

Calretinin-Containing Neurons in the Deeper Layers of the Hamster Superior Colliculus

Ye-Eun Kim¹, Jae-Sik Choi¹, Hye-Hyun Kim^{1,2}, Ji-Yeon Yeo¹ and Chang-Jin Jeon^{1*}

¹Department of Biology, College of Natural Sciences, Kyungpook National University, Daegu 702-701, S. Korea

²Agrobiotechnology Education Center, NURI

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Calcium-binding protein calretinin is thought to play important roles in calcium buffering. Recently, we reported on the distribution, morphology of calretinin-immunoreactive (IR) neurons and the effects of eye enucleation on the immunoreactivity of calretinin in the superficial layers of the hamster superior colliculus (SC). In the present study, we describe the distributions and types of labeled cells and effects of enucleation in the deeper layers by immunocytochemistry. We also compare this labeling to that of GABA, the major inhibitory neurotransmitter in the central nervous system. In contrast to the superficial layers, the deeper layers contained many calretinin-IR neurons which formed two tiers. The first tier, which was very distinctive, was found within the intermediate gray layer. The second tier was found in the deep gray layer. Labeled neurons varied dramatically in morphology and included vertical fusiform, stellate, round/oval, and horizontal neurons. In contrast to the superficial layers, enucleation appeared to have no effect on the distribution of calretinin immunoreactivity in the deeper layers. Two-color immunofluorescence revealed that none of calretinin-IR neurons were labeled with an antibody to GABA. The present results demonstrate that calretinin identifies unique neuronal sublamina organizations in the hamster SC. The present results also demonstrate that none of the calretinin-IR neurons in the hamster SC is GABAergic interneurons. As many calretinin-IR cells are GABAergic interneurons in most other brain areas, this phenomenon in hamster SC is exceptional.

Key words – Calretinin, immunocytochemistry, superior colliculus, calcium-binding protein, enucleation

Introduction

The mammalian superior colliculus (SC) is a laminated structure lying above the cerebral aqueduct in the midbrain. It is the sensory-motor integration center where sensory and motor maps interact to produce appropriate movements. The SC plays critical role in the neural control of saccadic eye movements. The mammalian SC is a seven-layered structure that can be divided into superficial and deeper layers. The dorsally located three superficial or "visual" layers (zonal, superficial gray and optic layers) appear to have a purely sensory role as they receive their major input directly from the retinal and indirectly from the visual cortical axons that form a topographic map. In contrast, the underlying four deeper or "motor" layers (intermediate gray, intermediate white, deep gray, and deep white) have both sensory and motor functions as they receive auditory, somatic, and visual inputs from numerous cortical and subcortical axons. The deeper layer

neurons can respond to auditory, somatic, or visual stimuli and many neurons receive converging modality-specific inputs. The neurons in "motor" layers also generate bursts of action potentials that command saccades[7,9,12].

Calcium is essential to maintaining a wide variety of cellular mechanisms such as cell proliferation, muscle contraction, bone growth, learning and memory, and neurotransmission. Calcium-binding proteins are known to control and modulate the actions of calcium[2,10,23,25]. Among the many calcium-binding proteins, calretinin is a 29 kDa EF-hand calcium-binding protein that was first isolated as a cDNA clone from chick retina[22]. Calretinin abundantly occurs in various types of neurons throughout the brain, spinal cord, and sensory ganglia. It is known to be present in distinct subpopulations of neurons and is a predominantly cytosolic protein[2,23]. Although the biochemical properties of this protein have been well characterized, the physiological roles of calretinin are still unclear. The functions of calretinin like calbindin D28K, include a role in neuroprotection against excitotoxicity in the brain or a calcium buffering function in the cells.

One of the principal organizing features of the SC is the

*Corresponding author

Tel : +82-53-950-5343, Fax : +82-53-953-3066

E-mail : cjeon@knu.ac.kr

topographical distribution of its afferents and efferents. Retinotopic organization in the SC in many vertebrates has been demonstrated amply[9]. Calcium-binding proteins show horizontal laminar segregation in the SC. Calbindin D28K is found in cells that are located in three layers of the cat SC[20]. Parvalbumin-immunoreactive cells formed a single dense band in the deep superficial gray and optic layers with loosely scattered cells in the deep layers in cat[21]. Calretinin forms a dense plexus of immunoreactive fibers in the superficial layers of cat[11], rat[24], mouse[5], and hamster SC[16,17]. However, there are significant species differences in the distribution of calcium-binding proteins in the SC. For example, instead of calretinin-immunoreactive fibers in most mammalian SC, many calretinin-immunoreactive cells are localized in the superficial layers in the rabbit SC[14].

