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Short Communication

Glycine-immunoreactivity in the auditory brain stem of the chick *

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Glycine-immunoreactivity (Gly-I) was studied in the auditory brain stem of chick using a polyclonal antiserum to glycine conjugated to bovine serum albumin (Wenthold et al., 1987). During embryonic development, little Gly-I is present in nucleus magnocellularis (NM), nucleus laminaris (NL), or nucleus angularis (NA). In posthatch chicks, a few Gly-I terminals are found on nerve cell bodies in NM. Gly-labeled terminals are rare in NL and NA. When present in NL, they appear to be apposed to neuronal somata and not to dendrites. Occasionally, a Gly-labeled cell can be found in NM. After unilateral cochlea removal, Gly-labeled terminals are still present in NM. Thus, the cochlea does not appear to be the source of the glycinergic afferents to NM.

The pattern of staining of Gly-I contrasts sharply with that of gamma-aminobutyric acid-immunoreactivity (GABA-I). The number of Gly-I terminals in NM, NL and NA appears to be much less than that of GABA-I. In addition, GABA-I terminals are very abundant around both the cell bodies and dendrites of neurons in NM and NL, whereas Gly-I terminals are found only on neuronal somata. Gly-I in NM also appears to be much less than that in its mammalian homologue, the anteroventral cochlear nucleus.

Nucleus magnocellularis; Nucleus laminaris; Immunocytochemistry; Cochlea removal

Introduction

The amino acid, glycine, is considered to be an inhibitory neurotransmitter in the vertebrate central nervous system (Aprison et al., 1975; Berger et al., 1977). High levels of glycine, glycine-immunoreactivity (Gly-I), and glycine receptors have been demonstrated in the mammalian auditory brain stem (Godfrey et al., 1977, 1978; Wenthold et al., 1987; Aoki et al., 1988; Altschuler et al., 1986; Peyret et al., 1987; Sanes et al., 1987). However, little is known about its distribution in the avian auditory brain stem.

Gamma-aminobutyric acid (GABA), another putative inhibitory neurotransmitter, has been immunocytochemically localized in nucleus magnocellularis (NM), nucleus laminaris (NL), and nucleus angularis (NA), the chick auditory brain stem nuclei (Müller, 1987; Code et al., 1987, 1989a). Other evidence suggests that GABA and glycine are co-localized in nerve terminals (Oberdorfer et al., 1987) and cells (Wenthold et al., 1987) of the guinea pig cochlear nucleus. In addition, at least two inhibitory amino acid receptor types, GABA- and glycine-preferring, are present in the anteroventral cochlear nucleus (Martin et al., 1982), the mammalian homologue of NM. In electron microscopic preparations of NM, two types of non-primary endings can be observed on the cell bodies (Parks, 1981). GABA-labeled afferents make synaptic contact with neuronal somata in NM (Code et al., 1989a) and thus probably correspond with one of these types of non-primary endings. Our hypothesis was that Gly-I terminals

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were also present in the auditory brain stem nuclei of the chick and may correspond to the second type of non-primary ending in NM.

Methods

The development of Gly-I was studied in NM, NL, and NA of 6 normal chick embryos (inbred White Leghorns, H & N Supplier) at embryonic days 10, 13, 17 and 19 (E10, E13, E17 and E19) and in 7 posthatch chicks from P1 to over 1 year of age. Embryonic ages were determined according to the Hamburger and Hamilton (1951) staging series. In addition, the distribution of Gly-I terminals was studied following cochlea removal.

Surgical procedures

Four posthatch chicks (P6) underwent unilateral cochlea removal. Animals were anesthetized with an intramuscular injection of ketamine hydrochloride (Vetalar; 80 mg/kg body weight) and an intraperitoneal injection of sodium pentobarbital (Nembutal; 20 mg/kg body weight). After a topical application of Xylocaine, the tympanic membrane was punctured, the columella (middle ear ossicle) was removed, and the basilar papilla (avian cochlea) was removed with fine watchmaker's forceps (Born and Rubel, 1985). Two of the animals were allowed to recover for 13 days and the other two survived for 21 days. In addition, one normal, unoperated, age-matched chick served as a control for the 21-day survival group.

