., 1980; Wylie et al., 1997). For the VbC injections, we targeted the granular layer of folium IXcd. None of the injections spread outside the cerebellar cortex, and the involvement of other folia was minimal to none. Previous studies have shown that the vast majority of the mossy fibers from the nBOR terminate in IXcd but some terminate in folia VI-IXab. The nBOR does not project to folium X (Brecha et al., 1980; Wylie et al., 1997; Pakan & Wylie, 2006).

PV and CR immunoreactivity in the nBOR

The expression of both PV and CR was abundant in the nBOR, with neurons clearly labeled (Fig. 1F–K). Both the PV+ve and CR+ve neurons in the nBOR were large and resembled those labeled from injections in the VbC. The cross-sectional area of the PV+ve and CR+ve was 448.5 $\pm$ 36.0 μm^2 (mean $\pm$ S.E.M., $n = 49$) and 430.9 $\pm$ 25.6 μm^2 ($n = 99$), respectively. The nBOR cells retrogradely labeled from the VbC injections were of similar size, 455.3 $\pm$ 32.5 μm^2 ($n = 32$), whereas those labeled from the IO injections were much smaller, 203.3 $\pm$ 13.6 μm^2 ($n = 40$). In sections that were reacted for both PV and CR, it was clear that many neurons were immunopositive for both calcium-binding proteins. Fig. 1F–H and I–J show two coronal sections through the nBOR processed for both PV (red) and CR (green). The section shown in F–H is from the midpoint of the nBOR, with the overlay (H) highlighting a collection of large cells. Such large cells typically project to the VbC (Wylie et al., 2007). The section shown in I–K was from the rostral margin of the nBOR. The overlays (H and K) show that many cells were immunopositive for both PV and CR (white arrows). In a survey through the rostrocaudal extent of the nBOR in two cases, we counted 1337 CR+ve and 1315 PV+ve nBOR neurons, 394 of which were double-labeled. That is, about 30% of CR+ve were also PV+ve, and 30% of the PV+ve neurons were also CR+ve. The double-labeled cells were not localized to any particular part of the nBOR, although there was a paucity of them in the dorsal part of the nBOR.

VbC-projecting nBOR neurons

Fig. 2 shows several examples of retrogradely labeled nBOR neurons, from injections of red LumaFluor in the VbC, in sections immunoreacted for CR (A, B), PV (C), and both PV and CR (D). In Fig. 2A and B, most of the retrogradely labeled cells were CR+ve. Across all five cases immunoreacted for CR, 1066 nBOR neurons were retrogradely labeled, and of these, 558 (52.3%) were CR+ve. Slightly fewer cells were PV+ve (Fig. 2C). Across all five cases immunoreacted for PV, 978 nBOR neurons were retrogradely labeled and 329 (33.6%) of these were PV+ve. In sections that were immunoreacted for both PV and CR, it was clear that the vast majority of those retrogradely labeled cells that were PV+ve were also CR+ve. From all these sections, of the 162 retrogradely labeled cells that were PV+ve, 146 (90.1%) were also CR+ve. Fig. 2D shows a section containing seven cells retrogradely labeled from an injection in the VbC, five of which were also PV+ve and CR+ve.

IO-projecting nBOR neurons

Fig. 3 shows examples of retrogradely labeled nBOR neurons from injections of BDA (red) in the IO in sections immunoreacted for PV (A–C) and CR (D–F). None of these cells expressed PV or CR. Across all cases, only 1.3% of the IO-projecting neurons were CR+ve (3 of the 224 retrogradely labeled cells) and 0.9% were PV+ve (2 of the 235 retrogradely labeled cells). In the sections

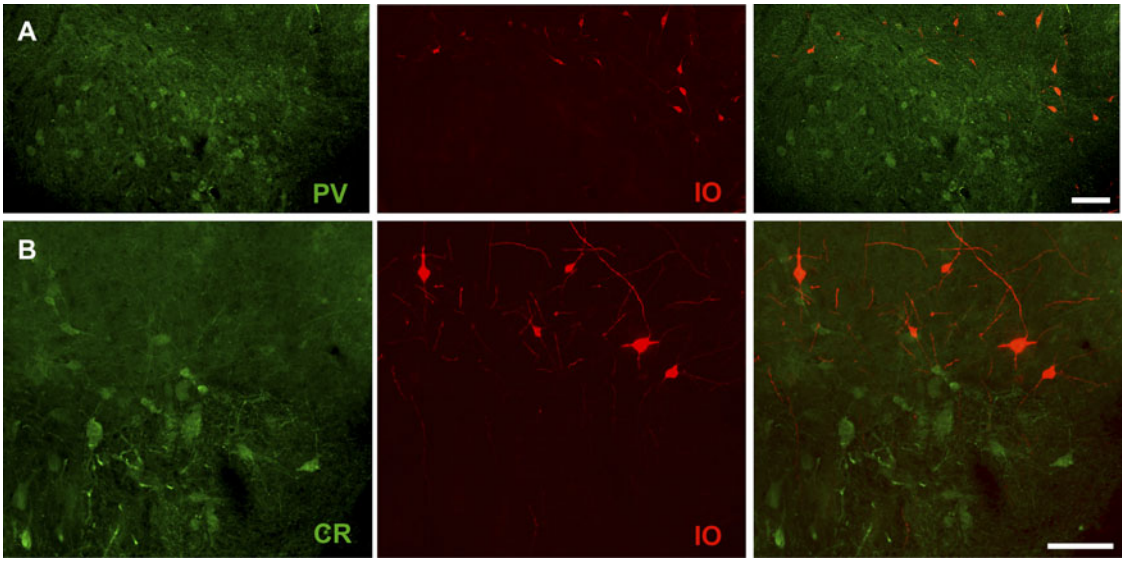

Fig. 3. Inferior olive (IO)-projecting neurons in the nucleus of the basal optic root (nBOR) do not express calcium-binding proteins. Two sections through the nBOR are shown. Cells were retrogradely labeled with red biotinylated dextran amine from injections into the IO (middle panels), and the sections were reacted for either calretinin (green, CR; **B**) or parvalbumin (green, PV; **A**) (left panels). The overlays are shown in the right panels. None of the retrogradely labeled cells were immunopositive for the calcium-binding proteins. Scale bars = 100 μm.

