Distribution and Morphology of Calretinin-Immunoreactive Neurons in the Intermediate and Deep Layers of Cat Superior Colliculus

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Calretinin is thought to play roles in calcium buffering. Its site of expression has been extensively studied in the central nervous system. We previously reported (Hong et al., 2002, Neurosci. Res., 44: 325-335) calretinin expression in the superficial layers of the cat superior colliculus (SC). In the present study, we studied the distribution of calretinin in the intermediate and deep layers by immunocytochemistry. We found striking differences in calretinin immunoreactivity among the superficial, intermediate, and deep layers. In contrast to the superficial layers, the intermediate and deep layers contained many calretinin-immunoreactive (IR) neurons. They formed two laminar tiers. The first tier, which was very distinctive, was found within the upper intermediate gray layers and formed clusters of labeled neurons in many sections. The second tier of calretinin-IR neurons was found in the deep gray layer. However, the second tier was not distinctive compared to the first tier and the labeled neurons did not form any clusters. Calretinin-IR neurons in the intermediate and deep layers varied dramatically in morphology and included vertical fusiform, pyriform, and stellate neurons. Neurons with varicose dendrites were also labeled. Most of the labeled neurons were small to medium in size. Enucleation appeared to have no effect on the distribution of calretinin immunoreactivity in the contralateral intermediate and deep layers of the SC. The results indicate that calretinin is present in various neurons, at different locations. These results should be applicable for better understanding of the functional organization of the SC.

The mammalian superior colliculus (SC) is a highly differentiated region of alternating fibers and cells lying on the roof of the midbrain. It is the center of visuo-motor integration and receives visual output from the retina through the retino-collicular pathway. Based on anatomical and physiological characteristics, the SC can be divided into superficial, intermediate and deep layers. The superficial layers that include zonal, superficial gray and optic layers are exclusively related with vision. They receive their major input from the retina and visual cortex. The intermediate (intermediate white and intermediate gray) layers and deep (deep white and deep gray) layers are related with motor behavior. They receive multisensory input from auditory, somatosensory and nociceptive pathways (for reviews, Huerta and Harting, 1984; Binns, 1999). One of the principal organizing features of the SC is the topographical distribution of its afferent fibers and efferent cells. Many afferents and efferents of the SC are segregated into specific laminae or have a patch-like organization. An example is the laminar segregation of calcium-binding protein-immunoreactive (IR) neurons. In the cat SC, there is a dense band of highly calretinin-IR fibers in the superficial gray layer (Hong et al., 2002). The calbindin D28K is found in neurons that are located in three tiers of the cat SC (Mize et al., 1992). Parvalbumin-IR neurons form a single dense band in the deep superficial gray and optic layers with loosely scattered neurons in the deep layers in cat. Thus, the distribution of parvalbumin-IR neurons in cat SC was complementary to that of calbindin D28K-IR neurons (Mize et al., 1991).

Calretinin is a member of the EF hand family of calcium-binding proteins. It is found in subpopulations of neurons throughout the nervous system. Although the exact role of this protein has not been established yet, it has been suggested that calretinin plays a role in calcium buffering to regulate the intracellular calcium. Misregulation of calcium by calretinin is closely related with many neuro-logical diseases (Heizmann and Braun, 1995). As investigation of specific neurochemical architecture is crucial to understand how the neurons

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work, the sites of calretinin expression in the central nervous system have been extensively studied. Calretinin is present in a number of visual structures, including retina (Pasteels et al., 1990; Ellis et al., 1991; Wässle et al., 1995; Goebel and Pourcho, 1997; Volgyi et al., 1997; Jeon and Jeon, 1998; Jeon et al., 2001), lateral geniculate nucleus (Arai et al., 1992; Lüth et al., 1993; Leuba and Saini, 1996; Yan et al., 1996; Soares et al., 2001), SC (Arai et al., 1993; Leuba and Saini, 1996; Jeon et al., 1998; Yang and Jeon, 1998; Hong et al., 2002; Kang et al., 2002), and visual cortex (Glezer et al., 1992; Leuba and Saini, 1996; Jeon and Park, 1997; Meskenaite, 1997; Leuba et al., 1998; DeFelipe et al., 1999; Park et al., 2000, 2002).

