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Unique Developmental Patterns of GABAergic Neurons in Rat Spinal Cord

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ABSTRACT

γ-aminobutyric acid (GABA)ergic neurons have been postulated to compose an important component of local circuits in the adult spinal cord, yet their identity and axonal projections have not been well defined. We have found that, during early embryonic ages (E12–E16), both glutamic acid decarboxylase 65 (GAD65) and GABA were expressed in cell bodies and growing axons, whereas at older ages (E17–P28), they were localized primarily in terminal-like structures. To determine whether these developmental changes in GAD65 and GABA were due to an intracellular shift in the distribution pattern of GAD proteins, we used a spinal cord slice model. Initial experiments demonstrated that the pattern of GABAergic neurons within organotypic cultures mimicked the expression pattern seen in embryos. Sixteen-day-old embryonic slices grown 1 day in vitro contained many GAD65- and GAD67-labeled somata, whereas those grown 4 days in vitro contained primarily terminal-like varicosities. When isolated E14–E16 slices were grown for 4 days in vitro, the width of the GAD65-labeled ventral marginal zone decreased by 40–50%, a finding that suggests these GABAergic axons originated from sources both intrinsic and extrinsic to the slices. Finally, when axonal transport was blocked in vitro, the developmental subcellular localization of GAD65 and GAD67 was reversed, so that GABAergic cell bodies were detected at all ages examined. These data indicate that an intracellular redistribution of both forms of GAD underlie the developmental changes observed in GABAergic spinal cord neurons. Taken together, our findings suggest a rapid translocation of GAD proteins from cell bodies to synaptic terminals following axonal outgrowth and synaptogenesis. J. Comp. Neurol. 456: 112–126, 2003.

Indexing terms: glutamic acid decarboxylase; GABA; commissural neurons; spinal cord; organotypic cultures

Although γ-aminobutyric acid (GABA)ergic neurons comprise a major percentage of total neurons in the central nervous system (CNS), the identity and synaptic targets of most of the ventral GABAergic spinal cord neurons are unknown. For reasons that are not well understood, the cell bodies of GABAergic neurons in postnatal and adult spinal cord generally do not contain enough glutamic acid decarboxylase (GAD), the rate-limiting enzyme for the synthesis of the GABA, to be localized by standard immunocytochemical methods. The first GAD localization in the adult rat spinal cord reported immunoreactive punctate structures concentrated in the superficial dorsal horn and throughout the gray matter that were shown with electron microscopy to correspond to synaptic terminals (McLaughlin et al., 1975; Barber and Saito, 1976). Only in colchicine-injected animals were numerous GAD-labeled somata identified in adult spinal cord (Barber et al., 1982). Little GAD immunoreactivity was detected in the surrounding white matter (McLaughlin et al., 1975; Barber and Saito, 1976; Mugnaini and Oertel, 1985), an observation that may have led to the assumption that GABAergic spinal cord neurons are interneurons. Subsequent studies of GABA localization in adult spinal cord did characterize abundant interneurons in the superficial dorsal horn, but the ventral GABAergic spinal cord neurons

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remain undefined (McLaughlin et al., 1975; Mugnaini and Oertel, 1985; Magoul et al., 1987).

In the mammalian CNS, there are two forms of GAD (GAD65 and GAD67) that are encoded by separate genes with 70% sequence homology (Kaufman et al., 1986; Erlander et al., 1991; Erlander and Tobin, 1991). GAD65 and GAD67 differ in their amino acid sequences, molecular weight, response to the cofactor pyridoxal phosphate, and subcellular distribution (Kaufman et al., 1991). Both in situ hybridization and protein localization studies in the hippocampus have shown that GAD65 and GAD67 colocalize in most GABAergic cells, but their expression levels vary in different subcellular compartments; GAD65 preferentially accumulates in axon terminals, whereas GAD67 is localized in both terminals and cell bodies (Esclapez et al., 1993, 1994).

Based on the report of punctate GAD localization in the adult spinal cord (McLaughlin et al., 1975), the early embryonic detection of GAD65 within numerous ventral commissural neurons was unexpected (Phelps et al., 1999; Tran and Phelps, 2000). GAD65 protein was detected in commissural neurons as early as E11 (Phelps et al., 1999), along with GAD65 mRNA in the cervical spinal cord (Somogyi et al., 1995). However, GAD65-containing embryonic commissural somata declined several days later as immunoreactive terminal-like structures increased (Phelps et al., 1999). The GABAergic identity of the ventral commissural neurons was confirmed with antibodies against GAD67 and GABA, but their apparent loss was enigmatic. A second unexplained feature of developing GABAergic neurons was the densely immunoreactive marginal zone axons (Lauder et al., 1986; Ma et al., 1992a; Phelps et al., 1999) that were not evident in adults (Barber et al., 1982; Mugnaini and Oertel, 1985). In this study, we explore both of these novel aspects of developing GABAergic spinal cord neurons.

After the detection of abundant GAD-containing neurons early in development, we examined the temporal and spatial changes in GAD65 and GABA expression throughout spinal cord development. Several possible interpretations of the apparent disappearance of developing GABAergic neurons are due to (1) a change in the phenotype of the neurons, (2) a down-regulation of the GAD proteins, or (3) a shift in the intracellular distribution of GAD65 within GABAergic neurons. By manipulating the localization of GAD65 and GAD67 in an organotypic model, we sought to determine whether both GAD65 and GAD67 shift their intracellular distribution from somata at early ages to terminal-like structures at later ages.

MATERIALS AND METHODS

Animal and tissue preparation

Sprague-Dawley male and female rats were placed together overnight for breeding, and vaginal smears were used to confirm insemination. Embryonic day 0 (E0) was designated as the day a positive smear was obtained. Animals studied ranged in age from E12 to postnatal day (P) 28. Pregnant rats were deeply anesthetized by means of intraperitoneal injections of ketamine (0.9 μl/g) and xylazine (0.5 μl/g) before the embryos were removed by Caesarean section. Postnatal animals were heavily anesthetized with Nembutal sodium solution (0.8 μl/g), or 18% chlorohydrate (0.01 μl/g) before perfusions. All procedures were approved by the Chancellor's Animal Research Committee at UCLA.

For localization of GAD65 and GABA, 12- to 13-day-old embryos were fixed by immersion, whereas E14 to P28 animals were fixed by vascular perfusion (Phelps et al., 1999) with 2% paraformaldehyde, 0.01 M periodate and 0.075 M lysine (PLP; McLean and Nakane, 1974), 4% paraformaldehyde in 0.12 M Millonig's phosphate buffer (pH 7.4), or 5% glutaraldehyde. Animals were immersed in the same fixative for 4–15 hours at 4°C. After buffer washes, cervical enlargements were blocked, infiltrated with 30% sucrose, and frozen for transverse cryosectioning at a thickness of 40 μm. Slide-mounted embryonic sections were surrounded by a silicone ring, whereas postnatal sections were processed free-floating in glass wells. All sections were maintained in a plastic chamber during immunocytochemistry.

GAD65, GAD67, and GABA immunocytochemical procedures

To localize GAD65, the monoclonal antibody GAD-6 supernatant (Chang and Gottlieb, 1988; Developmental Studies Hybridoma Bank, Dept. of Biological Sciences, University of Iowa, Iowa City, IA) was used at a 1:50 dilution. To localize GAD67, a polyclonal antiserum K2 (kind gift of Dr. D. Kaufman; Kaufman et al., 1986) was used at 1:16,000. GAD65 labeling was performed as described (Phelps et al., 1999; Orlino et al., 2000; Tran and Phelps, 2000). For GAD67 immunocytochemistry, the procedures were similar to those published (Phelps et al., 1999) with additional avidin-biotin blocking steps (diluted 1:1, Vector) included. A polyclonal antiserum (NT108, Eugene Tech International, Inc.) was used to localize GABA expression with 5% glutaraldehyde-fixed tissues as described (Phelps et al., 1999).

Culture preparations

Organotypic cultures (300 μm thick) were prepared according to methods described by Gähwiler et al. (1981), and modified for embryonic spinal cord preparations (Barber et al., 1993; Phelps et al., 1996). Slices of cervical and upper thoracic spinal cord were prepared and grown in defined media (EOL+10 ng NGF; Annis et al., 1990) on a roller drum in a 5% CO_2 incubator at 35 °C for 1–4 days. Before fixation, experimental slices were incubated in 10 μg/ml of colchicine for 3–6 hours to block axonal transport (Ribak, 1978; Ribak et al., 1978; Barber et al., 1982; Ribak and Roberts, 1990), while control slices remained in media. Slices remained histotypic after the brief colchicine treatment and GABAergic neurons in experimental slices were found in the same location as controls. Slices were fixed with 2% PLP for 2 hours at room temperature, then rinsed and stored in Millonig's buffer with 0.1% sodium azide (pH 7.4). Cultures were embedded and sectioned at a thickness of 40 μm as reported (Phelps et al., 1996).

