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Developmental Changes in the Distribution of Gamma-Aminobutyric Acid-Immunoreactive Neurons in the Embryonic Chick Lumbosacral Spinal Cord

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ABSTRACT

The development of γ-aminobutyric acid (GABA)-immunoreactive neurons was investigated in the embryonic and posthatch chick lumbosacral spinal cord by using pre- and postembedding immunostaining with an anti-GABA antiserum. The first GABA-immunoreactive cells were detected in the ventral one-half of the spinal cord dorsal to the lateral motor column at E4. GABAergic neurons in this location sharply increased in number and, with the exception of the lateral motor column, appeared throughout the entire extent of the ventral one-half of the spinal gray matter by E6. Thereafter, GABA-immunoreactive neurons extended from ventral to dorsal regions. Stained perikarya first appeared at E8 and then progressively accumulated in the dorsal horn, while immunoreactive neurons gradually declined in the ventral horn. The general pattern of GABA immunoreactivity characteristic of mature animals had been achieved by E12 and was only slightly altered afterwards. In the dorsal horn, most of the stained neurons were observed in laminae I–III, both at the upper (LS 1–3) and at the lower (LS 5–7) segments of the lumbosacral spinal cord. In the ventral horn, the upper and lower lumbosacral segments showed marked differences in the distribution of stained perikarya. GABAergic neurons were scattered in a relatively large region dorsomedial to the lateral motor column at the level of the upper lumbosacral segments, whereas they were confined to the dorsalmost region of lamina VII at the lower segments.

The early expression of GABA immunoreactivity may indicate a trophic and synaptogenetic role for GABA in early phases of spinal cord development. The localization of GABAergic neurons in the ventral horn and their distribution along the rostrocaudal axis of the lumbosacral spinal cord coincide well with previous physiological findings, suggesting that some of these GABAergic neurons may be involved in neural circuits underlying alternating rhythmic motor activity of the embryonic chick spinal cord.

Key words: interneurons, GABA, neural differentiation, immunocytochemistry

The inhibitory neurotransmitter γ-aminobutyric acid (GABA) is considered to play important roles in early processes of neural development. GABA is one of the earliest neurotransmitters detected in the developing central nervous system of various animals (Obata et al., 1978; Reitzel et al., 1979; Lauder et al., 1986; Maderdrut et al., 1986; Roberts et al., 1987; Chun and Shatz, 1989; Van Eden et al., 1989; Blanton and Kriegstein, 1991; Del Rio et al., 1992; Ma et al., 1992a,b; Meinecke and Rakic, 1992; Wu et al., 1992). Studies on rat superior cervical ganglia and cerebral cortex have demonstrated that, in addition to its well established neurotransmitter function, GABA also promotes neural development as a trophic and synaptogenetic factor (Wolff et al., 1978, 1979; Wolff, 1981). Other pharmacological studies indicate that GABA facilitates neurite outgrowth, neuronal differentiation and survival, as well as GABA receptor expression in cultured neurons (Spoerri and Wolff, 1981; Hansen et al., 1984; Meier et al., 1987, 1991; Spoerri, 1988; Lipton and Kater, 1989). Physiological recordings obtained in an isolated hemisected spinal cord preparation indicate that GABAergic neurons are also essential constituents of neural circuits generating

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spontaneous rhythmic motor activity in the embryonic chick spinal cord (O'Donovan, 1989; Sernagor and O'Donovan, 1991; O'Donovan et al., 1992) that is believed to play an important role in the development of motoneurons and muscles (Renaud et al., 1978; Pittman and Oppenheim, 1979; Toutant et al., 1979; McLennan, 1983). In addition, other embryonic studies have raised the possibility that these neural mechanisms controlling embryonic motility are the precursors of neural circuits underlying motor activity in the adult animal (Bekoff et al., 1975, 1989; Bekoff, 1976).

Because of its amenability to embryonic microsurgery and electrophysiological and neuropharmacological experiments, the chick embryo is a particularly useful subject for studies concerning embryogenesis. Many of the aforementioned experiments attesting to the fundamental role of GABA in early neurogenesis, especially those that focused on embryonic motility, have also been performed on chick embryos (Renaud et al., 1978; Pittman and Oppenheim, 1979; Reitzel et al., 1979; Toutant et al., 1979; Reitzel and Oppenheim, 1980; McLennan, 1983; Maderdrut et al., 1986; O'Donovan and Landmesser, 1987; O'Donovan, 1987, 1989; O'Donovan et al., 1992; Ho and O'Donovan, 1993). Despite many physiological, pharmacological, and behavioral experiments concerning GABA-mediated events in neural development, the neurochemical localization of GABA in the central nervous system of chick embryos has received little attention. Although transient GABA immunoreactivity has been reported in cranial nerves of the chick embryo (Bartheld and Rubel, 1989; Code et al., 1989), GABA-immunoreactive neurons have not been demonstrated within the chick spinal cord at early developmental stages.

Accordingly, in the experiments presented here, we investigated the distribution of GABA-immunoreactive neurons in the embryonic and posthatch chick lumbosacral spinal cord by using pre-and postembedding immunocytochemical methods. Since it has recently been reported that the rhythmogenic capacity of the rostral segments of the lumbosacral spinal cord is significantly greater than that of the caudal segments (Ho and O'Donovan, 1993), special attention was given to the search for regional differences in the distribution of GABA-immunoreactive neurons within the upper and lower lumbosacral spinal cord.