Recently, we reported the distribution of calretinin-IR fibers and origin of these fibers in the superficial layers of cat SC (Hong et al., 2002). In this study, we investigated the distribution of calretinin in the intermediate and deep layers of cat SC. Although our primary goal was to analyze the neurochemical specific regions and cell types in the intermediate and deep SC and to complete our model of chemoarchitecture of calretinin in the entire cat SC, we also wanted to compare our results with distribution of two other important calcium-binding proteins, calbindin D28K and parvalbumin.

Materials and Methods

Animals

Seven adult cats (2.5-3.0 kg) were used for these experiments. The animals were divided into three groups. First, intact cats (n = 3) were used to determine the normal distribution of immunoreactivity of calretinin. Second, unilaterally enucleated cats (n = 4) were produced in order to examine the effects of retinal deafferentation. Enucleation was performed under anesthesia with a mixture of ketamine hydrochloride (30-40 mg/kg) and xylazine (3-6 mg/kg) (supplemented as needed to maintain anesthesia).

The enucleated cats were allowed to survive for 10 (n = 2) and 20 (n = 2) d. The National Institute of Health guidelines for the use and care of animals were followed for all experimental procedures. All efforts were made to minimize animal suffering as well as the number of animals used.

Perfusion and tissue processing

Cats were anesthetized deeply with a mixture of ketamine hydrochloride (30-40 mg/kg) and xylazine (3-6 mg/kg) before perfusion. All cats were perfused intracardially with 4% paraformaldehyde and 0.3-0.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) with 0.002% calcium chloride added. After exposure of the thoracic cavity, 1 ml of 1% sodium nitrite and 0.2 ml of 25,000 units of sodium heparin were injected directly into the heart to dilate the blood vessels and reduce coagulation. Following a prerinse with approximately 500 ml of phosphate-buffered saline (PBS, pH 7.2) over a period of 3-4 min, each cat was perfused with 1700-1800 ml of fixative for 1-2 h via a syringe needle inserted through the left ventricle and aorta. The head was then removed and placed in the fixative for 2-3 h. The brain was then removed from the skull and stored 2-3 h in the same fixative and left overnight in 0.1 M phosphate buffer (pH 7.4) containing 8% sucrose and 0.002% $CaCl_2$. The SC was removed, mounted onto a chuck, and cut into 50 μm thick sections with a vibratome.

Immunocytochemistry

A polyclonal antibody against calretinin was obtained from Chemicon (Temecula). The tissue was processed free floating in small vials at 25°C with gentle agitation. The primary antiserum was diluted 1:500-1:1000. The immunocytochemical methods have been described in detail in our previous reports (Jeon et al., 1998; Hong et al., 2002). The tissue was examined and photographed on a Zeiss Axioplan microscope using conventional or differential interference contrast optics.

Results

Distribution of calretinin immunoreactivity

Calretinin immunoreactivity was very selectively distributed in the SC of all three normal cats (Fig. 1). As we previously reported (Hong et al., 2002), the calretinin immunoreactivity consisted of numerous well-labeled small fibers in the superficial layer (Fig. 2A). The fibers in the superficial layers were not homogeneously distributed. Some areas contained more labeled fibers than other areas. In the present study, we found striking differences in calretinin immunoreactivity between superficial, intermediate, and deep layers. In contrast to superficial layers, intermediate and deep layers contained many calretinin-IR neurons. They formed two laminar tiers in the SC. The first tier, which is very distinctive, was found within the upper intermediate gray layers (Fig. 1). Some neurons were localized in the lower optic layer. Its thickness was approximately 200-300 μm at the middle level. However, in many sections, labeled neurons in the intermediate gray layer did not form a continuous band. Rather, they formed clusters of labeled neurons (Fig. 2B, arrowheads). Figure 2B shows three calretinin-IR neuron clusters in the upper intermediate gray paye'. The second tier of calretinin-IR neurons was found in the deep gray layer. However, the second tier was not distinctive compared to the first tier and the labeled neurons did not form any clusters. The calretinin-IR neurons in these two tiers could be seen throughout the

