A classification of NOergic neurons in the inferior colliculus of rat according to co-existence with classical amino acid transmitters

By

Men-dar WU¹, Masaru KIMURA²,
Ishigami HIROMICHI³ and Robert H. HELFERT⁴

¹Research Complex for the Medicine Frontiers, ²Department of Anatomy

³Department of Otorhinolaryngology, Aichi Medical University School of Medicine

⁴Department of Surgery, Southern Illinois University School of Medicine

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Summary: Since the localization of nitric oxide synthase (NOS) can be identified by enzyme histochemistry for NADPH-diaphorase (NADPH-d), this method has been used widely for mapping NOS-containing (presumably NOergic) neurons in the central nervous system. So far several studies suggest that NADPH-d is present in distinct neuronal populations in the inferior colliculus (IC), a major processing center for both the ascending and descending auditory pathway, and NO may play an important role in audition. On one hand, there is evidence from several lines of research that the IC makes extensive use of the neuroactive amino acids, in particular the inhibitory transmitter γ -aminobutyric acid (GABA) and the excitatory amino acid glutamate (GLU). However, lacking is a description of the distribution of NOergic neurons to which traditional neurotransmitters may be linked. The present research utilized NADPH-d enzyme histochemistry in combination with immunocytochemistry to determine if NO may colocalize with either or both GABA and glutamate in distinct subpopulations of IC neurons.

The NADPH-d positive neurons were predominantly found in two main subdivisions of the IC: the external cortex (ECIC) and the dorsal cortex (DCIC). The large numbers of these NADPH-d positive neurons appeared immunostained for GLU while only a small number, seemed to belong to the small cells (somatic area $< 100 \mu\text{m}^2$) similarity to stellate cells group was positive for GABA throughout the cortex of the IC. Owing to no coexistence between GABA and GLU in the same NADPH-d positive neuron in the pairs of adjacent sections of the IC by the mirror-image technique, the present results consequently support that NOergic neurons could be subdivided into at least three distinct populations with a large proportion of about 77% being GLUergic, much lower frequency of about 11% being GABAergic and the remaining 12% expressing non-GABA and non-GLU.

In summary, the existence of two functionally distinct populations of NO/GABAergic and NO/GLUergic neurons in the NOergic neurons of IC suggest that at least two differential pattern of GLU-mediated excitatory NO transmission and GABA-mediated inhibitory NO transmission are involved in the networking of auditory communication in the cortex of IC.

Introduction

Since the first report suggesting its involvement in signal transduction in the brain¹⁵⁾, nitric oxide (NO) has been established as a neuroactive substance with a biochemical mechanism of action radically different from that of traditional neurotransmitters. Nitric oxide is an inorganic, gaseous free radical that mediates a variety of functions, including vasodilatation, neurotransmission and cytotoxicity. The nitric oxide synthase (NOS) fam-

ily catalyzes the oxidation of arginine to form L-citrulline and NO. Each NOS isoform possesses regions for the binding of calmodulin and reduced nicotinic-adenine-nucleotide phosphate (NADPH). NOS can be identified by histochemical staining for NADPH diaphorase (NADPH-d)^{3, 9)}. Indeed, the above approach has been used widely for mapping NOS-containing (presumably NOergic) neurons in the central nervous system^{3, 38)}. NADPH-d histochemistry has also been combined with immunohistochemistry to assess relationships between the distribu-

Corresponding author: Men-dar Wu, MD, Research Complex for the Medicine Frontiers, Aichi Medical University School of Medicine, 21 Karimata, Yazako, Nagakute-Cho, Aichi, 480-1195 Japan, Email: mendwugo@aichi-med-u.ac.jp

tion of NO and traditional neurotransmitters in various regions of the CNS^{1, 14, 30, 35, 37}.

Several studies suggest that NO and NOS play a role in audition. Nitric oxide synthase (not differentiated by isoform) was observed in cells and great numbers of synaptic boutons in nuclei of the lower auditory brainstem²⁰ and inferior colliculus (IC)⁴. In the cochlea, NOS expression has been described in the spiral ganglion and organ of Corti¹⁸. In mice, immunostaining for NOS increases with age in the spiral ganglion⁴⁰, suggesting that there is an elevation in NO synthesis in the primary afferent auditory neurons of aged animals.