Previously, we reported on the distribution, morphology of calretinin-immunoreactive (IR) neurons and effects of eye enucleation on the immunoreactivity of calretinin in the superficial layers of the hamster SC[16,17]. However, calcium-binding protein calretinin immunoreactivity has not been reported in the deeper (intermediate and deep) layers of the hamster SC. Thus, the main purpose of the current study is to provide knowledge of calretinin chemoarchitecture in the intermediate and deep layers of the hamster SC by analyzing the morphology, distribution, and effect of enucleation as the deeper layers of the SC are critical part of the SC that is involved in head, eye, and ear movements. The second aim was to identify whether calretinin-IR neurons in the deeper layers are GABAergic interneurons. In most brain areas calretinin-IR neurons also expressed GABA. Our results show that the organizational features of the calretinin-IR neurons found in the intermediate and deep layers of the hamster SC are strikingly different from those of the superficial layers. In addition, no calretinin-IR neurons contain GABA in the hamster SC.

Materials and Methods

Animals

Ten adult Golden hamsters (8-10 weeks, 25-30 g) were used for these experiments. The animals were divided into two groups. First, intact hamsters (n=6) were used to determine the normal distribution of immunoreactivity to the calcium-binding protein calretinin. Second, unilaterally (n=4) enucleated hamsters were produced in order to ex-

amine the effects of retinal deafferentation. Enucleation was performed under anesthesia with isoflurane (Ohmeda Caribe Inc., Guayama, USA). The eye-enucleated hamsters were allowed to survive for 10 (n=2) and 20 (n=2) days.

Perfusion and tissue processing

Hamsters were anesthetized deeply with isoflurane (Ohmeda Caribe Inc.) before perfusion. All hamsters 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 preinse with approximately 10 ml of phosphate-buffered saline (PBS, pH 7.2) over a period of 1-3 min, each hamster 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 hr. The brain was then removed from the skull and stored 2-3 hr in the same fixative and left overnight in 0.1 M phosphate buffer (pH 7.4) containing 8% sucrose and 0.002% CaCl₂. The SC was removed, mounted onto a chuck, and cut into 50 μ m thick sections with a vibratome.

HRP immunocytochemistry

A monoclonal antibody against calretinin (Chemicon MAB 1568, Temecula, USA) and a polyclonal antibody against GABA (Chemicon AB 131) were used in the present study. The tissue was processed free floating in small vials. For immunocytochemistry, the sections were incubated in 1% sodium borohydride (NaBH₄) for 30 min. Subsequently, these sections were rinsed for 3 x 10 min in 0.25 M Tris buffer, and incubated in 0.25 M Tris buffer with 4% normal serum (normal goat serum for calretinin and normal horse serum for GABA) for 2 hr with 0.5% Triton X-100 added. The sections were then incubated in the primary antiserum in 0.25 M Tris buffer with 4% normal serum for 48 hr with 0.5% Triton X-100 added. The primary antibodies were diluted 1:500-2000 (calretinin) or 1:100-200 (GABA). Following 3 x 10 min rinses in 0.25 M Tris buffer, the sections were incubated in a 1:200 dilution of biotinylated secondary IgG in 0.25 M Tris buffer with 4% normal serum for 2 hr with 0.5% Triton X-100 added. The sections were then rinsed for 3 x 10 min in 0.25 M Tris buffer and incubated in a 1:50 dilution of avidin-biotinylated horseradish peroxidase complex (ABC, Vector lab, Burlingame, USA) in 0.25 M Tris buffer for 2 hr. The

sections were again rinsed in 0.25 M Tris buffer for 3 x 10 min. Finally, the staining was visualized by reaction with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide in 0.25 M Tris buffer for 3-10 min using a DAB reagent set (Kirkegaard & Perry, Gaithersburg, USA). All sections were then rinsed in 0.25 M Tris buffer before mounting. As a control some sections were incubated in the same solution without addition of the primary antibody. Control tissues showed no calretinin or calbindin D28K immunoreactivity. In our recent study in several mammalian retinas[13] and SC[14], this antibody specifically labeled subpopulations of neurons. Following the immunocytochemical procedures, the tissue was mounted on Superfrost Plus slides (Fisher, Pittsburgh, USA) and dried overnight in a 37°C oven. The mounted sections were dehydrated, cleared, and coverslipped. The tissue was examined and photographed with a Zeiss Axioplan microscope, using conventional or differential interference contrast (DIC) optics.

