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# Distribution of Calretinin in the Superficial Layers of the Mouse Superior Colliculus: Effect of Monocular Enucleation

**Hye-Won Yang and Chang-Jin Jeon***

*Department of Biology, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea*

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**We localized a calcium-binding protein, calretinin, in the superior colliculus of the mouse and studied the distribution and effect of eye enucleation on the distribution of this protein. Calretinin was localized with immunocytochemistry. A dense plexus of anti-calretinin-labeled fibers was found within the superficial layers. The highest density was found in the deep superficial gray layer. Monocular enucleation produced an almost complete reduction of calretinin-immunoreactive fibers in the superficial layers of the superior colliculus contralateral to the enucleation. Furthermore, many calretinin-labeled cells appeared in the contralateral superior colliculus. These newly appeared neurons had small oval or round cell bodies. The results demonstrate that calretinin identify unique neuronal sublaminar organizations in the superior colliculus of the mouse. They also suggest that the retinal projection may control in part the content of calretinin in some neurons in the superficial layers of the mouse superior colliculus.**

Calcium controls a wide variety of cellular mechanisms. Many of these mechanisms of calcium are probably mediated by interactions with a variety of calcium-binding proteins (Heizmann et al., 1990; Rogers et al., 1990; Baimbridge et al., 1992; Schäfer and Heizmann, 1996). Among the many calcium-binding proteins, at least three types of calcium-binding proteins, calbindin D28K, calretinin, and parvalbumin abundantly occur in various types of neurons in the central nervous system (Baimbridge et al., 1992). These three calcium-binding proteins are known to be present in distinct subpopulations of neurons (Rogers et al., 1990; Baimbridge et al., 1992). Calretinin is a 29 kDa calcium-binding protein that was first isolated as a cDNA clone from the chick retina. The sequence of the chick calretinin is 58% homologous to that of chick calbindin D28K (Rogers, 1987). Although the physiological roles of calretinin have not been established, it may simply work as a calcium buffer or may actively work in calcium-mediated signal transduction.

The mammalian superior colliculus is the center of visuo-motor integration. It is a seven-layered structure that can be divided into superficial and deep layers. The three superficial layers (zonal, superficial gray, and optic layers), receiving their major input from the retina and the visual cortex, are exclusively related to vision. In contrast, the four deeper layers (intermediate gray, intermediate white, deep gray, and deep white), that receive auditory, somatic, and visual inputs from numerous cortical and subcortical areas, are involved in head, eye, and ear movements (Huerta and Harting, 1984; Grantyn, 1988).

Calcium-binding proteins show horizontal laminar segregation in the superior colliculus. Thus, the calcium-binding protein, calbindin D28K, is found in cells that are located in three layers of the cat superior colliculus (Mize et al., 1991). Parvalbumin-immunoreactive cells formed a single, dense band in the deep superficial gray and optic layers with loosely scattered cells in the deep layers of the cat (Mize et al., 1992). Calretinin has also been localized in some animals. It forms a dense plexus of immunoreactive fibers in the superficial layers of the cat (Jeon et al., 1996), rat (Rogers and Resibois, 1992), mouse (Gobersztejn and Britto, 1996), and hamster superior colliculus (Jeon et al., 1997). Calretinin-immunoreactive fibers, however, have not been found in the rabbit superior colliculus. Instead, many calretinin-immunoreactive cells are localized in the superficial layers of the rabbit superior colliculus (Jeon et al., 1998). These results indicate that there are significant species differences in the distribution of calcium-binding proteins in the superior colliculus.

The present study was undertaken to investigate the detailed organization of calretinin immunoreactive fibers and to investigate the changes of the distribution of this calcium-binding protein in the mouse superior colliculus by enucleation. Parts of data in this study have been reported before (Yang and Jeon, 1997).

