

Use of Calcium-Binding Proteins to Map Inputs in Vestibular Nuclei of the Gerbil

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ABSTRACT

We wished to determine whether calbindin and/or calretinin are appropriate markers for vestibular afferents, a population of neurons in the vestibular nuclear complex, or cerebellar Purkinje inputs. To accomplish this goal, immunocytochemical staining was observed in gerbils after lesions of the vestibular nerve central to the ganglion, the cerebellum, or both. Eleven to fourteen days after recovery, the brain was processed for immunocytochemical identification of calretinin and calbindin. After lesion of the vestibular nerve, no calretinin staining was seen in any of the vestibular nuclei except for a population of intrinsic neurons, which showed no obvious change in number or staining pattern. Calbindin staining was reduced in all nuclei except the dorsal part of the lateral vestibular nuclei. The density of staining of each marker, measured in the magnocellular medial vestibular nucleus, was significantly reduced. After the cerebellar lesion, no differences in calretinin staining were noted. However, calbindin staining was greatly reduced in all nuclei. The density of staining, measured in the caudal medial vestibular nucleus, was significantly lower. After a combined lesion of the cerebellum and vestibular nerve, the distribution and density of calretinin staining resembled that after vestibular nerve section alone, whereas calbindin staining was no longer seen. This study demonstrates that calretinin and calbindin are effective markers for the identification of vestibular afferents. *J. Comp. Neurol.* 386:317-327, 1997.

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Normal operations of the vestibular system result in stabilization of head position and the visual field via compensatory movements of the extremities, neck, and eyes. For example, if a head is passively rotated to the right, then vestibular reflexes result in deviation of the eyes to the left and an increased tone in the extensor muscles of the extremities on the right. The vestibulospinal and vestibuloocular responses (VSR and VOR, respectively) involve minimally a three-neuron arc. The first neuron is the vestibular afferent, the second is a neuron in the vestibular nuclei, and the third is a motoneuron in the spinal cord or oculomotor complex (Wilson and Melville Jones, 1979).

There is a need to characterize morphologically the interactions between various vestibular inputs on vestibulospinal and vestibuloocular neurons. Physiological studies have shown that the inputs to vestibulospinal and vestibuloocular neurons may differ (see, e.g., Goldberg et al., 1987; Highstein et al., 1987; Minor and Goldberg, 1991). This includes differences in the physiological properties of the vestibular afferents. Thus far, it has not been possible to examine directly these issues at synaptic levels.

We are investigating the staining patterns of various marker proteins in an effort to address these issues directly. Thus far, calbindin and calretinin are two proteins that may prove practical and effective in the effort. Calretinin immunoreactivity in the vestibular end organs is limited to calyx endings in the apex of the crista or to the striola (the line demarcating orientation of the kinocilium) of the macula (Desmadryl and Dechesne, 1992; Leonard and Kevetter, 1995). A count of the number of stained fibers and the inability to identify label in any bouton endings led these authors to suggest that calretinin immunoreactivity is limited to the population of "calyx-only" units in the mammalian crista (Desmadryl and Dechesne, 1992).

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Cerebellar Purkinje cells and their terminals also stain with some calcium-binding proteins (e.g., calbindin: Celio, 1990; Schwab et al., 1992). Some Purkinje cells project directly to neurons in the vestibular nuclear complex (VNC), bypassing the cerebellar nuclei (see, e.g., Ito, 1984; Tan et al., 1995; Umetani, 1992; Walberg and Dietrichs, 1988). Cerebellar input to the VNC is thought to convey, among others, inputs related to vision and eye movements (Chubb et al., 1984; Stone and Lisberger, 1990a,b). Eye-related cerebellar inputs travel in pathways that originate in portions of the inferior olive, the pontine nuclei, and, indirectly, the paramedian pontine reticular formation and accessory optic system, and pretectal areas, such as the nucleus of the optic tract (Balaban and Henry, 1988; Bernard, 1987; Blanks, 1990; Cohen et al., 1992b; Gerrits et al., 1984; Katayama and Nissimaru, 1988; Takeda and Maekawa, 1989a,b; Walberg et al., 1987; Yamada and Noda, 1987). The floccular-nodular lobe and adjacent areas also receive massive inputs from the vestibular nerve (e.g., nodulus: Barmack et al., 1993) and vestibular nuclei (Blanks, 1990; Epema et al., 1990). The brainstem-cerebellar-oculomotor circuits have been implicated in certain types of neuronal plasticity. For example, cells in the flocculus may be critical for the modification in gain of the VOR due to changes in visual input (Barmack and Peorossi, 1985; Broussard et al., 1992; Cohen et al., 1992a,b; Favilla et al., 1984; Lisberger et al., 1984; Nagao, 1992; Waespe and Cohen, 1983). Knowledge about the interaction of visual and vestibular input in both the cerebellar cortex and the vestibular nuclei is crucial for understanding sensory-motor integration in the VOR. To test the hypothesis that calretinin is a useful marker to study vestibular afferent input within the VNC and that calbindin stains only Purkinje cell and primary vestibular afferents within the VNC, lesions were made of the vestibular ganglion and/or the cerebellum, and fibers were allowed to degenerate.

MATERIALS AND METHODS

Gerbils were anesthetized with 20 mg/kg Nembutal, i.p., and 2.5 mg ketamine, i.m.; anesthesia was maintained with supplements of ketamine, if necessary. The animals were positioned in a stereotaxic frame. Surgery was performed by using sterile instruments.

To generate a lesion in the cerebellum, the skull overlying the cerebellum was removed. Subsequently, the cerebellar vermis was gently aspirated until the fourth ventricle was exposed; then, the suction was moved laterally. The animals were nursed during their survival of 11–14 days ($n = 5$).

To generate a lesion in the vestibular ganglion, the bulla was opened. The brainstem was approached laterally by using the lateral canal ampulla as an anatomical guide. When the vestibular, auditory, and facial nerves were exposed, the eighth nerve was sectioned close to the brainstem ($n = 10$). The animals survived for 11–14 days. Combined lesions used both procedures during the same surgery, and these animals ($n = 2$) survived for 11 days. These lesions had such debilitating effects on the animals that, when two animals survived and showed the same results, no more experiments were performed. These animals were not used in any statistical analyses.