The IC is a major processing center for both the ascending and descending auditory pathway. In rats, glucose utilization measurements indicate that the IC is one of the most metabolically active areas in the brain³⁴. Several studies suggest that NADPH-d is present in distinct neuronal populations in the IC^{8, 38}. However, lacking is a description of the distribution of NOergic neurons to which traditional neurotransmitters may be linked.

Despite the fact that the functional role of some neurotransmitters and neuromodulators in the IC is still a matter of dispute, there is evidence from several lines of research that the IC makes extensive use of the neuroactive amino acids, in particular the inhibitory transmitter γ -aminobutyric acid (GABA) and the excitatory amino acid glutamate (GLU)^{2, 10, 19, 22, 23, 26}. The present research utilized NADPH-d histochemistry in combination with immunocytochemistry to determine if NO may colocalize with either or both GABA and GLU in distinct subpopulations of IC neurons.

Materials and Methods

Tissue preparation

Thirteen 300–400 g, male Fischer-344 (F344) rats were used in this study. They were deeply anesthetized with a cocktail of ketamine (80 mg/kg body weight) and xylazine (10 mg/kg body weight) administered intraperitoneally, exsanguinated transcardially with ~50 ml of physiological saline containing 0.01% sodium nitrite, and perfused with ~750 ml of aldehyde-based fixative in Sorensen's sodium-potassium phosphate buffer, pH 7.4. For the experiments in which only NADPH-d histochemistry was performed, three animals were perfused with a fixative containing 4% w/v freshly depolymerized paraformaldehyde (PAF). For studies combining NADPH-d histochemistry and immunocytochemistry, ten animals were perfused with fixative containing 4% w/v PAF and 0.05–0.1% v/v glutaraldehyde. Following perfusion, the brains were quickly removed and the midbrain dissected, immersed in the same fixative for an additional 2 hr, and stored overnight at 4°C in 0.005 M phosphate-buffered saline (PBS, pH 7.4) containing 30% w/v sucrose. Using a sledge microtome (AO Spencer), 6 μ m frozen sections

were cut through the right and left ICs along sagittal and frontal planes, respectively, and collected in cold, 0.05 M Tris-HCl buffer (pH 8.0). All of the selected mixed-aldehyde-fixed sections were first reacted for NADPH-d, and then pairs of adjacent sections were respectively immunolabeled for GABA and GLU as described below.

NADPH-d histochemistry

The NADPH-d procedure was modified from Scherer-Singler *et al.*³³ Briefly, after washing in 0.1 M Tris-HCl buffer (pH 8.0) free-floating sections were incubated in a solution containing 0.25 mg/ml nitroblue tetrazolium (NBT), 1 mg/ml β -NADPH and 0.3% Triton X-100 in 0.1 M Tris-HCl buffer (pH 8.0) for 30–45 min at 37°C in the dark. The aforementioned chemicals were purchased from Sigma (St. Louis, MO). The reaction was terminated by transferring the sections through a series of graded acetones, which also proved useful in removing the residual formazan and reducing non-specific background signal. Finally, the sections were washed in PBS. Sections reacted with incubation media free of the substrate (β -NADPH) or NBT served as controls.

Immunocytochemistry for GABA and GLU

Following the NADPH-d reaction, alternate mixed-aldehyde-fixed sections were preincubated for one hour in a blocking solution of 10% normal goat serum (NGS, Vector Laboratories, Burlingame, CA) in PBS. The sections were then gently agitated in an affinity-purified polyclonal rabbit antiserum against GABA conjugate (provided by Robert J. Wenthold, Ph.D., NIDCD, NIH, Bethesda, MD)⁴¹, diluted 1:1000 in PBS containing 5% NGS, for 12–18 hr at 4°C. Adjacent sections were blocked for one hour in 10% normal horse serum (NHS, Vector Laboratories) in PBS and then incubated in a monoclonal mouse antiserum against GLU (abcam®, ab8893), diluted 1:1750 in PBS with 5% NHS under the same conditions and time period as those described for GABA. The primary immunolabeling was tagged with biotinylated horseradish peroxidase using Vectastain ABC kits (Vector Laboratories), and the peroxidase activity was visualized using a solution of 0.05 M 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.005% hydrogen peroxide in PBS. Control sections were incubated with 5% normal sera in PBS in the absence of primary antisera.

