Transient GABA Immunoreactivity in Cranial Nerves of the Chick Embryo

CHRISTOPHER S. von BARTHELD and EDWIN W RUBEL

Hearing Development Laboratories, University of Washington, Seattle, Washington 98195

ABSTRACT

The distribution and time course of γ-aminobutyric acid (GABA) immunoreactivity was investigated in the cranium of the chick embryo from 2 to 16 days of incubation (E2–16). A fraction of nerve fibers transiently stains GABA-positive in all cranial motor nerves and in the vestibular nerve. Cranial motor nerves stain GABA-positive from E4 to E11, including neuromuscular junctions at E8–11; labeled fibers are most frequent in the motor trigeminal root (E6–9.5). Substantial GABA staining is present from E4 to E10 in a subpopulation (1–2%) of vestibular ganglion cells. Their peripheral processes are labeled in the vestibular endorgan, predominantly in the posterior crista. Some GABA-positive fibers are present in the olfactory nerve (after E5) and in the optic nerve (after E9.5); their immunoreactivity persists throughout the period investigated. Transient GABA immunoreactivity follows "pioneer" fiber outgrowth and coincides with the formation of early synaptic contacts.

GABA-containing neurons may change their neuronal phenotype (loss of GABA expression) or they may be eliminated by embryological cell death. Periods of cell death were determined in cranial ganglia and motor nuclei by aggregations of pycnotic cells in the same embryonic material. The periods of embryonic cell death partly coincide with transient GABA immunoreactivity. The function(s) of transient GABA expression is unknown. Some lines of evidence suggest that GABA has neurotrophic functions in developing cranial nerves or their target tissue. In the developing neuromuscular junction, GABA may be involved in the regulation of acetylcholine receptors.

Key words: γ-aminobutyric acid, development, transmitter, vestibular, trigeminal, avian, neuromuscular junction, cell death

It has been suggested that several neurotransmitters have functions in the developing nervous system that differ from their "classical" role in synaptic transmission (Buznikov et al., '70; Olson and Seiger, '72; Lauder and Bloom, '74; McMahon, '74; Lanier et al., '76). Recent studies have described "neurotrophic" effects of serotonin, dopamine, noradrenaline (references in Madtes, '87), and of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Wolff et al., '78). In vitro studies indicate that GABA promotes protein synthesis (Campbell et al., '66) and influences neurite elongation (Meier and Jorgensen, '86; Hansen et al., '87; Michler-Stuke and Wolff, '87). Long-term application of GABA induces the formation of "free" postsynaptic thickenings, and GABA appears to be involved in the regulation of synaptogenesis (Wolff et al., '78, '79; Wolff, '81).

In the brain, GABA immunoreactivity appears at very early developmental stages, before the first synaptic connections are formed (Chronwall and Wolff, '80; Fung et al., '82; Hatten et al., '84; Lauder et al., '86; Roberts et al., '87; Peduzzi, '88). It has been suggested that GABA occurs transiently in some cerebral circuits (Chronwall and Wolff, '80), but because of temporal overlap of the presumed transient and the permanent GABA circuits, this hypothesis has not been adequately tested. Transient expression of GABA in horizontal cells of rabbit retina led Redburn and Keith ('87) to hypothesize that GABAergic cells may provide "pioneering" fibers that critically influence pathfinding and positioning of later-developing neurons.

Besides being an important inhibitory neurotransmitter in the central nervous system, significant levels of GABA are found in peripheral organs and in the peripheral nervous system (Erdö and Bowery, '86). However, little attention has been directed to the distribution and function of GABA in the developing peripheral nervous system.

In the present study, we investigate the distribution of GABA in cranial nerves of the chick embryo. We describe temporospatial patterns of transient GABA expression in all motor cranial nerves and in a restricted portion of the vestibular nerve. Such staining was completely lacking in the cochlear nerve and the sensory part of the trigeminal nerve. The time-course of GABA expression in cranial nerves is not correlated with "pioneer" fiber outgrowth but occurs after the first fibers have reached their target and begin to form early synaptic contacts. Termination of transient GABA immunoreactivity may be due to embryological cell death and/or to a change of neuronal phenotype. The temporally and spatially restricted patterns of GABA in different nerve components are suggestive for a specific function of GABA in subsystems of the developing nervous system.

MATERIALS AND METHODS

By means of standard immunocytochemical· techniques, the distribution of GABA immunoreactivity was studied in 23 chick embryos (*Gallus domesticus,* White Leghorn, H + N, Redmond, Washington) from embryonic day 2 (E2) to E16. Embryonic stages from E2 to E12.5 were investigated in half-day intervals. In addition, one E14 embryo and one E16 embryo were examined. All embryos were staged with reference to Hamburger and Hamilton ('51). Ages reported are the median for each stage. Embryos of advanced stages (>E7) were anesthetized with Nembutal prior to death. The animals were perfused intracardially (from stage E5 to stage E16) or through the ascending aorta (E2–4) with 0.1 M phosphate buffer (pH 7.3–7.4) containing 0.6% glutaraldehyde and 1.5% paraformaldehyde at room temperature. The heads were then postfixed for 1–28 days at room temperature and transferred overnight to a 30% sucrose solution for cryoprotection. Frozen sections were cut on a cryostat at 30 μm in a more-or-less transverse plane (cf. Fig. 1). From embryos of ages E2–E6.5, every section through the head was collected; from older embryos, every third, sixth, or tenth section was collected and processed for immunocytochemistry. The sections were placed on gelatin-coated slides and dried for 30 minutes. Alternating sections from each animal were stained for Nissl bodies with thionin.

Standard immunocytochemical procedures were applied by using the avidin-biotin-peroxidase (ABC) method (Hsu et al., '81) and reagents from Vectastain kits (Vector Labs.). In short, the sections were rinsed in 0.1 M phosphate buffer and incubated for 1 hour in a presoak solution (Tris buffer) containing 3% normal goat serum. Incubated tissue was kept on a shaker table at room temperature. The sections were incubated for 11–20 hours in rabbit-anti-GABA antiserum (INCSTAR Corp.), diluted 1:3,000 or 1:4,000 in the presoak solution, then in the secondary antiserum (biotinylated goat-antirabbit immunoglobulin G, 1:200 in phosphate buffer) for 1 hour, and then in avidin-peroxidase complex for another hour. The sections were processed in a 0.02% diaminobenzidine solution for 5–10 minutes. Most sections were lightly counterstained with thionin, dehydrated, and coverslipped with DPX mounting medium. One set of sections from each of two animals (E6.5 and E7.5) was not dehydrated; each was coverslipped with a hydrophilic glycergel mounting medium (DAKO). Sections were examined on a Leitz Orthoplan microscope with oil immersion and 63× and 100× objectives by using both brightfield and Nomarski optics.

Control sections incubated without the GABA antiserum showed no immunoreactive staining. The specificity of the
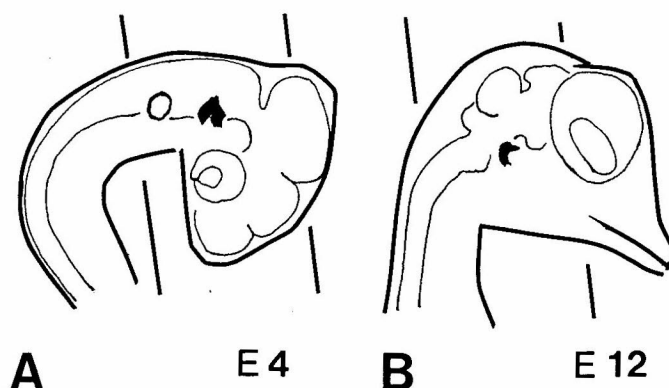

Fig. 1.   Schematic drawing of lateral aspect of a chick embryo's head and brain at embryonic ages E4 (A) and E12 (B). Heads are drawn to same size. Approximate plane of sectioning is indicated by black lines. The otocyst is represented by a heavy circle at the level of the hindbrain (A); the trigeminal ganglia are marked in black (A, B).

antiserum used has previously been tested in our own laboratory (Code et al., '89) and by others (e.g., Maley and Newton, '85).

Three embryos (E7, E7.5, and E14) were perfused with 1% paraformaldehyde and were immunoprocessed with an antiserum against glutamic acid decarboxylase (GAD, Gottlieb et al., '86; Code et al., '89), essentially as described above for the GABA antiserum.

For examination of embryological cell death, an alternate series of immunostained sections from the same animals was counterstained with thionin. Pycnotic nuclei (degenerating cells) are prominent in Nissl-stained sections (e.g., Hamburger, '75; Hamburger et al., '81; Linden and Pinon, '87). The method of observing degenerating cells, rather than counting absolute numbers of cells at different stages, provides the advantage that the onset and relative degree of degeneration can be accurately estimated, although the absolute amount of cell loss is not obtained (Hamburger et al., '81). In the present context, it sufficed to determine whether or not a particular time in development coincides with a period of extensive cell death. Periods of increased cell death were investigated only in those structures and stages relevant for comparison with patterns of transient GABA immunoreactivity (Table 2).

RESULTS

All embryos were staged according to the Hamburger and Hamilton ('51) series; to facilitate comparisons, we refer to the corresponding stages as days after incubation (E2–16). We use the expressions "GABAergic" and "GABA-immunoreactive" cells, although we have determined only "GABA-like immunoreactivity," and—theoretically—the antiserum could crossreact with a GABA-like antigen. In all cases examined, patterns of GABA immunoreactivity were similar on both sides of the brain.