For histochemical staining, the animals were anesthetized with an overdose of urethane (400 mg, i.p.) or

Nembutal (60 mg/kg, i.p.) and were perfused with 4% paraformaldehyde and 1% picric acid in phosphate buffer. The brain and vestibular ganglia were dissected and postfixed overnight (4°C) in 20–30% sucrose fixative. Then, 30–40 μm sections were cut with a freezing microtome, collected in phosphate-buffered saline (PBS), blocked in the appropriate normal serum, and processed for immunohistochemical staining of calbindin-D (1:400 mouse monoclonal antibody; Sigma, St. Louis, MO) or calretinin (1:2,000 rabbit polyclonal antibody; Chemicon, Temecula, CA). Sections were further processed with the avidin-biotin-peroxidase procedures for mouse or rabbit. A black or brown diaminobenzidine reaction was obtained.

Sections were analyzed with light microscopy and the density of staining was measured for the medial vestibular nucleus (MVN). Measurements were made in three portions of the MVN: the magnocellular portion (mc), the parvocellular portion (pc) near the genu of the seventh nerve, and the caudal portion (c) by using the cytoarchitectural distinctions of Gerrits (1990). We chose to concentrate on this nucleus because it contains both VOR and VSR neurons (Kevetter and Coffey, 1991; Kevetter and Hoffman, 1991). For a control, a section from the same brain that was processed together with the immunohistochemically stained sections but was not exposed to the primary antibody was also measured to determine background staining. Image analysis was performed with Sigma Scan analysis software (Jandel Scientific Software, San Rafael, CA). For each brain, measurements of average density of staining, as transmittance of light, were made of 184 μm^2 in three sections each for the MVNmc, the adjacent MVNpc, the MVNc, a nucleus with no staining (hypoglossal nucleus), and a nucleus with maximal staining (the inferior olive for calbindin and the dorsal cochlear nucleus for calretinin). In animals in which the vestibular ganglion was lesioned on one side, measurements of the nuclei on the intact side served as controls. The density of staining on each side was compared with a t-test of paired values.

Successful lesion of the vestibular ganglion was verified in 2 days. First, the dissection was performed from the lateral side of the brainstem, a procedure that normally leaves the ganglion attached to the brainstem. Visual observation confirmed that the ganglion was removed successfully on one side. Second, the vestibular nerve root was carefully analyzed for histochemical staining. Incomplete lesions always leave some vestibular afferents that stain positively with the antibodies. Three gerbils received complete lesions of the vestibular ganglion and were subsequently used for analysis.

In other animals, we aspirated one cerebellar hemisphere and the entire vermis. Lesions were verified by affirming that each peduncle was cut. This was done by observing the amount of cerebellum that remained in cross sections through the brainstem. Slight distortions of the dorsal portion of the lateral vestibular nucleus (LVN) often occurred with cerebellar lesion. In two animals, a small amount of the flocculus remained; therefore, it is possible that a small number of Purkinje cell afferents entered the VNC. However, all measurements were sufficiently different from controls, and the lesions were sufficiently debilitating, that we did not increase the number of animals used ($n = 3$). Because the entire vermis was aspirated, we could not use the vestibular nucleus on the nonlesioned side as a "control"; therefore, the density of staining in

these animals was compared with staining in normal, nonlesioned animals. To compare directly brains from different individuals, the measurements were normalized by determining the lightest and darkest pixel values in reference nuclei. The reference nuclei (hypoglossal and cochlear nuclei) were unaffected by the lesion. The difference between these two points was normalized to 100 units. All procedures using animals were approved by the institutional Animal Care and Use Committee and conformed to the Guidelines of the National Institutes of Health.

RESULTS

Normal distribution of calretinin and calbindin immunoreactivity in VNC

The normal distribution of calretinin and calbindin immunoreactivity in the VNC of gerbils has been described previously (Kevetter, 1996). Briefly, both calcium-binding proteins occur in vestibular afferents, but neither is present in all afferent fibers. Many fibers are labeled in the vestibular nerve and in fascicles within the descending vestibular nucleus (DVN), as well as ascending fibers in the superior vestibular nucleus (SVN) and fibers directed to the MVN. Stained terminals are present in the MVN, especially along the ventricular border (Fig. 1A,C); in the neuropil of the SVN (Fig. 1E); and scattered in the DVN and ventral portions of the LVN. Calbindin-stained, but not calretinin-stained, terminals are present in the neuropil of the dorsal LVN, especially surrounding the large neuronal somata. Some of these terminals are presumably from Purkinje cells, which are also labeled with calbindin.

In the VNC, some neurons are labeled with calretinin, but very few (if any) cells are stained with calbindin. The calretinin-labeled cells can be divided into two groups. The larger group is a cluster of small to medium-sized neurons located in a densely stained network, presumably including dendrites and terminals. This network is located in the MVN, adjacent to the ventricular border (i.e., the parvocellular portion; Fig. 1A). The smaller group of calretinin-labeled neurons are medium-sized cells with long dendritic domains scattered throughout the VNC. The number of calretinin-positive neurons is variable between animals despite otherwise comparable staining. However, in any individual the number of stained neurons always appears to be bilaterally symmetrical.

All calretinin fibers that enter the VNC can be attributed to vestibular afferents

After lesions of the vestibular nerve, almost no calretinin-labeled fibers are present in the VNC. On the lesioned side, calretinin-labeled fibers are only found contiguous with calretinin neurons in the vestibular nucleus. The plexus of labeled neurons adjacent to the ventricle appears unaffected by the lesion and remains densely stained (Fig. 1B). It was not possible to determine whether the number of terminals in this area had decreased. It was also not possible to follow axons directly from the calretinin-positive neuronal plexus. Small numbers of thin fibers appeared to travel ventral and medial from the MVNpc toward the midline.

