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Neuronal Nitric Oxide Synthase-Immunoreactive Neurons in the Hamster Visual Cortex: Lack of Co-localization with Parvalbumin

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Nitric oxide (NO) and calcium-binding proteins occur in various types of cells in the central nervous system. They are important signaling and calcium buffering molecules, respectively. In the present study, using immunocytochemistry we examined the distribution and the co-localization pattern of neurons containing neuronal nitric oxide synthase (nNOS) and parvalbumin in the visual cortex of hamster. The overall number of parvalbumin-immunoreactive (IR) neurons was 17 times higher than that of the nNOS-IR neurons in the hamster visual cortex. The highest differences were found in layer V, where parvalbumin-IR neurons were 54.7 times more abundant than nNOS-IR neurons. Many nNOS- and parvalbumin-IR neurons were similar in size, shape, and manner of distribution in the visual cortex. However, two-color immunofluorescence revealed that no neurons in the hamster visual cortex expressed both nNOS and parvalbumin. The present results indicate that there are subtle species differences in the co-localization pattern between nNOS and calcium-binding proteins. The present results also suggest not only the heterogeneity and functional diversity of nNOS-IR neurons in the visual cortex, but also the importance of understanding animal diversity

Key words – Nitric oxide, parvalbumin, distribution, cell type, double-labeling

Numerous studies have focused on the anatomical and physiological organizations of the visual cortex since Hubel and Wiesel's momentous works[24]. The visual cortex contains a precise, horizontally-arranged, six major laminar structure (layers I to VI) of afferents and efferents, along with vertically-oriented columns forming a precise organization into a functional cortical module. One of the key aspects in understanding the visual cortical function lies in understanding the neurochemical organization of its various neurons. Various types of neurotransmitters, receptors, and neuromodulators have been extensively visualized in the visual cortex through the use of antibodies[3,8,20,25].

Nitric oxide is a gaseous signaling molecule that acts as a neurotransmitter in the nervous system. It is produced by a group of enzymes called nitric oxide synthases (NOS). There are three isoforms of NOS: neuronal NOS (nNOS), endothelial NOS, and inducible NOS. nNOS is associated with post-synaptic density protein (PSD-95) in the neuronal membrane and plays many important biological roles including synaptic modulation, neuronal development, learning and memory, and neuronal death[9,31]. In response to increased intracellular calcium, nNOS interacts with calmodulin, a calcium-binding protein that can bind to and regulate a multitude of different protein targets[9,31]. The three 'EF-hand' family calcium-binding proteins - calbindin-D28K, calretinin, and parvalbumin - have been abundantly found in various types of neurons in the central nervous system [2,18,42,44]. Among these three calcium-binding proteins, parvalbumin is a calcium binding protein expressed in specific fast-firing neurons. It consists of a single, unbranched chain of linked amino acids and has a molecular weight near 12,000 daltons, and is water-soluble[18]. Parvalbumin has been proved to be particularly useful in identifying distinct subpopulations of neurons. It may protect neurons from calcium-induced cell damage. However, the physiological role of parvalbumin is not yet clear.

Neurons expressing NO have been studied in the visual cortex of human [38], monkey[1,43,47], hamster[32,48], and rat[7,14,51]. Several calcium-binding proteins have been localized in the visual cortex of a number of mammals including humans[5,12,21,34,35,36], monkeys[5,11,13,17,19, 21,39,49,50], dolphins[12,13], cats[10,22,27,46], flying foxes [26], hamster[32,40], and rats[6,14,15,16,37].