GABA immunoreactivity in cranial nerves

In the *olfactory nerve,* GABA-labeled fibers are very rare. At E4.5–6.5, we counted between two and five immunopositive fibers per animal on each side. The relatively thick fibers (1–2 μm) course in different fascicles of the olfactory nerve into the olfactory bulb. The origin of these fibers could not be determined. No cell bodies are convincingly labeled in the olfactory epithelium or in the olfactory nerve
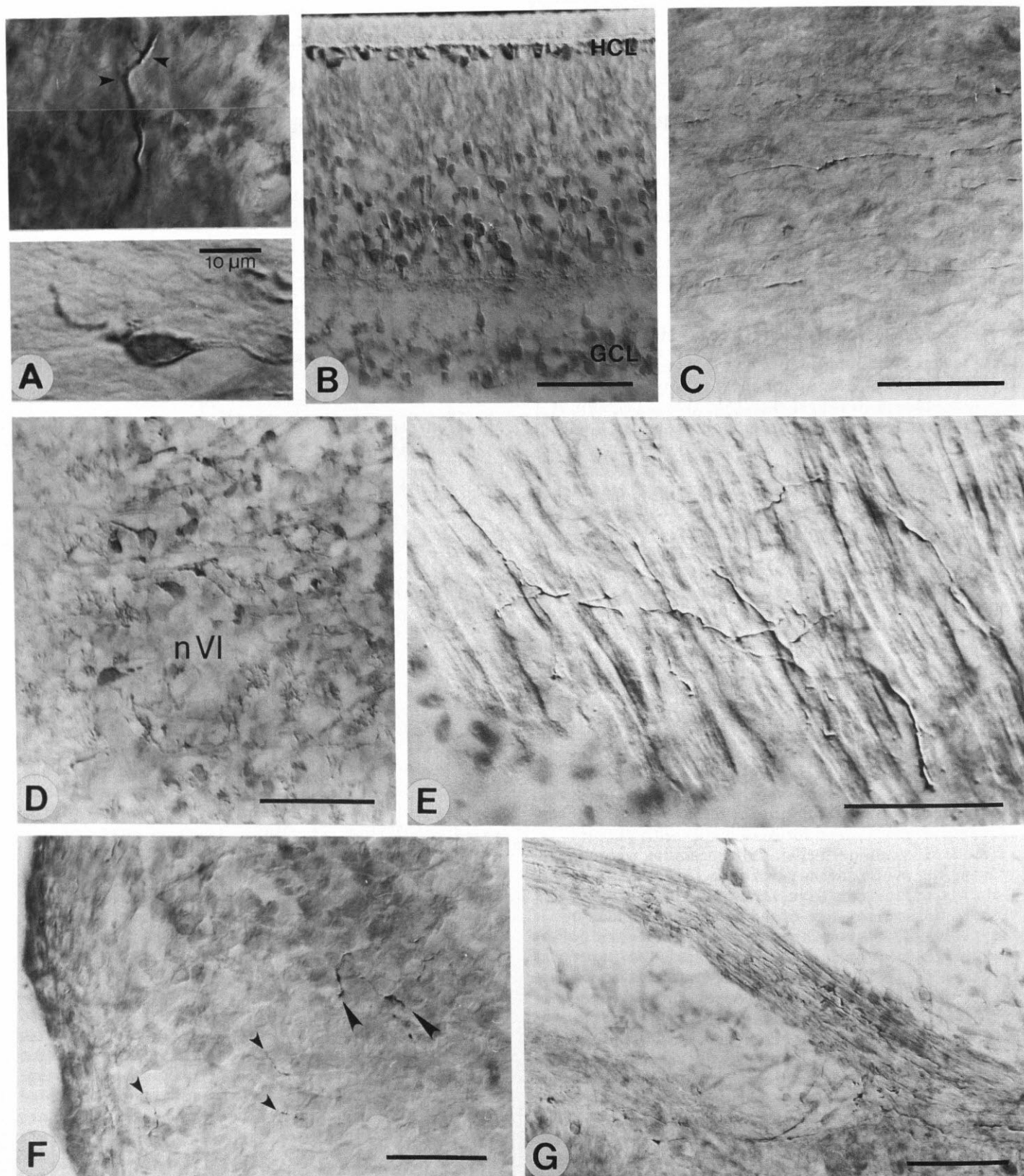
Fig. 2. A–G: Sections through the cranium of the chick embryo processed for GABA immunoreactivity. The sections were lightly counterstained with thionin and photographed with Nomarski optics. Scale bars: 50 μm, if not indicated otherwise. **A:** Section through the olfactory nerve showing a thick GABA-positive nerve fiber at E5 (**upper panel**) and a labeled bipolar cell at E14 (**lower panel**). Both panels at same magnification. *Arrowheads* indicate bifurcations of large-caliber fiber. **B:** Section through the retina at E9.5. Note intense labeling of the horizontal cell layer (HCL). GCL: ganglion cell layer. **C:** Section through the optic nerve showing GABA-positive fibers at E9.5. **D:** Section through the nucleus of the abducens nerve in an E6 embryo showing GABA-immunostained cells. **E:** GABA-positive motor fibers of the oculomotor nerve innervating the superior rectus muscle at E9.5. **F:** GABA-positive nerve fibers (*arrowheads*) of the oculomotor nerve in the ciliary ganglion at E6.5. **G:** Section through the trochlear nerve at E8.5 showing GABA-immunostained fibers.

at this stage. The labeled fibers seem to course centripetally, since they bifurcate (Fig. 2A) and send collaterals toward the brain. Considering their large diameter, they resemble fibers of the nervus terminalis (von Bartheld et al., '87) rather than fibers of the olfactory filae. The rostralmost part of the head with the olfactory pathway was investigated only at one later age (E14). At this stage, several bipolar cells are GABA-positive in the proximal olfactory nerve (Fig. 2A). They apparently give rise to labeled fibers in the olfactory nerve. The number of these cells was not determined. The distal olfactory nerve and epithelium were not examined at this stage.

In the *retina,* the first GABA-positive neurons appear as single neurons in the proliferating zone between the ganglion cell layer and the pigment layer at E6. Staining is more pronounced after E9: the monolayer of outer horizontal cells is intensely labeled (Fig. 2B). Some cells are labeled in the inner nuclear layer, and terminal puncta appear in the inner plexiform layer. Staining is more pronounced in the medial than in the lateral retina (E8–16). In the embryo, labeling of the inner plexiform layer is less intense than in the adult (Agardh et al., '87). Light labeling is present in the ganglion cell layer, but no immunoreactive fibers are found in the optic nerve before E9.5. At E8.5, some GABAergic fibers occur in the margin of the optic tract and chiasm, while after E9.5 immunoreactive fibers are present in the optic nerve (Fig. 2C). This proximodistal sequence suggests that GABAergic fibers in the optic nerve originate from neurons in the optic tract or from other areas of the brain and do not represent axons from GABAergic retinal ganglion cells. The GABA-positive fibers do not appear to originate from the isthmo-optic nucleus, which remains GABA-negative

TABLE 1. Number of GABA-Immunoreactive Fibers in Embryonic Nerves

| Age | Cranial nerves[1] | | | | |
	III	IV	V	VI	VIII[2]
E5.5	5–10	n.d.	50–70	n.d.	60–75
E6.5	25–30	10–15	90–120	n.d.	200
E8.5	40	20–30	150–180	10–15	n.d.
E9.5	40	15	200	150	n.d.

[1]n.d., not determined. [2]Labeled ganglion cells counted.

(Granda and Crossland, '87). GABAergic fibers are observed in the optic nerve up to the latest stages investigated (E16).

The *oculomotor nerve* contains GABA-immunoreactive fibers after E4 and until about E10. At early stages (E4), the immunoreactivity is diffuse and not confined to single fibers. Nuclear borders of the oculomotor nucleus are difficult to identify before E4. After E4.5, GABAergic cells can be identified in all eye muscle nuclei (cf. Fig. 2D). In the present material, it was not possible to demonstrate that these cells actually give rise to the immunoreactive fibers in the nerves. In later stages, the nerve fibers seem less immunoreactive in their proximal portion, while GABA immunoreactivity becomes more pronounced further distally (Fig. 2E). GABA may move from perikaryal to distal (axonic) sites during development, as has been observed in maturing cerebellar neurons (cf. Curtis and Stewart, '86).

From E5 to E8.5, some fibers of the oculomotor nerve are GABAergic as they enter the ciliary ganglion. These fibers ramify and appear to terminate in various portions of the ganglion (Fig. 2F). The cells of the ciliary ganglion are not seen to be GABAergic at any stage, and no GABAergic fibers

are observed to leave the ganglion in the ciliary nerve. Thus, GABA-positive fibers in the ciliary ganglion apparently represent visceral, preganglionic fibers of the oculomotor nerve. At E6.5, GABAergic neurons are frequent in the nucleus arciformis, the possible homologue of the Edinger-Westphal nucleus in mammals (Niimi et al., '58). During E6–9.5, we counted about 25–40 immunoreactive fibers in cross sections through the oculomotor nerve proximal to the ciliary ganglion (Table 1). GABAergic fibers enter the superior and the inferior rectus muscle at E8.5, and all the external eye muscles appear to be innervated to some extent by GABA-positive fibers at E9.5 (Fig. 2E). Labeled motor fibers form small terminal boutons which resemble the "en grappe"-type motor endplate (e.g., Hess, '61). In the eye muscles, GABAergic motor endplates are less frequent than in muscles innervated by the trigeminal nerve (see below). GABA-positive staining is not observed in any eye muscle investigated after E11.