No labeled fibers are present in the vestibular nerve on the lesioned side, and there are no labeled fibers in the DVN (Fig. 2A), the SVN (Fig. 1F), or the LVN. Also, no

labeled fibers are seen in the magnocellular portion of the MVN (Fig. 1D). In addition, the large-diameter, generally unbranched fibers that appear to project in normal brains from the vestibular nuclei into the juxtatrigenital reticular formation (Kevetter, 1996) are absent (Fig. 7A,B).

The density of calretinin staining was measured in the MVNc, MVNpc, and MVNmc. After neurectomy, staining in MVNmc is virtually eliminated (reduced to 1.8% of control value; Fig. 2B). This results in a significant difference in the density of staining between the lesioned and intact MVNmc ($P < 0.01$; Fig. 3A). There is no significant difference in the density of staining between the MVNc (Fig. 2B) and the MVNpc of the intact and deafferented sides. This probably reflects the staining of intrinsic neurons along the ventricle. The distribution of neurons along the ventricular border shows no obvious changes due to the lesion, although the number of these neurons is different in individual animals.

Lesions of the cerebellum do not produce any obvious changes in the distribution of calretinin staining (Fig. 2C). Confirming the qualitative observation, no significant difference was measured between the density of staining in any of the nuclei. Because calretinin is not found in projection neurons of the cerebellum (Floris et al., 1994; Kevetter, 1996), this result was expected. After combined lesion of the vestibular ganglion and cerebellum, staining is eliminated in the vestibular nuclei, except for the calretinin-positive neurons in the MVNpc, which remain stained (Fig. 6A). These are the same changes observed after neurectomy alone.

Calbindin staining in the MVN can be accounted for by Purkinje cell and vestibular afferents

After successful lesions of the vestibular nerve, calbindin-labeled fibers are greatly reduced in many portions of the vestibular nuclei, especially the MVNmc (Fig. 4A–D), the SVN (Fig. 4E,F), lateral parts of the MVNc, the DVN, and ventral portions of the LVN (not illustrated). Projections into the juxtatrigenital reticular formation are absent (Fig. 7C,D). Density measurements in the ventral portion of the MVNmc showed no staining on the lesioned side. When the medial and dorsal portions of the MVNmc are included in the measurements, staining on the side of the lesion is reduced to 15.5% (Fig. 3A) of the intact side ($P < 0.05$). No significant difference in density of staining is measured in the MVNpc and the MVNc after lesions of the vestibular nerve.

Calbindin projections in the dorsal portions of the LVN and DVN, where many cell perimeters were outlined by boutons, appeared unaffected by neurectomy. Fibers and terminals remained dense in the MVN adjacent to the ventricle, especially in dorsal portions. Although staining in the SVN was reduced, it was still extant throughout the nucleus. The pattern of staining seen within the vestibular nuclei after neurectomy is the same as reported projections of Purkinje cells into the vestibular nuclei (see, e.g., Alley, 1977; Angaut and Brodal, 1967; Balaban, 1984; De Zeeuw et al., 1994; Haines, 1977; Langer et al., 1985; Sato et al., 1982; Tan et al., 1995; Umetani, 1992; Wylie et al., 1994).

After lesion of the cerebellum, the density of calbindin staining in the MVN is also reduced. This is especially dramatic along the ventricular border (Fig. 5A–C). Because the staining in these animals was compared with the