The relationship between NO and calcium-binding proteins has been extensively studied[4,14,29,45]. Our previous stu-

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dies showed many nNOS-containing neurons in the hamster visual cortex also contained calbindin D28K or calretinin [32,40]. It is not known, however, whether nNOS-containing neurons also contain parvalbumin in the hamster visual cortex. Thus, we first investigated the features of the co-localization patterns of the nNOS with parvalbumin. Our previous research showed that there are some nNOS-containing neurons that also contain parvalbumin in the mouse[33]. However, the features of the co-localization patterns of the nNOS with parvalbumin were strikingly different between the mouse and rabbit. None of the nNOS-containing neurons contained parvalbumin in the rabbit visual cortex. Thus in this study we wanted to provide data on any species differences with regards to the distribution of nNOS and calcium-binding proteins. Understanding those differences is very important in understanding species diversity.

Materials and Methods

Perfusion and tissue processing

Twelve adult hamsters were anesthetized deeply with a mixture of ketamine hydrochloride (30-40 mg/kg) and xylazine (3-6 mg/kg) before perfusion. All hamsters were perfused transcardially 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 15 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 for 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% $CaCl_2$. The visual cortex was removed, mounted onto a chuck, and cut into 50 μm thick sections with a vibratome.

Horseradish peroxidase (HRP) immunocytochemistry

A monoclonal antibody against parvalbumin was obtained from Sigma Chemical (St. Louis, USA). A polyclonal antibody against nNOS was obtained from BD Biosciences (Franklin Lakes, USA). The tissue was processed free floating in small vials at 25℃ with gentle agitation. The primary antiserum was diluted 1:1000 (parvalbumin) or 1:500-1000 (nNOS). The immunocytochemical methods have been described in detail in our previous reports[23,28,30,32]. The tissue was examined and photographed on a Zeiss Axioplan microscope using conventional or differential interference contrast (DIC) optics.

Fluorescence immunocytochemistry

To double-label sections for both nNOS and parvalbumin, sections were incubated in the primary antiserum using the steps described above. For detection by immunofluorescence, the secondary antibodies were fluorescein (FITC) conjugated anti-mouse or rabbit IgG (Vector Lab., Burlingame, USA) for detecting the anti-parvalbumin antibody, and Cy5 conjugated anti-mouse (Jackson Immuo-Research Lab., West Grove, USA) for detecting the anti-nNOS antibody. Labeled sections were coverslipped with a Vectorshield mounting medium (Vector Lab., Burlingame, USA). Images were obtained on a Bio-Rad MRC 1024 laser scanning confocal microscope.

Quantitative analysis

nNOS- and parvalbumin-IR neurons were plotted in three animals with the aid of a Zeiss drawing tube attached to a Zeiss Axioplan microscope with a 20X objective. We sampled from nine different fields, each 1200 μm in width, from three different animals. The number of labeled neurons was expressed either as a percentage of the total population of labeled neurons or raw numbers for comparison between different cortical layers. Double-labeled neurons were counted from nine different sections, 1200 μm in width, and selected from three different animals across all layers. Double-labeled images were obtained on a Bio-Rad MRC 1024 laser scanning confocal microscope using a 20X objective. The morphological types of nNOS-IR cells were analyzed using 3,3'-diaminobenzidine (DAB)-reacted sections. All analyses were done with a 40X Zeiss Plan-Apochromat objective. We sampled from nine tissue sections, each 1200 μm in width, from each of three animals across all layers. 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 obtain an estimate of each morphological cell type, no attempt was made to assess the total cell numbers of each neuronal subpopulation.