The *trochlear nerve* becomes immunoreactive at E5. We counted between ten and 30 immunoreactive fibers in cross sections through the nerve in animals at E6–E9.5 (Table 1). Trochlear nerve fibers are immunoreactive in distal as well as proximal portions of the nerve at E8.5 (Fig. 2G). GABA-positive fibers are traced to distal portions of the nerve at E9.5, but the superior oblique muscle was not examined. GABA immunoreactivity could not be observed after E11.

The *trigeminal nerve* shows no GABA staining in the purely sensory portions at any age, including the ophthalmic nerve and the entire sensory ganglion. Considerable portions of the trigeminal motor root, however, are immunoreactive from E4.5 to E11. At E11.5 staining is very faint, and by E12 it is no longer detectable.

At E4, neurons are immunoreactive at the floor of the brainstem (Fig. 3A); these GABA-positive neurons may be young trigeminal motor neurons in the process of migration and neurite outgrowth (Heaton and Moody, '80). Some of their processes extend laterally, toward the exit of the trigeminal nerve from the brainstem (Fig. 3A). At later stages (after E4.5–5.0), it is impossible to identify the origin of GABAergic nerve fibers, because too many GABAergic elements are present in the brainstem. Some favorable cases reveal labeled fibers that are traced from ventral portions of the brainstem to exit through the medial portion of the trigeminal nerve; they continue their course through and along the ventromedial portion of the trigeminal ganglion (Fig. 3B). The labeled fibers are contained in nerve fascicles which invade masses of undifferentiated myoblasts. In early stages, some labeled fibers appear to end in growth cones.

Most of the GABAergic fibers are contained in the medial fascicle of the trigeminal nerve as it leaves the brain, but a few are found in the two adjacent fascicles. The axon diameters range from approximately 0.5 μm to 2.5 μm. Distal to the trigeminal ganglion, the motor root divides into several fascicles. A disproportionately large number of GABA-positive fibers follow the fascicle into the developing adductor muscles of the beak (E6–9). In all stages, only a small fraction of fibers are labeled in each nerve fascicle (Fig. 3C). We counted approximately 200 GABAergic fibers in cross sections of the entire motor root of the trigeminal nerve on each side at E9.5 (Table 1).

Following the stage of fasciculation and branching in the muscle (E6), numerous GABA-positive motor endplates are formed (E7–10). Labeled terminals resemble the en grappe type rather than the "en plaque" motor endplate. The
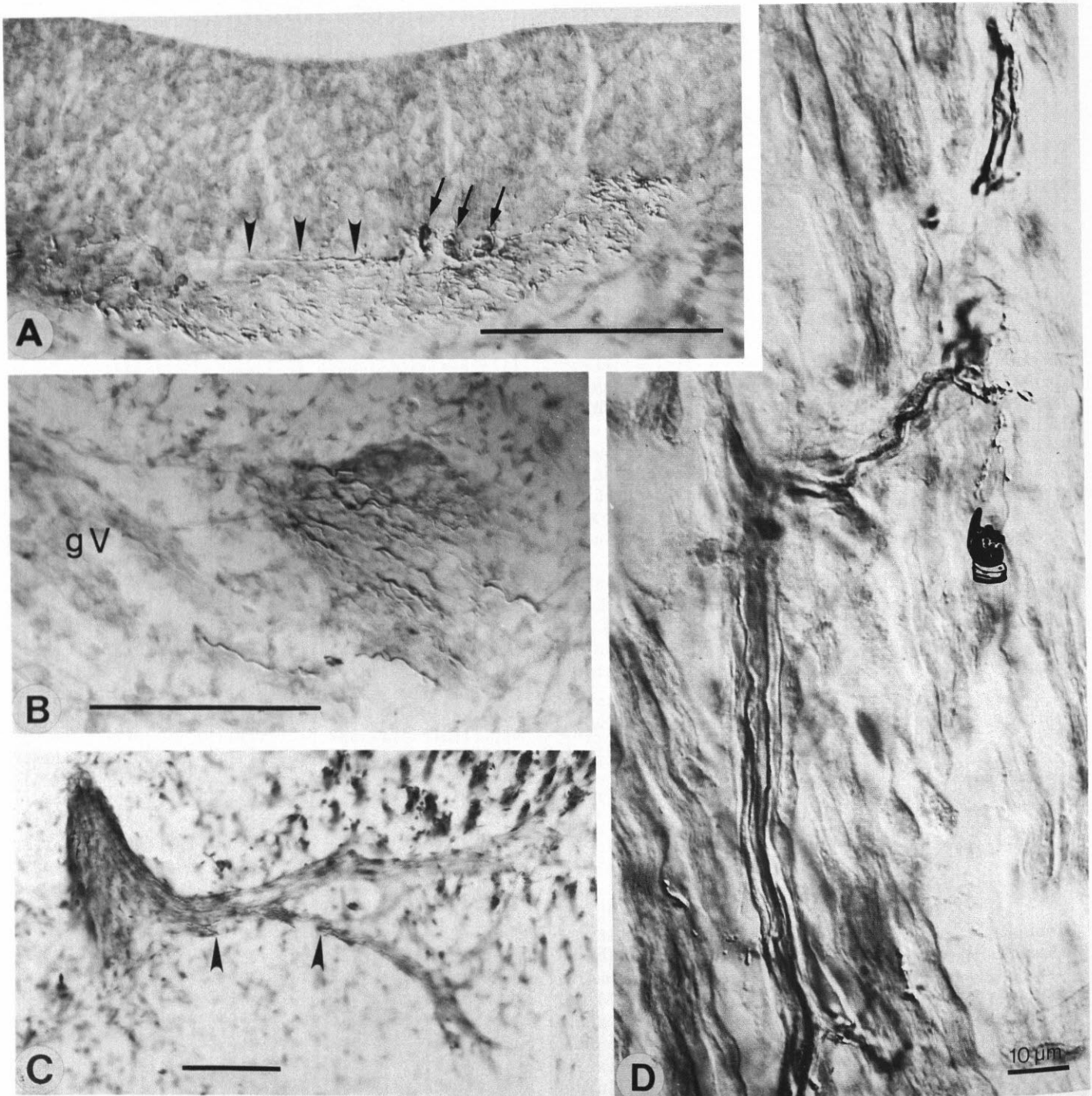
Fig. 3. GABA immunoreactivity in the embryonic trigeminal motor pathway. Sections are lightly counterstained with thionin and photographed with Nomarski optics. Scale bars: 100 μm, if not indicated otherwise. A: Section through the brainstem close to the exit of the trigeminal nerve at E4. Note GABA-positive cells (arrows) and labeled processes extending laterally (arrowheads). B: Several dozen nerve fibers are GABA-positive in the motor root of the trigeminal nerve at E6.5; such staining is lacking in the sensory ganglion cells of the trigeminal nerve (gV). C: GABA-positive fibers (arrowheads) occur in several fascicles of the ramifying motor trigeminal root at E6.5. D: Section through the inferior palpebral muscle showing GABA-positive motor endplate (pointer) and nerve fibers of the trigeminal nerve at E9.5.

adductor muscles of the beak are the first in which differentiated GABAergic contacts develop (E7–9). At E9.5, GABAergic innervation of the inferior palpebral muscle, which develops relatively early in the chick embryo (Adelmann, '27), is also abundant (Fig. 3D). GABA staining of

nerve fibers and endplates is lacking after E11.5.