Fluorescence immunocytochemistry

To generate two simultaneous labels, the sections were incubated in the primary antiserum by using the appropriate steps described above. For detection by immunofluorescence, the secondary antibodies were fluorescein conjugated anti-rabbit IgG (Vector Lab.) to detect the anti-calretinin antibody and Cy5 conjugated anti-mouse IgG (Jackson Immuno Research Lab., West Grove, USA) to detect the anti-GABA antibody. Labeled sections were coverslipped with Vectashield mounting medium (Vector Lab.). Images were obtained, and viewed with a Zeiss LSM510 laser scanning confocal microscope.

Quantitative analysis

The morphological types of calretinin-IR cells were estimated on DAB-reacted sections of normal animals. We sampled from five sequential fields, each 310 μm X 310 μm in area, across the intermediate and deep gray layers of calretinin-IR cells of both sides of the SC. Cell types were analyzed from the six (two rostral, two middle, and two caudal SC) best labeled sections from each of two normal animals (total 120 fields). To obtain the best images, we analyzed cells under DIC optics. Only cell profiles containing a nucleus and at least a faintly visible nucleolus were included in this analysis. Since the goal of the present study was to estimate each morphological cell type, no attempt

was made to assess the total cell numbers of each neuronal subpopulation. Double-labeled neurons were also counted from six different sections selected from two different animals. Double-labeled images were obtained on a Bio-Rad MRC 1024 laser scanning confocal microscope using a 40X objective.

Results

Distribution of anti-calretinin immunoreactivity

As previously reported[17], calretinin immunoreactivity consisted of numerous well-labeled fibers in the superficial layers (Fig. 1B). We found striking differences in calretinin immunoreactivity between superficial and deeper layers. Calretinin immunoreactivity in the deeper layers of the hamster SC was selectively distributed. In contrast to the superficial layers, deeper layers contained many calretinin-IR cells. The labeled neurons in the deeper layers were not homogeneously distributed. Some areas contained more labeled neurons than other areas within the deeper layers. They formed two laminar tiers in the SC. The first tier was found within the intermediate gray layer (Figs. 1B, 2). Its thickness was approximately 200-300 μm at the middle level. The second tier of calretinin-IR neurons was found in the deep gray layer (Figs. 1B, 2). The thickness of this tier was similar to that of the first tier. In general, the tier was thicker at the lateral portion than the medial portion both in the intermediate and deep gray layers. These tiers of labeled neurons were found throughout the rostral-caudal extent of the hamster SC.

Morphology of anti-calretinin-IR neurons

Calretinin labeling in the hamster SC was intense in various types of neurons. In the intermediate gray layer, the large majority of anti-calretinin-IR neurons were round or oval cells with many dendrites coursing in all directions. Figure 3A and C show representative small to medium-sized multipolar round or oval neurons. The other types of neurons found in the intermediate gray layer in the present study were stellate, vertical fusiform, and horizontal neurons. Figure 3B shows a horizontal cell that displays a horizontally-oriented small, fusiform cell body and horizontally-oriented processes. Figure 3D shows a cluster of various types of calretinin-IR neurons. Figure 3E shows a vertical fusiform cell that displays a vertical fusiform cell body with a main, long process ascending towards the pial

