Colocalization of Enkephalin-like and Choline Acetyltransferase-like Immunoreactivities in Olivocochlear Neurons of the Guinea Pig

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The guinea pig lateral superior olive was examined immunocytochemically using antisera against enkephalin and choline acetyltransferase sequentially on the same sections. A colocalization of choline acetyltransferase-like and enkephalin-like immunoreactivities was found in cells of the lateral superior olive that give rise to the lateral system of olivocochlear efferents. Only choline acetyltransferase-like immunoreactivity was observed in the group of olivary nuclei that give rise to the medial group of olivocochlear fibers.

KEY WORDS: Guinea pig; Lateral superior olive; Olivocochlear fibers; Enkephalin; Acetylcholine; Choline acetyltransferase; Immunocytochemical colocalization.

We have reported enkephalin-like immunoreactivity in the guinea pig cochlea (14,22) in olivocochlear (OC) efferents, a system for which there is much evidence that it is cholinergic (2-4,11-13,15-18,21,24,25,29,31). This raises the question whether there are two distinct cholinergic and enkephalinergic systems of OC efferents or colocalization of acetylcholine and enkephalin within OC neurons. In a recent study we have demonstrated that the OC neurons in the guinea pig lateral superior olive show both enkephalin-like-immunoreactivity (ELI) and acetylcholinesterase (AChE) staining (1), which suggests that there is cocontainment of the two neurotransmitter candidates. These neurons are the cells of origin of OC fibers belonging to the lateral system of efferents, following the classification of Warr (36-38). Cell bodies of OC fibers of the medial system (38) showed AChE staining but not ELI (1). AChE staining, however, is not considered an adequate marker for cholinergic neurons (7,20,26,28,34). We have therefore extended our studies using antisera to choline acetyltransferase (ChAT), an enzyme that is a specific marker for cholinergic neurons (7,20,26,28,32,34). In the present study antisera to ChAT and methionine enkephalin were used to determine if the lateral OC neurons of the guinea pig also show immunoreactive staining for ChAT in addition to enkephalin.

Materials and Methods

Antisera. Two antisera raised against methionine enkephalin conjugated to bovine thyroglobulin were used in this study. Antiserum RA164, raised in rabbit, has been described in detail previously (1). Antiserum GP121 was raised in the same manner but in guinea pig. Two antisera raised against ChAT from pig brain were used. One antiserum was raised in mouse as described (6). A second antiserum, raised in rabbit against a conjugate of pig brain ChAT and mouse anti-Chat immunoglobulin G (lgG), has also been described (6,7). This second Chat antiserum was used in most of the study.

Immunocytochemistry. Twelve female National Institutes of Health strain guinea pigs, weighing 100-200 g, were used. The guinea pigs were anesthetized with chloral hydrate and perfused through the heart with 0.1 M sodium cacodylate buffer, pH 7.2, followed by 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4°C. Brains were immersed for 1 hr in the fixative at 4°C and then rinsed 16-20 hr at 4°C in either phosphate buffered saline (PBS), pH 7.2, with 6% sucrose or in 0.1 M sodium cacodylate buffer, depending on which of two protocols was to be followed. Brains from PBS/sucrose were frozen onto cryostat chucks and 10 μm cryostat sections through the brainstem at the level of the lateral superior olivary complex were cut. Rabbit antiserum to Chat, at a 1:400 dilution in PBS with 0.3% Triton X-100, was applied and sections were incubated for 16-20 hr at 4°C. The indirect immunofluorescence technique of Coons (5), modified as described previously (1,14,15) was followed. OC cells showing Chat-like immunofluorescence were photographed through a Zeiss microscope under epifluorescent illumination. Coverslips were then floated off under PBS. The method of Tramu (35) to remove antibodies from sections was then followed. Sections were briefly dipped in a solution of 0.0015 M potassium permanganate with 0.02 M H2SO4 added (0.5 ml/100 ml). Sections were then rinsed in PBS. Antiserum to enkephalin, either RA164 diluted 1:200 or GP121 diluted 1:300, was then applied, sections were incubated 16-20 hr at 4°C, and indirect immunofluorescence techniques followed. Areas on sections previously photographed were then examined and sites of ELI were compared with sites of Chat-like immunoreactivity. In the
second protocol, brains from the sodium cacodylate buffer rinse were
Vibratome sectioned at 20 μm and antisem GP121 to enkephalin
was applied at a dilution of 1:300 to free-floating sections for 16–20
hr at 4°C. Indirect immunofluorescence was then carried out as before.
Areas containing OC neurons were photographed under epifluores-
cent illumination, cover slips were floated off, and the rabbit antisera
to ChAT diluted 1:400 applied. The free-floating sections were in-
cubated 16–20 hr at 4°C and the Vectastain biotin–avidin immuno-
peroxidase staining procedure (23) (Vector Laboratories) was carried
out. Diaminobenzidine HCl was used as a chromagen. Sections were
then reexamined and sites of ChAT immunoreactivity were compared
with sites of ELI.