MATERIALS AND METHODS

Animals and fixation

Experiments were carried out on white Leghorn chick embryos maintained in a forced-draft incubator at 37°C. Fifty-five embryos ranging from 4 to 20 days of incubation (E4–E20) and five newly hatched chicks 4 weeks posthatching (P28) were studied. Embryos were removed from the shell and decapitated, whereas posthatch chicks were deeply anaesthetized with sodium pentobarbital (35 mg/kg i.p.). E4–E6 embryos were placed into an oxygenated Tyrode solution. After evisceration, the spinal cord was removed and inserted into a fixative containing 2.5% glutaraldehyde, 0.5% paraformaldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (PB; pH 7.4) for 5–6 hours. E7–E20 embryos and posthatch chicks were perfused transcardially with Tyrode solution (oxygenated with a mixture of 95% O_2, 5% CO_2), followed by the same fixative used for younger embryos. The spinal cord was removed and postfixed in the same fixative for 1–2 hours.

Preembedding immunocytochemistry

Blocks of the upper (LS 1–3) and lower (LS 5–7) lumbosacral segments of the spinal cord were dissected and immersed in 10% and 20% sucrose dissolved in 0.1 M PB until they sank. Tissue blocks were freeze-thawed in liquid nitrogen, sectioned at 50 μm on a vibratome, and extensively washed in 0.1 M PB. The free-floating sections were first incubated with a polyclonal antibody against GABA raised in rabbit (code 9; diluted 1:3,000–1:6,000) for 2 days at 4°C. The immunological and immunocytochemical characteristics of the antibody have been extensively tested and published earlier (Hodgson et al., 1985; Somogyi et al., 1985). The sections were then transferred to biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA; diluted 1:200) for 6–8 hours at room temperature, followed by an overnight incubation in avidin-biotinylated horseradish peroxidase complex (ABC; Vector; diluted 1:100) at 4°C. The immunoreaction was completed with a diaminobenzidine chromogen reaction (0.05% diaminobenzidine and 0.01% H_2O_2 in 50 mM Tris buffer, pH 7.4). All of the antibodies were diluted in 10 mM phosphate-buffered saline (PBS; pH 7.4) to which 0.5% Triton X-100 and 1% normal goat serum (Vector) were added. Immunostained sections were collected on chrome alum-gelatin-coated slides and mounted with Permount neutral medium.

Postembedding immunocytochemistry

Slices (1–2 mm thick) were cut from the upper and lower lumbosacral spinal cord. Following several washes in 0.1 M PB, tissue blocks were treated with 1% OsO_4 for 1 hour, dehydrated, and embedded in Durcupan ACM resin (Fluka). Semithin sections were cut at 0.5 μm from the embedded tissue blocks and dried on a hot plate onto slides coated with chrome alum-gelatin. The resin was etched using ethanolic sodium hydroxide for 30 minutes, and then the sections were treated with 1% sodium metaperiodate to remove the OsO_4. Thereafter, sections were incubated with the same rabbit anti-GABA serum used in the preembedding procedure (diluted 1:1,000) for 2 hours. Subsequently, biotinylated goat antirabbit IgG (Vector; diluted 1:200) was layered over the slides for 50 minutes, followed by ABC (Vector; diluted 1:100) for 1 hour. All of the antibodies were diluted in 10 mM Tris-phosphate-buffered isotonic saline (TPBS; pH 7.4) to which 1% normal goat serum (Vector) was added. The immunoreaction was visualized by treating the sections with diaminobenzidine as a chromogen. The reaction end-product was intensified with OsO_4, dehydrated, and covered with Permount neutral medium.

Specificity of the immunohistochemical procedure

Method specificity was tested by omitting the diaminobenzidine from the incubation medium and also by replacing the primary antiserum with normal rabbit serum (dilution 1:100). Under these conditions, no peroxidase reaction was observed in the sections.

Antiserum specificity was tested by treating the diluted anti-GABA serum with glutaraldehyde-GABA conjugates. The conjugation of GABA with glutaraldehyde was performed according to the method of Storm-Mathisen et al. (1983). The glutaraldehyde-GABA complex (500 μM) and the anti-GABA serum (diluted 1:1,000) were mixed, left at 4°C for 16–18 hours, and then centrifuged. Semithin sections were incubated according to the postembedding

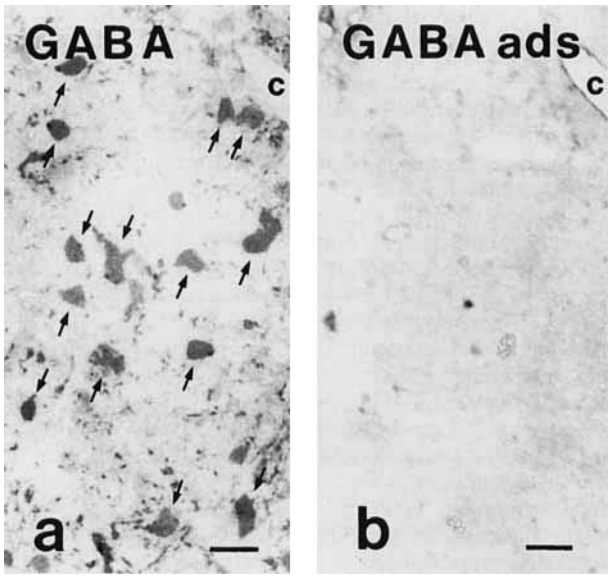

Fig. 1. Photomicrographs from consecutive semithin sections showing the effect of pretreating the GABA antiserum with glutaraldehyde-GABA conjugates in the dorsal horn of the spinal cord at E10. **a:** Section treated with GABA antiserum. Perikarya show intense immunostaining for GABA. **b:** Section incubated with GABA antiserum pretreated with glutaraldehyde-GABA conjugate. The pretreatment results in an almost complete loss of staining. Arrows in a point to immunoreactive perikarya. c, Capillary serving as a landmark in fitting the micrographs. Scale bars = 10 μm.