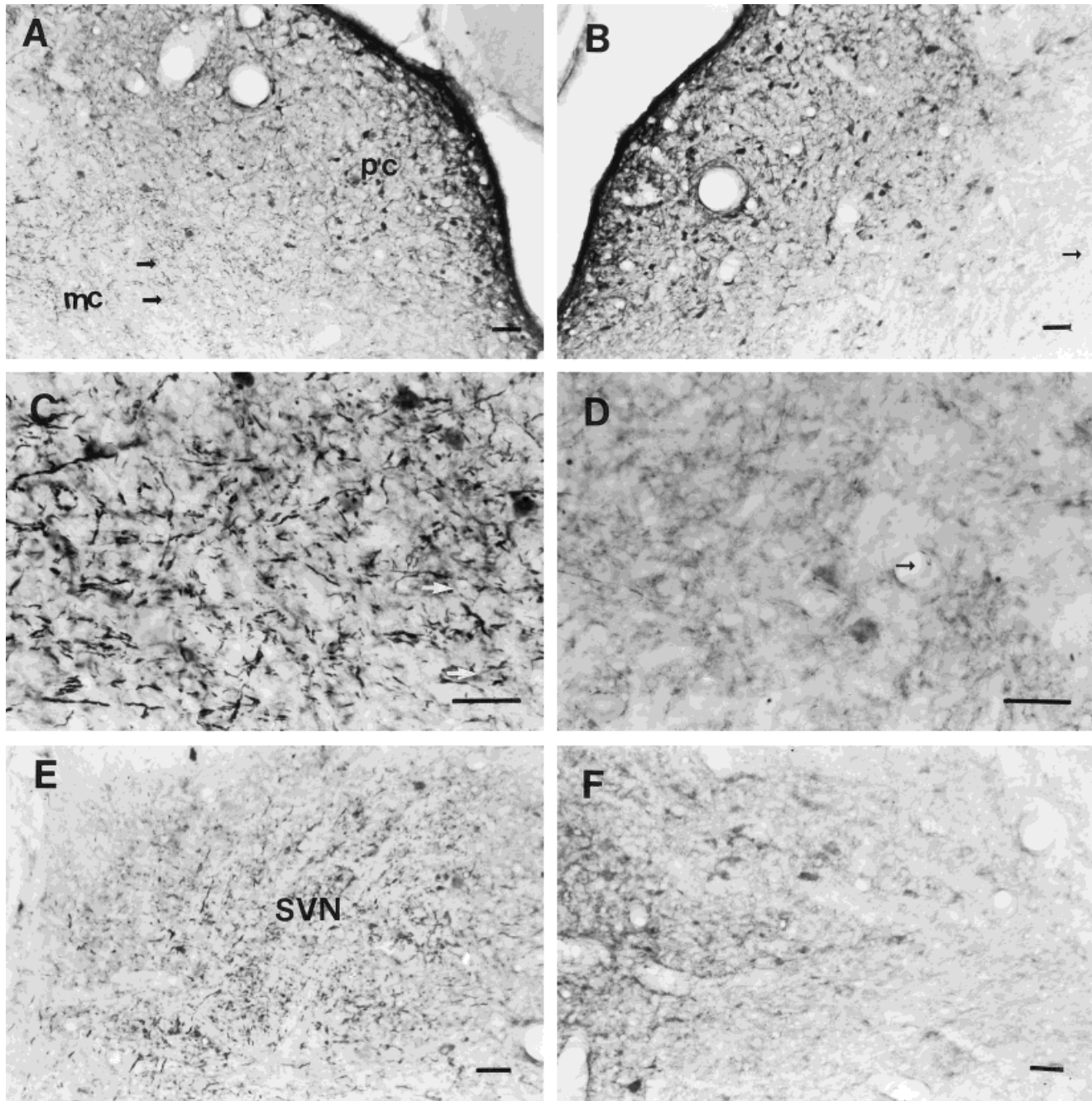


Fig. 1. Photomicrographs of calretinin-immunostaining in the vestibular nuclear complex (VNC). **A:** Parvocellular portion (pc) of the medial vestibular nucleus (MVNpc) with a plexus of labeled cells adjacent to the ventricle and the magnocellular portion (mc) of the MVN more laterally on the intact side. **B:** Same area on the side of a

neurectomy. **C:** Higher power of the MVNmc shown in A. Arrows indicate the same positions in A and C. **D:** Higher power of the MVNmc shown in B. Arrows denote the same structures in B and D. **E:** Superior nucleus (SVN) on the intact side. **F:** SVN on the lesioned side. Scale bars = 50 μ m.

staining in nonlesioned, control animals, the differences are probably underestimated. However, staining in the MVNpc is reduced to an average of 56.5% of the intact side (ranging from 20.7% to 75%). This reduction is appreciable (Fig. 3B) but is not statistically significant ($P = 0.136$). Staining in the MVNc is reduced (Fig. 3B) to 22.4% ($P < 0.002$). Staining in the MVNmc is not significantly altered. Combined with the loss of staining after neurectomy, this result indicates that all of the calbindin staining in the

ventral part of the MVNmc is from the vestibular nerve. These results indicate that, whereas the relative contribution from either varies by region, the overwhelming majority of the calbindin staining in the MVN originates either in Purkinje cells or in vestibular ganglion.

This suggestion was confirmed by combined lesions of the vestibular ganglion and cerebellum. After these lesions, no calbindin-labeled staining was seen in any vestibular nuclei (Fig. 6B). This result indicates that all

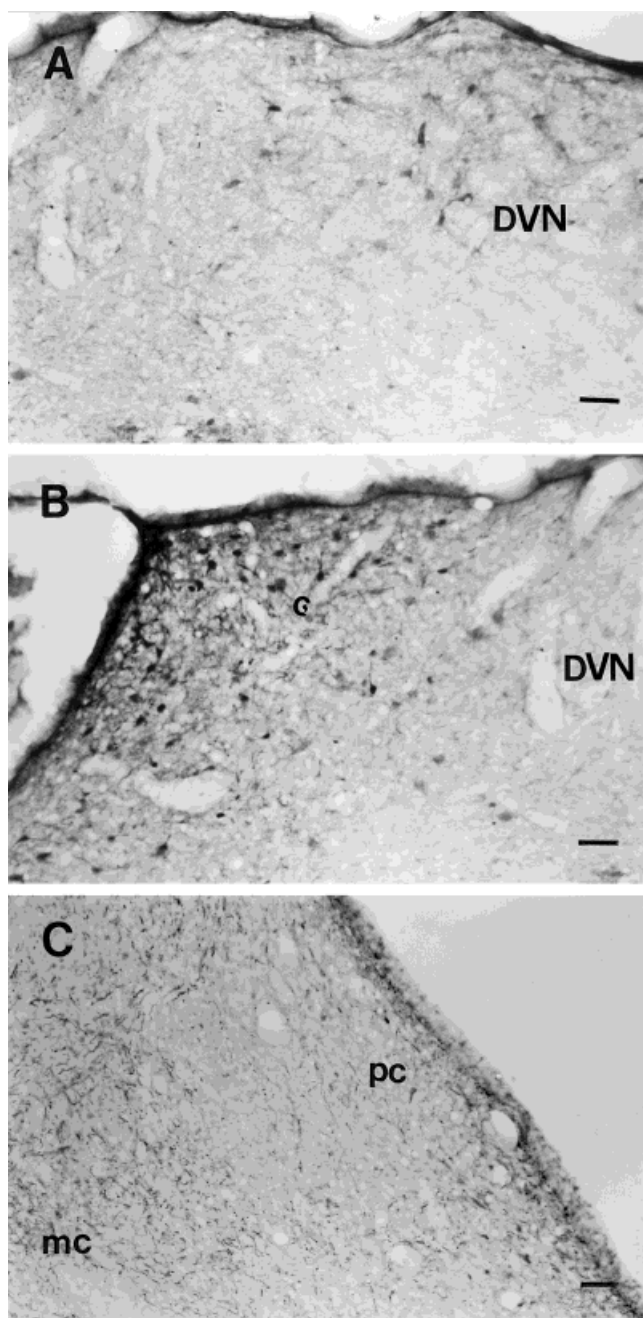


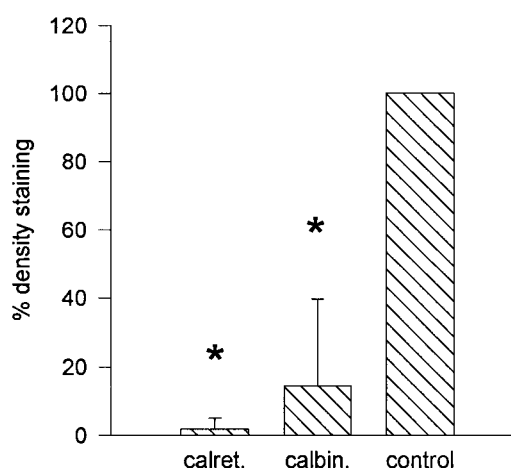
Fig. 2. Photomicrographs of calretinin immunostaining in the VNC after lesions. **A:** Absence of staining in the descending vestibular nucleus (DVN) after lesion of the vestibular ganglion. **B:** Staining in caudal portion (c) of the MVN after lesion of the vestibular ganglion. **C:** Staining in the MVNpc (pc) and MVNmc (mc) after lesion of the cerebellum. Scale bars = 50 μ m.

calbindin projections originated either in the cerebellar cortex or in vestibular ganglion.