The abducens nerve is GABA immunoreactive from E4.5 to E11.5. The abducens motor nucleus contains some GABAergic cells in early stages (E6.5–7.5, Fig. 2D). The peripheral nerve shows diffuse staining in this period, and
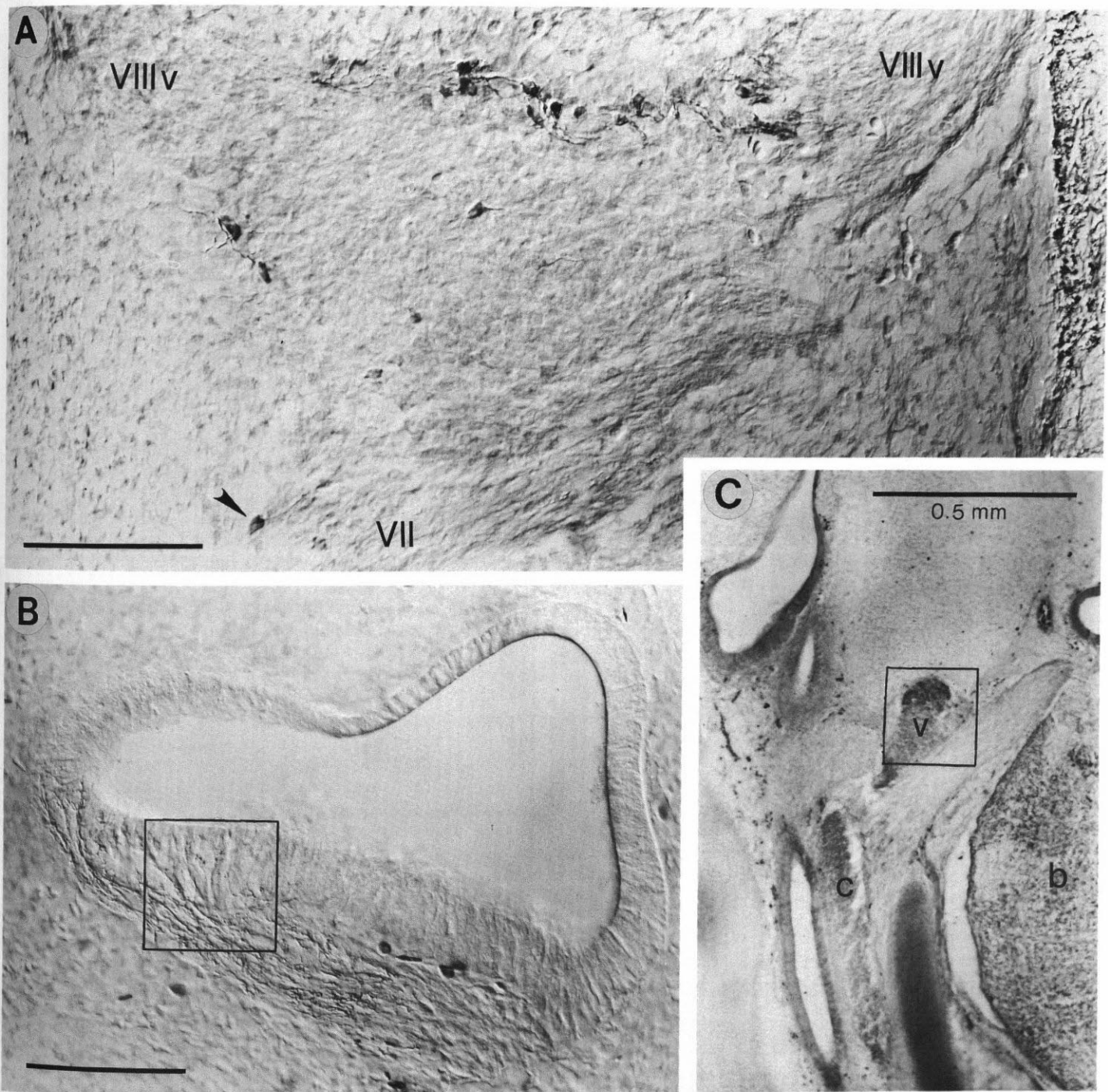
Fig. 4.   GABA immunoreactivity in the primary vestibular pathway at E7. **A:** Section through the vestibular ganglion and vestibular nerve (VIIIv). Most GABA-positive ganglion cells are located in a dorsal position; few occur in a farther ventral position and one labeled cell (*arrowhead*) is located close to the facial nerve (VII). The lateral edge of the brainstem is visible on the right side of the photomicrograph. Scale bar: 100 µm. **B:** Section through the developing posterior crista. Note GABA-positive fibers in lateral portion of the sense organ (left). Marked area is shown at higher magnification in Figure 5A. Scale bar: 100 µm, counterstained with thionin. **C:** Low-power photomicrograph of embryonic brainstem and inner ear. Marked area is shown at higher magnification in Figure 5B. b, brainstem; c, cochlear ganglion; v, vestibular ganglion.

the label does not appear confined to single nerve fibers. In later stages (E9.5), the abducens nerve contains approximately 150 intensely GABA-stained motor axons. The lateral rectus muscle was not examined after E9.5; up to this stage, GABAergic motor endplates were not observed. GABA immunoreactivity is lacking in the abducens nerve after E12.

The *facial nerve* contains some immunoreactive fibers from E4.5 to E10. While the vast majority of sensory ganglion cells are not immunoreactive, in young embryos

(E5–6.5, data not shown) a few cells appear lightly GABA-positive in the distal ganglion of the facial nerve (D'Amico-Martel and Noden, '83). Their staining intensity is not comparable to that seen in vestibular ganglion cells (see below). The proximal ganglion of the facial nerve is contained in the vestibular ganglionic complex; here, approximately ten to 15 single ganglion cells are GABA-positive at E6.5. Most of these bipolar ganglion cells seem to project laterally, into the vestibular endorgan (see below), but others obviously have processes that course ventrally into the facial nerve. In
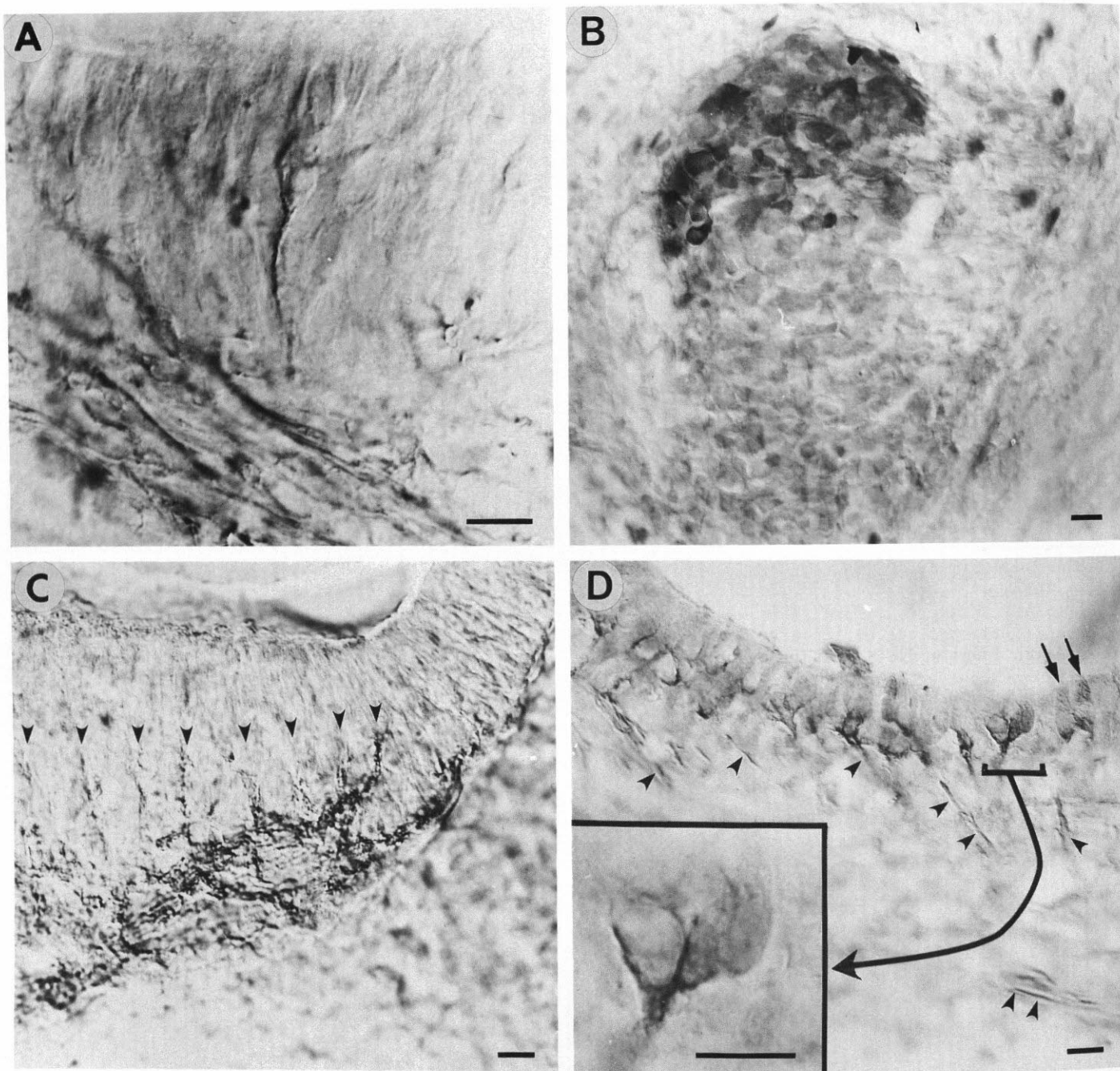
Fig. 5. GABA immunoreactivity in the embryonic primary vestibular pathway. Scale bars: 10 μm. **A:** GABA-positive fibers in the posterior crista at E7. Note labeled fiber approaching and embracing a hair-cell-like structure in the sensory epithelium. **B:** GABA-positive cells in the vestibular ganglion at E7. Note the clustering of labeled cells in the dorsal portion of the ganglion. **C:** GABA-positive fibers of the vestibular nerve innervating the posterior crista at E7. Note that labeled fibers occur at consistent intervals (*arrowheads*) as they approach the epithelium. The lateral side is to the right. **D:** GABA immunoreactivity in primary afferent fibers (*arrowheads*) at E9.5. Note formation of calyxlike afferent endings (**insert**) on hair cells in sensory epithelium. Hair cells are GABA-positive at this stage (*arrows*).

one case, we counted five such labeled fibers in distal portions of the facial nerve. The portion of the facial nerve which innervates the paratympanic organ contains rare GABA-positive fibers (von Bartheld, '89); a few such fibers are seen below the sensory epithelium at E5.5 and E7. Motor fibers of the facial nerve are GABAergic in the anlage of the facial (ventral) muscles at E5.5. The posterior ramus of the facial nerve contains some GABAergic fibers at E8–9.5. No immunostaining was observed in the facial nerve after E11.5.

The *cochlear nerve* and ganglion cells are not GABA-positive at any of the stages investigated, between E5 (the earliest stage in which the cochlear ganglion is separate from the vestibular ganglion) and E16.