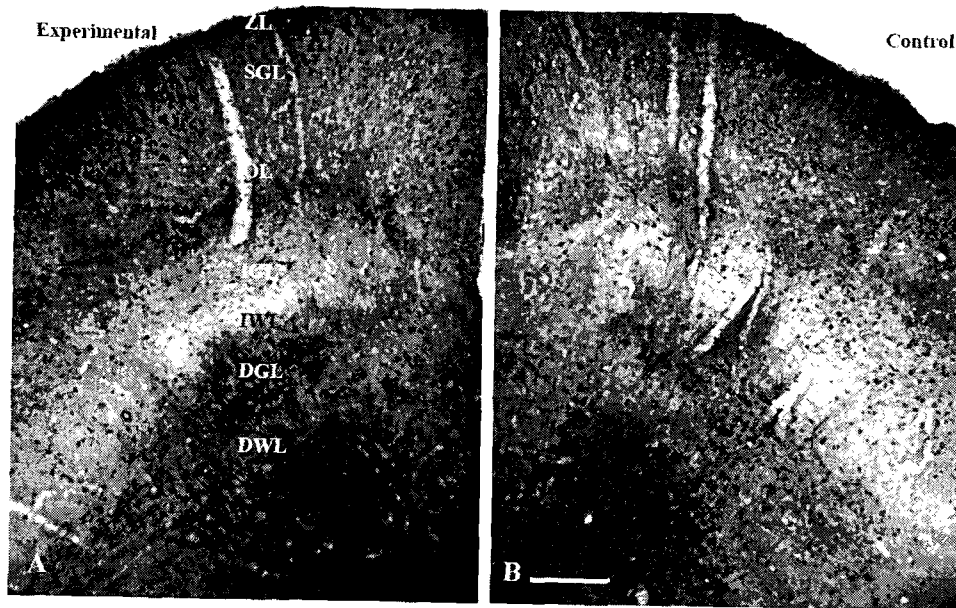


Fig. 1. Calretinin immunoreactivity in the normal control (B) and enucleated experimental (A) hamster SC. Many calretinin-IR neurons were found in the deeper SC. ZL, zonal layer; SGL, superficial gray layer; OL, optic layer; IGL, intermediate gray layer; IWL, intermediate white layer; DGL, deep gray layer. Scale bar = 200 μ m.

surface and a long descending process. Figure 3F shows a stellate cell. Stellate cells had polygonally-shaped cell bodies with numerous dendrites coursing in all directions. Quantitatively, 73.6% of anti-calretinin labeled neurons were round or oval, 12.6% were stellate, 10.9% were vertical fusiform neurons in the intermediate gray layer, and 2.9% were horizontal neurons in the intermediate gray layer (Table 1).

In the deep gray layer, the large majority of anti-calretinin-IR neurons were also round or oval cells. Figures 4A and C show representative small to medium-sized multipolar round or oval neurons. Figure 4B shows a vertical fusiform cell that displays vertical fusiform cell body with a main, long process ascending towards the pial surface and a long descending process. The other types of neurons found in the present study were stellate neurons. Figure 4D and E show medium to large in size stellate cells that have polygonally-shaped cell bodies with numerous den-

drites coursing in all directions. Horizontal cells were not found in the deep gray layer in the present study. Quantitatively, 76.7% of anti-calretinin labeled neurons were round or oval, 11.7% were stellate, and 11.7% were vertical fusiform neurons (Table 1).

Calretinin immunoreactivity after eye enucleation

Marked reduction of calretinin immunoreactivity was previously reported in the superficial SC after monocular enucleation (Fig. 1A)[16]. In contrast to the superficial layers, enucleation had no apparent effect on calretinin immunoreactivity in the intermediate and deep gray layers in the contralateral experimental SC (Fig. 1A). Although we saw occasional variability in immunoreactivity in the contralateral experimental and ipsilateral control SC, there were no consistent differences on labeled neurons on the two sides after monocular enucleation.

Table 1. Distribution of calretinin-IR neurons in the intermediate and deep gray layers of the hamster SC

Morphology	Intermediate gray layer	Deep gray layer
Round or oval neurons	73.56±1.03%	76.69±2.71%
Stellate neurons	12.64±1.75%	11.66±0.98%
Vertical fusiform neurons	10.92±1.60%	11.66±1.17%
Horizontal neurons	2.87±0.75%	0

Numbers are mean ± S.D.