Data analysis of ventral marginal zone degeneration

Slices designated as +0 are equivalent to spinal cord sections from embryos, whereas those fixed after 1 or 4 days in culture were designated as +1 or +4 days in vitro. To standardize the region of the GAD65-labeled ventral marginal zone (VMZ) axons measured, a line was drawn perpendicular to the ventricle that extended to the outer

edge of the widest point of the ventral spinal cord. A second line was drawn from the center of the first line to the outer edge of the VMZ, and measurements were taken at the intersection of the line and the VMZ. For each embryonic age examined, samples of 7–10 cultures (two sections per culture) were measured by two independent evaluators. For each section analyzed, the width of the VMZ was measured with a calibrated eyepiece micrometer by using a 10× objective. The measurements of the evaluators were averaged, analyzed statistically with the Student's *t* test, and plotted with Excel software.

Digitization and animation

Optimal cervical enlargement sections of embryonic and postnatal spinal cord labeled with GAD65 immunocytochemistry were chosen and photographed with a 4.0 objective and a 4.0 ocular to ensure accurate sizing for constructing the animation. Sections were digitized into high-resolution images with a Nikon LS-1000 35-mm slide scanner and imported into Adobe Photoshop formatted as 800 dpi PICT images. All images were proportionally reduced to 20% of their original size to a final 800 × 411 pixel image. The images were minimally processed in Photoshop for color consistency and for reduction of scanning artifacts. The Morph™ version 2.5 animation program (Gryphon Software Corp.) was used to create links between 50 and 100 pairs of designated key points between each pair of digitized images. This program takes common features of two different images and interpolates between them by adding additional frames. The GAD65 animation was composed of a total of 14 chain-sequenced spinal cord sections, each consisting of two linked images. The first sequence links the image E12 with E13, the second links E13 with E14, and so on. The final animation was exported as a QuickTime movie that can be played with the MoviePlayer application, by using either Macintosh or PC computers.

RESULTS

Developmental expression pattern of GAD65 and GABA

Both GAD65 and GABA immunocytochemistry were performed at the same ages to determine whether the unique changes observed in the subcellular localization of the GAD65 protein could be confirmed with antibodies against GABA. The temporal sequences of GAD65 and GABA were compared (Figs. 1, 2), and the GAD65-labeled sections from these and additional ages (E12–P28) were used to construct an animation of GABAergic spinal cord development (available at http://www.interscience.wiley.com/jpages/0021-9967/suppmat/index.html).

On E14, densely packed GAD65- and GABA-positive cell bodies were detected ventromedially in the same location as those previously identified as commissural neurons (Phelps et al., 1999). Many of these ventromedially located immunoreactive neurons projected their axons contralaterally into the ventral commissure (Fig. 1, E14, arrows). As early as E13, GAD65-labeled axons also emanated from ventrolaterally located neurons and coursed into the ipsilateral marginal zone (Fig. 3a,b). Similarly, ventrolaterally located GABA-labeled neurons were detected with axons that extend toward the ipsilateral marginal zone (Fig. 3c). In addition, numerous GAD65- and GABA-labeled axons were observed in the ventral and lateral marginal zones (Fig. 1, E14).

By E15, ventrally located, contra- and ipsilaterally projecting GAD65-positive axons and their somata still were detected but now many more GAD65-positive somata were distributed throughout the dorsal intermediate zone (Fig. 1, E15, left side). Although the pattern and intensity of GABA-labeled neurons in the ventral spinal cord were similar to that detected with GAD65, the intensity of GABA immunoreactivity in dorsal spinal cord was greatly reduced (Fig. 1, E15, right side). Somatic motor neurons were GAD65 and GABA-negative at all ages examined, whereas the adjacent ventral and lateral marginal zones contained strongly immunoreactive axons. By E15, the dorsal marginal zone (future dorsal columns) contained both GAD65- and GABA-positive axons (Fig. 1, E15, open arrows), whereas the dorsal root ganglion (not shown) and dorsal root entry zone (asterisks) remained unlabeled.

On E17, distinct GAD65-labeled cell bodies were found within the receding ventricular zone but, otherwise, few immunoreactive cells were observed. When compared with younger ages, large numbers of GAD65-labeled terminal-like structures were present throughout the E17 intermediate zone (Fig. 3d, small arrows). In contrast to the primarily terminal-like GAD65 immunoreactivity, several GABA-positive cell bodies (Fig. 1, E17 and Fig. 3e, arrowheads) were detected throughout the spinal cord. Additionally, cross-cut bundles of GAD65- and GABA-immunoreactive axons were detected in the reticulated area of the deep dorsal horn (Fig. 1, E17, long arrows). GAD65- and GABA-positive axons filled much of the ventral and lateral marginal zones with GAD65-labeled axons being more concentrated at the inner than the outer marginal zone borders, whereas GABA-labeled axons were distributed homogeneously. The expanded dorsal marginal zone now was enfolded into its more ventromedial position with GAD65- and GABA-positive axons located in the inner midline regions.

From E19 until birth, both GAD65 and GABA were primarily contained within terminal-like structures distributed rather homogeneously throughout the future gray matter, whereas marginal zone axons remained immunoreactive (Fig. 2, P0). More GAD65-immunoreactive axons continued to be detected along the inner than the outer edges of the ventral and lateral marginal zones, whereas GABA-labeled axons were uniformly distributed (Fig. 2, P0).

By P14, the overall pattern of GAD65 and GABA terminal-like labeling was similar, including the dense band of immunoreactivity within the superficial dorsal horn (Fig. 2, P14). However, differences were noted such as the small immunoreactive cell bodies (Fig. 2, P14, arrowheads) that were found in the dorsal horn of GABA- but not GAD65-immunoreactive sections. In addition, between P0 and P14, both GAD65 and GABA immunoreactivity was lost gradually within the axons of the funiculi (Fig. 2, P0 and P14, animation), although GABA immunoreactivity was detected for a longer period of time. By P28, virtually no GAD65-positive cell bodies were detected, whereas small GABA-positive somata remained in the dorsal horn (Fig. 2, P28). In both preparations, numerous GABAergic terminal-like structures were distributed throughout the gray matter and were most densely concentrated in the superficial dorsal horn, whereas the funiculi were essentially devoid of GAD65 and GABA immunoreactivity (Fig. 2, P28, animation). Thus,