The *vestibular nerve* contains GABA-positive cells and fibers from E4 to E11. The first GABA-positive cells are found at E4 in the portion of the vestibular ganglion immediately adjacent to the basal lamina of the otocyst. At E4–4.5, labeled processes are short and grow along the basal lamina. No fibers are labeled in the proximal vestibular

nerve before E4.5, but at this stage labeled fibers already enter the epithelium of the otocyst. At E5–8, a subpopulation of labeled ganglion cells forms clusters in the dorsal half of the ganglion (Figs. 4C, 5B), along the rostral two-thirds of the vestibular ganglion. A few isolated labeled cell bodies are also observed in further ventral parts of the ganglion (Fig. 4A). Often, the bipolar shape of the labeled cells is apparent (Fig. 4A). We counted 200 labeled ganglion cells on each side in one case at E7, which is 1.5–2% of the vestibular ganglion cells (cf. Ard and Morest, '84). Most of the central GABAergic processes enter the brainstem as a distinct GABAergic fiber bundle within the dorsal part of the vestibular nerve. In the brainstem, the labeled fibers could not be traced to their terminations because of the large number and diversity of GABAergic fibers and neurons in the brain.

Peripheral processes of labeled vestibular ganglion cells can be followed into developing portions of the vestibular endorgan (Figs. 4B, 5A,C,D). In some cases, the processes of single ganglion cells are immunolabeled within the same section for 200–300 μm and can be traced individually into sensory areas of the developing labyrinth. Such cases indicate that most GABAergic neurons in lateral components of the vestibular ganglionic complex are vestibular, while others may belong to the proximal portion of the facial ganglion, which is contained within the vestibular ganglionic complex (D'Amico-Martel and Noden, '83).

The distribution of GABAergic fibers to the sensory endorgan is not uniform; the posterior cristae receive more GABAergic fibers at E6–8 than the maculae, the anterior cristae, and the lateral cristae. Within the posterior crista, the distribution of GABAergic fibers is restricted to the lateral side (Fig. 4B). Often, the labeled fibers grow laterally for quite a distance along the basal lamina, before they enter the developing sensory epithelium. Occasionally, the tips of the fibers resemble growth cones (Fig. 5A). The GABA-positive fibers which have penetrated the basal lamina end about one cell-length short of the otocyst lumen (Fig. 5A). Some fibers seem to "embrace" unstained cells in the epithelium, presumably future hair cells (Fig. 5B). At this stage (E6–7), the hair cells have not yet developed GABA immunoreactivity.

From E9 to E10, GABA staining is still present in some vestibular ganglion cells and fibers, but the staining begins to fade by E11. Immunostaining is still clearly above background in the endorgan, especially in nerve fibers and in conspicuous afferent endings of vestibular fibers (Fig. 5D). Due to their thickness and their calyxlike endings on hair cells in the cristae, these fibers are easily identified as the colossal fibers of the vestibular nerve which grow into the cristae at E6.5–8 (Fink and Morest, '77). The stage in which endings of colossal fibers are GABAergic (E9.5–11) corresponds with the stage of "protocalyces" as described by Fink and Morest ('77). The hair cells of the vestibular and cochlear endorgans become GABA-positive at E7–9 (Figs. 5D, 8).

The *glossopharyngeal and vagal* nerves contain some GABAergic fibers from E4.5 to at least E9.5. The overall staining characteristics are similar to those observed in the facial nerve. Only very few, if any, ganglion cells appear to be specifically labeled in the proximal or the distal ganglia. Several GABA-positive fibers pass through the sensory ganglia. As with the facial nerve, it is difficult to tell parasympathetic, preganglionic, and other divisions apart. At E11.5, GABAergic fibers are contained in several myenteric ganglia which circumscribe the foregut (esophagus) in a circular arrangement.

The *hypoglossal nerve* contains a considerable number of GABAergic fibers at E5 and E6. At these stages, no endplates are labeled in the muscle anlagen of the tongue. The peripheral targets of this nerve were not investigated at later stages. No label was detected in the proximal hypoglossal nerve at E14.

A few of the most cranial *spinal nerves* were examined at stages E4 to E7.5. Some ventral root fibers are GABAergic from E5.5 to E7.5. The staining characteristics and temporospatial distribution appear principally similar to those of the trigeminal motor root, although the number of GABAergic fibers in nerve fascicles and of labeled motor endplates on muscle cells is smaller. The dorsal root ganglia of spinal nerves also contain some GABA-positive fibers, but ganglion cell bodies were not convincingly labeled. No label was found in the proximal spinal nerves at E14.

The periods of transient GABA immunoreactivity in cranial nerves and ganglia, as well as periods of cell death (see below), are synoptically summarized in Figure 6.

Cell death: distribution and time course

In order to evaluate the possibility that transiently GABAergic neurons are eliminated by embryological cell

TABLE 2. Periods of Cell Death in the Cranium of the Chick Embryo[1]

	Age of embryonic (E) incubation (day)														References
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
Optic stalk		*	*	*											
Paratympanic organ		*	*	*	*	*	*								
Otocyst epithelium		*	*	*	*	*	*	*	*	*	*	*	*		
Vestibular nuclei		*	*	*	*	*	*	*							
Trigeminal ganglion		*	*	*	*	*	*	*	*						
Spinal ganglia				*	*	*		*	*	*					Hamburger et al. ('81)
Facial ganglion				*	*	*	*	*	*						
Ciliary ganglion				*	*	*	————————								Landmesser and Pilar ('76)
Oculomotor nucleus				*	*	*		*	*	*					
Trigeminal nuclei				*											
Spinal motor column				*	*	*	*	*	*						Hamburger ('75)
Vestibular ganglion					*	*	*		*						Ard and Morest ('84)
Cochlear ganglion					*	———————————————									Ard and Morest ('84)
Retina						*	*	*	*	*	*	*	*	*	————— *	Rager ('80)
Trochlear nucleus							*	*	*	———————					Cowan and Wenger ('67)

[1] * – determined in the present study; ___ – as reported in the literature.
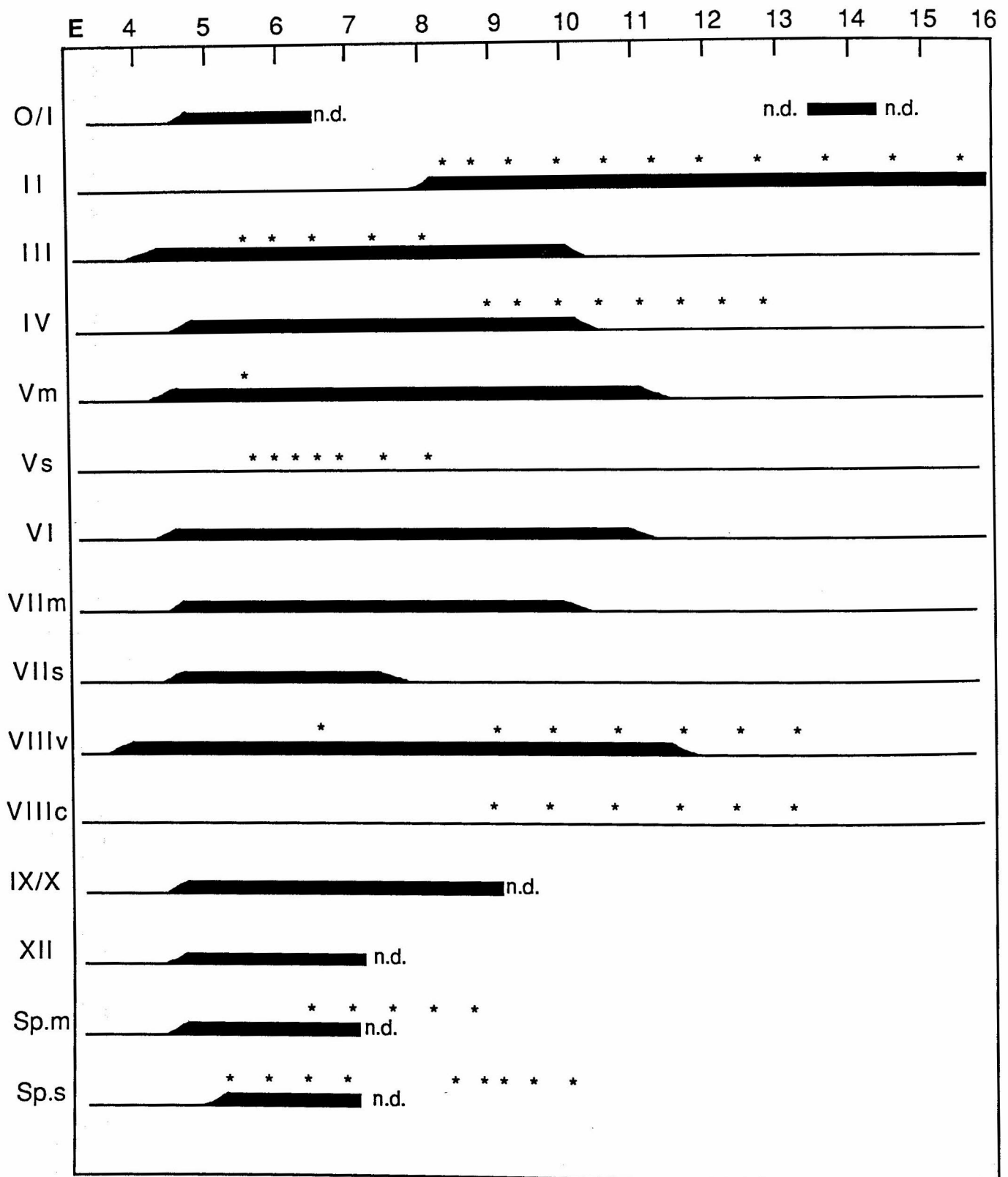
Fig. 6. Summary of GABA immunoreactivity in cranial nerves of the chick embryo. Thick black bars indicate period of GABA immunoreactivity during development; thin lines indicate the lack of GABAergic fibers. Asterisks indicate periods of cell death which were derived from the same material, and from the literature (cf. Table 2). Abbreviations: m, motor; s, sensory; v, vestibular; c, cochlear; Sp., spinal; n.d., not determined.
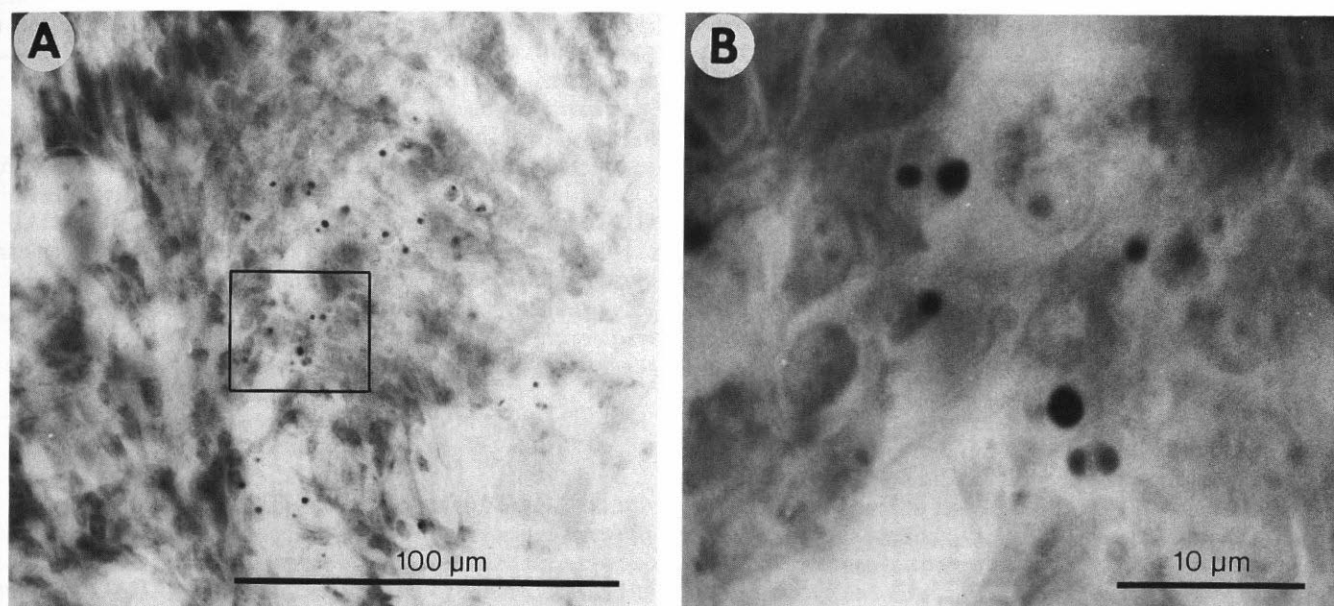
Fig. 7.   Pycnotic (degenerating) cells in vestibular brainstem nuclei at E5.5 in the chick embryo. Nissl-(thionin)-stained cryostat section. Marked area in **A** is shown at higher magnification in **B**.