DISCUSSION

A major deterrent to studying cell populations in the vestibular nuclei and/or their afferent fibers has been the lack of chemoarchitectonic markers for these populations. In other sensory systems, these are available. For ex-

A. Calretinin and Calbindin after Neurectomy MVN magnocellular



B. Calbindin after Cerebellar Lesion MVN caudal & parvocellular

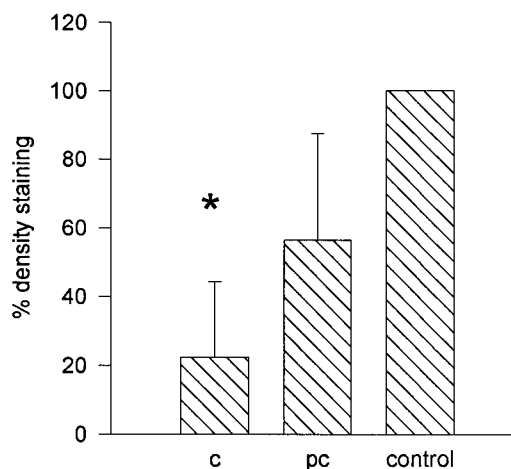


Fig. 3. Histograms of staining density measured on control and lesioned sides. **A:** Calretinin (calret.) and calbindin (calbin.) staining in the MVNmc after lesion of the vestibular ganglion. **B:** Density of calbindin staining in the MVNpc (pc) and MVNc (c) after cerebellar lesion. Asterisks indicate measurements that were significantly different from control.

ample, in the primate auditory system, parvalbumin and calbindin stain two separate populations of thalamocortical neurons (Jones et al., 1995; Molinari et al., 1995). There may be at least two brainstem auditory pathways with totally different patterns of calcium-binding proteins (De Venecia et al., 1995). In the somatosensory system, the lemniscal and nonlemniscal pathways can also be distinguished with different calcium-binding protein antibodies (Jones and Hendry, 1989; Rausell and Jones, 1991a,b). All of these studies argue the feasibility of pursuing such a goal in the vestibular system. In monkey, parvalbumin immunostaining selectively labels burst neurons in the pons that are involved in horizontal saccades: This tool has been utilized to locate and study these neuronal popula-