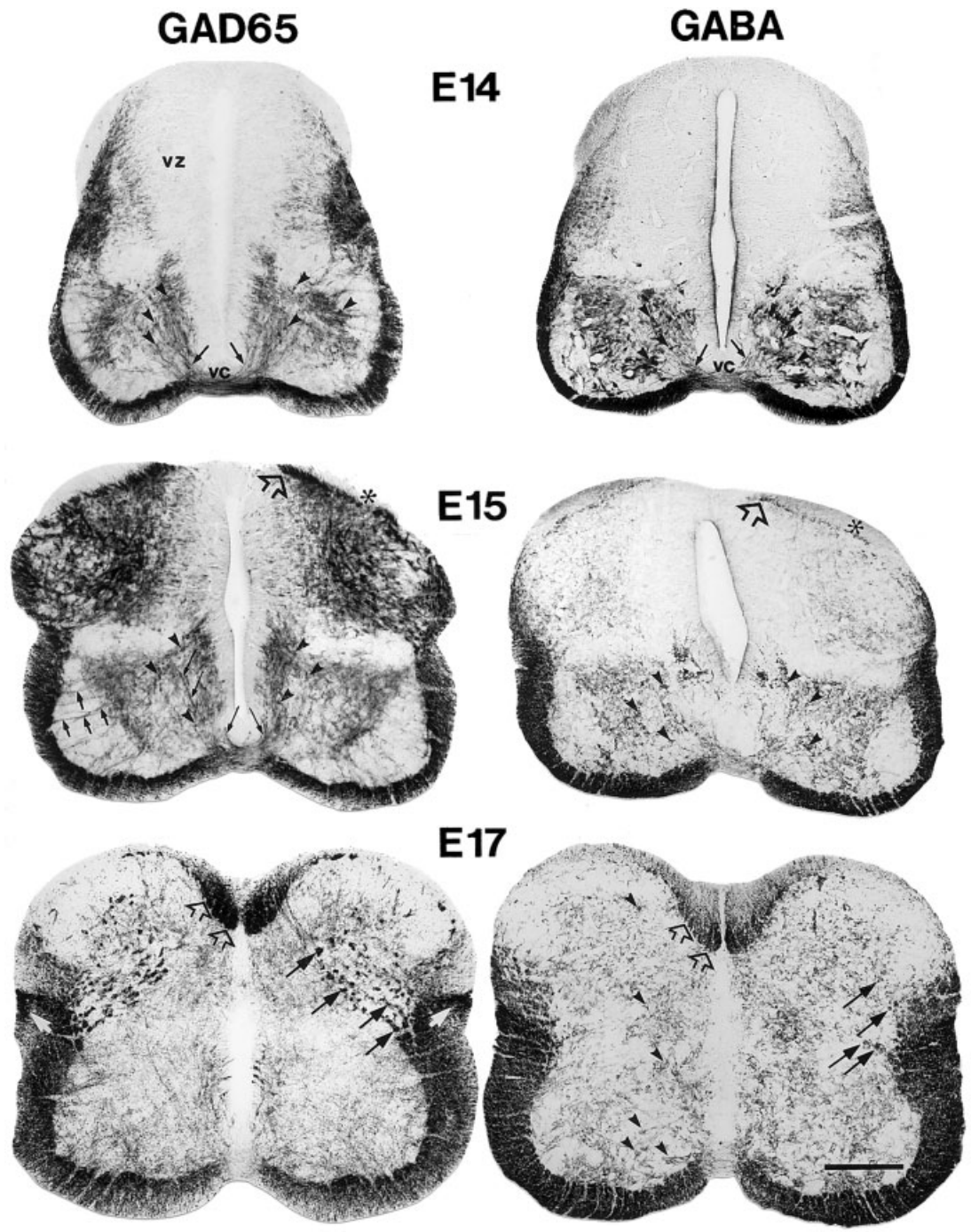


Fig. 1. Developmental sequence illustrates the pattern of glutamic acid decarboxylase 65 (GAD65; left) and γ-aminobutyric acid (GABA; right) immunoreactivity in embryonic day (E) 14, 15, and 17 cervical spinal cords. Somatic motor neurons are GAD- and GABA-negative at all ages examined. **E14:** Ventrally located GAD65- and GABA-labeled commissural neurons (arrowheads) extend their axons (arrows) into the ventral commissure (vc). Numerous GAD65- and GABA-positive axons fill the ventral and lateral marginal zones, whereas the ventricular zone (vz) remains unlabeled. **E15:** GAD65 is detected in ventrally located neurons (arrowheads) that give rise to both contra- (longer arrows) and ipsilaterally projecting axons (shorter arrows), and also in numerous dorsally located somata. Although GABA-immunoreactive somata (arrowheads) and proximal axons are found ventrally, few GABA-positive somata are detected dorsally. Although both GAD65- and GABA-labeled axons fill the ventral and lateral marginal zones, only a few immunoreactive axons are present in the dorsal marginal zone (future dorsal columns, open arrows) and none are detected in the dorsal root entry zone (asterisks). **E17:** Two days later, there are very few GAD65- but many GABA- (arrowheads) immunoreactive somata. Many GAD65- and a few GABA-positive axon bundles (long arrows) are present in the reticulated area of the dorsal horn. The intensely GABA immunoreactive ventral and lateral marginal zone axons appear uniformly distributed, whereas GAD65-labeled axons are more intensely labeled along the outer border in the dorsolateral marginal zone (white arrows). By E17, the dorsal marginal zone has folded into a midline position, and contains numerous GAD65- and GABA-immunoreactive axons (open arrows) that may be derived from immunoreactive dorsal horn neurons. Scale bar = 200 μm in E17 (applies to E14–E17).

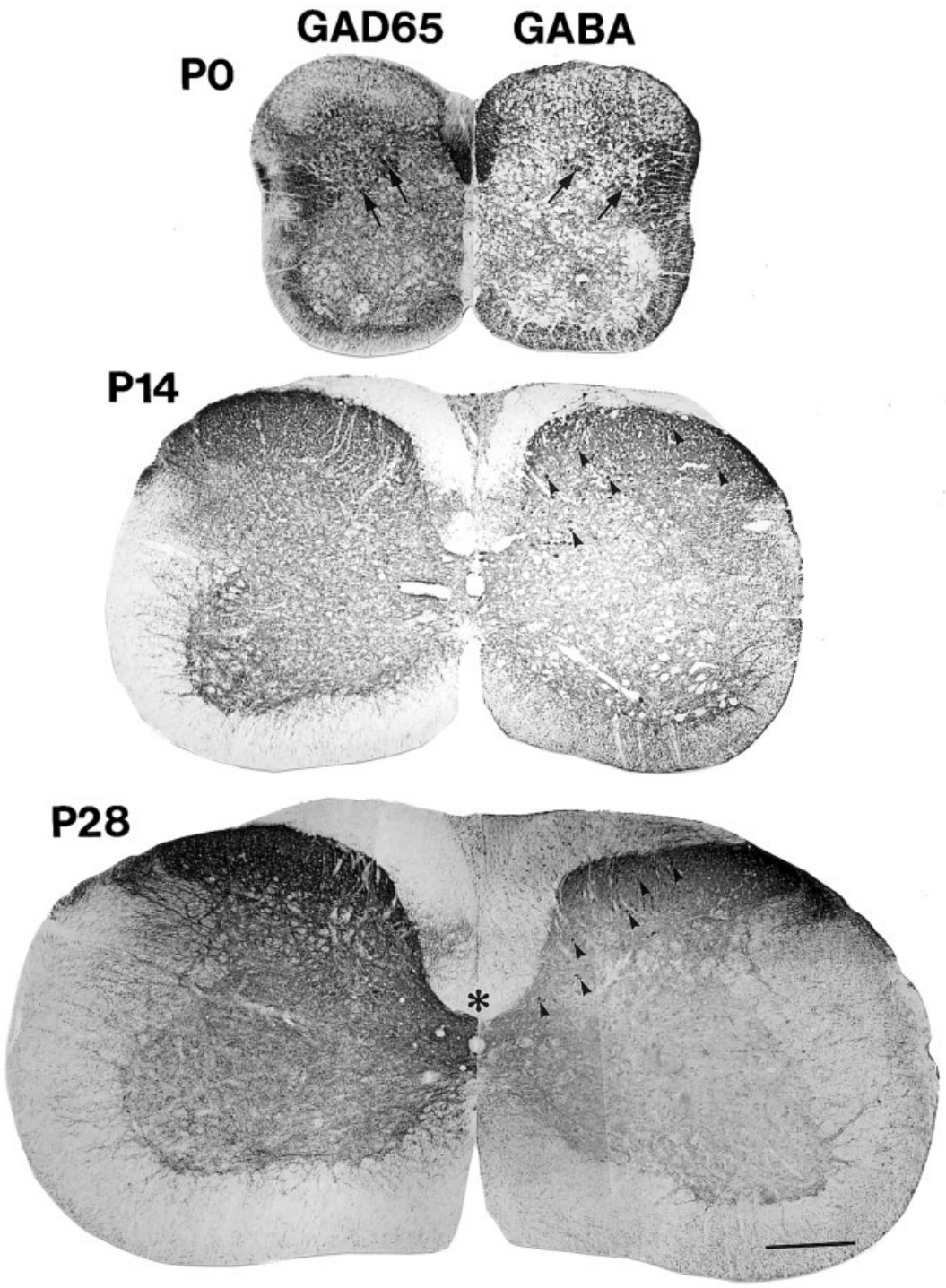


Fig. 2. This developmental sequence illustrates the pattern of glutamic acid decarboxylase 65 (GAD65; left half) and γ-aminobutyric acid (GABA; right half) immunoreactivity in postnatal day (P) 0, 14, and 28 cervical spinal cords. **P0:** Few GAD65- or GABA-labeled somata are detected, but rather numerous terminal-like structures fill the gray matter. However, many immunoreactive axons are found within the funiculi with GAD65-positive axons concentrated along the inner border, whereas GABA-labeled axons appear uniformly distributed. GAD65- and GABA-immunoreactive axon bundles still are detected in the reticulated area of the dorsal horn (arrows). **P14:** Most somata detected at P14 are small GABA-positive cells (arrowheads) interspersed throughout the dorsal spinal cord. Similarly, most immunoreactive funicular axons detected at P14 are labeled with GABA. The gray matter is densely packed with GAD65- and GABA-positive punctate structures that are most highly concentrated in the superficial dorsal horn. **P28:** GAD65- and GABA-immunoreactive terminal-like varicosities are distributed throughout the gray matter in a similar pattern, and are most densely concentrated in the superficial dorsal horn. GABA-positive cell bodies (arrowheads) still are detected in the dorsal horn. The levels of GAD65- and GABA-immunoreactive funicular axons now have decreased to near adult levels. The corticospinal tract (asterisk) remains unlabeled. Scale bar = 500 μm in P28 (applies to P0–P28).