Evaluation of labeled neurons

After completion of the above procedures, the labeled sections were rinsed in PBS, mounted on gelatin-coated slides and allowed to air dry. On the following day, slides with tissue reacted for NADPH-d only were counterstained with neutral red. After the slides were coverslipped, the IC sections were examined and photographed using a Leitz Diaplan photomicroscope equipped with bright field optics (Leica, Wetzlar, Germany). With the

same microscope, camera lucida images were traced from five respective pairs of sagittal sections obtained from a mediolateral extent of the IC corresponding roughly to that of "lat. 2.0 mm" in Faye-Lund and Osen¹¹⁾. Each pair consisted of one section colabeled for NADPH-d and GABA, and the other for NADPH-d and GLU. Adjacent surfaces were examined as mirror-images to determine if any of the NADPH-d-positive coimmunolabeled for GABA and GLU. The camera lucida images were then optically scanned and digitized using a G4 Power Mac (Apple Computer, Inc., Cupertino, CA) running the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at). The distribution of labeled neurons was mapped, with each neuron categorized into one of three classes: NADPH-d+/0, NADPH-d+/GABA+, and NADPH+/GLU+. Somatic areas were measured from those NADPH-positive neurons possessing discernible nuclear profiles.

Results

The NADPH-d staining pattern observed in the IC of rat was consistent with that observed in a study by Druga and Syka⁸⁾, in which tissues were fixed with a combination of 4% PAF and 0.1% glutaraldehyde. However, we found that the intensity of NADPH-d staining was lower when 0.1% glutaraldehyde was contained in the fixative rather than 0.05% glutaraldehyde. On the other hand, fixative containing 0.05% glutaraldehyde produced an intensity of NADPH-d staining comparable to that observed in tissues fixed only with 4% paraformaldehyde while retaining a level of antigenicity adequate for good GABA and GLU immunolabeling.

Distribution of NADPH-d activity in the IC

All profiles positive for NADPH-d were characterized by the dark blue-violet formazan reaction product contained in their cytoplasm. Intensely stained neurons, fibers, and puncta were distributed mainly in the cortical subdivisions of the IC (Fig. 1-a). Throughout the dorsal and external cortices (DCIC and ECIC, respectively), small (< 100 μm^2), labeled neurons, primarily fusiform or oval in shape, were distributed from layer 1 to layer 3. Especially in transverse sections, many of the layer-1 neurons were oriented parallel to the cortical surface.

Medium-sized (100–150 μm^2), NADPH-d positive neurons predominated in the deeper layers, and they possessed fusiform, polygonal or triangular somata with multipolar dendritic arbors, morphological features similar to those of stellate cells (Fig. 1-b, -c). It was apparent from the neutral red counterstaining that the labeled neurons composed only a fraction of the total population of "stellate" neurons. The largest (> 200 μm^2) stellate cells, located in the deeper layers of the DCIC and ECIC, were

unlabeled (Fig. 1-b, -c).

A uniformly dense staining pattern was evident in the neuropil of the DCIC (Fig. 1-b). In comparison, the labeling of fibers and puncta in the ECIC was discontinuous, particularly in the deeper layers. The distribution of NADPH-d-stained cells, fibers and boutons overlapped one another and appeared to be patchy in comparison to the DCIC (Fig. 1-c).

NADPH-d staining was sparse in the central nucleus of the IC (CIC). A small number of weakly stained, medium-sized, bipolar and multipolar neurons were scattered mostly in the dorsomedial half of the CIC (Fig. 1-a). The labeled bipolar cells did not exhibit preferential alignment along any particular axis.