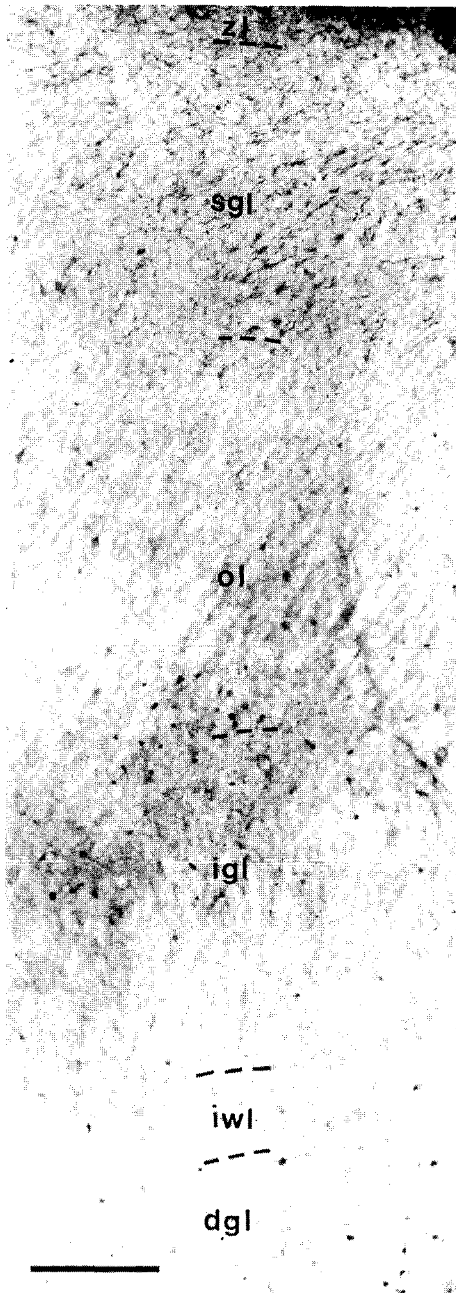

Fig. 1. Distribution of anti-calretinin immunoreactivity in the cat superior colliculus. Calretinin-IR fibers were distributed in the upper part of the superficial layers. The majority of calretinin-IR neurons were distributed in two tiers, one in the intermediate gray layer, and the other in the deep gray layer. zl, zonal layer; sgl, superficial gray layer; ol, optic layer; igl, intermediate gray layer; iwl, intermediate white layer, dgl, deep gray layer. Bar=200 μm.

rostrocaudal extent of the SC.

Morphology of calretinin-IR neurons

Calretinin-IR neurons in the intermediate and deep layers varied dramatically in morphology. Most of the labeled

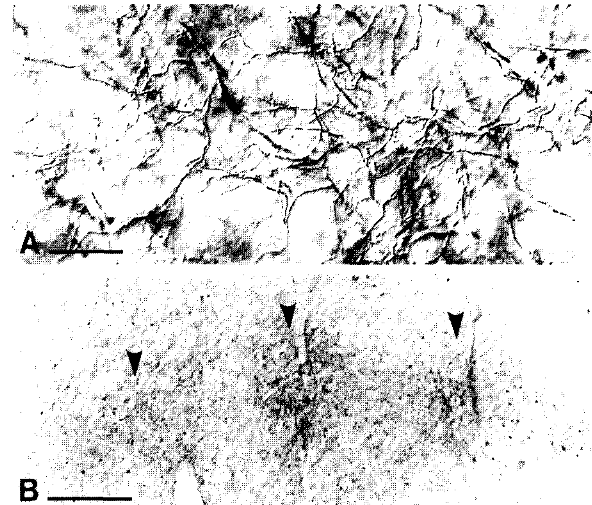

Fig. 2. A, High magnification Nomarski differential interference contrast photomicrograph of calretinin-IR fibers in the superficial gray layer. Almost all of the labeled fibers are small with few varicosities. B, Medium magnification of calretinin-IR neurons in the intermediate gray layer. Arrowheads indicate clustering of calretinin-IR neurons. Bars=40 μm (A) and 150 μm(B).