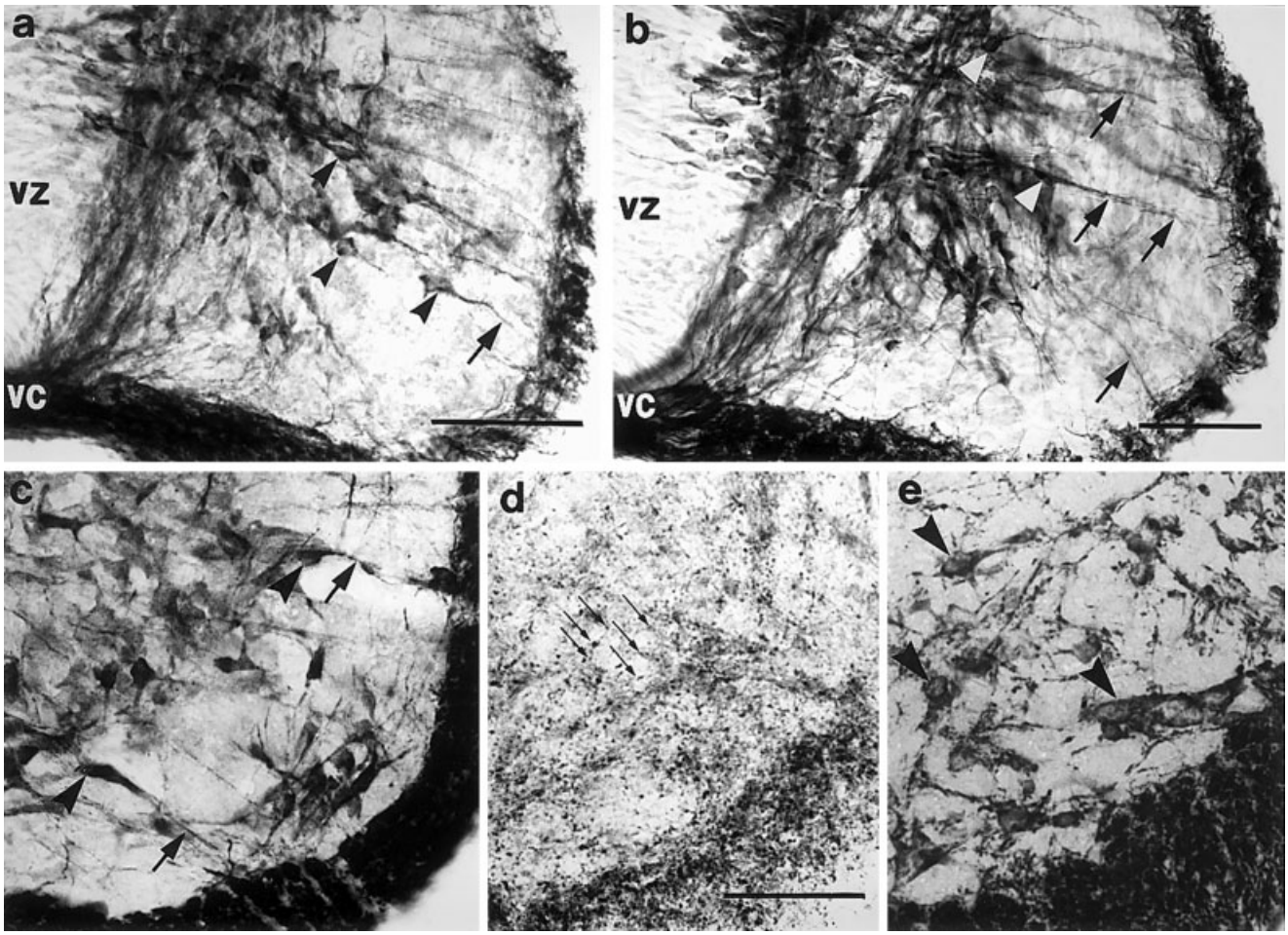


Fig. 3. Embryonic γ-aminobutyric acid (GABA)ergic neurons are detected with anti–glutamic acid decarboxylase 65 (anti-GAD65; a,b,d) or anti-GABA (c,e) antibodies in embryonic day (E) 13 (a), E14 (b,c), and E17 (d,e) ventral spinal cord. The ventricular zone (vz) is oriented to the left in a–c, and to the right in d,e. The immunoreactive ventral marginal zone is located at the bottom of all panels. **a:** Cell bodies of GAD65-positive neurons (arrowheads) are detected in ventral E13 spinal cord. The axon (arrow) of one of the GAD65-positive neurons is shown to extend into the ipsilateral marginal zone. GAD65-labeled axons also project contralaterally into the ventral commissure (vc). **b:** One day later, the ventral spinal cord contains GAD65-immunoreactive neurons (white arrowheads) that project axons (arrows) into the ipsilateral marginal zone. **c:** Several ventrolaterally located GABA-labeled neurons (arrowheads) also extend processes (arrows) toward the ipsilateral marginal zone. **d:** Primarily GAD65-labled terminal-like structures (small arrows) fill the neuropil of this enlargement of ventromedial aspect of E17 spinal cord section illustrated in Figure 1. **e:** Many GABA-immunoreactive cell bodies (arrowheads) were detected in the ventromedial region of the E17 spinal cord section illustrated in Figure 1. Scale bar = 60 μm in a,b,d. (Scale bar in d applies to c and e.)

at P28, the distributions of GAD65 and GABA immunoreactivity closely resembled previous reports of the adult spinal cord (McLaughlin et al., 1975; Barber et al., 1982; Mugnaini and Oertel, 1985).

The developmental changes illustrated in the animation (http://www.interscience.wiley.com/jpages/0021-9967/suppmat/index.html) provide the first dynamic representation of the growth of the spinal cord, as well as a temporal view of the development of the GABAergic elements reported above. The shift in the intracellular localization of GAD65 from cell bodies to terminal-like structures is temporally and spatially displayed, and includes a ventral to dorsal developmental gradient of the GAD65-labeled neurons. The gradual increase in the overall dimensions of the developing spinal cord also is evident. An additional feature noted in the animation is the apparent rotation of the dorsal spinal cord and enfolding of the future dorsal columns as the ventricular zone recedes and transforms into the central canal. GAD65-labeled axons first are detected in the inner border of the dorsal marginal zone on E15, and then, as development proceeds, these axons expand in number and reside primarily in the midline area of the dorsal columns.

Histotypic in vitro expression pattern of GAD65

To examine and manipulate the presence and subsequent loss of immunoreactive GABAergic soma and proximal axons during embryonic development, we used an organotypic slice model of developing spinal cord (Barber et al., 1993; Phelps et al., 1996). Initial experiments were conducted to determine whether the pattern of GAD65