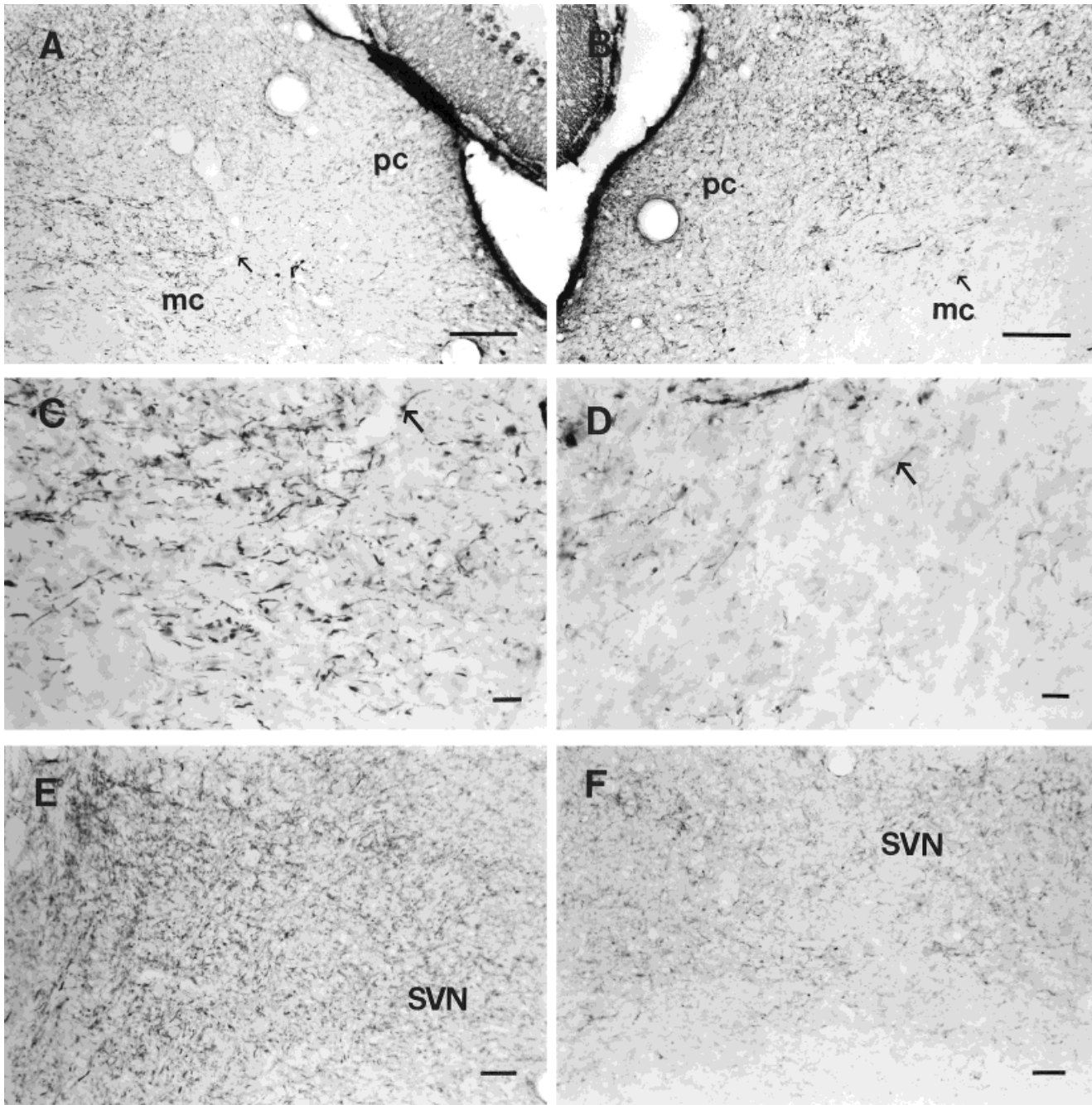


Fig. 4. Photomicrographs of calbindin-immunostaining in the VNC. **A:** MVNpc (pc) and MVNmc (mc) more laterally on the intact side. **B:** MVNpc and MVNmc after a lesion of the vestibular ganglion. **C:** Higher power of the MVNmc shown in A. Arrows indicate the same

structure in A and C. **D:** Higher power of the MVNmc shown in B. Arrows indicate the same structure in B and D. **E:** SVN on the intact side. **F:** SVN on the lesioned side. Scale bars = 50 μ m.

tions in human (Horn et al., 1995). We believe that our study shows that calretinin and calbindin may partially fulfill the goal of chemical identification of vestibular afferent populations.

The distribution of calretinin and calbindin in the vestibular ganglion and nuclei has been described previously (Arai et al., 1991; Kevetter, 1996). Particularly striking has been the staining of ganglion cells and staining in the vestibular nerve with both markers. Calbindin

staining has also been localized in Purkinje cells and their terminals, especially in the dorsal LVN and cerebellar nuclei (Baurle and Grusser-Cornehls, 1994; Celio, 1990; Kevetter, 1996). Lack of calbindin staining in the dorsal LVN in mutant mice that lack Purkinje cells has already demonstrated that calbindin is an excellent tool for studying Purkinje cell innervation of this nucleus (Baurle and Grusser-Cornehls, 1994). After lesion of the vestibular nerve, we see calbindin staining in all four vestibular

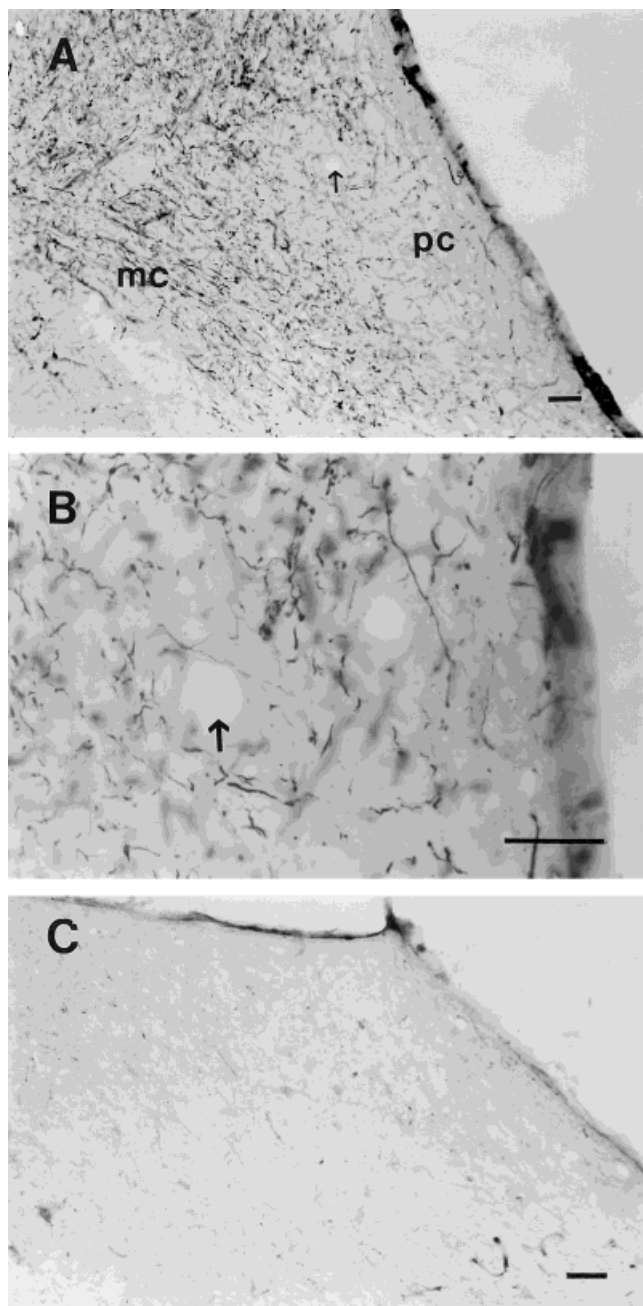


Fig. 5. Photomicrographs of calbindin-stained sections through the MVN after cerebellar lesions (compare with normal staining in Fig. 4A,C). **A:** MVNpc (pc) and MVNmc (mc) on the side with lesion. **B:** Higher magnification of A showing reduced staining in the MVNpc. Arrows indicate the same structure in A and B. **C:** MVNc with very little staining. Scale bars = 50 μ m.

nuclei. The pattern of calbindin stain remaining is the same as that reported previously for Purkinje cell input to the vestibular nuclei in a variety of species (see, e.g., Alley, 1977; Angaut and Brodal, 1967; Balaban, 1984; De Zeeuw et al., 1994; Haines, 1977; Langer et al., 1985; Sato et al., 1982; Tan et al., 1995; Umetani, 1992; Wylie et al., 1994). Conversely, after lesions of the cerebellum, the distribution of calbindin staining within the VNC resembles the