immunocytochemical procedure by using the anti-GABA serum treated with glutaraldehyde-GABA conjugate as primary serum. Under these conditions specific immunostaining was completely abolished (Fig. 1).

Quantitative analysis

In order to obtain comparable figures concerning the numbers of immunoreactive neurons in the lumbosacral spinal cord at different developmental stages, stained perikarya were counted in randomly selected semithin sections (three sections from three animals at each investigated developmental stage) in the dorsal and ventral gray matters. The dorsal and ventral gray matters were defined as the dorsal and ventral one-halves of the spinal cord at E4–E6. From E8 onwards, when the presumptive dorsal horn became obviously separable from the other regions of the gray matter, the primordium of laminae I–VI and presumptive laminae VII–X (Martin, 1979) were regarded as the dorsal and ventral gray matters, respectively. At E8–P28, the upper (LS 1–3) and lower (LS 5–7) lumbosacral segments were analyzed separately. In younger embryos, no distinction was made between the upper and lower lumbosacral segments, and the entire extent of the lumbosacral spinal cord was analyzed as a common entity.

RESULTS

By immunocytochemical detection of GABA, numerous perikarya and punctate profiles were visualized in several regions of the embryonic and posthatch chick lumbosacral spinal cord. Since no attempt was made in this study to identify the origin, ultrastructure, and synaptic relations of immunostained punctate profiles that are considered to represent primarily axons and axon terminals (Magoul et al., 1987; Roberts et al., 1987), the present paper focuses on the development of immunoreactive perikarya.

E4–E7

The first GABA-immunoreactive cells were detected in the ventral one-half of the spinal cord dorsal to the lateral motor column (LMC) at the border of the presumptive gray and white matters at E4 (Figs. 2, 3a). They presented rounded or ovoid perikarya with a diameter of 5–8 μm. The number of immunoreactive cells in this location was significantly increased at E5 (Figs. 2, 3b), and they were also found at the dorsomedial and medial aspects of the LMC at E6 (Figs. 2, 3c). By E6–E7, with the exception of the LMC, GABA-immunoreactive cells were distributed throughout the entire ventral one-half of the spinal gray matter in a remarkably high density (Fig. 4). Stained neurons, however, were only occasionally found within the LMC and the dorsal one-half of the gray matter. This distribution of immunoreactive neurons was characteristic of both the upper and the lower segments of the lumbosacral spinal cord at this developmental stage.

E8–E12

The general distribution of immunoreactive perikarya was fundamentally changed during this period of embryogenesis. The number of immunoreactive perikarya declined in the ventral horn, while stained neurons appeared and then gradually accumulated in the dorsal horn.

Ventral horn. The number of GABA-immunoreactive neurons in the ventral horn peaked at E8 (Fig. 4). Subsequently, however, the numbers of stained cells gradually declined throughout the entire length of the lumbosacral spinal cord. By E12, GABA-immunoreactive neurons were present in a low but still substantial number in the ventral horn at the level of the upper lumbosacral spinal cord, whereas they were found in a considerably lower density at the lower lumbosacral segments (Figs. 4, 5). The majority of immunoreactive neurons were distributed at the dorsomedial aspect of the LMC, and only some of them were located in the vicinity of the dorsal or medial boundaries of the LMC or within the motor column (Fig. 5).

Dorsal horn. GABA-immunoreactive neurons emerged and accumulated in the dorsal horn at these developmental stages, and the pattern of GABA-immunoreactivity followed a mediolateral gradient of maturation. Faintly stained perikarya appeared in the medial one-half of the presumptive dorsal horn at E8 (Fig. 5). Subsequently, their number and staining intensity were increased, while they gradually appeared also in the lateral regions of the dorsal horn (Figs. 5, 7a,b). By E12, the entire extent of the presumptive dorsal horn had become densely packed with GABA-immunoreactive neurons at both the upper and the lower lumbosacral segments (Fig. 5).

E13–P28

The general pattern of GABA-immunoreactivity achieved by E12 was retained in the last one-third of embryonic life. Slight alterations, however, occurred when cytoarchitectonic laminae characteristic of mature animals (Martin, 1979) became progressively delineated in the gray matter.