* To whom correspondence should be addressed.
Tel: 82-53-950-5343, Fax: 82-53-953-3066
E-mail: cjjeon@kyungpook.ac.kr

## Materials and Methods

### Animals

Eight adult mice (C57/BL-6, 8-10 wk) were used for these experiments. The animals were divided into two groups. First, intact mice (n=4) were used to determine the normal distribution of immunoreactivity to calretinin. Second, unilaterally (n=4) enucleated mice were produced in order to examine the effects of retinal deafferentation. Enucleation was performed under anesthesia with isoflurane (Ohmeda Caribe Inc.). The eye-enucleated mice were allowed to survive for 10 (n=2) and 20 (n=2) d.

### Perfusion and tissue processing

Mice were anesthetized deeply with isoflurane (Ohmeda Caribe, Inc.) before perfusion. All mice 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. Following a prerinse with approximately 10 ml of phosphate-buffered saline (PBS, pH 7.2) over a period of 1-3 min, each mouse was perfused with 20-30 ml of fixative for 5-10 min 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 superior colliculus was removed, mounted onto a chuck, and cut into 50 μm thick sections with a vibratome.

### Horseradish peroxidase immunocytochemistry

A polyclonal antibody against calretinin purified from guinea pig was obtained commercially from Chemicon.

The tissue was processed free floating in small vials. For immunocytochemistry, the sections were incubated in 1% sodium borohydride ($NaBH_4$) for 30 min. Sections were rinsed 3×10 min in 0.25 M Tris, incubated in 0.25 M Tris with 4% normal goat serum for 2 h with 0.5% Triton X-100 added. Sections were then incubated in the primary antiserum diluted 1:1000-1:2000 in 0.25 M Tris with 4% normal goat serum for 48 h with 0.5% Triton X-100 added. Following three times for 10 min rinses in 0.25 M Tris, sections were incubated in a 1:200 dilution of biotinylated goat anti-rabbit secondary IgG in 0.25 M Tris with 4% normal goat serum for 2 h with 0.5% Triton X-100 added. Sections were then rinsed three times for 10 min in 0.25 M Tris and incubated in a 1:50 dilution of avidin-biotinylated horseradish peroxidase (HRP, Vector lab) in 0.25 M Tris for 2 h. The sections were again rinsed in 0.25 M Tris three times for 10 min. Finally, tissues were visualized by reacting with 3,3-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide in 0.25 M Tris for 3-10 min using a DAB reagent

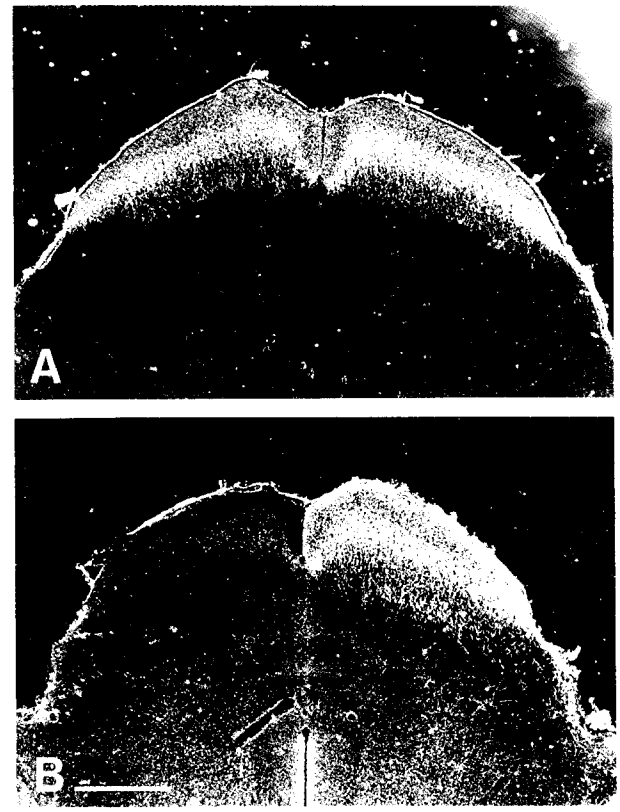


**Fig. 1.** Calretinin immunoreactivity in the normal (A) and enucleated (B) mouse superior colliculus. On the contralateral superficial layer to the enucleation (B), there was almost complete reduction of anti-calretinin-immunoreactive fiber density. The mouse had its right eye removed 10 days before sacrifice. Dark field photomicrographs. Scale bar=500 μm.