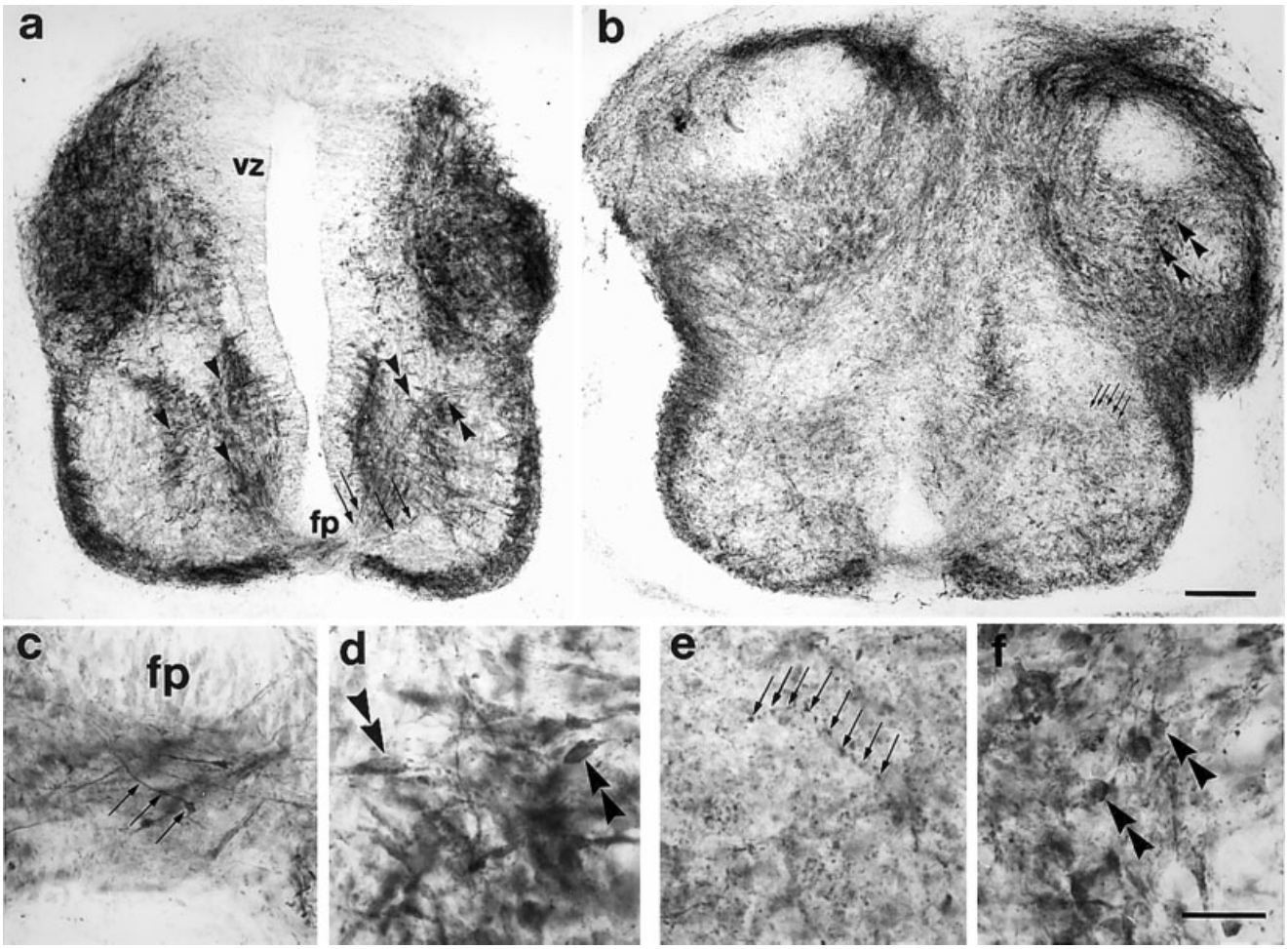


Fig. 4. Spinal cord slices maintain a histotypic glutamic acid decarboxylase 65 (GAD65) expression pattern after 1 (a,c,d) or 4 (b,e,f) days in vitro. Both the ventricular zone (vz) and the floor plate (fp) are glutamic acid decarboxylase 65 (GAD65) -negative in embryonic slices. **a:** GAD65-positive somata (arrowheads, double arrowheads enlarged in d) are present in both the ventral and dorsal spinal cord in a pattern similar to that seen in 14- (E14) to 15-day-old embryos. GAD65-positive axons (arrows) and axonal growth cones project through the ventral commissure (enlarged in c). Both the ventral and lateral marginal zones are densely packed with immunoreactive axons. **b:** Primarily terminal-like structures (arrows, enlarged in e) are present in the E14+4 slice in a pattern similar to that seen at later developmental stages. In addition, GAD65-positive cell bodies (double arrowheads, enlarged in f) are detected in the dorsal spinal cord. Other normal developmental features seen in these cultures are the contraction of the ventricular zone and the expansion of the dorsal horn. **c:** GAD65-positive commissural neurons project axons with growth cones (arrows) into the ventral commissure. **d:** Many GAD65-positive cell bodies (double arrowheads, see a for location) are detected in the ventral spinal cord. **e:** After 4 days in vitro, numerous terminal-like structures (multiple arrows, see b for location) are detected in the ventral spinal cord. **f:** Many GAD65-positive cell bodies (double arrowheads, see b for location) still are detected in the dorsal spinal cord. Scale bar = 100 μm in b (applies to a,b), 20 μm in f (applies to c–f).

immunoreactivity within different ages of spinal cord slices would mimic equivalent embryonic sections. Embryonic day 14 slices were cultured for up to 4 days and processed for GAD65 immunocytochemistry. After 1 day in vitro (E14+1), ventrally located GAD65-positive commissural neurons (Fig. 4a, arrowheads) were intensely immunoreactive, including their axons (Fig. 4a, arrows) and growth cones in the ventral commissure (Fig. 4c). Additionally, the E14+1 cultures contained many other GABAergic neurons (Fig. 4a,d, double arrowheads) just as observed in 15-day-old embryos (Fig. 1, E15). After 4 days in vitro (E14+4), primarily terminal-like structures (Fig. 4b,e, arrows) were present in the ventral spinal cord, a pattern similar to that observed in late embryonic development (Fig. 1, E17 and Fig. 3d). Some somata still were detected in the dorsal horn of E14+4 slices (Fig. 4b,f, double arrowheads). Thus, the intracellular changes observed in GAD65 localization from early to late embryonic development were recapitulated in vitro.

Marginal zone axons slowly degenerate in vitro

The large number of GABAergic axons detected in the marginal zones was unexpected and could be derived from intrinsic and/or extrinsic sources. We reasoned that, if the GABAergic marginal zone axons originated from either supraspinal sources or other spinal cord levels, they would degenerate in an isolated spinal cord slice, whereas axons

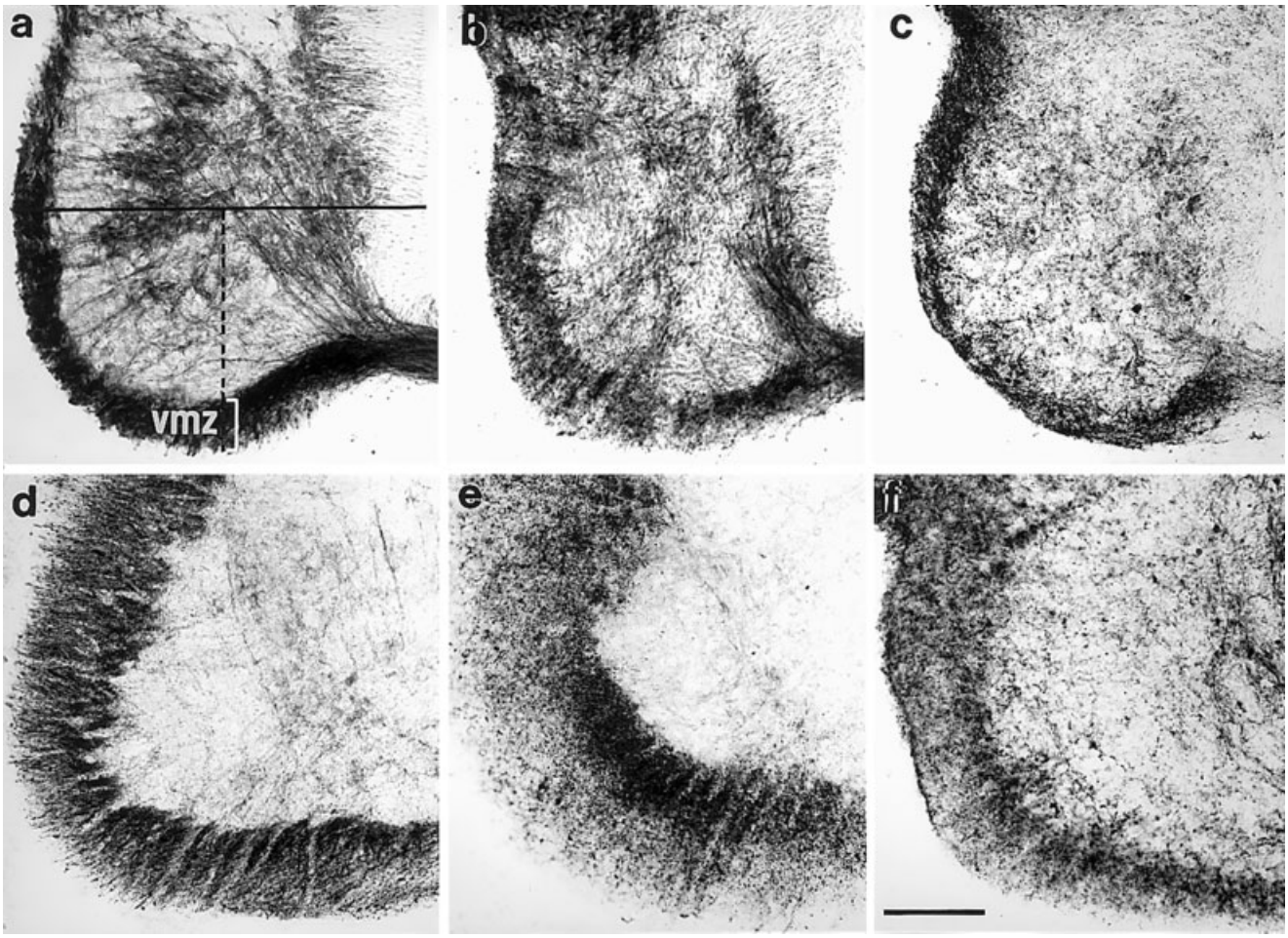


Fig. 5. Extrinsically derived glutamic acid decarboxylase 65 (GAD65) -labeled axons degenerate in vitro. Spinal cord slices prepared from embryonic day (E) 14 (a–c) and E16 (d–f) embryos were grown for 0 (a,d), 1 (b,e), or 4 (c,f) days in vitro. **a:** Ventral and lateral marginal zones of E14 embryos (E14+0) are densely packed with GAD65-labeled axons. To measure the width of the ventral marginal zone (vmz), a solid line was drawn perpendicular to the ventricle through the widest part of the lateral marginal zone. Then a vertical line (dashed) was drawn from the midpoint of the solid line to the outer edge of the vmz, the site of the measurement (delineated by the white bracket). **b:** After 1 day in vitro (E14+1), many GAD65-labeled axons are maintained in the ventral and lateral marginal zones. **c:** Many fewer marginal zone axons are GAD65-positive after 4 days in vitro (E14+4). **d:** The ventral marginal zone of older embryos (E16+0) is almost twice as wide and contains many more GAD-positive axons than seen in younger embryos (a). **e:** After E16, slices are cultured for 1 (E16+1) day, numerous loosely arranged, GAD65-labeled axons remain in the marginal zones. **f:** After 4 days in vitro, significantly fewer GAD65-labeled marginal zone axons are detected. Scale bar = 100 μm in f (applies to a–f).

derived from cells within the slice might be maintained. Thus, to address the origin of the numerous GAD65- and GABA-positive axons observed during embryonic development, we grew spinal cord slices isolated at different embryonic ages. After E14, slices were grown for 1 day in vitro, numerous GAD65-immunoreactive axons within the ventral and lateral marginal zones were detected similar to those present in embryonic sections (Fig. 5a,b). Older spinal cord slices (E16; Fig. 5d) that contained larger numbers of axons also were cultured to determine whether more dramatic axon degeneration would be observed. We determined that E16+1 slices (Fig. 5e) contained numerous GAD65-labeled marginal zone axons, a finding that suggests that the cut ends of these axon segments may reseal, a phenomenon similar to that reported in several invertebrate species (Yawo and Kuno, 1985; Spira et al., 1993; Bedi and Glanzman, 2001). Upon closer examination, GAD65-labeled marginal zone axons within E14+0 and E16+0 spinal cord sections (Fig. 5a,d) were densely packed, whereas axon bundles in E14+1 and E16+1 slices (Fig. 5b,e) were more loosely arranged. We suspected that these GAD65-labeled axons would further degenerate in slice cultures if the time in vitro was increased. Both E14+4 and E16+4 cultures (Fig. 5c,f) were prepared and found to contain significantly fewer GAD65-labeled axons when compared with embryonic sections or +1 day cultures, thus suggesting that many GABAergic marginal zone axons originate from cells extrinsic to the slice.