Ventral horn. The density of immunoreactive perikarya slightly regressed, and the regional differences between the

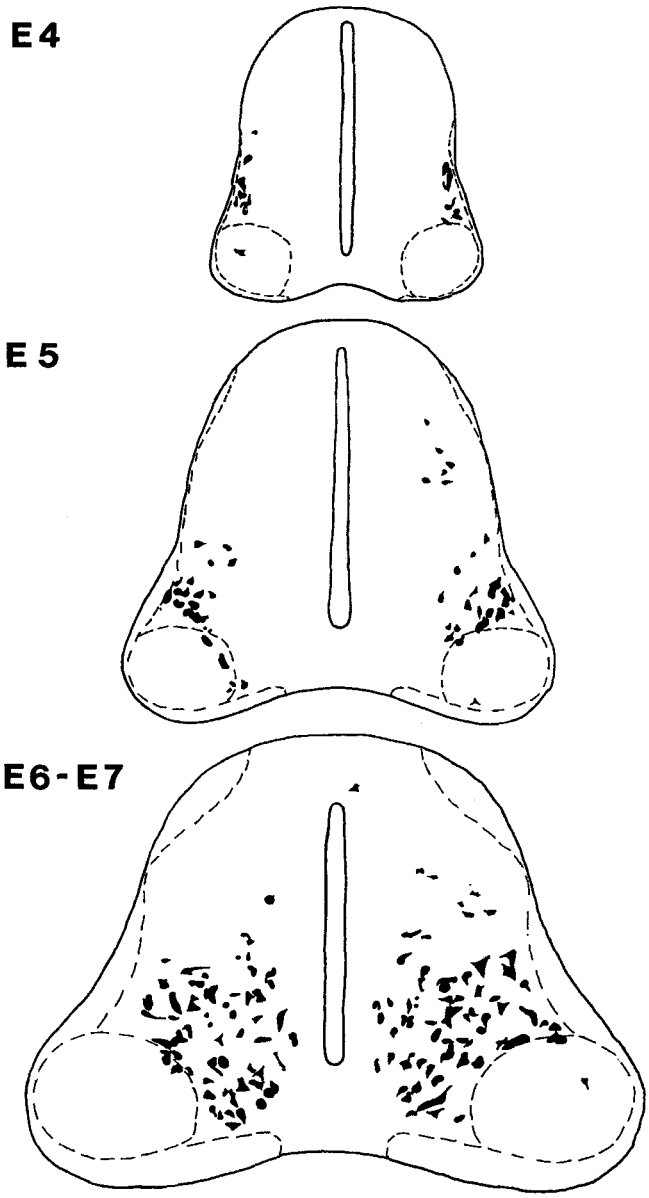

Fig. 2. Schematic representation of the distribution of GABA-immunoreactive perikarya at the level of the lumbosacral spinal cord at E4–E7. The borders of the gray and white matters as well as the LMC are drawn with dashed lines.

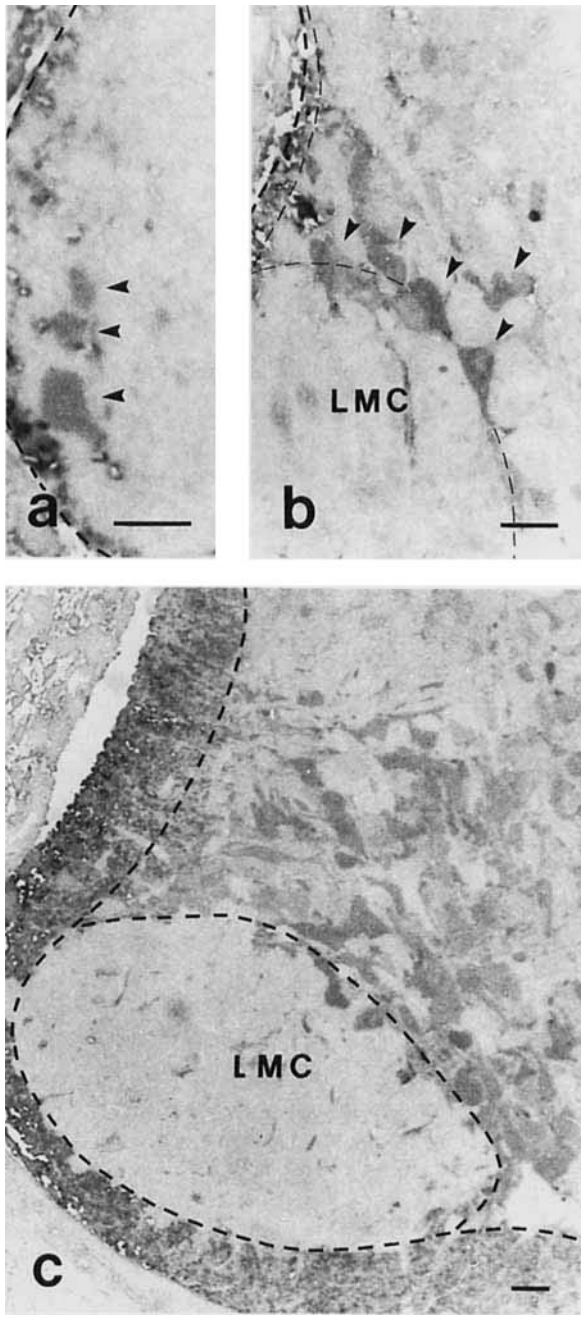

Fig. 3. Photomicrographs showing GABA-immunoreactive neurons in the ventral horn at E4–E6. **a:** Immunostained neurons at the border of the presumptive gray and white matters dorsal to the LMC at E4. **b:** Immunoreactive neurons dorsal to the LMC at E5. **c:** GABA-immunoreactive neurons distributed throughout the entire ventral horn dorsal as well as medial to the LMC. The borders of the gray and white matters as well as the LMC are drawn with dashed lines. Arrowheads in a and b point to immunoreactive perikarya. LMC, lateral motor column. Scale bars = 10 μm.