reacted for both PV and CR, none of the retrogradely labeled cells were positive for both CR and PV. In the dorsal regions of the nBOR where the IO-projecting neurons reside, perikarya that were PV+ve and CR+ve were sparse compared to the rest of the nBOR.

Discussion

The nBOR conveys optic flow information to the VbC *via* two pathways. The nBOR projects to the medial column of the IO, which in turn projects to the VbC as climbing fibers (Clarke, 1977; Gamlin & Cohen, 1988; Arends & Voogd, 1989; Lau et al., 1998; Wylie, 2001). A homologous pathway exists in mammals. The mammalian homologues of nBOR, the MTN/LTN, project to the dorsal cap of the IO, which in turn projects to the VbC as climbing fibers (Mizuno et al., 1973; Alley et al., 1975; Holstege & Collewijn, 1982; Ruigrok et al., 1992). The existence of a mammalian homologue of the direct nBOR–VbC mossy fiber projection is a subject of debate: a projection from the MTN/LTN to the VbC has been found in chinchillas (Winfield et al., 1978) and tree shrews (Haines & Sowa, 1985) but not cats (Kawasaki & Sato, 1980), rats, and rabbits (Giolli et al., 1984, 1985, 1988). In other vertebrates, a homologous pathway has been described in turtles (Reiner & Karten, 1978) and fish (Finger & Karten, 1978) but not in frogs (Montgomery et al., 1981).

The complex spike activity of Purkinje cells in the VbC (which reflects climbing fiber input; Eccles et al., 1966) responds best to patterns of optic flow resulting from either self-translation or self-rotation (Simpson et al., 1981; Graf et al., 1988; Leonard et al., 1988; Wylie & Frost, 1991, 1993; Wylie et al., 1993, 1998, 1999*a*; Van der Steen et al., 1994; Wylie & Frost, 1999*b*; Winship & Wylie, 2001), and VbC Purkinje cells are critical for mediating the optokinetic response (Robinson, 1976; Zee et al., 1981; e.g., Ito et al., 1982; Nagao, 1983; Waespe et al., 1983; Lisberger et al., 1984). The VbC cells responsive to translational optic flow have been linked to head-bobbing (Wylie et al., 1993; Wylie & Frost, 1999*a*), an optokinetic response that is stereotypical in pigeons and some other birds (Friedman, 1975; Frost, 1978). Winship et al. (2005) suggested that the two pathways have complimentary roles in maintaining the optokinetic response. These pathways originate from morphologically distinct nBOR neurons: large multipolar neurons throughout the nBOR project to the VbC, whereas small fusiform neurons in the dorsal part of the nBOR project to the IO (Wylie et al., 2007). The visual Wulst, the avian homologue of primary visual cortex (Karten & Shimizu, 1989; Medina & Reiner, 2000), projects to the dorsomedial region of the nBOR (Miceli et al., 1979, 1987). Thus, this telencephalic pathway might influence the nBOR–IO pathway but not the nBOR–VbC mossy fiber pathway.

In the present study, we showed that the IO- and VbC-projecting neurons can also be differentiated with respect to their expression of calcium-binding proteins. We found that CR and PV are both expressed in the somata of nBOR neurons, generally in larger neurons. In addition, many nBOR neurons express both CR and PV; of the PV+ve neurons, about 30% also expressed CR. Although the colocalization of calcium-binding proteins is rare in the mammalian neocortex (see reviews in DeFelipe, 1997; Leuba & Saini, 1997), it is higher in subcortical structures, including the superior colliculus (Leuba & Saini, 1997) and other areas (Rogers & Resibois, 1992). Thus, the degree of colocalization of PV and CR in the nBOR neurons is similar to that reported for other subcortical structures in vertebrates.

In the present study, very few of the IO-projecting nBOR neurons were either CR+ve or PV+ve ($<$2% each), whereas about half of the VbC-projecting nBOR neurons were CR+ve. This is in agreement with De Castro et al. (1998) who reported that about half of the VbC-projecting neurons in the chicken (*Gallus gallus*) nBOR were CR+ve and the fact that CR is strongly expressed in mossy fiber terminals of the cerebellum (reviewed in Schwaller et al., 2002). As previously mentioned, we also found that some (about 30%) of the VbC-projecting nBOR neurons were PV+ve, but most of them also expressed CR (90%). Thus, the PV+ve nBOR neurons that coexpress CR generally project to the VbC. About two-thirds of the PV+ve nBOR neurons are CR$-$ve, and these do not project to the VbC. Like CR, PV is also expressed in mossy fiber

terminals in the cerebellum but not as extensively or as intensely as CR (reviewed in Schwaller et al., 2002).

In summary, immunostaining for calcium-binding proteins differentiated the nBOR–IO and nBOR–VbC pathways; the nBOR–IO pathway expressed little of either PV or CR, whereas the nBOR–VbC pathway expresses a greater amount of CR than PV. The functional significance of this differential expression will, however, remain uncertain until the role of PV and CR in neural system's functioning is better understood.

Acknowledgments

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