neurons were small in size. Figure 3 shows calretinin-IR neurons in the intermediate gray layer. Figures 3A and B show the first type of calretinin-IR neuron, a vertical fusiform neuron. These neurons had a thick proximal dendrite oriented toward the pial surface. Dendritic branches originated from this thick primary dendrite. The second type was a varicose dendrite neuron (Fig. 3C). This is a unique class of neurons in the intermediate gray layer. The neurons had numerous prominent varicosities along their dendrites (Fig. 3C, arrowheads). The varicosities often appeared at fairly regular intervals, and were quite thick relative to the intervaricose segments. These varicose dendrite neurons were small and had oval-shaped cell body. The third type of calretinin-IR neuron as the stellate neurons (Fig. 3D). These neurons had medium-sized stellate-like cell bodies with multiple dendrites. Neurons in the deep gray layer also varied considerably in morphology. Figure 4A (right cell) shows a calretinin-IR pyriform neuron. These neurons usually had pear- shaped cell bodies with a thick, proximal dendrite directed towards the pial surface. Secondary branches from the proximal dendrite formed a dendritic bouquet. There was a variety of stellate-like neurons with multiple dendrites (Figs. 4B, D). Their morphology was similar to the stellate neurons in the intermediate gray layer. Figure 4C shows a vertical fusiform neuron that is very similar to the vertical fusiform neuron in figure 3A in the intermediate gray layer.

Calretinin immunoreactivity after eye enucleation

We previously reported a marked reduction of calretinin immunoreactivity in the superficial layers of the SC

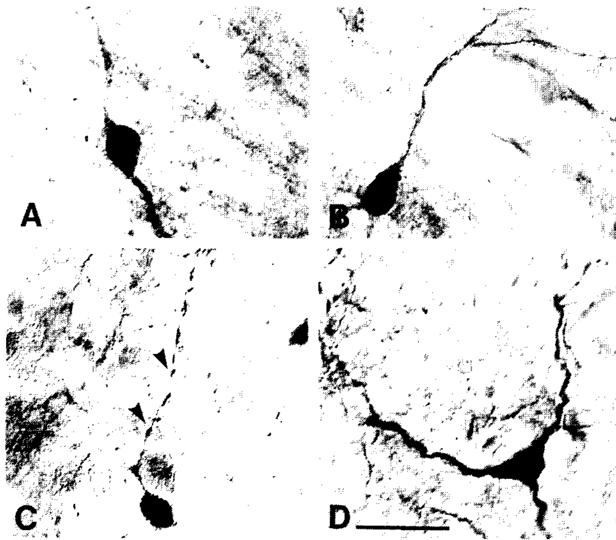

Fig. 3. High magnification Nomarski differential interference contrast photomicrographs of calretinin-IR neurons in the intermediate gray layer. A and B, Vertical fusiform neurons with proximal dendrite projecting superficially toward the pial surface. C, Calretinin-IR neuron with varicose dendrites. The varicosities (arrowheads) often appeared at fairly regular intervals. D, Multipolar stellate neuron. Bar=40 μm.