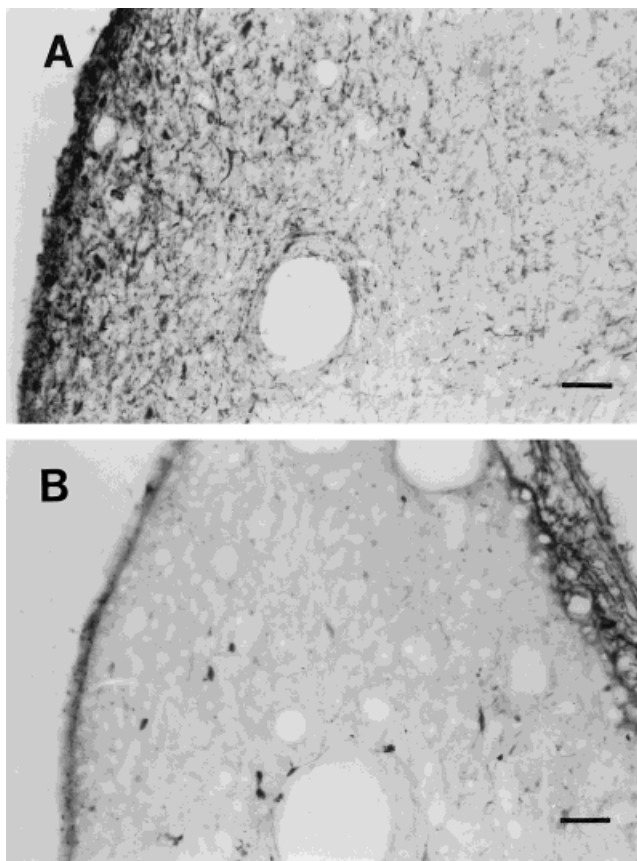


Fig. 6. Staining in the MVN after combined lesions of ganglion and cerebellum. **A:** Calretinin staining is seen only in neurons adjacent to the ventricle. **B:** Absence of calbindin staining. Scale bars = 50 μ m.

distribution of vestibular afferents (see, e.g., Kevetter and Perachio, 1986). All staining in the vestibular nuclei is depleted after combined neurectomy and cerebellar aspiration, demonstrating that calbindin staining originates solely from these two sources. If the ultrastructural characteristics of calbindin boutons from the Purkinje cells and the vestibular nerve are different, then it will be possible to use this marker to study Purkinje cell input as well as vestibular afferent input simultaneously.

In the peripheral vestibular system, calretinin staining has been studied most. Calretinin appears (and disappears) from hair cells during certain defined stages of development (Deschesne et al., 1994). In rodents at birth and throughout normal life, calretinin staining is not present in hair cells but, instead, is confined to a subset of afferent terminals (Dechesne et al., 1994). Calyx endings, confined to the apex of the crista, are the only terminals that stain positive for calretinin (Dechesne et al., 1994; Desmadryl and Dechesne, 1992; Leonard and Kevetter, 1995, 1996).

In the vestibular ganglion, approximately 16% of the cells stain for calretinin, and these cells also stain with calbindin (Dememes et al., 1992; Raymond et al., 1993). In fact, the area of calretinin-positive somata overlaps with the size of intracellularly injected otolith afferents with the most irregular firing rate (Leonard and Kevetter, 1996; Perachio et al., 1988). This is good evidence that these