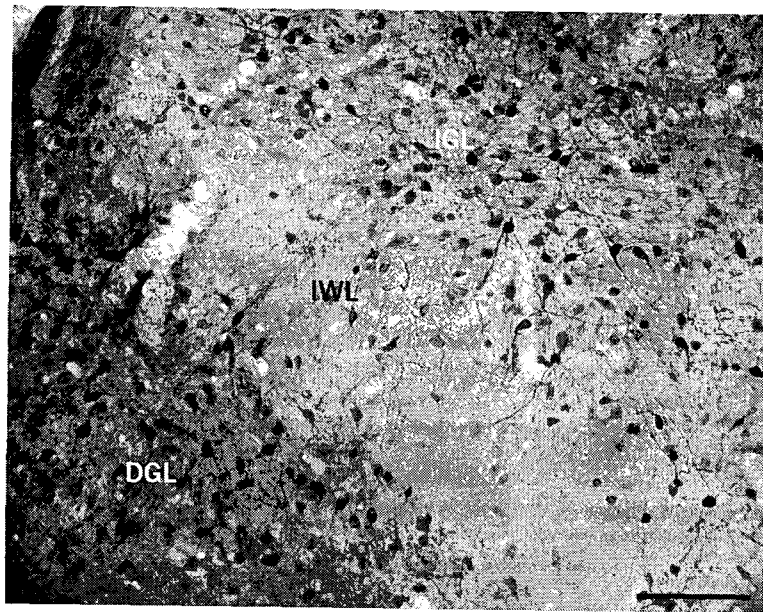


Fig. 2. Distribution of anti-calretinin immunoreactivity in the deeper hamster SC. Anti-calretinin-IR neurons in the deeper layers were concentrated in two layers: one within the intermediate gray layers, and a second within the deep gray layers. IGL, intermediate gray layer; IWL, intermediate white layer; DGL, deep gray layer. Scale bar = 100 μ m.

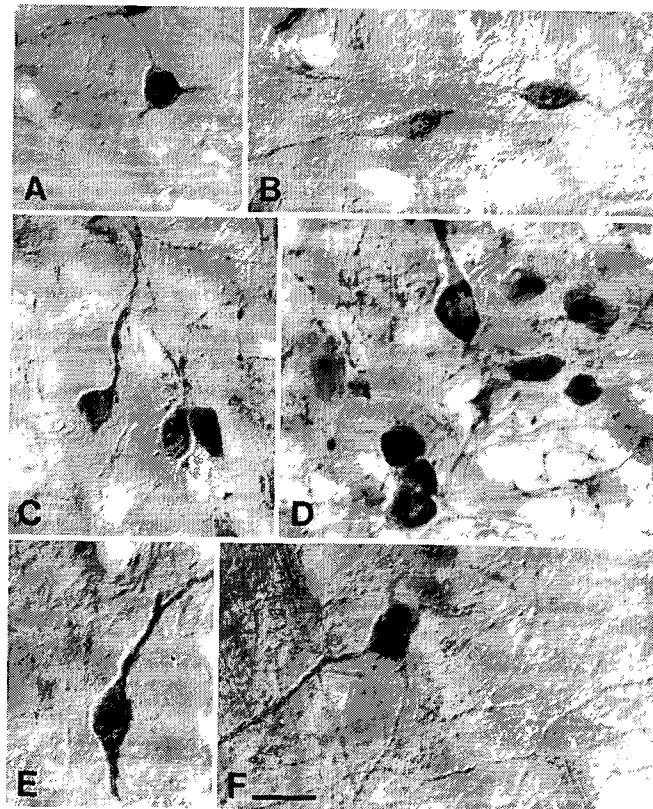


Fig. 3. High-power differential interference contrast (DIC) photomicrographs of some calretinin-IR cells in the intermediate gray layer of hamster SC. (A, C) Multipolar round or oval neurons. The large majority of anti-calretinin-IR neurons were round or oval cells with many dendrites coursing in all directions. (B) Horizontal neuron with a horizontal fusiform cell body with horizontally oriented processes. (D) Clusters of anti-calretinin-IR neurons in the intermediate gray layer. Various types of cells can be identified from this figure. (E) Vertical fusiform cell with its longitudinal axis perpendicular to the pial surface. (F) A stellate multipolar cell. Scale bar = 20 μ m.