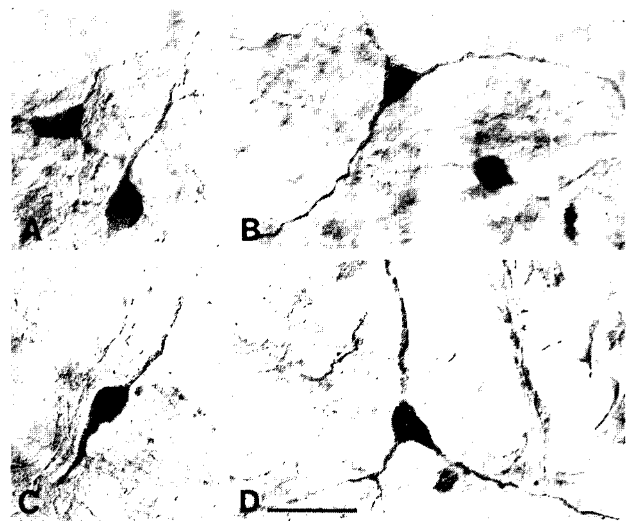

Fig. 4. High magnification Nomarski differential interference contrast photomicrographs of calretinin-IR neurons in the deep gray layer. A, Calretinin-IR pyriform neuron with a thick, proximal dendrite directed towards the pial surface. B and D, Multipolar stellate neurons. C, Vertical fusiform neuron. Bar=40 μm.

contralateral to the enucleation (Figs. 5A, B) and a new appearance of many calretinin-IR neurons after enucleation (Hong et al., 2002). To determine whether enucleation affects the distribution of calretinin immuno-reactivity in the deeper SC, we performed monocular enucleations in some animals. Enucleation appeared to have no effect on the distribution of calretinin-immunoreactivity in the contralateral intermediate and deep layers of the SC. Although we saw occasional variability in immunoreactivity in the contralateral and ipsilateral SC, there was no consistent difference in labeled neurons on the two sides in calretinin-IR sections after enucleation (Figs. 5C, D). We also found no apparent differences in antibody labeling between 10 and 20 d of monocular enucleation.

Discussion

Calretinin immunoreactivity has been reported in the human (Leuba and Saini, 1996), cat (Hong et al., 2002), rabbit (Jeon et al., 1998), rat (Arai et al., 1993), and hamster (Kang et al., 2002) SC. However, except in the human, these reports focused on the chemoarchitecture of calretinin immunoreactivity in the superficial layers. In addition, previous reports primarily focused on the origin of calretinin-IR fibers and changes of calretinin immunoreactivity after enucleation. Leuba and Saini (1996) briefly described calretinin immunoreactivity in intermediate and deep layers of human SC. They found a broad band of calretinin-IR neurons in the intermediate gray layer and neurons in the deep gray. Thus, the location of calretinin-IR neurons in the intermediate and deep layers of cat SC is similar to that of human. However, important differences in calretinin distribution exist between the human and rabbit SC. In the rabbit SC, the neurons in the intermediate gray layer formed clusters. These differences in distributional pattern may reflect the functional differences of specific efferent and afferent connections of the SC.

Our results show that the majority of calretinin-IR neurons were found in two layers: one within the intermediate gray layer and the other within the deep gray layer. The calcium-binding protein calbindin D28K is found in neurons located in three tiers of the cat SC: the upper one half of the superficial gray layer, the lower portion of the optic layer extending to the intermediate gray layer, and the deep gray layer (mize et al., 1991). similar results have been reported in some other mammalian sc (behan et al., 1992; mize and luo, 1992; schmidt-kastner et al., 1992; leuba and saini, 1996; gonzález-soriano et al., 2000). some calbindin d28k-ir neurons in the rabbit sc are distributed in clusters in the intermediate gray layer (gonzález-soriano et al., 2000). thus, the laminar location of calretinin in the intermediate and deep layers is similar to that of calbindin d28k. As we reported previously (Hong et al., 2002), calretinin forms a dense band of IR fibers in the superficial layers. Thus, the patterned distribution of calretinin n the superficial layer is strikingly different from that of calbindin D28K in the cat SC. The parvalbumin-IR neurons are concentrated in a dense tier within the deep superficial gray and upper optic layers. Scattered parvalbumin-IR neurons are also found in the deep layers of SC (Mize et al., 1992; Cork et al., 1998). The distribution of parvalbumin-IR neurons in the cat and rat is thus