Controls. Absorption controls were used to determine specificity
of staining. Antisera to enkephalin were preabsorbed by adding 25
μg of methionine enkephalin to 25 μl of antisera and incubated for
16–20 hr at 4°C. These absorbed antisera were then used at the same
dilution as the nonabsorbed antisera on adjacent sections. The rabbit
antisem to ChAT was preabsorbed with pig brain ChAT, 20 μg of
protein per microliter of antisem, and used on adjacent sections in
two animals. In other animals normal rabbit serum was used as a
control on adjacent sections. Both types of controls were used to
define background staining of cells and neuropil. The labeling intensity
of cells considered to show specific immunoreactivity in sections in-
cubated with non-absorbed antisem had to be visibly greater than
background staining in controls. In addition, because of the nature of
immunoreactive staining wherein an antisem can bind to substances
other than the antigen to which it is directed if they share antigenic
sites in common, we use the term “like” in our description of im-
munoreactive labeling. Procedural controls for the two colocalization
protocols were also performed. For the first protocol performed on
cryostat sections using the method of Tramu, PBS was substituted for
antisem to enkephalin on many sections and then normal procedures
followed. Similarly for the second protocol, PBS was substituted for
the antisem to ChAT on many sections. Mouse antisem to ChAT
was used along with rabbit antisem to ChAT on two animals and
the results compared.

Results

Colocalization

ELI and ChAT-like immunoreactivity were found in the same
cells in the lateral superior olive (Figures 1A–D, 2A,B). This
result was obtained using both protocols for colocalization.
Most of the cells of the lateral superior olive that had im-
munoreactive staining for one substance showed colocalization
for the other. This colocalization was not observed in the
medial OC nuclei, where only ChAT-like immunoreactivity
was observed. These results are similar to those of the earlier
study using immunostaining for ELI followed by AChE stain-
ing (1).

Controls

No staining of cells was seen after using preabsorbed antisera,
normal rabbit serum or after the substitution of PBS for antisi-
era to ChAT or enkephalin in the second step of the colo-
calization procedures. Antisem to enkephalin RA164 and GP121
gave similar results when applied to adjacent cryostat sections.
Also, when the rabbit and mouse antisem to ChAT were ap-
plied on adjacent sections they showed the same staining.

The fact that medial olivary nuclei cells showed ChAT-like
immunoreactivity but not ELI served as a built-in control. In
the protocol where enkephalin immunostaining followed ChAT
immunostaining, ELI was seen only in the cells of the lateral
superior olive, demonstrating that there was no nonspecific
staining due to reactivity with unremoved ChAT antibodies.

Discussion

This study shows colocalization of ChAT-like immunoreactiv-
ity and ELI in neurons of the guinea pig lateral superior olive. A
previous study has shown that these cells correspond to the
cells of origin of the lateral OC fibers (1). While we (14) and
others (9,10) have reported ELI at the bases of outer hair cells,
corresponding to terminals of the medial group of efferents,
our recent observations (unpublished observations) show that
most antisem to methionine enkephalin produce immuno-
reactive labeling only of the lateral group of efferents in the
cochlea. In the present study only ChAT-like immunoreactiv-
ity and never ELI was seen in the cells that give rise to the
medial group of OC fibers. The colocalization of ChAT-like
immunoreactivity and ELI is a feature of the lateral and not
the medial OC system in the brainstem. The ELI that we have
previously reported in unmyelinated fibers in the intergang-
glomerular spiral bundle (14) is then likely to be a part of the
lateral system. It is possible that a neuropeptide sharing an-
tigenic sites with methionine enkephalin is present in medial
OC terminals in the cochlea, however, no ELI was seen in
medial OC neurons in this study nor in a previous study where
other antisem to methionine enkephalin were applied (1). The
classification of the OC fibers into medial and lateral systems
was based on morphological cri-
teria, particularly origin and termination of fibers. The differ-
ence between the lateral and medial OC systems in colocali-
zation of ChAT-like Immunoreactivity and ELI adds to an
increasing list of features that further differentiate the two OC
systems, including differences in uptake (8,9,19,33), develop-
ment (27,30), and aspartate aminotransferase-like immu-
noreactivity (15).

We have not attempted to exactly quantify the percentage
of lateral OC neurons of the lateral superior olive that showed
colocalization because the possibility of false negatives re-
sulting from the method of Tramu and the other procedures
applied could easily make such a quantitation inaccurate. How-
ever, over 75% of cells that showed staining for ChAT-like
immunoreactivity or ELI had staining for both. The lateral OC
neurons not showing colocalization could all be false negatives
or might represent a subpopulation of the lateral OC system.
Utrastructural immunocytochemical studies in the organ of
Corti would help resolve the question of whether there are
subpopulations of lateral OC fibers and terminals with differ-
ces in immunostaining for ChAT-like immunoreactivity or
ELI.

The colocalization of ChAT-like immunoreactivity and ELI
in the cells of origin of the lateral OC efferents suggests that
there may be cocontainment of the two neuroactive sub-
stances, acetylcholine and enkephalin, in these efferent fibers
and that both of these substances may be playing a role in
synapses at the bases of inner hair cells in the cochlea.
Figure 1. 10 μm cryostat sections through the guinea pig lateral superior olivary complex showing choline acetyltransferase (ChAT) immunoreactivity (A,C) using rabbit anti-ChAT or enkephalin-like immunoreactivity (B,D) using rabbit anti-enkephalin antiserum 164. B and D show the same sections as A and C, respectively, restained for ELI after the first set of antibodies were removed using the method of Tramu (35). The same cells that show ChAT-like immunofluorescent labeling also show ELI (arrowheads). Original magnification × 225. Bar = 40 μm.
7. Eckenstein F, Sofroniew MV: Identification of central cholinergic neurons containing both choline acetyltransferase and acetylcholine esterase (ChAT) using indirect immunofluorescence techniques and then restained using immunoperoxidase techniques to show choline acetyltransferase (ChAT)-like immunoreactivity (rabbit anti-ChAT antiserum). The same cells (arrowheads) show both ELI and ChAT-like immunoreactive staining. Original magnification ×250. Bar = 40 μm.

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