upper and lower lumbosacral segments became even more prominent during the final stages of embryogenesis (Figs. 4, 6). By E20, when a pattern of immunoreactivity characteristic of the adult animal was achieved, GABA-immunoreactive neurons were sparsely scattered in lamina VII dorsomedial to the LMC at the level of the upper lumbosacral segments (Fig. 6). At the lower lumbosacral segments, however, stained neurons in the ventral horn were aggregated in the dorsalmost regions of lamina VII, whereas the ventral regions of lamina VII were almost completely devoid of labeling (Fig. 6). In addition to stained perikarya in lamina VII, GABA-immunoreactive neurons were occasionally found also within the LMC both at the upper and at the lower segments of the lumbosacral spinal cord, and they persisted even in the mature animal (Figs. 6, 7d,e).

Dorsal horn. Immunoreactive perikarya gradually became concentrated in the most superficial laminae of the dorsal horn at these developmental stages, while their

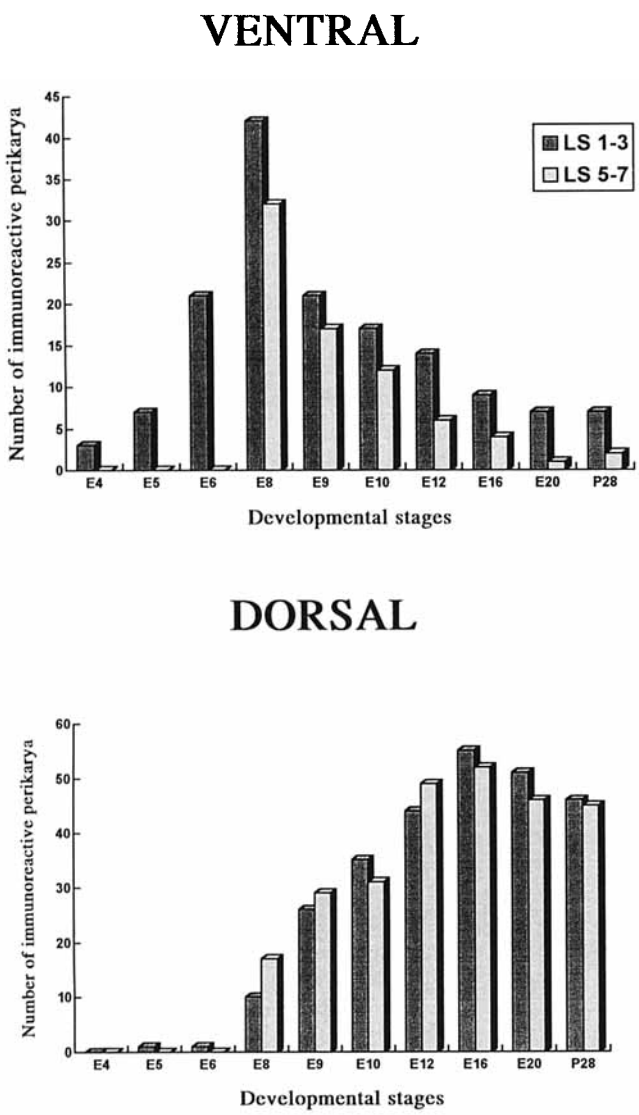

Fig. 4. Histograms showing the numbers of GABA-immunoreactive perikarya in the ventral and dorsal gray matters at the level of the upper (LS 1–3) and lower (LS 5–7) lumbosacral spinal cord at different developmental stages. The columns indicate the mean numbers of immunoreactive neurons in a 0.5 μm semithin transverse section. See text for details.

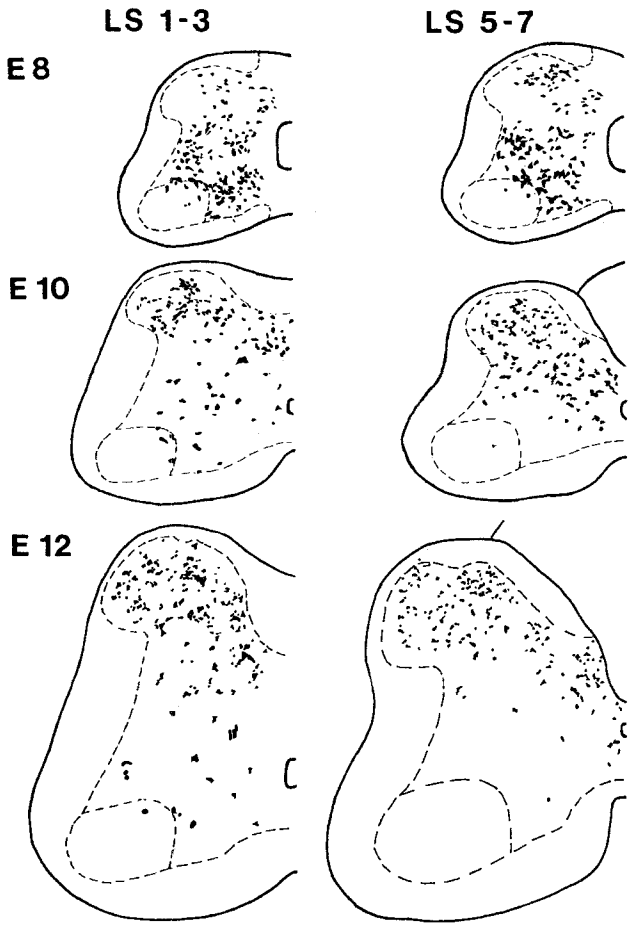

Fig. 5. Schematic representation of the distribution of GABA-immunoreactive perikarya at the level of the upper (LS 1–3) and lower (LS 5–7) lumbosacral spinal segments at E8–E12. The borders of the gray and white matters as well as the LMC are drawn with dashed lines.