Results

Distribution of nNOS and parvalbumin immunoreactivity

Fig. 1B and 1C show nNOS and parvalbumin immunoreactivity in the hamster visual cortex, respectively, while Fig. 1A shows thionin-stained section for cortical lamination. In the present study we presented raw numbers of IR-neurons for direct comparison between nNOS-IR neurons and parvalbumin-IR neurons, while our previous study only showed the relative percentage frequency in each layer. The frequency of the labeled neurons varied in each layer (Fig. 2). The densest concentration of nNOS-IR neurons was found within layer VI. Thus, 0.11±0.33 (mean ±S.D.) (0.70%) of the labeled neurons were found in layer I, 0.88±0.93 (5.8%) in layers II/III, 1.11±0.60 (7.1%) in layer IV, 5.44±2.30 (34.9%) in layer V, and 8.00±1.73 (51.5%) in layer VI (Table 1). As previously reported[40] parvalbumin was present in many neurons within the adult hamster visual cortex (Fig. 2). The frequency of the labeled neurons varied in each layer. The densest concentration of the parvalbumin- IR neurons was found within layer V. Thus, 0.22±0.44 (mean ±S.D.) (0.07%) of the labeled neurons were found in layer I, 41.00±11.48 (15.83%) in layers II/III, 60.78±9.89 (23.50%) in layer IV, 111.33±17.07 (42.99%) in layer V, and 45.56± 13.16 (17.59%) in layer VI (Table 1). The overall number of parvalbumin-IR neurons was about 17 times higher than that of the nNOS-IR neurons in the hamster visual cortex. The greatest differences were found in layer V. Thus, the number of parvalbumin-IR neurons was 2.00 times higher in layer I, 46.13 times in layers II/III, 54.70 times in layer IV, 20.45 times in layer V, and 5.69 times in layer VI.

Morphology of nNOS- and parvalbumin-IR neurons

The morphology nNOS-IR neurons in the hamster visual

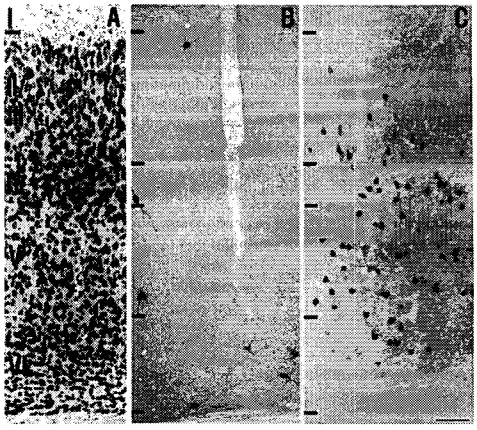

Fig. 1. Low power photomicrographs of the distribution of nNOS- and parvalbumin-IR neurons in the hamster visual cortex. (A) Thionin-stained section shows the cortical lamination. (B) nNOS-IR neurons. (C) Parvalbumin-IR neurons. Scale bar = 100 μm.

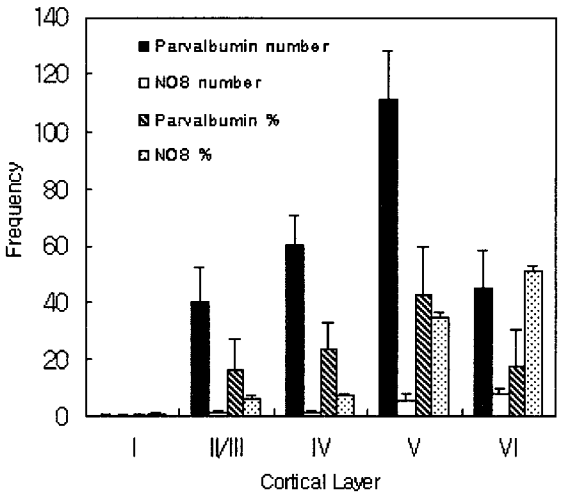

Fig 2. Histogram showing distribution of nNOS- and parvalbumin-IR neurons in the hamster visual cortex. The highest concentration of intense nNOS-IR neurons was located in layer VI, while the parvalbumin-IR neurons were predominantly located in layer V.