set (Kirkegaard & Perry). All sections were then rinsed in 0.25 M Tris before mounting. As a control, some sections were incubated in the same solution without the addition of the primary antibody. These control tissues showed no calretinin or calbindin D28K immunoreactivity. In our recent study of several mammalian retinas (Jeon and Jeon, 1998) and rabbit superior colliculus (Jeon et al. 1998), this antibody specifically labeled subpopulations of neurons. Following the immunocytochemical procedures, the tissue was mounted on Superfrost Plus slides (Fisher) and dried overnight in a 37℃ oven. The mounted sections were dehydrated through alcohol, cleared with xylene, and coverslipped with Permount (Fisher). The tissue was examined and photographed on a Zeiss Axioplan microscope using conventional or differential interference contrast (DIC) optics.

## Results

### Distribution of anti-calretinin immunoreactivity

Anti-calretinin immunoreactivity was very selectively distributed in the superior colliculus of all four normal mice. In the superficial layers, the calretinin immu-

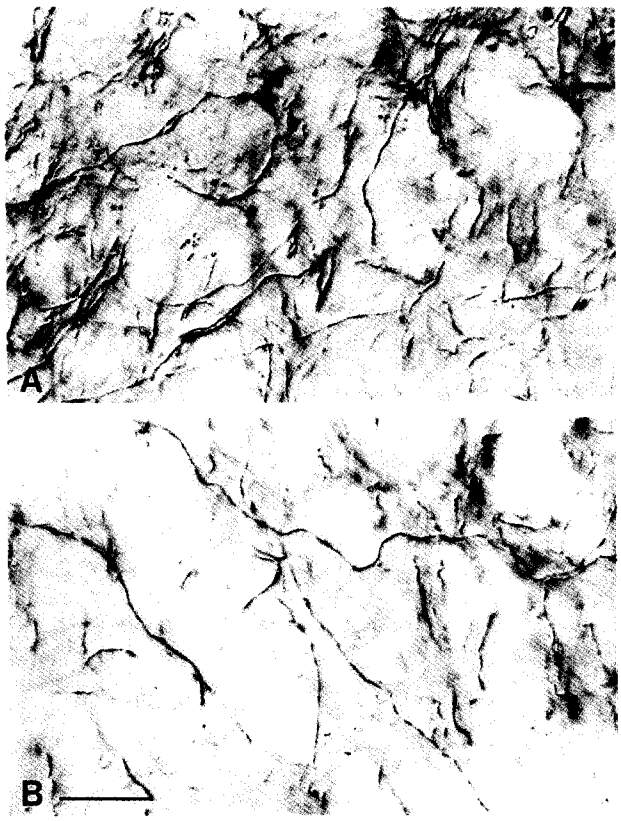


**Fig. 2.** Higher magnification of calretinin-immunoreactive fibers found within the superficial layers. Fiber density was more intense in the deep superficial gray layer (A) than the upper superficial gray and optic layers (B). Almost all these fibers were thin. DIC optics. Scale bar=20 μm.

noreactivity consisted of numerous, well-labeled fibers (Figs. 1, 2, 3A). This tier of labeled fibers was found throughout the rostral-caudal extent of the mouse superior colliculus. These fibers were found in the zonal, superficial gray and optic layers. In this plexus of anti-calretinin-labeled fibers, except for a few lightly labeled cells in the optic layer, no calretinin-immunoreactive cell bodies were localized.