Combined Staining of NADPH-d Histochemistry with GABA Immunocytochemistry

Neurons intensely immunopositive for GABA were scattered throughout the IC. These neurons varied considerably in size. The pattern of distribution was similar to that observed in F344 rats by Caspary *et al.*⁵⁾ using the same antibody. All GABA-immunolabeled neurons possessed a homogeneous staining profile in which both the nucleus and perikaryon were filled with brown DAB labeling. Immunonegative neurons possessed no staining in either compartment. Thus, in the double-labeled sections, cells exhibiting co-labeling for GABA and NADPH-d were readily distinguishable by the blue-black NADPH-d reaction product contained in the perikarya and the brown GABA immunostaining located in the nuclei.

The majority of GABA/NADPH-d-colabeled neurons were small cells (< 100 μm) (Table 1) possessing round or oval somata, usually with a bipolar dendritic organization. They were distributed mainly in layer 2 of the DCIC and ECIC (Fig. 2-a, -b). There were no NADPH-d positive cells in the central nucleus of the IC (CIC) that co-labeled for GABA. Cell counts from midsagittal IC sections obtained from five F344 rats (Fig. 3) revealed

Table 1. Cell size distribution of neurons double-labeled with NADPH-d and GABA.

All NADPH-d positive neurons (NADPH-d+/0) are composed of cells ranging in size less than 200 μm^2 , especially more than 60% are smaller than 100 μm^2 . NADPH-d/GABA colabeled neurons (NADPH-d+/GABA+) measure no more than 130 μm^2 and the majority of them (over 80%) are found less than 100 μm^2 . There are also some occasional medium cells in layer 3 containing both GABA and NADPH-d, however the most of NADPH-d/GABAergic neurons seemed to belong to the small cells group (somatic area < 100 μm^2).

	Somatic area (μm^2)				
	< 100	100–129	130–149	150–199	≥ 200
NADPH+/0 (5 animals, n = 1070)	685 (64.0%)	315 (29.4%)	52 (4.9%)	17 (1.6%)	1 (0.1%)
NADPH+/GABA+ (5 animals, n = 119)	96 (80.7%)	23 (19.3%)	0	0	0

Fig. 3. A diagram of double labeling for NADPH-d and GABA.

Showing the relative percentages of NADPH-d/GABA+ colabeled neurons (NADPH-d+/GABA+) and NADPH-d single-labeled neurons (NADPH-d+/0) in the total population of NADPH-d containing neurons (set to 100%). Cell count is carried out for each section through the mediolateral middle of the IC of 5 animals and reveals that a small population; approximate 11.2% of NADPH-d containing neurons are also GABA immunoreactive.

Fig. 4. A diagram of double labeling for NADPH-d and GLU.

Showing the relative percentages of NADPH-d/GLU+ colabeled neurons (NADPH-d+/GLU+) and NADPH-d single-labeled neurons (NADPH-d+/0) in the total population of NADPH-d positive neurons (set to 100%). Cell count is carried out for each section through the mediolateral middle of the IC of 5 animals and reveals that the greater part, approximate 76.6% of NADPH-d containing neurons are also GLU immunoreactive.

Table 2. Cell size distribution of neurons double-labeled with NADPH-d and GLU.

Similar to the somatic distribution of NADPH-d positive neurons (NADPH-d+/0), a little more than 60% of NADPH-d/GLU colabeled neurons (NADPH-d+/GLU+) measure less than 100 μm^2 , and about 30% measure from 100 to 129 μm^2 . There are also some larger cells ranging from 130 to 200 μm^2 , however no one is found greater than 200 μm^2 , in somata.

	Somatic area (μm^2)				
	< 100	100–129	130–149	150–199	≥ 200
NADPH+/0 (5 animals, n = 1079)	676 (62.7%)	320 (29.7%)	61 (5.7%)	22 (2.0%)	0
NADPH+/GLU+ (5 animals, n = 827)	506 (61.2%)	257 (31.1%)	49 (5.9%)	15 (1.8%)	0

that $11.2\% \pm 0.92\%$ (S.E.M) of the total population of NADPH-d containing neurons also were GABA immunoreactive.