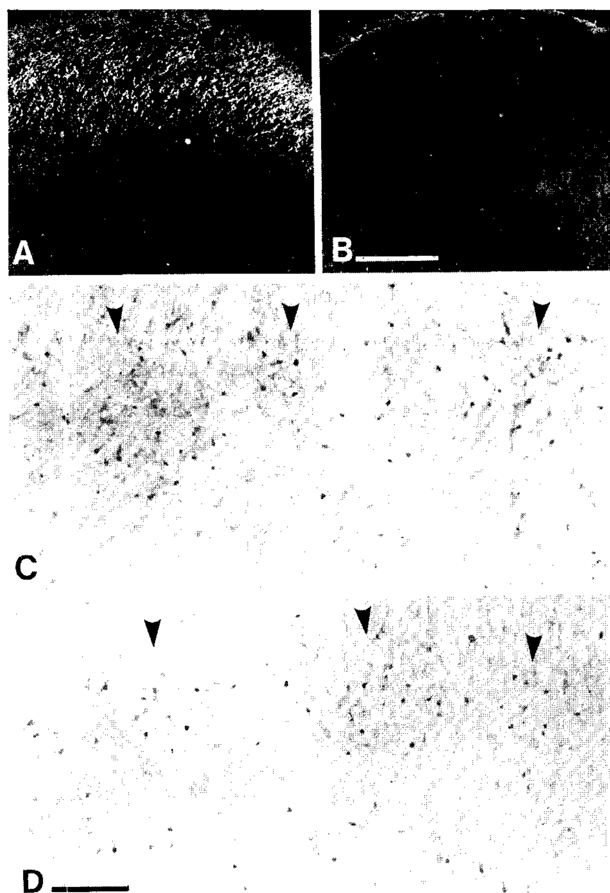

Fig. 5 Calretinin immunoreactivity in the normal and in the side of SC contralateral to the enucleation. A and B, Low magnification of darkfield photomicrographs of calretinin-IR fibers in the cat SC. A, Calretinin-IR fibers were concentrated within the upper part of the superficial layers in the normal SC. B, The fiber staining was almost completely reduced in the side of SC contralateral to the enucleation. C and D, Medium magnification bright-field photomicrographs from the intermediate and deep layers in C, normal and D, contralateral SC to the enucleation. The arrowheads indicate calretinin-IR neurons in clusters. Bars = 250 μm (A, B) and 100 μm (C, D).

complementary to that of calbindin D28K-IR neurons. The two dorsal tiers of calbindin-IR neurons border the dense tier of parvalbumin-IR neurons. The deepest calbindin D28K-IR neurons also lie in a region where there are relatively few parvalbumin-IR neurons (Mize et al., 1992; Cork et al., 1998). Therefore, the present results also indicate that the distribution of parvalbumin-IR neurons forms a complementary pattern to that of calretinin.

The calretinin-IR neurons in human intermediate and deep layers of SC included small- to large-sized pyramidal bipolar, and multipolar neurons (Leuba and Saini, 1996). Although some calretinin-IR neurons were similar to those of human, we found no large pyramidal cells in the present study. These differences may reflect functional variations of collicular cells among different species. In the present study, we found neurons with small cell bodies and distinctive varicose dendrites. This type of neuron has also been observed in calbindin-IR neurons in the cat SC (Mize et al., 1992). Whether these varicosities are related to vesicle containing dendrites can only be answered by electron microscopy. However, there is evidence that presynaptic density has been described in the intermediate gray layer of the cat SC (Norita, 1980). The calretinin-IR neurons in the cat SC had varied morphology. Many of the calbindin-IR neurons in the intermediate and deep layers included nonvaricose stellate and large neurons, while many of the parvalbumin-IR neurons included medium sized stellate neurons (Mize et al., 1991; 1992). Therefore, there are similarities and differences in morphology between calretinin-IR neurons and calbindin- and parvalbumin-IR neurons in the intermediate and deep layers. Most of the calbindin-IR neurons in the cat SC were interneurons while most of the parvalbumin-IR neurons were projection ones. Neurons showing double immunoreactivity against calretinin and calbindin D28K or parvalbumin were distributed in the intermediate and deep layers in the human SC (Leuba and Saini, 1997). These results and the heterogeneity of calretinin-IR neurons indicate that the calretinin-IR neurons are a mixture of interneurons and projection neurons.