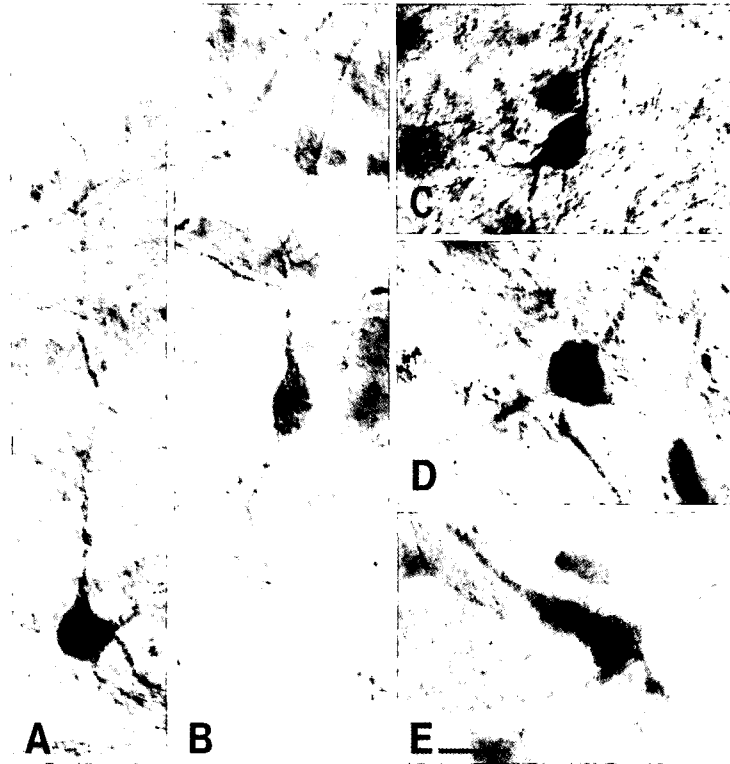


Fig. 4. High-power differential interference contrast (DIC) photomicrographs of some calretinin-IR cells in the deep gray layer of hamster SC. (A, C) Multipolar round or oval neurons. The large majority of anti-calretinin-IR neurons were round or oval cells with many dendrites coursing in all directions. (B) Vertical fusiform cell with its longitudinal axis perpendicular to the pial surface. (D, E) Stellate multipolar cells. Scale bar = 20 μ m.

Co-localization of calretinin and GABA

To determine whether calretinin-IR cells co-localize with GABA, we labeled calretinin with fluorescein and GABA with Cy5. We checked more than 500 calretinin-IR neurons within the intermediate and deep layers from six sections of two animals. Although some neurons with GABA or calretinin were similar in size, shape, and manner of distribution in the intermediate and deep layers, no neurons (0 of more than 500 neurons or 0%) were labeled with both anti-calretinin and anti-GABA antibodies (Fig. 5).

Discussion

The present results demonstrate that calretinin is contained in many neurons in the hamster SC. Calretinin is very much segregated in the deeper layers and occupies specific areas. Calretinin immunoreactivity is not changed by monocular enucleation. Finally, in the deeper hamster SC calretinin-containing neurons are not GABAergic interneurons.

The topographical distribution of SC afferents and effer-

ents such as laminar segregations, clusters, or domain organizations has been associated with functionally separate pathways in the visual system. Patchy ipsilateral retinotectal projection has been first described to describe one of the principal organizing features[8]. The present results demonstrate that anti-calretinin immunoreactivity is very selectively distributed in the hamster SC. In contrast to the superficial layers where the calretinin immunoreactivity consisted of numerous well labeled fibers[17], calretinin-IR neurons were segregated into two tiers, one in the intermediate gray and the other in the deep gray layer. So far, the laminar distribution of calretinin-IR neurons in the deeper layers has only been briefly described in human SC. Scattered cells were widely distributed in the deeper layers[18]. The distribution pattern of calretinin-IR cells in deeper hamster SC is somewhat different from that in humans. The patterned distribution was also different from that of mouse SC[15]. In the mouse SC, calretinin-IR cells in the intermediate gray layer formed clusters while the calretinin-IR cells in the hamster SC formed continuous band. The functional implication of species differences and