numbers were not significantly altered after peaking at E16 (Figs. 4, 6). Most of the stained neurons were observed in laminae I–III, while the deeper layers of the dorsal horn (laminae IV–VI) contained GABA-immunoreactive cells in a significantly lower number in prehatching (E20) and mature animals (P28; Figs. 6, 7c).

DISCUSSION

Early expression of GABA immunoreactivity

As demonstrated in this study, GABA immunoreactivity is expressed at a very early stage of neural development in the embryonic chick lumbosacral spinal cord. GABA-immunoreactive neurons first appear at E4, reach a remarkably high density by E6, and peak at E8 in the ventral horn. Electron microscopic studies, however, have shown that the first synaptic specializations can be observed at E5–E6, and synapse formation advances only after E7 in the lumbosacral spinal cord of chick embryos (Foelix and Oppenheim, 1973; Oppenheim et al., 1975). Because of the low incidence of synaptic contacts, it appears to be unlikely that GABA would act exclusively as a classical neurotransmitter at these early stages of neurogenesis.

Although the physiological significance of the early expression of GABA remains to be elucidated, it is interesting to note that the early appearance of GABA coincides with several events having crucial importance in the development of the lumbosacral spinal cord. Axons of segmental and intersegmental propriospinal neurons develop at E3–E4.5 in the chick spinal cord (Holley, 1982; Oppenheim et al., 1988), while descending fibers of brainstem origin first reach the lumbosacral spinal cord at E5 (Okado and Oppenheim, 1985). Following injections of dorsal root ganglia or peripheral nerves with HRP or DiI, it has also been demonstrated that ingrowing primary afferents penetrate the spinal gray matter at E5.5-E6 (Lee et al., 1988; Mendelson and Frank, 1991). The birth of motoneurons, and presumably of a significant proportion of spinal interneurons (Kanemitsu, 1982), is nearly completed by E4

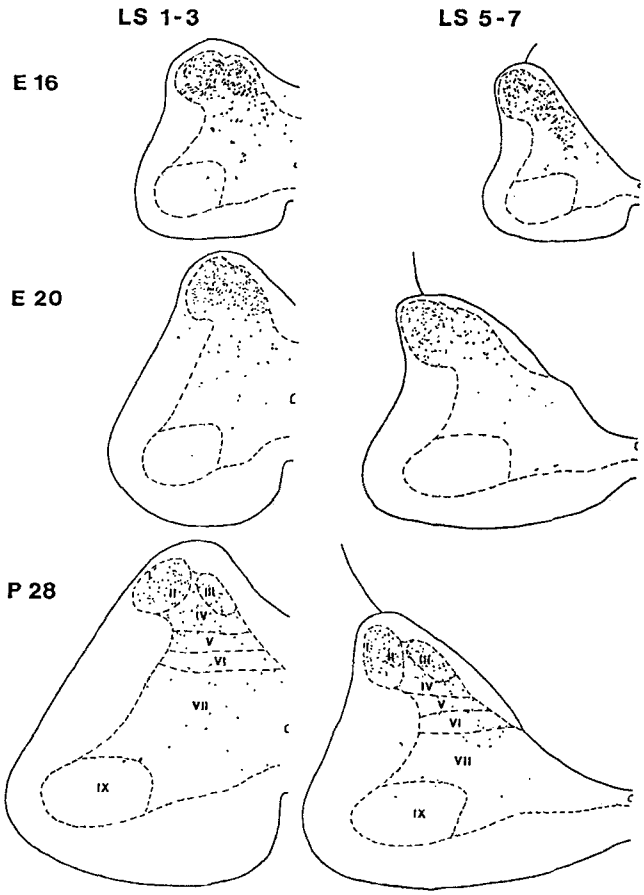

Fig. 6. Schematic representation of the distribution of GABA-immunoreactive perikarya at the level of the upper (LS 1–3) and lower (LS 5–7) lumbosacral spinal segments at E16–P28. The borders of the gray and white matters as well as the cytoarchitectonic laminae of the gray matter at P28 are drawn with dashed lines.

(Hamburger, 1975; Hollyday and Hamburger, 1977), and maturation of their dendritic arbor is initiated immediately afterwards (Kanemitsu and Matsuda, 1985; Lee et al., 1988).