The width of the GAD65-labeled axons within the VMZ was used to determine the extent of GABAergic axon degeneration; therefore, measurements were made after different times in vitro and analyzed statistically (Fig. 6). The width of the VMZ for both E14+0 and E14+1 were

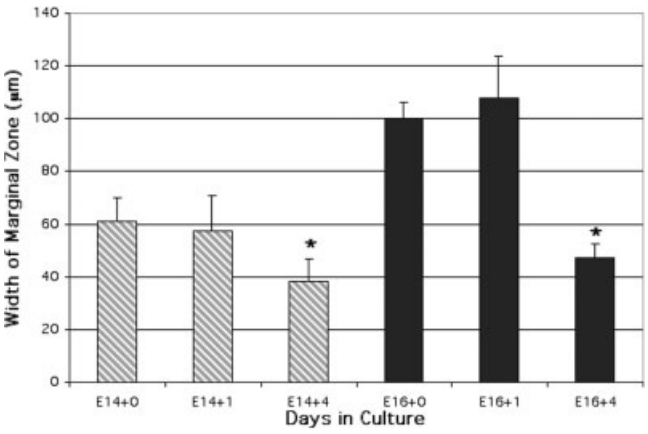


Fig. 6. Axonal degeneration quantified by ventral marginal zone width measurements in vitro. In both younger (embryonic day [E] 14, diagonal shading) and older (E16, black) slice cultures, there were no significant differences between embryos (+0) and 1 day (+1) old slices. However, there were significant differences (asterisks) in the ventral marginal zone widths after 4 days in vitro compared with both embryonic sections and 1-day cultures for both E14 and E16 slices ($P < 0.0001$).

similar and no significant differences in size were found (E14+0: $\bar{x} = 61.1 \pm 8.8$ μm; E14+1: $\bar{x} = 57.2 \pm 13.7$ μm; $P = 0.34$). Similar results were found in older cultures, with no significant differences in VMZ widths between E16+0 and E16+1 slices, although the VMZ width at E16+1 was slightly wider than that of E16+0 (E16+0: $\bar{x} = 100 \pm 6.1$ μm; E16+1: $\bar{x} = 108 \pm 15.8$ μm; $P = 0.075$). However, after 4 days in vitro, the VMZ was significantly reduced in size when compared with both E14+0 or +1 (E14+4: $\bar{x} = 38.3 \pm 8.5$ μm; $P < 0.0001$), or E16+0 or +1(E16+4: $\bar{x} = 47.1 \pm 5.2$ μm; $P < 0.0001$). The significant decrease in the width of the GAD65-labeled VMZ after 4 days in vitro provided evidence that many extrinsically derived GABAergic axon bundles within the marginal zones were lost. In addition, these findings demonstrated that there are a significant number of intrinsic GABAergic neurons that give rise to the GAD65-positive marginal zone axons that remained after 4 days in vitro.

GABA-immunoreactive somata are maintained in organotypic slices

More GABA-positive somata were observed in spinal cord sections during later development (E17–P28) than were identified with GAD65 (Figs. 1, 2). To determine whether GABA was maintained within somata longer than GAD65, organotypic spinal cord slices derived from E15 embryos were grown for 4 days in vitro and processed for GABA immunocytochemistry. Many GABA-positive somata and axons were detected in both ventral and dorsal regions of these spinal cord slices (Fig. 7a,b), suggesting that GABAergic neurons are present and able to synthesize GABA in vitro. This result contrasted with the few GAD65-positive cell bodies that were detected in E14+4 slices (Fig. 4b). The difference in the temporal expression between GABA and GAD65 prompted further investigation into the expression patterns of both GAD65 and GAD67.

Developmental expression patterns of GAD65 and GAD67 can be inhibited in vitro

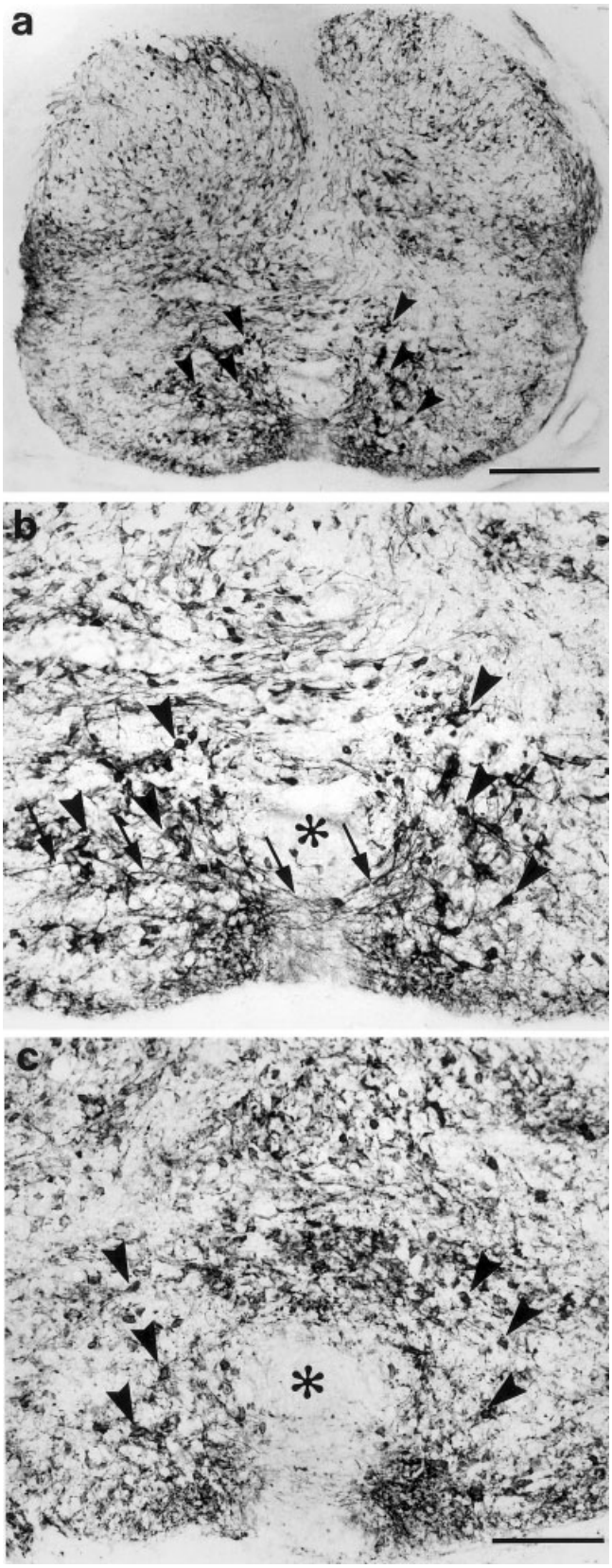
Initial experiments were designed to determine whether we could reverse the abrupt developmental changes in the intracellular distribution of GAD65 from a primarily somal distribution to one of terminal-like structures. Axonal transport was blocked within our slice culture model by adding colchicine (10 μg/ml) to the defined media for 6 hours before fixation. Many more GAD65-positive somata were detected in E14+2 slices treated with colchicine (Fig. 8b,c) than observed in colchicine-free controls (Fig. 8a). The intracellular shift of GAD65 expression also was tested at later times during development by using slices from 16-day-old embryos when few GAD65 somata were normally detectable. Untreated E16+4 slices resembled GAD65-labeled P0 specimens (Fig. 2) as the gray matter was filled with terminal-like structures and most GABAergic cell bodies were found adjacent to the receding ventricular zone (Fig. 8d,f). After colchicine treatment, numerous GAD65 somata were detected throughout E16+4 slices (Fig. 8e,g). Thus, the developmental changes observed in the expression pattern of GAD65 could be blocked in vitro.

During early spinal cord development, GAD65 is detected at higher levels than GAD67 (Somogyi et al., 1995; Phelps et al., 1999) and several studies suggest that the two forms of GAD are found in different subcellular compartments and may have different functional roles (Erlander and Tobin, 1991; Esclapez et al., 1994). Thus, it was important to evaluate if the intracellular shift detected in GAD65 immunoreactivity also occurred in developing GAD67-positive neurons. We repeated the axon transport blocking experiments and examined the changes in GAD67 expression. In E16+2 control slices, virtually no GAD67-labeled cell bodies were detected, whereas prominent immunoreactive somata were evident in colchicine-treated slices (data not shown). In E16+4 colchicine-free control cultures, primarily terminal-like structures were detected (Fig. 9a,b), but numerous somata were filled with GAD67 reaction product in colchicine-treated cultures (Fig. 9c,d). Thus, the developmental changes in the expression pattern of GAD67 from primarily somal to terminal-like structures also could be blocked in vitro.

Finally, the effect of colchicine on the localization of GABA was examined. In contrast to the findings reported with GAD65 and GAD67, the level of GABA expression within somata was similar in both control (Fig. 7a,b) and colchicine-treated cultures (Fig. 7c). The only difference detected was that more GABA-positive axons were present in the control than in the experimental cultures.