The fibers in the superficial layers were not homogeneously distributed. Some areas contained more labeled fibers than other areas within the superficial layers. Fiber density was higher in the deep superficial gray layer (Fig. 2A) than in the upper superficial gray and the optic layers (Fig. 2B). Almost all calretinin-immunoreactive fibers in the superficial gray layer were small in diameter and had a few varicosities (Fig. 2). Some calretinin-immunoreactive fibers in the deep superficial gray layer were often loosely surrounded by small to medium-sized unlabeled neuronal somata (Fig. 2A).

*Anti-calretinin immunoreactivity after enucleation*

To determine whether the calretinin-labeled fibers in the superficial layers originate from the retina and

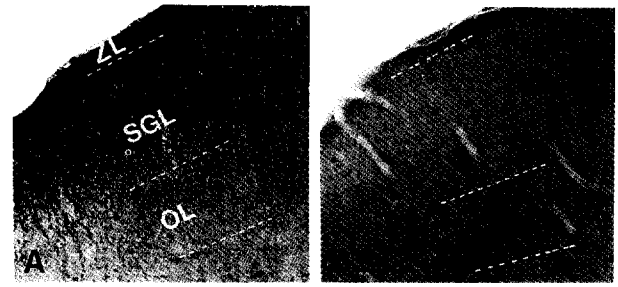


**Fig. 3.** Anti-calretinin immunoreactivity in the superior colliculus in normal (A) and enucleated (B) mouse. On the contralateral superficial layer to the enucleation (B), many calretinin-labeled cells were observed. The mouse had its right eye removed 10 days before sacrifice. ZL, zonal layer; SGL, superficial gray layer; OL, optic layer. Scale bar=100 μm.

whether the enucleation affects the distribution of calretinin immunoreactivity in the mouse superior colliculus, we performed enucleations in some animals. Figs 1A and 3A show calretinin immunoreactivity in the normal mouse superior colliculus while Figs 1B and 3B show calretinin immunoreactivity after monocular enucleation. A marked reduction of calretinin immunoreactivity was produced on the superficial layers of the superior colliculus contralateral to the enucleation. Calretininlabeled fibers were almost completely eliminated following enucleation. By contrast, in the superficial layers of the superior colliculus contralateral to the enucleation, many anti-calretinin-labeled cells were

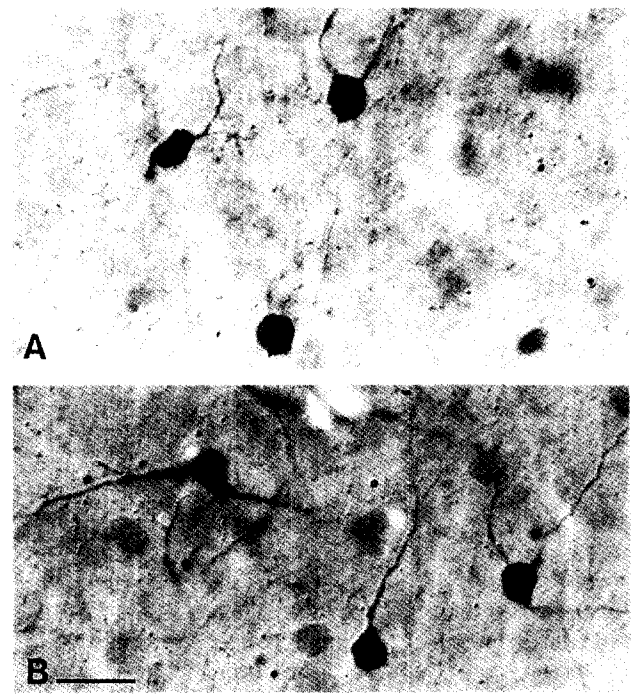


**Fig. 4.** Higher magnification of calretinin-labeled neurons on contralateral superficial gray layer to the enucleation. A, Most of these cells had small, round or oval or vertical pyriform cell bodies with main ascending dendrites toward the pial surface. B, A horizontal cell is also seen. Scale bar=20 μm.