A growing body of evidence from in vitro and in vivo studies indicates that GABA may play morphogenetic roles in the central nervous system. Studies using cell cultures have demonstrated that GABA promotes neurite outgrowth (Hansen et al., 1984; Spoerri and Wolff, 1981) and facilitates GABA receptor expression (Meier et al., 1987) as well as synaptogenesis (Wolff et al., 1978; Wolff, 1981). It has also been reported that GABA evokes depolarization and activates voltage-sensitive Ca^{2+} channels on developing neurons (Cherubini et al., 1991; Yuste and Katze, 1991; Wu et al., 1992), through which it may operate to produce an appropriate level of Ca^{2+} within the cytoplasm for neuronal viability and outgrowth (Kater et al., 1988; Lipton and Kater, 1989). At the ultrastructural level, GABA treatment leads to an increased density of cytoplasmic organelles including neurotubules, endoplasmic reticulum, Golgi apparatus, and various vesicles (Spoerri, 1988). The possibility that GABA may exert trophic influences on developing neurons also in vivo is reinforced by the finding that glutamic acid decarboxylase (GAD) immunoreactivity is present in growth cones in the developing cerebellum (McLaughlin et al., 1975) and other reports suggesting that neurotransmitters can be spontaneously released from growth cones (Hume et al., 1983; Young and Poo, 1983). The temporal coincidence of early GABA expression demonstrated in this study with the morphogenesis of fundamental neural constituents of the chick spinal cord may imply that GABA exerts some trophic and synaptogenetic influence on the development of neurons also in the chick lumbosacral spinal cord.

Transient expression of GABA immunoreactivity

We have demonstrated in this paper that the sequence of GABA expression follows a ventral-to-dorsal gradient in the chick spinal cord. This is in agreement with the findings concerning the development of GABAergic neurons in the rat spinal cord (Lauder et al., 1986; Ma et al., 1992) and the general neurogenesis of the spinal cord revealed by classical ^{3}H-thymidine autoradiographic studies (Hollyday and Hamburger, 1977; Kanemitsu, 1982; Altman and Bayer, 1984). GABA expression peaks in the ventral horn at E8, and then a marked loss of GABA-immunoreactive neurons begins in this location at E9 and progresses afterwards. The transitory expression of GABA in this region may be explained in at least to ways: 1) naturally occurring cell death eliminates these neurons; 2) the neurons change their neurotransmitter, decreasing or terminating GABA synthesis, after which they are no longer detectable. Naturally occurring cell death is a well described phenomenon common to many parts of the developing nervous system (for review, see Oppenheim, 1991). It seems, however, that there are some regions of the central nervous system in which cell death is insignificant or does not occur at all. Cell counts in embryos and postnatal animals show no cell loss in the developing CA1 region of the rat hippocampus (Boss et al., 1987) or in the pontine nuclei of the chick (Armstrong and Clarke, 1979). In addition, McKay and Oppenheim (1991) reported an apparent absence of cell death among spinal cord interneurons in the chick embryo. In light of this finding, it appears unlikely that naturally occurring cell death would significantly account for the regression of GABA immunoreactivity in the ventral horn of the chick lumbosacral spinal cord.

The other possibility, that neurons would change GABA to another neurotransmitter during development, seems to be more plausible. GAD, the GABA-synthesizing enzyme, is encoded by two genes (Erlander et al., 1991; Kaufman et al., 1991). These two genes encode two distinct forms of GAD proteins (classified as GAD1 and GAD2) that have different molecular weights and can be detected by different antisera (Erlander et al., 1991; Kaufman et al., 1991). A recent study on spinal cord cells derived from embryonic and postnatal rats demonstrated that cells from early developmental stages can be stained only with GAD1 antiserum (Behar et al., 1990). GAD1 immunoreactivity is expressed by 75% of cells at E13, but this percentage decreases to 45% by E15. GAD2 immunoreactivity, on the other hand, is expressed only at the end of embryonic development, showing a high degree of colocalization with GAD1 (Behar et al., 1990). Similar events may take place also in the chick embryo. At E4–E8, GABA is presumably synthesised by GAD1 in the chick lumbosacral spinal cord. Soon afterwards, a proportion of these cells may abandon production of GAD1 and consequently discontinue the synthesis of GABA, the concentration of which in the cytoplasm drops below the level