Table 1. Distribution of parvalbumin- and nNOS-IR neurons in the hamster visual cortex

	Animal no.	NO. sections	Number of cells in each layer					
			I	II/III	IV	V	VI	Total
PV	1	3	1	120	195	323	134	773
	2	3	0	95	168	311	97	671
	3	3	1	154	184	368	179	886
	PV total	9	2	369	547	1002	410	2330
	(mean±S.D.)		0.22±0.44	41.00±11.48	60.78±9.89	111.33±17.07	45.56±13.16	
nNOS	1	3	1	2	4	15	23	45
	2	3	0	2	2	11	23	37
	3	3	0	4	4	23	26	58
	nNOS total	9	1	8	10	49	72	140
	(mean±S.D.)		0.11±0.33	0.88±0.93	1.11±0.60	5.44±2.30	8.00±1.73	

PV=parvalbumin

cortex has been described in our previous study[32]. In the present study we present the percentage of each type of cells and some representative nNOS-IR neurons for comparison with parvalbumin-IR neurons. Fig. 3A shows nNOS-IR round or oval neurons with many dendrites coursing in all directions found predominantly in layers IV, V, and VI. The large majority (61.45±2.55%) of nNOS-IR neurons were round or oval cells (Table 2). Fig. 3A (arrow) and 3C show vertical fusiform neurons (27.71±1.94%). These neurons had a medium (10~15 μm in diameter), vertical fusiform cell body with a main, long process (Fig. 3A arrows) ascending towards the pial surface, and a long descending process. The other types of neurons found in the present study were stellate (6.02±0.73%) and horizontal (4.82±0.73%) neurons. Stellate cells had polygonally-shaped cell bodies with numerous dendrites coursing in all directions (Fig. 3B). They were typically medium to large in size (>15 μm in diameter). Fig. 3D shows a horizontal neuron. The horizontal cells display horizontally oriented processes with horizontally oriented small, fusiform cell bodies. Horizontal cells were rarely encountered in the present study (Table 2).

Table 2 shows the percentage of parvalbumin-IR neurons in the hamster visual cortex. Fig. 4A shows representative medium round to oval (48.44±3.35%) multipolar neurons found in layer VI, while Fig. 4B shows representative medium to large-sized stellate (19.27±1.17%) multipolar neurons found in layer V. These round or oval and stellate multipolar neurons were also found all other layers except layer I. The other types of neurons found in the present study were vertical fusiform (22.4±1.27%) (Fig. 4C arrows) and pyriform (4.69±3.38%) (Fig. 4D) neurons. These cells had vertical fusiform or pyriform cell bodies with a thick, proximal dendrite directed towards the pial surface. Fig. 4E shows a horizontal cell (5.21±0.71%) that displays horizontally oriented dendrites with horizontally oriented fusiform cell body. Thus, many parvalbumin-IR neurons were similar to nNOS-IR neurons in morphology.

Co-localization of nNOS and parvalbumin in the visual cortex

To determine whether the nNOS-IR cells in the visual

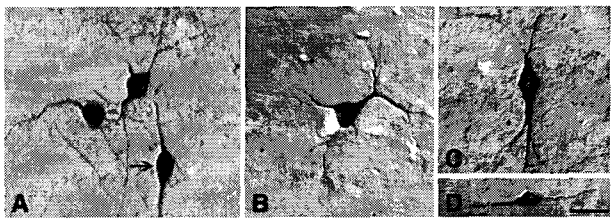

Fig 3. High-power DIC photomicrographs of some nNOS-IR cells in the hamster visual cortex. (A) Multipolar round or oval and vertical fusiform (arrow) neurons. The large majority of nNOS-IR neurons were round or oval cells with many dendrites coursing in all directions. (B) Medium-sized multipolar stellate neurons. (C) Vertical fusiform cell with its longitudinal axis perpendicular to the pial surface (D) Horizontal neuron with a horizontal fusiform cell body with horizontally oriented processes. Scale bar = 20 μm.