Combined Staining of NADPH-d Histochemistry with GLU Immunocytochemistry

Neurons moderately-to-intensely immunoreactive for GLU were distributed extensively throughout the IC. They existed in a wide variety of shapes and sizes, but mostly they were small-to-medium sized, and round or polygonal in shape. The density of glutamate-immunopositive neurons was greatest in the DCIC, followed by the ECIC and CIC in decreasing order. These findings

agree with previous biochemical and immunohistochemical showing that the cortical regions of the IC contained higher transmitter levels of GLU^{2,19}.

The immunostaining properties of GLU were similar to those observed with GABA. The cytoplasm and nuclei of GLU immunoreactive neurons were uniformly stained. Again, neurons coexpressing NADPH-d and GLU were detectable by virtue of a dark blue cytoplasmic precipitate and an immunostained nucleus.

Most of the NADPH-d positive neurons colabeled for GLU ($76.6\% \pm 0.53\%$; Fig. 4). This was most evident in the cortical regions of the IC where, as previously mentioned, the majority of NADPH-d positive neurons are located (Fig. 5-a, -b). From a cytoarchitectonic standpoint, the NADPH/GLU colabeled neurons possessed a wider range of somal sizes (small to large (Table 2) and shapes (fusiform to polygonal) than did the NADPH-d/GABAergic neurons, and included the population of neurons that could be characterized as stellate cells.

We were unable to detect GABA or GLU in a small percentage of the NADPH-d positive neurons, and we did not observed NADPH neurons that were intensely colabeled with both transmitters (Fig. 6-A, -a, Fig. 6-B, -b). Thus, NOS-containing IC neurons can be subdivided into three groups based on transmitter specificity, with the largest proportion ($\sim 77\%$) containing GLU, but not GABA. The remainder is divided into roughly equal portions of neurons immunopositive for GABA but not glutamate, and those containing neither (Fig. 7).

Fig. 7. A diagram showing the relative proportions of neurons colabeled for NADPH-d and GABA (NADPH-d+/GABA+), and for NADPH-d and GLU (NADPH-d+/GLU+).

For each bar, the percentages of both colabeled groups of NADPH-d+/GABA+ and NADPH-d+/GLU+ were referred to previous Fig. 3 and Fig. 4 respectively, and the remainder of percentage point was counted for NADPH-d single-positive group (NADPH-d+/0). Because that no coexistence of GABA and GLU could be found in the same cells according to the mirror-images technique, the mean corresponding ratio from 5 individual animals between these three subpopulations of NOergic neurons is NADPH-d+/GLU+, 8 (76.6%); NADPH-d+/GABA+, 1 (11.2%); and NADPH-d+/0, 1 (12.2%).

Discussion

Recently several investigators have described the distribution of NOergic neurons in relation to aminergic neurons in different regions of the central nervous system, including the zona inserta¹⁷⁾, prefrontal cortex¹³⁾, medulla oblongata²¹⁾, hippocampus³⁷⁾, cuneate nucleus³⁵⁾, olfactory bulb¹⁾, lateral geniculate nucleus¹⁴⁾, spinal cord³¹⁾, and cerebral cortex³⁶⁾. Nevertheless, little is known about the relationship between NO and neuroactive amino acids in the auditory nuclei. In the present study, the combined staining of NADPH-d histochemistry with GABA or GLU immunohistochemistry was utilized to investigate a potential co-existence of NO with GABA and GLU in the IC of rat.

Formaldehyde fixation has been established as a reliable preservation technique for the histochemical detection of NADPH-d. Indeed, a previous study by the authors demonstrated complete overlap between NADPH-d labeling and immunoreactivity for brain NOS (bNOS) in sections of IC perfusion fixed with buffered 4% paraformaldehyde³⁹⁾. Nevertheless, the present study has demonstrated that fixatives containing small concentrations (0.05%) of glutaraldehyde in addition to paraformaldehyde permit the immunodetection of amino acid

conjugates without seriously compromising NADPH-d staining. However, with progressively higher concentrations of glutaraldehyde, we found that the staining for NADPH-d became increasingly less intense such that increasing numbers of false negatives would certainly result. On the other hand, the low levels of glutaraldehyde in our fixatives produced patterns of GABA and GLU immunolabeling in the IC comparable to those previous reports^{2, 5, 26)}.