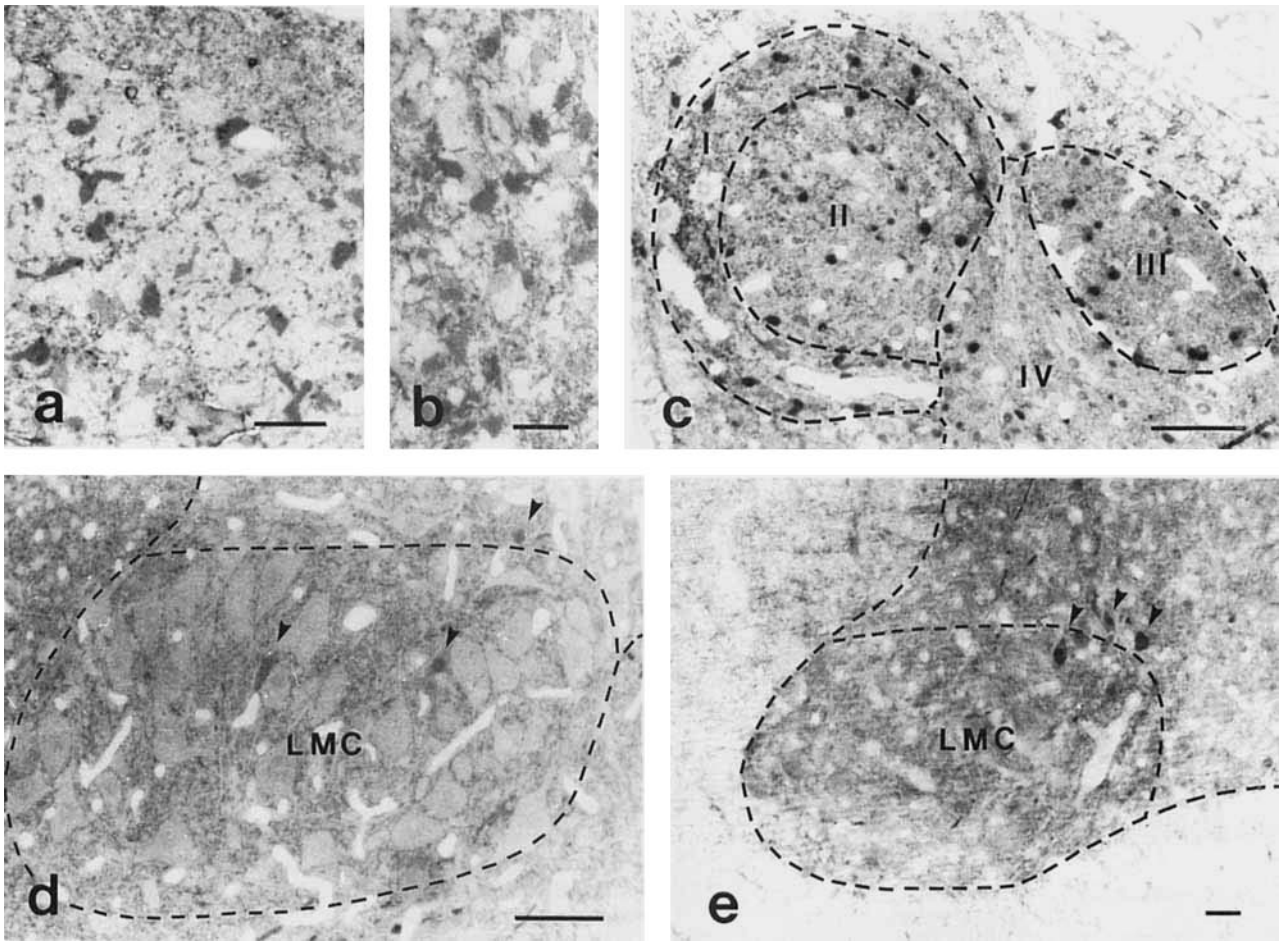

Fig. 7. Photomicrographs of GABA-immunoreactive neurons in various regions of the lumbosacral spinal cord at E12, E20, and P28. **a:** Immunostained perikarya in the superficial dorsal horn at E12. **b:** Immunoreactive perikarya in the deep dorsal horn at E12. **c:** GABA-immunoreactive neurons in the dorsal horn at E20. **d,e:** Neurons immunoreactive for GABA in the ventral horn at E20 (d) and P28 (e). The borders of the gray and white matters as well as laminae I–IV in c and the LMC in d and e are drawn with dashed lines. Arrowheads in d and e point to immunoreactive perikarya. LMC, lateral motor column. Scale bars = 20 μm in a and b, 50 μm in c–e.

of immunocytochemical detectability. In the absence of GAD, glutamic acid may accumulate in these cells, which can be utilized as the new neurotransmitter by the neurons. These notions are speculative and require exact experimental verification, although they are supported by previous reports describing neurotransmitter plasticity in peripheral sympathetic neurons (for review, see Landis, 1990).

GABAergic neurons in neural circuits underlying spontaneous rhythmic motor activity of the embryonic chick spinal cord

It is now well established in various animals that neural circuits underlying spontaneous rhythmic motor activity are localized within the spinal cord. The alternating pattern of flexor and extensor discharge is produced by a delicate spatiotemporal activation of spinal motoneurons that is controlled by a rhythm-generating neural network comprising both excitatory and inhibitory spinal interneurons (Reitzel et al., 1979; Grillner et al., 1986; Maderdrut et al., 1986; Roberts et al., 1986; O'Donovan, 1989; Ho and O'Donovan, 1993). In addition, a recent ablation experiment performed by Ho and O'Donovan (1993) shows that the critical neural components for motoneuron alternation in the chick embryo are located immediately dorsomedial to the LMC. This is the region where the majority of GABA-immunoreactive neurons are distributed in the ventral horn of the upper lumbosacral spinal cord in the chick embryo after E8. Ho and O'Donovan (1993) have also demonstrated that, similar to the case in other vertebrate species (Deliagina et al., 1983; Mortin and Stein, 1989), the rhythmogenic capacity of the upper lumbosacral spinal cord of the chick embryo is significantly greater than that of the lower lumbosacral segments, and this rostrocaudal difference in motor activity becomes more pronounced with development. It is an interesting coincidence that the density of GABA-immunoreactive neurons at the dorsomedial aspect of the LMC is significantly higher at the upper than at the lower segments of the lumbosacral spinal cord from E8 onwards, and this difference in GABA immunoreactivity becomes more pronounced with development. Because of their localization in the ventral horn and distribution along the rostrocaudal axis of the lumbosacral spinal cord, it is tempting to suppose that GABA-immunoreactive neurons at the dorsomedial aspect of the LMC may be involved in neural circuits underlying alternating rhythmic motor activity of the chick embryo.

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