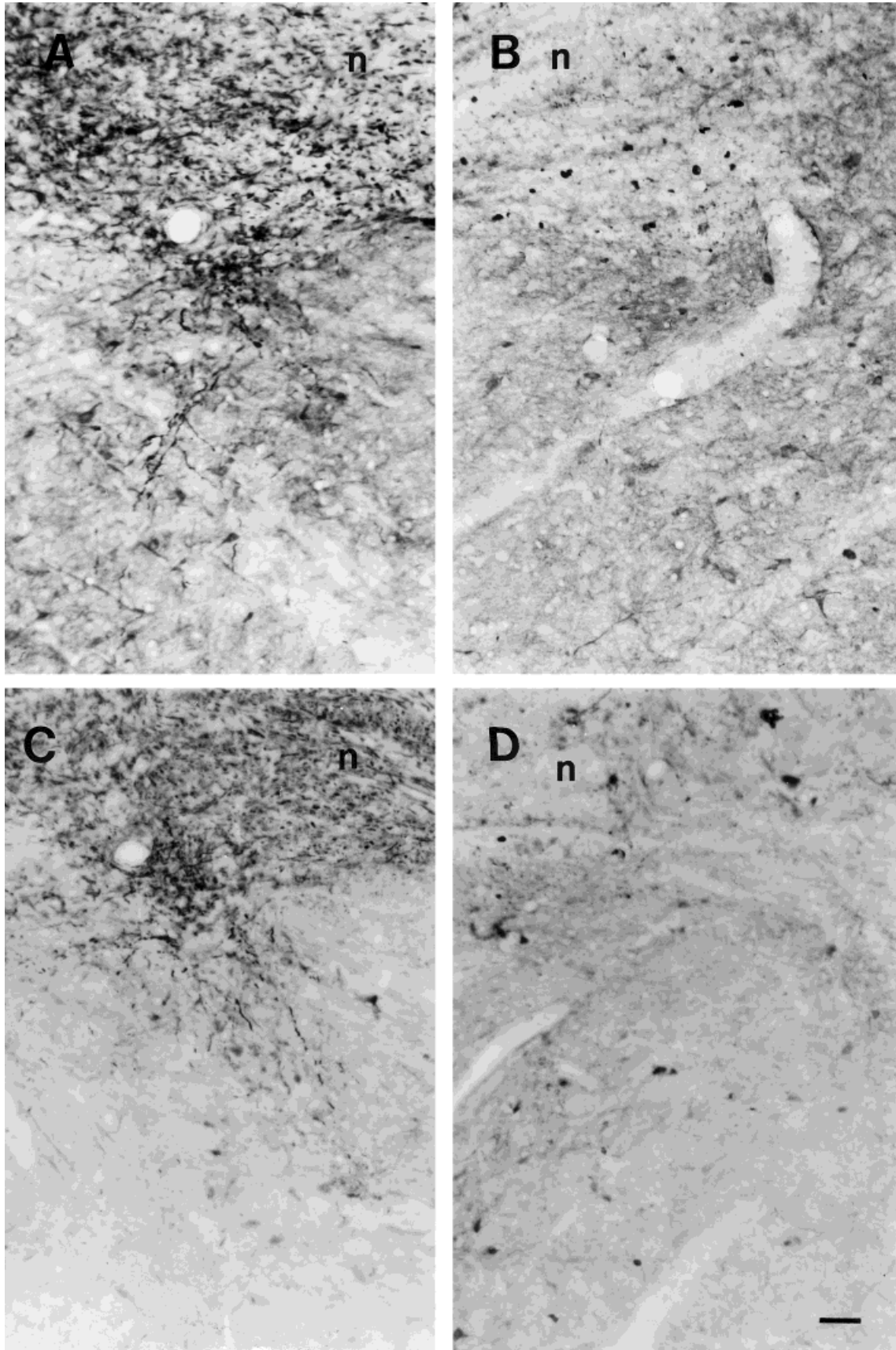


Fig. 7. Photomicrographs of entering eighth nerve and fibers in the reticular formation adjacent to the spinal nucleus of V. **A:** Normal calretinin staining in the intact side. **B:** Section on the side of vestibular ganglion removal. No staining is present in the nerve or in

descending fibers. **C:** Normal calbindin staining on the intact side. **D:** Lack of staining after lesion of vestibular ganglion. n, Vestibular nerve. Scale bar = 50 μ m in D (also applies to A-C).

calcium-binding proteins stain a selective population of afferents.

After vestibular neurectomy, calretinin staining is eliminated in all vestibular nuclei except a small area adjacent to the ventricle in the MVN. This result demonstrates that calretinin staining can be used to examine aspects of vestibular afferent innervation of the vestibular nuclei. Because calretinin staining is not present in projection neurons of the cerebellum (Floris et al., 1994; Kevetter, 1996), and we saw no change in density of staining after cerebellar lesion, studies utilizing calretinin staining would not be hindered by two different origins.

One particular projection that might prove more easily identified and studied with immunohistochemical labeling than with other procedures is the projection to the juxtatrigenital reticular formation. These calretinin-positive fibers were eliminated after lesions of the vestibular ganglion. Vestibular nerve fibers have been traced to this location by using degeneration and transganglionic transport techniques (see, e.g., Carleton and Carpenter, 1984; Kevetter and Perachio, 1986; Korte, 1979). Earlier anatomists proposed (discussed in Korte, 1979; Lindeman, 1969) that the geniculate ganglion might be the origin of seventh nerve fibers that project through the vestibular ganglion and nerve to the brainstem. If this were true, then injections of HRP or tritiated proline into the labyrinth would not label these fibers. Because these fibers are labeled after injections of tracer into the vestibular periphery, these fibers in the reticular formation can be ascribed definitively to the vestibular nerve. The use of a marker, such as calretinin, may make this projection more accessible for study.

After lesions of the vestibular nerve, calretinin staining remains along the ventricle (MVNpc), where a population of neurons stain. There is great variation in the number of these neurons in different animals but, qualitatively, they appear bilaterally symmetric, and the staining density was not different between sides in neurectomized, cerebellectomized, or normal animals. The functional characteristics of these cells could not be determined in this study. These neurons might be projection neurons. They might project to the cerebellum; however, no definitive axonal projections to the cerebellum were seen on the side of the neurectomy. A small number of thin fibers that appeared to exit the MVNpc and travel toward the midline could be seen after neurectomy. These fibers could originate from the calretinin-positive neurons. They might be commissural projections to the MVNpc on the contralateral side, or they might project either rostrally or caudally in the MLF. These possibilities should be explored in additional studies.

There appears to be a species variation in the population of VNC neurons that stain with calretinin. In guinea pig and, to a lesser extent, in rat, neurons that stain for calretinin mRNA are located mainly in the MVN, with the largest and most dense group near the ventricle (Sans et al., 1995). Consistent with this is the general population in gerbil. However, the number and distribution of calretinin-positive cells is smaller and more confined in gerbil than in rat (Kevetter, 1996) or guinea pig.

Calretinin may be involved in plastic changes that occur after lesions of components of the eighth nerve. For example, in the auditory system, short- and long-term changes in the mRNA and immunocytochemical staining for calretinin are observed after cochlear ablation (Winsky

and Jacobowitz, 1995). Increased immunocytochemical staining was preceded by a transient increase in mRNA in the anterior ventral cochlear nucleus, accompanied by a decrease in both mRNA and immunocytochemical staining of projection neurons of the posterior ventral cochlear nucleus (Winsky and Jacobowitz, 1995). Sans et al. (1995) reported a decrease in the numbers of neurons that stain for calretinin mRNA in the MVN after a hemilabyrinthectomy. Although we have not attempted to quantitate the labeled cells, there was no obvious decrease in staining on the lesioned side in our material.

With regard to the function of calcium-binding proteins, Kasai (1993) has proposed that they may play a role in pre- and postsynaptic plasticity. Different proteins might be involved in either depression or potentiation of synaptic efficacy, depending on their location in the cell and their affinity to Ca²⁺. After infection with recombinant adenovirus containing calbindin cDNA, cultured hippocampal neurons express calbindin (Chard et al., 1995). In these neurons, posttetanic potentiation is suppressed, although transmitter release, per se, remains functionally unchanged (Chard et al., 1995). Because long-term changes are present in the cerebellum (for review, see Ito, 1989) and in vestibular nuclei after tetanic stimulation (Capocchi et al., 1992), it is possible that the calcium-binding proteins play a role in the plasticity that occurs during vestibular compensation and/or plasticity within the VOR.

In this paper, we have shown that calretinin and calbindin are useful markers for studies of vestibular connectivity. We conclude that calretinin stains two populations of vestibular neurons: a population of primary afferents and a group of intrinsic neurons. The distribution of intrinsic neurons is always symmetric, although there is great variability between individual animals. Calretinin can be used to study vestibular afferents in all vestibular nuclei except the MVNpc and the MVNc. Calbindin staining is selectively decreased, depending on which lesion is made and the location studied. Calbindin stains a population of vestibular afferents and all Purkinje cell projections to the vestibular nuclei. Terminals from these two sources occur in overlapping, as well as predominantly segregated, parts of the vestibular nuclei.

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