Immunocytochemistry

The animals were anesthetized as above and perfused transcardially with cold 0.15 M cacodylate buffer, pH 7.4, followed by a 1% paraformaldehyde and 1.25% glutaraldehyde fixative in 0.1 M cacodylate buffer. The brain was removed from the skull, and either placed in fix for 4 h then into cold 0.15 M buffer overnight, or only into cold fix overnight. The brain was cut on a Vibratome (30–50 μm thick), and sections were collected in 0.1 M phosphate buffered saline (PBS). Standard immunocytochemical procedures were followed using the avidin-biotin-peroxidase complex (ABC) method (Hsu et al., 1981) and reagents from Vectastain kits (Vector Labs). The sections were rinsed at least 3 times over 1 h in PBS then

incubated in a presoak solution (3% normal goat serum) for 1 h at room temperature. The sections were then incubated in a polyclonal antiserum to glycine, diluted 1:60–1:250 in the presoak solution, at 4°C from 17–48 h (Wenthold et al., 1987). After 3 rinses of PBS over 1 h, the sections were incubated in biotinylated goat anti-rabbit IgG, diluted 1:100 in PBS, at room temperature for 1 h. Following 3 rinses of PBS (5 min each), the sections were incubated in ABC for 1 h at double the recommended concentration, i.e., 2 drops of A and B/5 ml PBS, then reacted for 10–15 min in a 0.02% di-aminobenzidine (DAB) solution with 0.003% hydrogen peroxide. The sections were mounted onto chrome-alum subbed slides, dehydrated and coverslipped with DPX. Immunocytochemical procedures using the GABA antiserum (Immunonuclear, Inc.) are detailed in a previous report (Code et al., 1989a).

Controls

Some sections from most experiments were processed as above except that the presoak solution was substituted for the glycine antiserum. This procedure resulted in the absence of any staining in the tissue for Gly-I. Other control experiments to determine the specificity of this glycine antiserum have been performed by other investigators (Wenthold et al., 1987).

Results

Development of Gly-I

In NM of chicks aged E10 to over 1 year old posthatch, Gly-I is present in NM as small, round or oval immunostained structures which surround nerve cell bodies (Fig. 1A). These structures are believed to be glycine-containing nerve terminals. Gly-I terminals appear to be uniformly distributed within NM. There does not appear to be any change in the distribution or density of Gly-I terminals in NM during development. The absolute number of Gly-I terminals in NM appears to be much less than the number of GABA-I terminals (compare Fig. 1A with B; see also Code et al., 1989a).

The number of Gly-labeled terminals in NL appears to be less than that in NM (Fig. 1C). Gly-I terminals were only observed apposing nerve