death, we determined periods of cell death by examining Nissl-stained sections for degenerating, pycnotic neurons. Pycnotic cells have a dropletlike shape and stain bright blue with thionin (Fig. 7A,B, cf. Hamburger, '75; Hamburger et al., '81; Linden and Pinon, '87). Degenerating cells can occasionally be observed in nearly every embryonic tissue, but they are found in high concentrations at particular ages. Clusters of pycnotic cells in sensory ganglia and in some motor nuclei correlate with periods of cell death as determined by cell or fiber counts reported by Cowan and Wenger ('67), Landmesser and Pilar ('76), Rager ('80), and Ard and Morest ('84). In some cases, e.g., in the ciliary ganglion (Landmesser and Pilar, '76) and the vestibular ganglion (Ard and Morest, '84), we determined the onset of degeneration to be 1–2 days earlier than in reports based on cell counts (Table 2).

The vestibular and paratympanic endorgans show periods of cell death between E4.5 and E7. Most cranial ganglia contained increased numbers of degenerating cells between E5 and E8. Degeneration appeared somewhat later in the vestibular ganglion (Fig. 7A,B), in which pycnotic cells were observed only after E6.5. The distribution of cell death in the vestibular ganglion was not uniform; more degeneration occurred in ventrocaudal than in rostrodorsal components (the latter contains the GABA-positive ganglion cells). Periods of cell death in sensory cranial ganglia are similar to those in spinal ganglia (Table 2). Motor nuclei showed a more variable pattern of cell death, occuring between E5.5 and E10. From E4.5 to E6, we found a marked peak of degeneration in the brainstem vestibular nuclei (Fig. 7A,B, Table 2). In the retina, cell death was observed between E8 and E16 in both the bipolar cell layer and the ganglion cell layer.

GABA immunoreactivity in the developing vestibular endorgan

The different components of the acousticovestibular endorgan develop from three proliferation areas (Knowlton,

'67): The anteroventral region gives rise to the anterior and lateral cristae ampullares and the macula utriculi; the ventromedial area differentiates into the macula sacculi, acustica basalis, and the lagena; and the posteroventrolateral area forms the posterior crista and the macula neglecta. Their development gradually progresses through four partly overlapping stages.

1. Stage of early epithelial differentiation (E4–5). Three epithelial areas, located at the posterior, anterior, and anteromedial aspect of the otocyst, thicken and aggregate pycnotic cells; they can thus be recognized as proliferating areas and are distinct from the rest of the otocyst epithelium. All three proliferating areas are approached by GABAergic fibers at E5 (Fig. 8). A fourth ventral area, the future cochlea, is recognized in ventral portions of the otocyst at E5–6, but it is not approached by GABAergic fibers. Vestibular ganglion cells proliferate in the otic placode adjacent to the otocyst; some ganglion cells are already GABA-positive at E4.

2. Ingrowth of GABAergic nerve fibers (E5–7.5). In the vestibular endorgans, GABAergic fibers grow along the basal lamina of the epithelial thickenings, penetrate the membrane, and enter the epithelium. At E5–6, similar numbers of GABAergic fibers enter the proliferating sensory areas. From E6.5 to E8, such fibers are rare in the anterior endorgan but are frequent in the posterior crista. Within this area, the distribution of GABA-positive innervation is asymmetric, the lateral half of the area receiving the vast majority of GABAergic fibers (Fig. 4B). In the medial half, there are about as many nerve fibers (data not shown), but they are not GABAergic. At this stage, the epithelia forming the cristae begin to enlarge, and the matrix of the future otoliths can be identified.