Relationship between NADPH-d staining and cell types in the IC

The greatest number of intensely NADPH-d-stained neurons were located in layers 2 and 3 of the DCIC and ECIC. Based on their cytoarchitectural features, these neurons appear to be stellate cells that have been identified as the predominate neurons in the IC cortices^{11, 24)}. The responses of stellate cells could reflect the synthesis of information from several synaptic domains suggesting that this type of cells may contribute to the intrinsic axonal plexus of the IC and also play a part in efferent projections²⁵⁾. As like the cortex of IC has been established to be a unique auditory relay station in which, much different from CIC, both of the ascending and descending inputs from the lower auditory brainstem and the telencephalon terminated, there was a suggestion of stellate cells playing an important role in connections of ascending and descending auditory pathways. And also ECIC was characterized as an acousticomotor nucleus, extensively interconnecting with the superior colliculus, cerebellum and somatosensory and vocalization systems^{6, 16, 25, 32)}. As determined by our previous quantitative study³⁹⁾, NADPH-d-stained neurons account for approximately 35% of the total neuronal population in the IC cortices. Thus, we believe that a prominent subpopulation of stellate neurons in the central and cortical IC is NOergic. It is presumptive that the NOergic neurons in the IC cortices, mostly corresponding to stellate cells, may participate in integrating the ascending and descending auditory pathways as well as possibly contribute to multiple connections between the auditory system and other somatosensory or motor systems, however, the precise neurophysiologic role of these neurons, along with the significance of NO in this capacity, remains to be determined.

The overlapping, patchy distribution of NADPH-d-labeled cells, fibers and puncta characterized the ECIC from the other IC subdivisions. Interestingly, the discontinuous distribution of the labeled fibers and boutons is reminiscent of that observed for the somatosensory projections to the same region¹¹⁾. However, the coincident appearance of NADPH-d labeled cells along with the fibers and puncta in discrete areas of the ECIC suggest that NOergic neurons function here as local circuit neurons.

Co-localization of NADPH-d and amino acid neurotransmitters in the same cells

Three types of NOS-containing neurons were identified in the IC based on whether or not they co-contained GABA or GLU. Approximately 77% of the NOergic neurons colabeled for GLU and ~11% were immunopositive for GABA, while the remainder appeared to contain neither transmitter (Fig. 7).

It is now evident that GABA is present at high concentrations in the IC, supporting a possible role of GABA as an inhibitory transmitter in this nucleus^{2, 12}. Immunohistochemical studies have shown that GABAergic neurons are found scattered throughout the IC, and they appear to be a subpopulation of small- to medium-sized stellate neurons²⁷ that are thought to be involved in local circuit activity^{28, 29}. Perhaps a small percentage of these neurons are also NOergic, capable of modifying intrinsic inhibitory circuits which shape the processing of acoustic information in the IC cortices⁵.

As with GABA, high concentrations of GLU are also found in the IC^{2, 10, 26} that has proposed a role of GLU as a candidate for the afferent excitatory transmitter in the IC where excitatory amino acid application has been examined to induced a reduction of the acoustic threshold in several IC neurons. On the other hand, several studies have suggested that NO may co-exist with GLU in neuronal groups in the cerebellum³, medulla²¹ and thalamus¹⁷ suggesting a complex interaction between these two messenger molecules and implying another different excitatory mode of NO transmission from what was inhibitorily modulated by GABA. However, given their smaller size and morphologic similarity to stellate cells NO/GLUergic cells may function as interneurons in the IC. With antagonizing to the inhibitory effects of NO/GABAergic neurons, a GLU-mediated excitatory NO transmission is presumed to be involved in the networking of auditory communication in the cortex of IC.