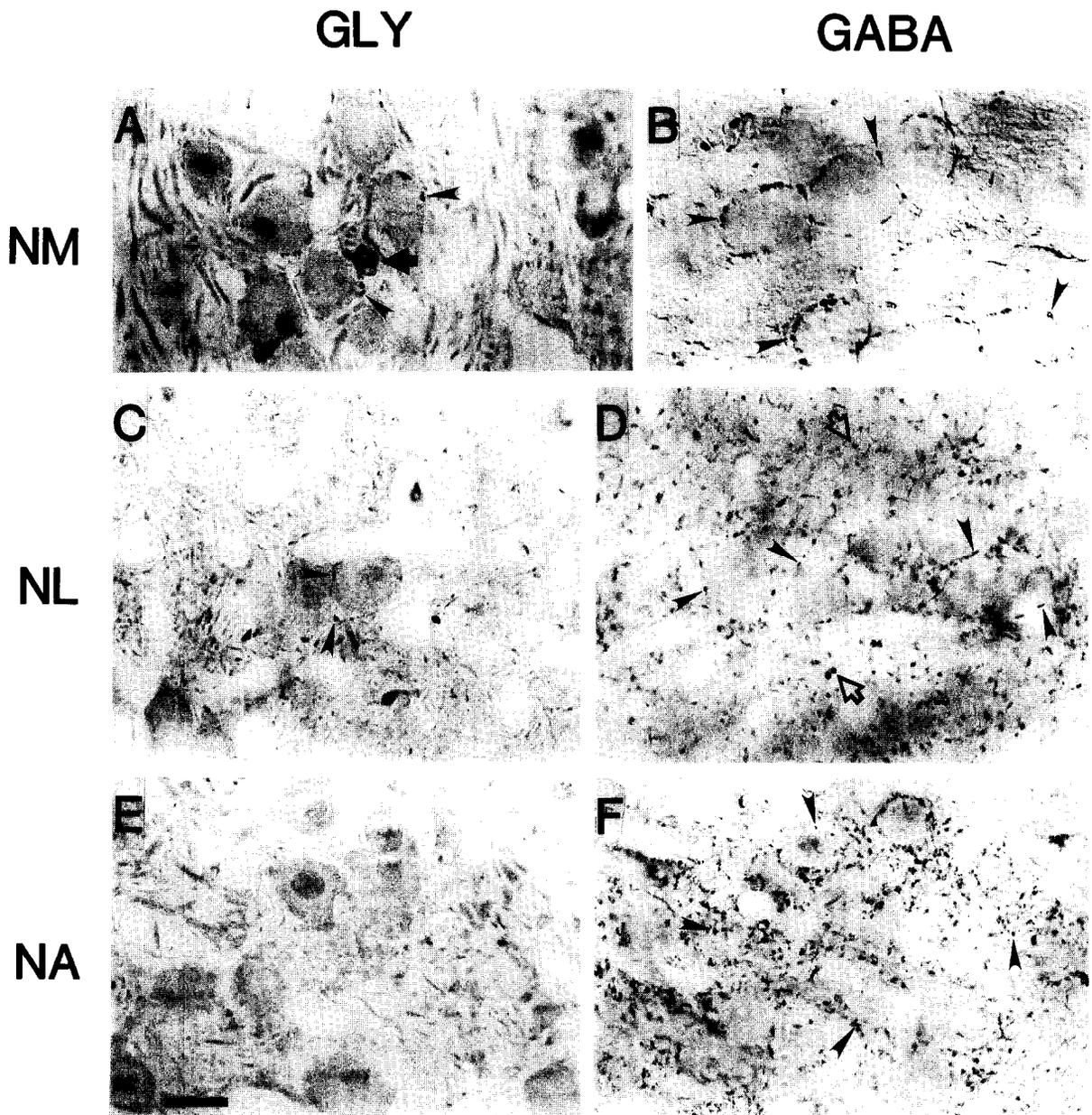


Fig. 1. Left column: immunostaining in posthatch (P26) chicks using the glycine antiserum. Right column: immunostaining in posthatch chicks using the GABA antiserum. Arrowheads indicate examples of immunostained terminals. (A) Gly-I terminals surrounding nerve cell bodies in NM. A Gly-labeled cell (arrow). (B) Numerous GABA-I terminals in NM of a P21 chick. (C) A few Gly-I terminals apposed to a nerve cell body in NL. (D) GABA-I terminals on cell bodies in NL (arrowheads) and in the neuropil (open arrows) of a P8 chick. (E) Gly-I terminals are rare in NA. (F) Numerous GABA-I terminals in NA of a P8 chick. Scale bar = 20 μ m.

cell bodies in NL and were rarely seen on dendrites. In contrast, GABA-labeled terminals are very abundant on both the somata and dendrites

of neurons in NL (compare Fig. 1D with C; see also Code et al., 1989a).

Gly-I terminals are rare in NA and their num-



Fig. 2. Gly-I terminals (arrowheads) in the ipsilateral NM 21 days after cochlea removal. Scale bar = 10 μ m.

ber appears much less than that of GABA-I terminals (Fig. 1E, F). There is no discernible change in either the distribution or density of Gly-I terminals in NL and NA during development.

Occasionally, a Gly-labeled cell was observed within NM (Fig. 1A). These cells are much smaller than the large, round, unlabeled NM neurons, and their processes are not usually labeled. Gly-labeled cells in NL or NA were less frequent than those in NM. In contrast, both Gly-I terminals and cell bodies in the medial vestibular nucleus were intensely labeled.