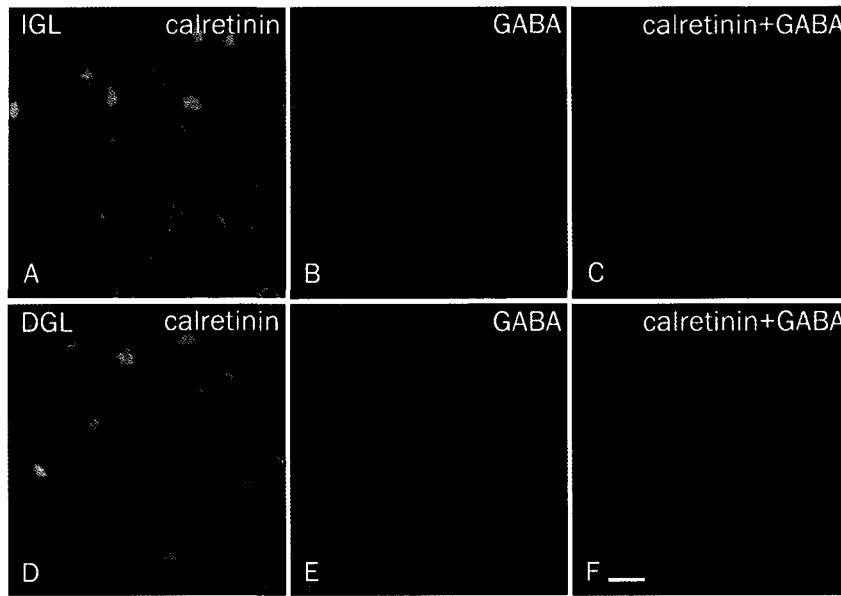


Fig. 5. Fluorescence confocal photomicrographs of intermediate gray (A-C) and deep gray layers (D-F) hamster SC immunostained for (A, D) calretinin and (B, E) GABA. (C, F) Superimposition of images in calretinin and GABA. Calretinin or GABA are expressed in different sets of neurons both in the intermediate and deep gray layers. IGL, intermediate gray layer; DGL, deep gray layer. Scale bar = 20 μ m.

similarities in calretinin expression in the mammalian SC is not clear yet.

However, the present data of distinctive laminar organization of calretinin-IR neurons in the deeper layers provide further evidence that the spatial distribution of calretinin-IR neurons may be correlated with the internal modular organization of multisensory areas.

The patterned distribution of calbindin D28K and parvalbumin, the two most important neuronal calcium-binding proteins, has been described in the mammalian SC. In the cat and hamster deeper SC, calbindin D28K is located in the upper intermediate gray and deep gray layers[3,20]. Parvalbumin did not form distinctive laminar pattern in the deeper SC. Rather, scattered parvalbumin-IR neurons were found in the deeper layers[4,21]. Thus, the present and the previous result support the notion that calretinin-IR neurons in different compartments from calbindin D28K and parvalbumin in deeper SC are associated with functionally different pathways.

In contrast to the superficial layers, calretinin-IR neurons in the deeper layers had varied morphology. The majority of the labeled neurons both in the intermediate and deep gray layers were small to medium-sized round or oval neurons. However, many medium to large sized stellate and vertical fusiform neurons that were not found in the

superficial layers were also labeled. The diversity of labeled neurons in the deeper layers reflects the functional diversity. In the human deeper SC, many pyramidal neurons expressed calretinin[18]. However, in the present study no calretinin-IR pyramidal neurons were found in the hamster SC. Beside, no neurons showed varicose dendrites that were found in calretinin-IR neurons in the deeper mouse SC[13]. These differences reflect functional variations of calretinin-expressing cells among different animal species and the variety of animal kingdom.

Previous studies of the several mammalian SC showed a new appearance of calretinin-immunoreactive neurons in the superficial SC after enucleation[1,11,17]. This suggests that genes for calretinin in some neurons in the superficial layers may not work in the normal state but may work in different situations. However, in the present study monocular enucleation appeared to have no effect on the distribution of calretinin-IR neurons in the deeper layer. Taken together the present and the previous results, thus, indicate that the expression of calretinin genes might be activity-dependent in the superficial layers while the expression of calretinin genes might be independent of retinal inputs in the deeper SC. Similar to the present results, there was no reduction of calbindin D28K in the monkey and rat SC after monocular enucleation.