3. Onset of GABA expression in hair cells (E6.5–8). First, some hair cells in the saccule develop light GABA immunoreactivity in their cytoplasm (data not shown). Hair cells in the utricle and in the anterior and lateral cristae become GABAergic at E7, and those in the lagena follow after E8. At
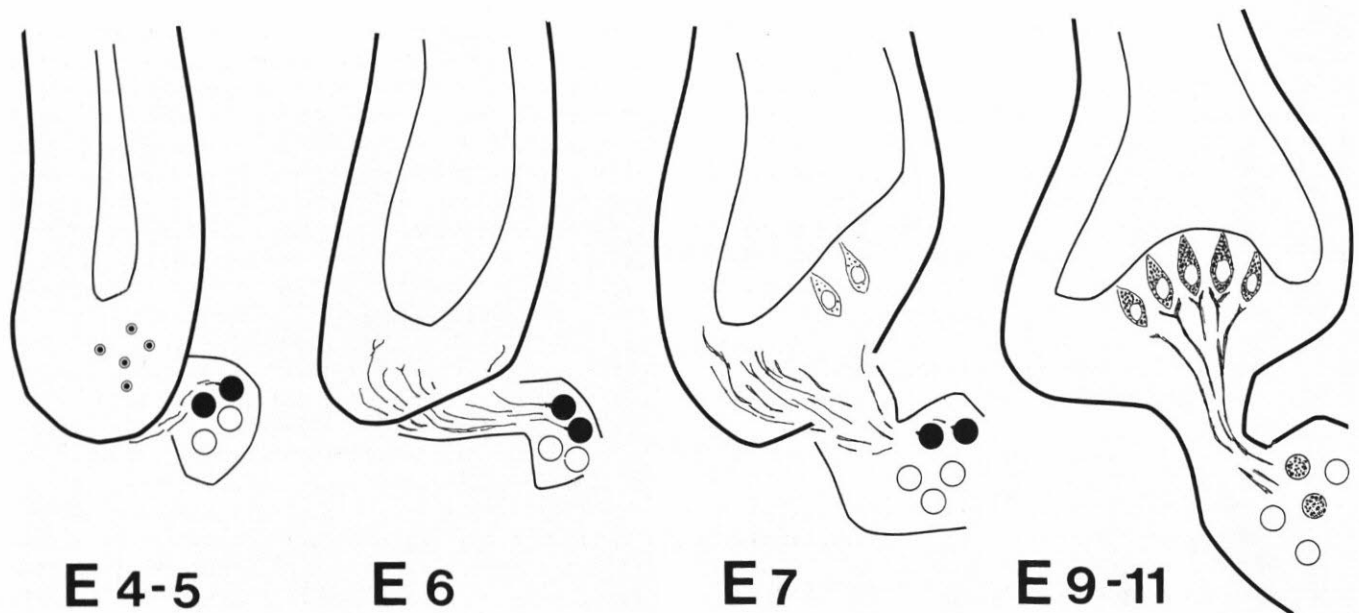
Fig. 8.　Summary of transient GABAergic innervation of the vestibular endorgan (posterior crista) in the chick embryo from E4 to E11. Small dots: pycnotic cells. The degree of GABA staining is indicated from stippling to black (ganglion cells and hair cells). Lateral is to the left.
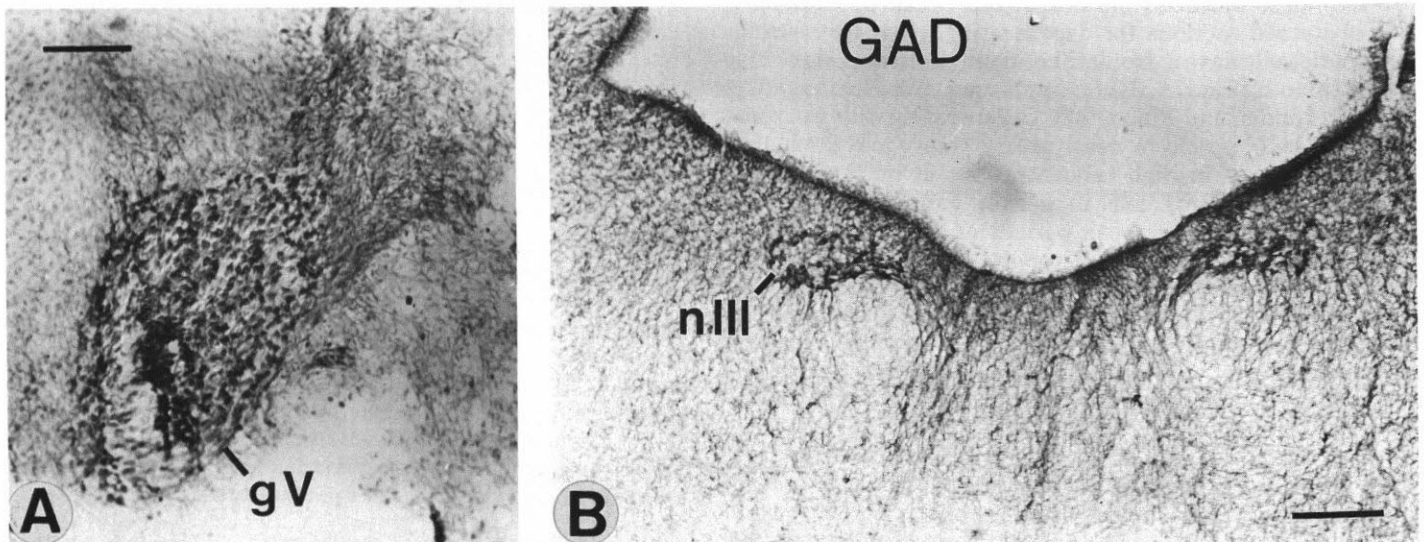

Fig. 9.　Transverse sections through the head of an E7 embryo immunoreacted with an antibody to GAD. Chromogen: DAB. Scale bars: 100 μm. **A:** Cell bodies are labeled throughout the sensory trigeminal ganglion (gV). **B:** Many neurons are immunolabeled in the oculomotor nucleus (nIII).

E9, most hair cells in the vestibular and cochlear endorgan show strong GABA immunoreactivity in their cytoplasm (cf. Usami et al., '87).

4. Formation of GABAergic calyxlike endings (E9–11). After E8.5, many large-caliber vestibular fibers are strongly GABA-positive in their distal portions; these obviously are colossal fibers which form spoonlike endings (calyces) on the hair cells in the anterior, lateral, and posterior cristae. A comparable GABAergic innervation of the utricle and saccule is lacking. Many vestibular ganglion cells still appear weakly GABA-positive at this stage. GABAergic innervation of the endorgan was not seen after E12.

This sequence of events during the innervation of the vestibular endorgan is schematically summarized in Figure 8.

Further observations with the GAD antibody

Sections from three embryos (E7, E7.5, and E14) processed with an antibody to GAD revealed the following results. At E7 and E7.5, the majority of cell bodies are GAD-positive in the trigeminal (Fig. 9A), facial, vestibular, glossopharyngeal, and vagal ganglia as well as in the dorsal root ganglia of the spinal cord; in the cochlear ganglion, a few cells are labeled. Some neurons are labeled in motor nuclei. In the oculomotor nucleus, in particular, the majority of cells appears GAD-positive (Fig. 9B). In the olfactory nerve and in the retina, diffuse label is not confined to nerve fibers or cell bodies. A similar diffuse staining for GAD is observed in the olfactory epithelium and in the acousticovestibular endorgan.

In the E14 embryo, GAD label was detected in neither cell bodies of cranial nerve ganglia nor in motor neurons. Neurons in the retinal ganglion cell layer are lightly stained, but no label was detected in the horizontal cell layer. In all three stages investigated, axonal GAD immunoreactivity was very faint or lacking, including the inner plexiform layer, in which GAD-immunoreactive fibers are abundant in the adult chicken retina (Agardh et al., '87).

GAD expression in sensory and motor nuclei apparently changes during development. While most GABA-positive cells appear to also express GAD, some structures (e.g., the trigeminal sensory ganglion) immunoreact transiently for GAD, but not for GABA. Such differences demonstrate that, at least in development, GABA and GAD expression are not necessarily parallel.

DISCUSSION

The present study demonstrates transient GABA immunoreactivity in several regions of the developing nervous system. In the following sections, we will discuss how the timing of GABA expression relates to other events in the developing nervous system. Since GABA expression appears similar in all motor nerves, we will discuss them in one section before we deal with transient GABA expression in the primary vestibular pathway. In a third section, we will discuss the distribution and possible functions of GABAergic nerves in the developing nervous system.

GABA expression in embryonic motor nerves

GABA-immunoreactive fibers occur in all cranial motor nerves of the chick embryo. However, the time course and frequency of GABAergic motor axons vary among the cranial nerves (Fig. 6, Table 1). The first motor nerve fibers grow out between E2 and E4 (Windle and Austin, '36; Romanoff, '60; Heaton and Moody, '80; Moody and Heaton, '81). GABA immunoreactivity appears in motor axons after the earliest motor axons have grown out and therefore does not seem to be a feature of "pioneering fibers" (Redburn and Keith, '87).

It is known that motor axons spontaneously release acetylcholinelike molecules and perhaps other substances from the growth cone while they approach their target (Young and Poo, '83). Similarly, it has been reported that GABA is taken up and released from GABAergic growth cones (Gordon-Weeks et al., '84). The first morphologically mature neuromuscular junctions are reported at E4–9 in the chick embryo (Atsumi, '71; Sisto Daneo and Filogamo, '73, '74; Atsumi, '77). At about E5, the first muscles appear to be functionally innervated (Ripley and Provine, '72; Landmesser and Morris, '75). At early stages (E4.5–5.5), some GABAergic motor axons seem to end in growth cones, but the major expression of GABA is seen when the fibers have reached their target and GABA is still expressed when specialized contacts (motor endplates) are forming. Transient GABA expression thus coincides with the stage in which cranial nerves form synaptic contacts.

The morphology of GABA-positive motor terminals in embryonic muscle resembles that of en grappe motor endplates which innervate slow (tonic) extrafusal muscle fibers (e.g., Hess, '61). In the chick embryo, en grappe terminals can be distinguished ultrastructurally from en plaque terminals at early stages (Atsumi, '77). Further studies are necessary to determine whether transient GABAergic innervation is confined to the slow muscle fiber type.

While GABA receptors seem to occur on most or all neuronal membranes (Bowery, '86), they have not been reported to occur on sceletal muscle cells in vertebrates, and their presence on smooth muscle cells is controversial (Erdö, '85; Amenta, '86; Hill and Bowery, '86; Jessen et al., '86; Kerr and Ong, '86). It would be of considerable interest to determine whether GABA receptors are transiently expressed on embryonic skeletal muscle cells. Motor nerves innervating sceletal muscle are generally believed not to use GABA as a transmitter and do not seem to be sensitive to GABA in the embryonic or adult vertebrate nervous system (Florey and McLennan, '55; Obata et al., '78; Smart, '80; Brown et al., '81; but see Hofmann et al., '62). Thus, it seems rather unlikely that embryonic motor axons use GABA for synaptic information transmission. On the other hand, GABA is known to interact with cholinergic systems. It coexists with acetylcholine in some hypoglossal motor neurons (Davidoff and Schulze, '88) and possibly in some hippocampal nerve terminals (Bonnano and Raiteri, '86). It also modulates acetylcholine release (e.g., Bonnano and Raiteri, '86; Farkas et al., '86), increases the number of nicotinergic acetylcholine receptors (Kasa et al., '85), and is necessary for the formation of synaptic contacts after implantation of the hypoglossal nerve into the superior cervical ganglion (Dames et al., '85; Wolff et al., '87). Our findings of transient GABA expression in embryonic motor axons, therefore, are consistent with the concept that GABA has synaptogenetic functions (Wolff et al., '78, '79; Wolff, '81).

Another interesting feature and possible function of transient GABA expression in motor axons concerns the clustering of acetylcholine receptors. It has been suggested that ascorbic acid is a factor which increases acetylcholine receptors in the developing neuromuscular junction (Knaack and Podleski, '85, Knaack et al., '86; Salpeter, '87; Schuetze and Role, '87). Since GABA induces the release of ascorbic acid in brain tissue (Bigelow et al., '84) and ascorbic acid is synthesized in the brain of the chick embryo up to the seventh day of incubation (Fabro and Rinaldini, '65), it is possible that GABA is involved in the regulation of acetylcholine-receptor aggregation in the developing neuromuscular junction.