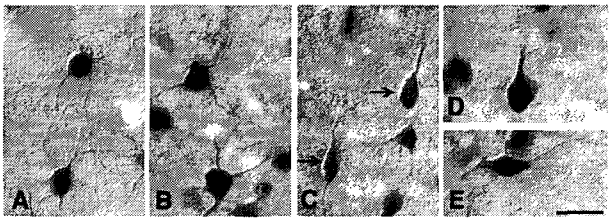

Fig 4. High-power DIC photomicrographs of some parvalbumin-IR neurons in the hamster visual cortex. (A) Multipolar round or oval neurons. The large majority of parvalbumin-IR neurons were round or oval cells with many dendrites coursing in all directions. (B) Medium to large-sized multipolar stellate neurons. Stellate neurons had polygonally-shaped cell bodies with numerous dendrites coursing in all directions. (C) Vertical fusiform cells (arrows) with their longitudinal axes perpendicular to the pial surface. (D) Pyriform cell with a thick primary dendrite oriented toward the pial surface. (E) A horizontal cell with a horizontal fusiform cell body with horizontally oriented processes. The horizontal type of neurons was rarely found. Scale bar = 20 μm.

Table 2. Distribution of the NOS- and parvalbumin-IR neurons in the hamster visual cortex

Morphology	NOS-IR neurons	Parvalbumin-IR neurons
Round or oval neurons	61.45±2.55%	48.44±3.35%
Vertical fusiform neurons	27.71±1.94%	22.4±1.27%
Stellate neurons	6.02±0.73%	19.27±1.17%
Horizontal neurons	4.82±0.73%	5.21±0.71%
Pyriform neurons	0	4.69±3.38%

Numbers are mean±S.D.

cortex co-localize with parvalbumin, we labeled nNOS with Cy5, and labeled parvalbumin with fluorescein. We counted the numbers of nNOS-IR neurons and double-labeled cells within a 1200 μm wide area, and selected from three different animals across all layers. Although many neurons with nNOS or parvalbumin were similar in size, shape, and manner of the distribution in the visual cortex, no neurons were labeled with both nNOS and parvalbumin antibodies (Fig. 5).

Discussion

Our results indicate that there are similarities in the morphology of the nNOS-IR with parvalbumin-IR neurons in the hamster visual cortex. However, no neurons in the hamster visual cortex expressed both nNOS and parvalbumin.

Our present study showed that the overall number of parvalbumin-IR neurons was 17 times higher than that of the nNOS-IR neurons. This distributional phenomenon is very similar to other calcium-binding proteins. Although no quantification data is available to date, the number of other EF-hand calcium-binding proteins calretinin- and calbindin D28K-IR is much higher than that of nNOS-IR neurons in the hamster visual cortex[32]. Previous studies in mouse and rabbit also showed that there are many more parvalbumin-IR neurons than nNOS-IR neurons[33,40]. These results may indicate that calcium-binding proteins are more actively involved than NO in the visual perception processing in the visual cortex.

The laminar position of cells containing nNOS and parvalbumin varies. The highest density of nNOS-IR neurons is located in layer VI in the hamster visual cortex. The expressional pattern of nNOS in the hamster visual cortex is similar to that of the monkey[1] and mouse and rabbit[33].

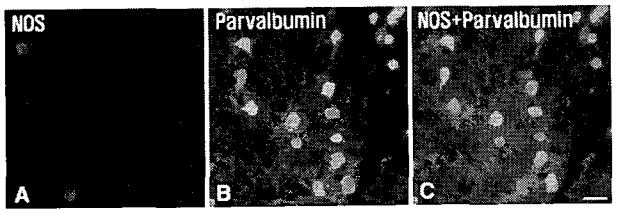

Fig 5. Fluorescence confocal photomicrographs of the hamster visual cortex immuno-labeled for nNOS (A) and parvalbumin (B) and superimposition of images in nNOS and parvalbumin (C). None of the nNOS-IR cells were double-labeled with parvalbumin. Scale bar = 20 μm.