In the present study of the deeper hamster SC, no neurons were immunoreactive for both calretinin and GABA. The results in the deeper layers were similar to those of superficial layers. No neurons were immunoreactive for both calretinin and GABA in the superficial hamster SC[16]. Similar to the present results, no neurons were immunoreactive for both calbindin D28K and GABA[3]. Thus, the present and the previous results of calbindin D28K demonstrate that calretinin and calbindin D28K expressing neurons both in the superficial and deeper layers of the hamster SC are not GABAergic interneurons. The co-localization pattern of calretinin and GABA in the hamster SC is substantially different from many other brain areas studied to date. In many other brain areas, many calretinin-IR neurons also contained GABA. For example, in the rat and monkey visual cortex[6,19], more than 90% of calretinin-positive neurons were GABAergic interneurons. The functional significance of the different co-localization patterns of calretinin and GABA in different brain areas is not yet clear. However, it appears that the subpopulations of calretinin expressing neurons in different brain areas are able to deal with different functional contents.

No report has been published so far in any mammalian SC confirming whether calretinin-IR neurons in the SC are projection neurons or not. The heterogeneity of calretinin-IR cells in the deeper layers in the present study suggests that part of the calretinin-IR cells are projection neurons. In cat SC, most calbindin D28K-IR neurons were interneurons while most of the parvalbumin-IR neurons were projection neurons[20,21]. Backfilling of deeper hamster SC neurons with tracer and co-localization with calretinin-IR cells needs to be done to determine whether calretinin-IR cells were projection neurons.

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 though its capacity of calcium buffering and transport[22]. Neurons in the deeper SC play a role in integrating sensory information into motor signals that help orient the head toward various stimuli and play an important role in saccadic eye movements. Thus, it is suggested that calretinin in neurons in the deeper SC would seem to deal with integration of multiple inputs in fast jumps from one eye to another position involving in sharpening the timing of action potentials. However, the function of calretinin is far from obvious yet, and will be the subject of our future

research.

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초록 : 햄스터 상구의 deeper layers에서 calretinin이 함유 신경세포

김예은¹ · 최재식¹ · 김혜현^{1,2} · 여지연¹ · 전창진*

(¹경북대학교 자연과학대학 생물학과, ²경북대학교 생물건강 농업생명 융합형 인재양성 사업단)

칼슘 결합 단백질 calretinin은 칼슘의 완충작용에 중요한 역할을 한다고 알려져 있다. 최근에 우리는 햄스터 상구의 superficial layer에서 calretinin과 면역반응(immunoreactive)을 일으키는 신경세포의 형태, 분포와 안구적출 후 calretinin 면역반응의 영향에 대해 보고한 바 있다. 본 연구에서는, 상구의 deeper layer에서 면역세포화학 방법을 이용하여 면역표지 된 세포의 분포와 유형 그리고 안구적출 후 변화의 양상을 기술한다. 또한 중추 신경계에서 주요 억제성 신경전달물질인 GABA를 사용하여 calretinin 면역표지 된 세포와 비교하였다. Superficial layer와 비교하여, deeper layer는 calretinin 면역 반응을 일으키는 많은 신경세포들이 분포한다. 이 신경세포들은 두 층을 형성하며, 그 중 첫 번째 층은 intermediate gray layer에서 뚜렷한 층 구조를 나타내었다. 두 번째 층은 deep gray layer에서 발견되었다. 면역표지 된 신경세포는 형태학적으로 매우 다양하며, 수직 방추모양, 성장, 둥근/타원형 그리고 수평 신경세포를 포함한다. Superficial layer와 비교하여, 안구적출은 deeper layer에서 calretinin 면역반응의 분포에 영향이 없는 것으로 보여진다. 두 가지 색을 이용한 면역 형광법은 calretinin 면역반응 신경세포들이 하나도 GABA항체와 함께 표지 되지 않는 것을 보여준다. 본 연구 결과는 햄스터 상구에서 calretinin 함유 신경세포는 특이한 sublaminar 구조를 이루고 있는 것을 보여준다. 본 연구 결과는 또한 햄스터 상구에서 calretinin 면역 반응 신경세포들은 GABAergic interneuron이 하나도 없는 것을 증명한다. 많은 calretinin 면역 반응 세포들은 대부분 뇌의 다른 부분에서는 GABAergic interneuron 인데 비해, 햄스터 상구에서의 본 연구 현상은 예외적이다.