At present, we do not know whether transiently GABAergic motoneurons express acetylcholine at the same time. Recent studies suggest that GABA and acetylcholine are synthesized simultaneously in some adult hypoglossal motor neurons (Chan-Palay et al., '82a,b; Davidoff and Schulze, '88). An age-dependent effect on the generation of endplate potentials has been described after exogenous application of GABA in nerve-muscle co-cultures from chick embryos (facilitation at E6–8; inhibition at E10; Obata et al., '78). A decrease of motility in the embryo has been reported after systemic administration of GABA in vivo, which is believed to be due to effects on spinal motor neurons or interneurons (Reitzel et al., '79).

Studies on GABA and other transmitter systems show that many neurons can go through transient expression of different transmitters in early development (are "plastic" with respect to their transmitter phenotype; Patterson, '78, '79; Black and Patterson, '80; Black et al., '84; Potter et al., '86; Redburn and Keith, '87; Landis et al., '88). Possibly some—or all—motor axons go through a stage of transient GABA expression. The duration of GABA expression in each nerve fiber could be very short (in the range of minutes or hours), which would be difficult to demonstrate. On the other hand, it is also possible that only a subpopulation of

motor neurons expresses GABA and that this population is eliminated by embryological cell death. The timing of cell death and the termination of transient GABA immunoreactivity in motor axons (Fig. 6) do not rule out the possibility of a causal link between these two events.

GABA expression in the embryonic vestibular nerve

The vestibular nerve is the only sensory cranial nerve in which we could clearly demonstrate substantial transient GABA immunostaining. The immunoreactivity was restricted to a subpopulation of vestibular ganglion cells. Such a pattern of transient GABA immunoreactivity was not observed in any other sensory cranial ganglion and was entirely lacking in the cochlear, ciliary, and trigeminal ganglion. In the facial ganglion, GABA-positive cell bodies were very rare. The believed lack of efferent, centrifugal fibers in the early stages of the vestibular endorgan (Cohen, '87) offers the opportunity to study transient GABA patterns in a purely afferent, sensory axon population.

The GABA-positive cells in the vestibular ganglion are not comparable to GABAergic interneurons in the superior cervical ganglion (Wolff et al., '86), because the vestibular neurons possess central and peripheral GABAergic processes that reach the brain and the vestibular endorgan, respectively. This feature also makes it extremely unlikely that the GABA-positive cells are glial cells, some of which possess a GABA-uptake system (e.g., Chronwall and Wolff, '80). Transiently GABAergic cells are located mainly in the dorsolateral portion of the vestibular ganglion. This spatial distribution of GABAergic cells indicates that only a subpopulation of vestibular cells expresses GABA and that not all the cells go through a GABAergic stage in development. The distribution and timing of cell death in the vestibular ganglion suggest that GABAergic cells are not eliminated by embryological cell death, but, rather, change their transmitter phenotype. The vestibular ganglion contains mainly cells of placodal origin, but some cells appear to be of neural-crest origin, especially in the rostral ventrolateral portion of the ganglion (D'Amico-Martel and Noden, '83). Their position does not correspond with the location of GABAergic cells.

Does the timing of GABA expression correspond with fiber outgrowth, with the formation of contacts, or with the maturing of contacts in the endorgan? The innervation of the vestibular endorgan has been investigated at the light and electron microscopic level in the chick embryo (e.g., Fink and Morest, '77; Ginzberg and Gilula, '80), and we will briefly review the main findings and relate them to transient GABA expression.

The vestibular ganglion cells send central processes toward the rhombic lip (E2–4) before the peripheral processes reach the neuroepithelium of the otocyst (E3–4) (Knowlton, '67). Peripheral fibers enter the sensory epithelium (E4) when the hair cells have not yet differentiated (Ginzberg and Gilula, '80). At E5–8, early contacts are formed between differentiating hair cells and afferent vestibular fibers (Ginzberg and Gilula, '80). From E6 to E8, colossal fibers of the vestibular nerve grow into the epithelium and form large calyces on receptor cells (Fink and Morest, '77). Efferent fibers and acetylcholinesterase are first observed at E11 in the endorgan (Cohen, '87).

The timing of GABA immunoreactivity in a subpopulation of vestibular ganglion cells correlates with the formation of contacts between afferent vestibular fibers and differentiating hair cells in the endorgan (E6–11). Gap-junction-like contacts are formed between hair cells and afferent fibers at early embryonic stages (E7–8) (Ginzberg and Gilula, '80), and amino acids are known to be able to pass through gap junctions (Dennis, '81). Therefore, afferent vestibular fibers may influence hair cells via GABA transfer through gap junctions without "release" into the extracellular space. In the foregoing section, we have suggested a function of GABA in the regulation of postsynaptic acetylcholine receptors. Afferent neurons do not seem to be necessary for gross differentiation of the sensory epithelium (Van de Water, '86). Nevertheless, GABA may play a role in the regulation of acetylcholine receptors in the vestibular endorgan, since hair cells and afferent dendritic processes are innervated by acetylcholinergic efferents (Meza, '85; Cohen, '87). On the other hand, hair cells of the basilar membrane (cochlea) are also innervated by cholinergic fibers, and no GABAergic staining of acoustical ganglion cells or fibers was observed at any time during development.

GABAergic nerves: distribution and possible function

The distribution of transient GABA immunoreactivity seems at first rather confusing. GABA-positive fibers are present in all motor nerves and in some of the sensory nerves: the olfactory (or terminal nerve), the optic nerve, and in some facial and some vestibular ganglion cells. The optic nerve will not be further considered, because the immunoreactive fibers appear to be "permanently" GABAergic (Granda and Crossland, '87) and seem to arise from cells located in the optic tract. The rare GABA-positive cells and fibers in the olfactory nerve may belong to the terminal nerve (cf. von Bartheld et al., '87) and thus could represent a preganglionic (vasomotor?) circuit (Larsell, '50). The GABA-positive facial ganglion cells might actually be "displaced" vestibular cells. Thus, only one clearly sensory nerve with transient GABA immunoreactivity remains: the vestibular nerve. At present, we do not have an explanation of why some vestibular ganglion cells, but no cochlear ganglion cells, express GABA.

In the brain of the chick embryo, GABA may be formed via several metabolic pathways other than the GAD-catalyzed reaction (e.g., Sobue and Nakajima, '78; Seiler and Sarhan, '83). The occurrence of GAD immunoreactivity in cells of most sensory ganglia at E7 but not at E14 indicates that many GAD-positive cells do not express GABA and that early GAD immunoreactivity in cranial nerves also is transient.

The predominance of GABA in embryonic motor as opposed to sensory nerves is interesting, because some motor nerves in the adult vertebrate and invertebrate nervous system are GABAergic. In invertebrates, GABA is the main transmitter of inhibitory motor nerves (Fatt and Katz, '53; Florey, '54; Atwood, '67; Takeuchi and Takeuchi, '72; Nistri and Constantini, '79). In vertebrates, some hypoglossal motor neurons (Davidoff and Schulze, '88) and some myenteric neurons are GABAergic (Jessen et al., '86). To date, there is no evidence that such neurons innervate smooth muscles directly, although GABA-receptor sites have been demonstrated on smooth muscle (Amenta, '86). GABAergic nerve fibers may influence gut motility via GABA receptors located presynaptically on cholinergic motor terminals or on motor axons (Hill and Bowery, '86; Jessen et al., '86; Kerr and Ong, '86). Myenteric GABAergic nerve fibers are assumed to modulate the release of other transmitters

(Jessen et al., '86), a function that may be similar to that of transiently expressed GABA in embryonic cranial nerves.

As already mentioned, it has been speculated that the early expression of neurotransmitters has regulatory influences on the differentiation of central and peripheral structures (Buznikov et al., '70; Olson and Seiger, '72; Lauder and Bloom, '74; McMahon, '74; Lanier et al., '76; Zimmermann and Wee, '84). Our findings of transient GABA expression in embryonic cranial nerves are consistent with the concept that GABA has developmental/trophic functions (Wolff et al., '79; Chronwall and Wolff, '80; Wolff, '81; Redburn and Schousboe, '87).

The heterogenous distribution of transient GABA expression among different motor nerves and in the vestibular nerve is striking. Why are so many GABAergic nerve fibers involved in the innervation of the muscles of the beak, the inferior palpebral muscle of the eyelid, and the posterior crista in the inner ear? What do these organs have in common? Possibly they are of special functional importance in embryonic development. The young embryo performs movements in the egg mainly by rotating along the transverse body axis. Muscles of the beak are needed for breaking the shell and normal hatching, and the eyelid-closing muscle may be important to protect the developing eye. Does GABA have the function of accelerating the maturation of such critical organs? This interpretation is, of course, highly speculative. Nevertheless, GABAergic systems in developing cranial nerves may provide a promising new model for the study of developmental functions of GABA.

ACKNOWLEDGMENTS

NOTE ADDED IN PROOF

While this manuscript was set in print, we investigated the possibility of transient expression of GABA-A receptors in cranial muscles of the embryonic chicken, using an antibody to the GABA-A receptor (courtesy of Dr. A. de Blas, SUNY, Stony Brook, N.Y.). Neither of the three ages investigated (E5, E8.5, and E11.5) revealed positive immunostaining in muscle tissue.

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