In Addition, it is also interesting to note that the CA1 pyramidal neurons in the hippocampus, supposed to be the source of long-term potentiation (LTP) has been identified as NO/GLUergic^{7, 26}, while an exclusive local circuit population also proposed to be related to the LTP induction were found NO/GABAergic³⁶. In the cerebellum, two types of inhibitory interneurons, i.e. the stellate and basket cells, presumably concerned in regulating the effect of long-term depression (LTD) were also found NO/GABAergic³⁶. At present, the knowledge concerning the synaptic plasticity such as LTP and LTD in auditory pathways is still deficient, however, NO/GABAergic and NO/GLUergic interneurons respectively provide a possibility that an inhibitory GABA- or an excitatory GLU-mediated form of synaptic plasticity may be associated with the progresses of acoustic learning or recognition memory in the IC, whereas different effects are probably produced by NOergic simple neurons which contain neither GABA nor GLU.

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Fig. 2. Combined staining of NADPH-d histochemistry with GABA immunohistochemistry in the IC.

A small number of double-labeled NADPH-d/GABAergic neurons, mostly possessing small round or oval soma (arrows) are found in the layer 2 of DCIC (2-a) and ECIC (2-b). In the layer 3 of ECIC, some occasional medium cells (arrowheads) also exhibit colocalization of NADPH-d and GABA; however the giant neurons (open arrowhead) are labeled with GABA immunohistochemistry but lack NADPH-d reactivity (2-b). Scale bar = 50 μ m.

Fig. 1. NADPH-d histochemistry in the IC.

1-a: The NADPH-d positive neuropil and neurons, which are characterized by the blue-violet formazan formation throughout their cytoplasm with a Golgi-like aspect, are predominantly found in the dorsal cortex (DCIC) and the external cortex (ECIC). Only a few NADPH-d labeled neurons thinly disperse in the central nucleus of the IC (CIC), however a number of labeled fibers are observed in this area bridging the DCIC and ECIC. **1-b:** A part of deeper layer of DCIC enlarged. NADPH-d positive neurons with small (arrows) to medium-sized (arrowheads) somata and irregular dendritic orientations, and neuropil are rich in the DCIC. The largest stellate cells (open arrowheads) are unlabeled. **1-c:** In comparison to the DCIC (1-b), cells (the small are indicated by arrows and the medium-sized by arrowheads), fibers and boutons are less marked in the ECIC and had a tendency to be arranged in clusters especially in layer 2. The largest stellate cells (open arrowheads) are unlabeled. Scale bar = 50 μ m all photomicrographs.

Fig. 5. Combined staining of NADPH-d histochemistry with GLU immunohistochemistry in the IC.

A majority of NADPH-d/GLUergic neurons showing NADPH-d reactive and intensive or moderate (arrows) GLU-immunoreactivity are detected in the DCIC (4-a) and ECIC (4-b). From the cytoarchitectonic perspective, these NADPH-d/GLUergic neurons possess a wider range of somal sizes (small to large) and shapes (fusiform to polygonal) than did the NADPH-d/GABAergic neurons, and include the population of neurons that could be characterized as stellate cells. Scale bar = 50 μ m.

Fig. 6. The pair adjacent sections consisted of one section colabeled for NADPH-d and GABA, and the other one for NADPH-d and GLU. Adjacent surfaces of the pairs of section **6-A** and **6-a**, and the pairs of section **6-B** and **6-b** are examined as mirror-images. **6-A** and **6-B** are colabeled for NADPH-d and GABA, and **6-a** and **6-b** are colabeled for NADPH-d and GLU respectively. Note that all of NO/GABAergic neurons (arrows) seen in **6-A** and **6-B** do not show any GLU immunoreactivity in **6-a** and **6-b**. In contrast, the NO/GLUergic neurons (arrowheads) seen in **6-a** and **6-b** are all GABA immunonegative. Asterisks indicate profiles of blood vessels as landmarks. Scale bar = 50 μ m for all photomicrographs.