observed (Fig. 3B). These cells were found in all superficial layers:zonal, superficial gray, and optic layers. The staining of calretinin was robust in certain small neurons in the superficial layers of the mouse superior colliculus. These anti-calretinin-labeled cells had small, round or oval cell bodies (Fig. 4). We identified at least two distinct morphologies based upon their dendrites. The first type in the superficial layer was composed of round or oval neurons with proximal dendrites projecting superficially toward the pial surface (Fig. 4). The majority of calretinin-immunoreactive neurons which appeared after eye enucleation, were this type of neuron. The second type also had round or oval cell bodies. However, these cells had horizontally oriented processes (Fig. 4B). Horizontal cells were very rarely found. We found that both 10 and 20 days of monocular enucleation had pronounced effects upon calretinin immunoreactivity. We also found no apparent differences in antibody labeling between 10 and 20 days of monocular enucleation.

## Discussion

The patterned distribution of calretinin-immunoreactive fibers found in the mouse superior colliculus in the present study is similar to that observed in previous studies in the rat (Arai et al., 1993), cat (Jeon et al., 1996), mouse (Gobersztejn and Britto, 1996), and hamster (Jeon et al., 1997). In all these animals, the antibody against calretinin formed a dense plexus of labeled fibers in the superficial layers of the superior colliculus. The previous studies of the rat and mouse, however, did not describe the fiber morphology in detail. Our findings indicate that the type of calretinin fibers that exist in the superficial layers of the mouse superior colliculus are small diameter fibers with a few varicosities. The highest density of labeled fibers were located in the deep superficial gray layer. No large fibers were observed. Even though many retinal ganglion cells were labeled by anti-calretinin antibody (Pasteels et al., 1990), recently in our lab Jeon and Jeon (1998) found that large ganglion cells in the retina were not labeled by antibody against calretinin. These results combined with our present result suggest that subtypes of calretinin-containing retinal ganglion cells may project to the superior colliculus.

The pattern of calretinin-immunoreactive fibers found in the present and previous studies was different from that described in the rabbit. In recent studies from this laboratory (Jeon et al., 1998), we found that calretinin did not form a plexus of labeled fibers in the superficial layers of the rabbit superior colliculus. By contrast, many anti-calretinin-labeled neurons were localized in the superficial layers. This result indicates that there are some species with differences in calretinin immunoreactivity in the superior colliculus.

We have observed a new appearance in calretinin-immunoreactive cells in the superficial layers of the mouse superior colliculus after enucleation. Even though Goberstzjn and Britto (1996) also observed calretinin positive neurons on the experimental side of the mouse superior colliculus, they did not describe its cell morphology. Their study (see their Fig. 1) did not give any useful information regarding the detailed morphology of calretinin-labeled neurons. The present study is the first detailed description of the newly localized calretinin-immunoreactive cells found in the mouse superior colliculus after enucleation. We found that the newly appeared calretinin-containing cells were localized in all superficial layers. All of these cells were small rounded or oval-shaped cells. The large majority of these cells had dendrites projecting toward the pial surface. Horizontal cells with horizontally oriented dendrites were very rarely found. There are many morphologically different types of cells in the superior colliculus. It is important to know which types of neurons express calretinin for physiological roles in the future. No anti-calretinin-immunoreactive stellate-shaped or large neurons were observed in the superficial layers after enucleation. The present localization of calretinin-immunoreactive neurons after enucleation suggests that genes for calretinin in some neurons in the superficial layers may not work in a normal state. These cells may express calretinin genes after enucleation. Thus, the expression of calretinin genes may be activity-dependent in the superficial layers of the mouse superior colliculus. Localization of mRNA by in situ hybridization will be necessary to prove activity-dependent expression of calretinin gene in the mouse superior colliculus after enucleation. Activity-dependent changes of calretinin mRNA has been reported in the auditory system (Sans et al., 1995; Winsky and Jacobowitz, 1995).

The physiological role of the calcium-binding proteins is still unclear. It has been suggested that calretinin is involved in sharpening the timing of action potentials by having the capacity of calcium buffering and transport (Rogers, 1987). However, the function of calretinin is not clearly understood yet.

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