One of distinctive features of the SC is its compartmental architecture. Our results show calretinin-IR neurons form clusters within the intermediate gray layer of the cat SC. It is possible that specific afferents converge onto these calretinin-specific clusters. The quite precise modular organization of efferent cell clusters and afferent patches has been reported in the cat SC. Thus, the cell clusters that project to the cuneiform region were found to overlap precisely with the cholinergic terminal patches in the intermediate gray layer of the cat SC (Jeon et al., 1993; Jeon and Mize, 1993). This previous study was the first demonstration of the relationship between an efferent cell group and the transmitter-identified afferents. As the prefrontal cortex and substantia nigra afferents converge to cholinergic patches, Jeon and Mize (1993) suggested that neuronal clusters in the cholinergic terminals were related to eye movements. Illing and Graybiel (1986) have suggested that "certain sensory and motor functions may be spatially separated in the intermediate collicular layers, each being represented by a domain of its own." Illing (1992) found at least two compartments in the intermediate gray layer of mammalian SC. These are the zones with high levels of acetylcholine and those with low levels of acetylcholine. The high acetylcholine zone overlaps with the enkephalin, NADPH-diaphorase, and substantia nigral input, while the low acetylcholine zone overlaps with innervations from the trigeminal nucleus and the somatosensory and visual association cortex. Thus, each zone is preferentially innervated with neurochemically and

anatomically specific afferents. Although it is hard to determine from the present study if calretinin-IR clusters belong to either high or low zones of acetylcholine, the calretinin-IR neuron clusters in the present study must serve to segregate the information of collicular afferents. Many neurochemical specific efferent and afferent systems within the SC are segregated into specific laminae and/or patches. For example, glutamate (Jeon et al., 1997), glutamate receptor (Cirone et al., 2002), nitric oxide synthase (Scheiner et al., 2000; González-Soriano et al., 2002), adhesion molecules (Yamagata et al., 1995), and enkephalin-IR cells and/or fibers (Graybiel et al., 1984; Mize, 1989) are distributed in specific laminae in the SC. The calcium-binding proteins calbindin D28K and parvalbumin are also distributed in specific laminae in the SC (Mize et al., 1991; 1992; Behan et al., 1992; Jeon et al., 1998; González-Soriano et al., 2000). Thus, specific laminae, or patches or cluster organizations constitute a key element of the collicular organization.

Enucleation appeared to have no affect on the calretinin-IR neurons in the intermediate and deep layers of cat SC. Similarly, there was no reduction of calbindin D28K immunoreactivity in the monkey and rat after monocular enucleation. In contrast to cells in intermediate and deep layers, a marked reduction in calretinin-immunoreactive fibers was produced in the superficial layers of the cat SC contralateral to the enucleation (Hong et al., 2002). Additionally, many calretinin-IR neurons appeared in the superficial SC. These results combined with the present results suggest that the calretinin-containing neurons in the superficial SC may be more plastic than those in the intermediate and deep layers after enucleation.

The precise role of calretinin in the SC is far from obvious yet. It has been suggested that calretinin is involved in sharpening the timing of action potentials via its capacity of calcium buffering and transport (Rogers, 1987). The present results show clustering and heterogeneity of calretinin-IR neurons. In this way, collicular neurons in the intermediate and deep gray layers may be able to segregate complex multisensory and motor pathways.

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