However, the highest density of the parvalbumin-IR neurons was located in layer V. The results demonstrated in hamster are very similar to those found in rat[14] and mouse[40]. In contrast to parvalbumin, the highest number of calbindin D28K-IR neurons was located in layers V, while the highest number of calretinin-IR neurons were located in layers II/III in hamster visual cortex[32]. The expressional pattern of calbindin D28K and calretinin in the hamster visual cortex is similar to that of other rodents such as rat[14] and mouse [41]. These results indicate that the laminar expressional pattern of the highest density of three major EF-hand calcium-binding proteins, calbindin D28K, calretinin, and parvalbumin, does not overlap with that of nNOS. Although the functional significance of different laminar distributions and the expressional pattern of nNOS and parvalbumin along with calbindin D28K and calretinin in the hamster visual cortex is not yet completely understood, different calcium-binding proteins may have some subtle different calcium buffering action in different cortical layers.

The nNOS-IR neurons do not contain parvalbumin. nNOS- and parvalbumin-IR neurons had round or oval, stellate, vertical fusiform, or horizontal cell bodies with multipolar or bitufted dendritic arrangements. Although our current study clearly showed that the types of nNOS-IR neurons and cells labeled for parvalbumin were very similar, no co-localization phenomenon is obvious. By contrast, the percentages of nNOS-IR neurons containing calbindin D28K (14.7%) and calretinin (27.5%) were moderate in our previous study in hamster visual cortex[32]. In contrast to hamster, none of the nNOS-IR neurons co-expressed calretinin or parvalbumin, while only approximately 1% of the nNOS-IR neurons co-expressed calbindin D28K in the rat visual cortex [14]. However, 16.7% of the nNOS-IR cells were double-labeled with calbindin D28K while more than half of the nNOS-IR cells were double-labeled with calretinin in the mouse visual cortex. One forth of the nNOS-IR cells were double-labeled with parvalbumin in the mouse visual cortex, too. In the rabbit visual cortex, more than 90% of the nNOS-IR neurons contained calbindin, while only 2.5% of nNOS-IR neurons contained calretinin. Similar to the hamster visual cortex, none of the nNOS-IR neurons contained parvalbumin in the rabbit visual cortex[33]. These results may indicate that there are subtle species differences in the co-localization pattern between nNOS and calcium-binding proteins. These results also suggest not only the heterogeneity and functional diversity of nNOS-IR neurons in the visual cortex, but also

the importance of understanding animal diversity.

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초록 : 햄스터 시각피질에서 Neuronal nitric oxide synthase-면역반응성 뉴런: parvalbumin과의 co-localization 부재

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산화질소(NO)와 칼슘 결합 단백질은 중추신경계의 다양한 세포들에서 나타나며, 이들은 각각 중요 신호전달 분자와 칼슘 완충 분자이다. 본 연구는 햄스터의 시각피질에서 뇌산화질소 합성효소 (nNOS)와 parvalbumin을 포함하는 뉴런들의 분포와 이들의 co-localization 양상을 면역세포화학적 기법을 이용하여 알아보았다. 햄스터 시각피질에서 parvalbumin에 대한 면역 반응성을 나타내는 뉴런들의 전체 수는 nNOS에 대한 면역 반응성을 보이는 뉴런들의 수보다 17배나 많았다. 가장 큰 차이는 시각피질 제5층에서 발견되었으며, 이곳에서 parvalbumin-면역 반응성 뉴런이 nNOS-면역 반응성 뉴런들의 수보다 54.7배나 높았다. nNOS- 또는 parvalbumin-면역 반응성 뉴런들은 크기와 형태, 분포 방식이 시각피질에서 유사하게 나타났다. 그러나 이색 면역형광 기법은 햄스터 시각피질에서 nNOS와 parvalbumin을 모두 발현하는 뉴런은 없음을 보여주었다. 본 연구의 결과는 nNOS와 칼슘 결합 단백질 사이의 co-localization 양상이 종간에 차이가 존재함을 나타내며 또한 시각피질에 있는 nNOS-면역 반응성 뉴런들의 다양성과 이질성뿐만 아니라 동물 다양성 이해의 중요성을 함께 제시한다고 볼 수 있다.