DISCUSSION

In the present study, the cellular localization of GABAergic spinal cord neurons evolved from one characterized by somatic and proximal axon labeling during early ages to a pattern of immunoreactive distal axons and terminal-like varicosities at later ages. As these changes were recapitulated in our in vitro system, we were able to experimentally block axonal transport and to determine that the intracellular changes in the developmental pattern of GAD65 and GAD67 distribution were reversed in older cultures. These perturbation studies provide strong



evidence that GABAergic spinal cord neurons are maintained throughout development and that the temporal changes in the markers of the GABAergic phenotype represent a shift in the intracellular localization of GAD proteins that occurs during the period of axon outgrowth and synaptogenesis (Fig. 10). Thus, we suggest that the apparent loss of GAD immunoreactivity in cell bodies and axons represents a rapid translocation of GAD proteins for local GABA synthesis at the synaptic terminals.

Intracellular shift of GAD65 and GAD67 within developing GABAergic neurons

Previous investigators have reported that GAD and GABA are transiently expressed in certain neuronal populations during spinal cord development (Ma et al., 1992a,b). Important to their argument in support of transient expression were observations that cell populations known to be immunonegative in the adult spinal cord, such as somatic motor neurons, expressed GAD and GABA during development (Ma et al., 1992a). These investigators illustrated GAD and GABA immunoreactivity within somatic motor neurons and the dorsal root entry zone (fasciculus ovalis) before but not after birth (Ma et al., 1992a). In the present study, the somatic motor neurons, the dorsal root ganglia and the fasciculus ovalis were all GAD65- and GABA-negative throughout development in sections that contained other strongly immunoreactive processes. Thus, our findings argue against the previous interpretation that GAD and GABA are transiently expressed in non-GABAergic neurons during development. Differences in the preparation of the embryos and the use of different antibodies most likely account for the discrepancies between our present data and those of Ma et al. (1992a,b).

The apparent loss of GABAergic neurons during spinal cord development is different from the developmental course of other neuronal transmitter phenotypes seen in similar studies. For example, the expression of the acetylcholine-synthesizing enzyme, choline acetyltransferase, is known to increase within the cell bodies and axons of cholinergic neurons during development and to be maintained in adulthood (Barber et al., 1984; Phelps et al., 1984, 1990). In contrast, GAD65 and GABA immunoreactivity was detected for only 3–4 days within ventral embryonic neurons. The changes associated with the presence and subsequent loss of GAD protein in spinal cord neurons could be interpreted as (1) a transient expression of GAD in neurons that eventually adapt a different neurotransmitter phenotype, (2) a developmental down-regulation of the GAD proteins, or (3) a shift of GAD within the intracellular compartments of the developing

Fig. 7. γ-Aminobutyric acid (GABA) -immunoreactive somata are maintained in organotypic slices. Cultures derived from 15-day-old embryos (E15) were grown for 4 days in vitro in normal media (a,b) or treated with colchicine for 6 hours before fixation (c). Asterisks mark the ventral midline. **a:** Control E15+4 slice shows numerous GABA-positive somata (arrowheads) and axons present in both the ventral and dorsal spinal cord. **b:** Many GABAergic somata (arrowheads) and proximal axons, including commissural axons (arrows), are illustrated in this enlargement of the ventral midline of a. **c:** Colchicine-treated E15+4 slice contains numerous GABA-positive somata (arrowheads) but fewer axons than observed in the control (b). Scale bars = 200 μm in a, 100 μm in c (applies to b,c).

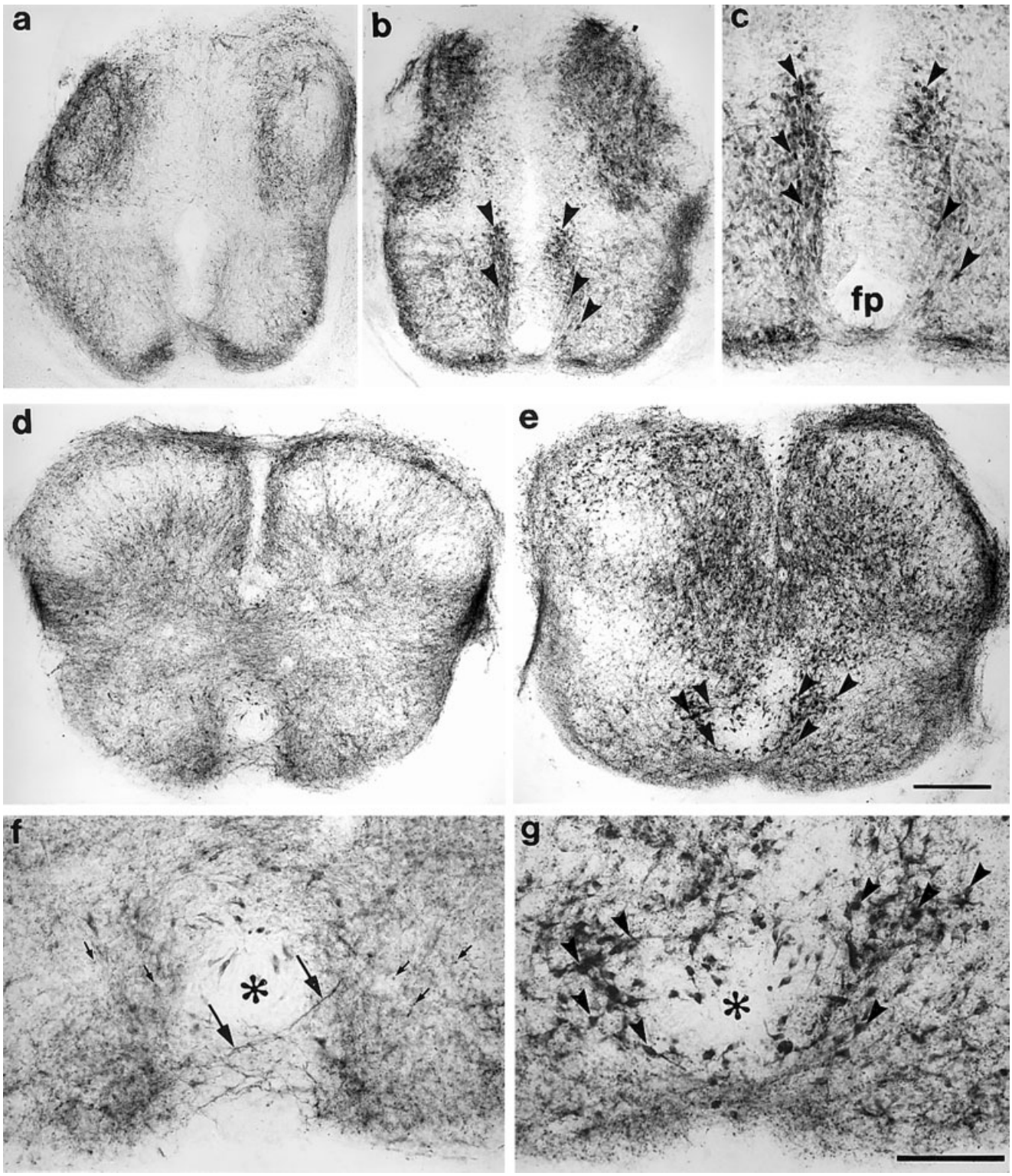


Fig. 8. Changes in the developmental expression pattern of glutamic acid decarboxylase 65 (GAD65) are reversible in vitro. Organotypic cultures derived from embryonic day (E) 14 (a–c) or E16 (d–g) spinal cords were grown for 2 (a–c) or 4 (d–g) days in vitro. Axonal transport was blocked by adding colchicine for 6 hours to the defined media of experimental (b,c,e,g) but not the control (a,d,f) cultures. Asterisks mark the ventral midline in f,g. **a:** Relatively few GAD65-labeled somata are detected in this E14+2 control culture. **b:** E14+2 colchicine-treated culture reveals numerous GAD65-labeled somata (arrowheads) in both the ventral and dorsal spinal cord. **c:** An enlargement of b illustrates GAD65-labeled neurons (arrowheads) in positions similar to those previously identified as commissural neurons. The GAD65-negative floor plate (fp) is identified in the ventral midline. **d:** The GAD65-labeled E16+4 control slice is filled with terminal-like varicosities. **e:** Numerous GAD65-labeled somata (arrowheads) are detected in this colchicine-treated E16+4 culture. **f:** An enlargement of d illustrates a few GAD65-labeled cell bodies as well as commissural axons (large arrows) projecting into the ventral commissure. Numerous terminal-like varicosities (small arrows) are distributed throughout the gray matter. **g:** Enlargement of e shows numerous ventrally located somata (arrowheads) filled with GAD65-immunoreactive product after colchicine treatment. Scale bars = 200 μm in a,b,d,e, 100 μm in c,f,g.