Cochlea removal

To determine whether Gly-I terminals originate from the cochlea, 4 posthatch chicks (P6) underwent unilateral cochlea removal. After survival times of 13 or 21 days, Gly-I terminals are still present in NM on both sides of the brain stem (Fig. 2). Qualitative observations suggest that the density of Gly-I terminals in NM ipsilateral to cochlea removal may have increased relative to that in the unoperated NM or controls. However, it is not clear if this apparent increase in the density of Gly-I terminals is due to decreases in the volume, number of cells and cell size in NM as

a result of cochlea removal (Born and Rubel, 1985). There was no observable change in the distribution of Gly-I terminals following cochlea removal.

Discussion

There is relatively little Gly-I in the auditory brain stem nuclei of embryonic or posthatch chicks. Gly-labeled terminals are infrequent, but usually can be found surrounding nerve cell bodies in NM and NA. In NL, Gly-I terminals appear to be apposed to neuronal somata and not to dendrites. A Gly-labeled cell can occasionally be observed in NM. There does not appear to be any change in the distribution or density of Gly-I terminals during development.

We do not believe that these data are the result of false negative staining using the glycine antiserum because the adjacent medial vestibular nucleus in the same tissue section is heavily stained. In addition, the lumbar spinal cord from one normal chick (P2) was dissected and processed for Gly-I. Its gray matter was intensely stained with the glycine antiserum.

The number of Gly-I nerve terminals in NM, NL and NA appears to be much less than the number of GABA-I terminals which are abundant at all ages past E15 (Code et al., 1987, 1989a). Since both glycine and GABA are presumably inhibitory neurotransmitters, the relatively few Gly-I terminals in NM (compared to GABA-I terminals) suggests that their contribution to any inhibitory processing within NM is probably very small.

In NL, Gly-stained terminals are apposed to cell bodies only and not to dendrites. In contrast, GABA-labeled terminals circumscribe neuronal somata and extend for some distance out onto the dendrites of NL neurons (Code et al., 1989a). Thus, the influence of GABA-I terminals in NL is also expected to be much greater than that of Gly-I terminals. The physiological role of both the GABA- and glycine-labeled terminals in these auditory brain stem nuclei remains to be determined.

In summary, while most of the non-primary afferents to NM, NL and NA are GABA-I, some appear to be Gly-I. In NM, the GABA-I and

Gly-I terminals may correspond to the 2 types of non-primary terminals seen in electron microscopic preparations (Parks, 1981). These 2 types of endings occupy 13% and 5%, respectively, of the cell surface area in NM. Using EM-immunocytochemistry, the GABA-I endings have been shown to make synaptic contact with NM neurons (Code et al., 1989a). If the GABA-I and Gly-I terminals segregate into the 2 types described by Parks, then the relative density of the GABA-I terminals indicates that they correspond to Parks's first type of non-primary ending and that the Gly-I terminals correspond to the less numerous non-primary ending in NM. An EM-ICC study of the Gly-I terminals needs to be done to definitively determine whether the Gly-I inputs correspond to the second type of non-primary endings observed in NM.

Comparison with mammalian cochlear nuclei

Gly-I in NM of the chick differs sharply with that observed in its mammalian homologue, the anteroventral cochlear nucleus (AVCN). The AVCN of the guinea pig (Wenthold et al., 1987; Peyret et al., 1987) and rat (Aoki et al., 1988) stain heavily with glycine antisera. In addition, glycine receptors are dense in the AVCN of the gerbil (Sanes et al., 1987), guinea pig and rat (Altschuler et al., 1986). Interestingly, the density of these receptors is greatest in high frequency regions of the gerbil AVCN (Sanes et al., 1987). In contrast, there was little or no staining in NM of the chick using an antiserum to the glycine receptor (Dan Sanes, personal communication). This difference in the Gly-I between NM and the AVCN may reflect the increased complexity of inhibitory processing in the mammalian cochlear nucleus.

Source of the Gly-I terminals

The origin of the Gly-I terminals to NM is unknown. The cochlea may be excluded as a significant source of the Gly-I afferents to NM since the density and distribution of Gly-I terminals appeared unchanged in NM 13 and 21 days after unilateral cochlea removal.

On the other hand, the occasional Gly-labeled cell in NM may provide enough local, intrinsic connections to NM neurons to account for the few Gly-labeled terminals observed. The possibility of

extrinsic sources of the Gly-I input to NM also cannot be excluded at this time.

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