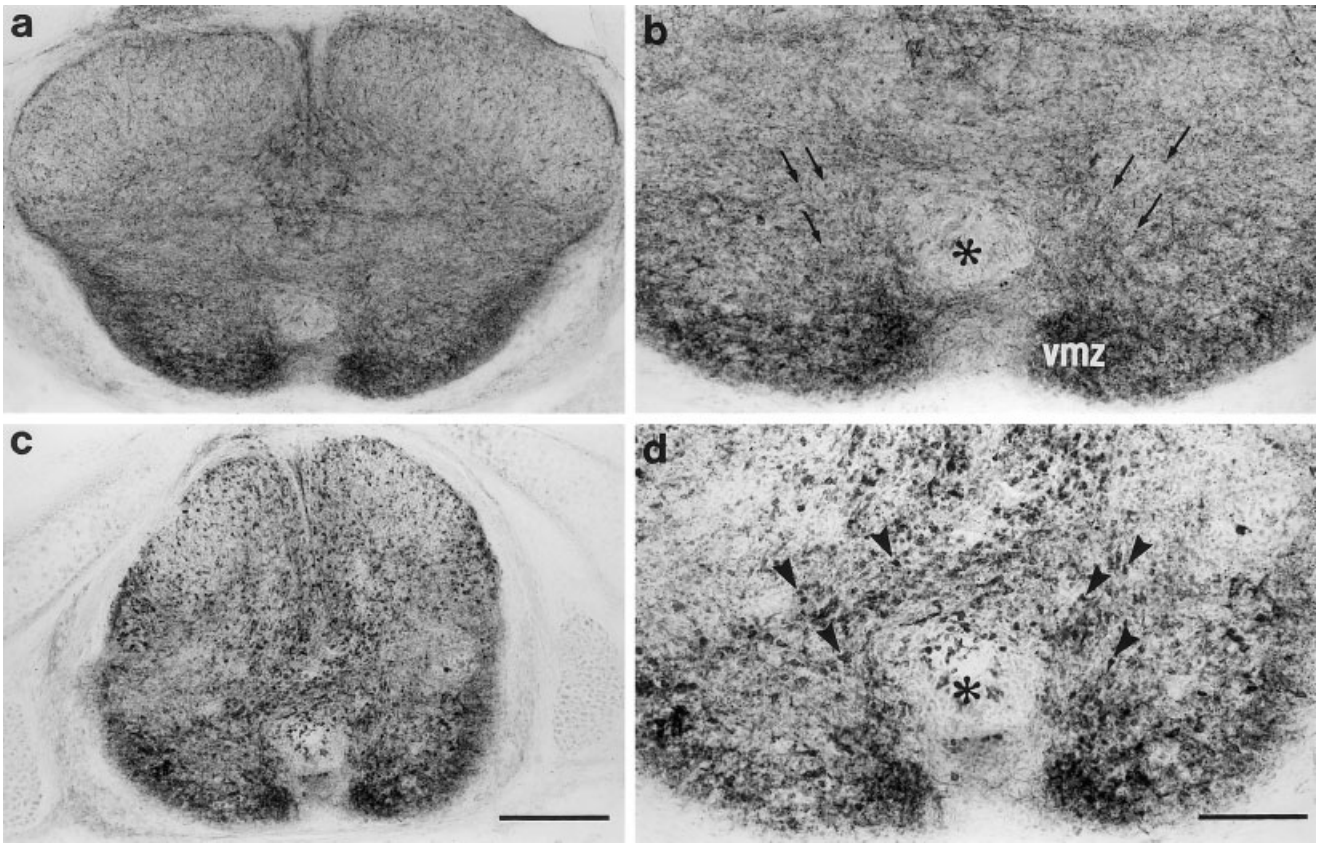


Fig. 9. Changes in the developmental expression pattern of glutamic acid decarboxylase 67 (GAD67) -positive somata are reversible in vitro. A cervical embryonic day (E) 16 control slice (a,b) cultured for 4 days was compared with a thoracic slice (c,d) from the same embryo treated with colchicine. Asterisks delineate the ventral midline in b and d. **a:** Very few GAD67-positive somata are detected in this E16+4 control culture. **b:** Enlargement of the ventral midline of a illustrates primarily terminal-like structures (arrows) in the ventral gray matter and GAD67-positive axons in the ventral marginal zone (vmz). **c:** GAD67-positive somata are detected throughout this E16+4 slice treated with colchicine. **d:** Enlargement of c illustrates numerous distinct cell bodies (arrowheads) containing the GAD67-immunoreactive product. Scale bars = 200 μm in c (applies to a,c), 100 μm in d (applies to b,d).

neurons (Phelps et al., 1999). Based on adult immunocytochemical studies of GAD with colchicine pretreatment (Barber et al., 1982) and postnatal and adult in situ studies (Ma et al., 1994), GAD-labeled neurons are maintained throughout life and are found in all spinal cord lamina, except for lamina IX that contains somatic motor neurons. Studies localizing GABA expression in adult spinal cord have primarily localized cells in the superficial dorsal horn (Magoul et al., 1987; Todd and Lochhead, 1990). These studies, in combination with our results from blocking axonal transport in organotypic slices, argue against the interpretation that either form of GAD is transiently expressed or down-regulated and suggest that the most likely explanation is that these changes are due to a shift in the intracellular compartmentalization of GAD65 and GAD67.

Results from our colchicine-treated cultures indicated that blocking axonal transport in vitro produced detectable concentrations of both GAD isoforms in somata that were not detected in equivalent aged embryos. For example, the commissural neurons detected during development between E12 and E15 (Phelps et al., 1999) were observed in colchicine-treated slices at all ages but not in control slices. Additional groups of ipsilaterally projecting, ventral GAD-positive spinal cord neurons also were detected in vitro, as well as in numerous immunoreactive cells in the dorsal horn. Reversing the expression pattern of GAD65 and GAD67 from primarily terminal-like structures to cell bodies provides strong evidence for the mechanism of these changes being an intracellular shift of GAD65 and GAD67 localization (Fig. 10).

A similar shift from predominantly GAD-containing cell bodies to terminal-like varicosities was reported in the developing hippocampal formation (Dupuy and Houser, 1996, 1997). The similarity of findings in both the hippocampal formation and the spinal cord suggests that such changes in the distribution of GAD within cell bodies during development may be a common phenomenon in developing GABAergic neurons. Although the functional significance of these early GAD-containing neurons is not known, the timing of this developmental shift in spinal cord is consistent with our working model that GAD protein accumulates in the somata of young neurons during axon outgrowth but once these neurons establish their synaptic connections, GAD is preferentially shipped to the axon terminals (Fig. 10).

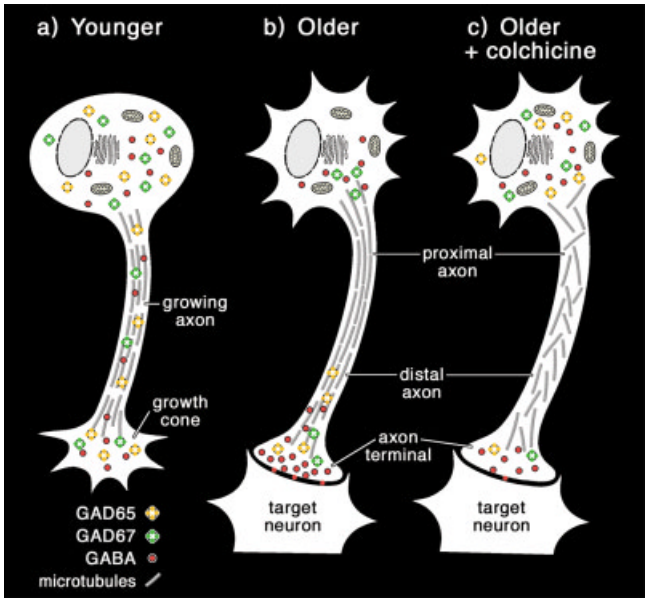


Fig. 10. Schematic model of the developmental changes of γ-aminobutyric acid (GABA)ergic spinal cord neurons. **a:** An immature GABAergic neuron extends its axon toward its synaptic target. Newly synthesized glutamic acid decarboxylase 65 (GAD65), GAD67, and GABA are associated with vesicular structures in the soma, growing axon, and growth cone. **b:** A more mature GABAergic neuron that has established synaptic connections preferentially ships GAD65 and GAD67 to the terminal for neurotransmitter synthesis. Some GAD67 and GABA remain in the soma. **c:** Colchicine disrupts microtubule polymerization and, thus, blocks the mechanism of axonal transport. Newly synthesized GAD65, GAD67, and GABA accumulate in the soma and present a cellular immunocytochemical profile similar to that of an immature GABAergic neuron.

Comparison of GAD65 and GABA expression patterns

The overall expression pattern of GAD65 and GABA in the developing spinal cord was similar, but several differences were noted. Based on both our spinal cord sections and in vitro data, GABA is detected and maintained within cell bodies and axons for a longer period of time than GAD65. One interpretation of these contrasting results is that the temporal and spatial regulation of GABA differs from that of the two forms of GAD. For example, physiological experiments using K^+ and glutamate to depolarize GABAergic neurons are reported to activate two different intracellular pools of GABA: one that is free in the cytoplasm, and the other that is compartmentalized in vesicles (Belhage et al., 1993). Additional support for the differential regulation of GAD and GABA can be drawn from our in vitro data in which the number of GABA-positive cell bodies detected in colchicine-treated cultures was similar to controls rather than increased as found with the GAD proteins.

Another difference between the expression pattern of GAD65 and GABA was found in the marginal zone axons. GAD65-labeled axons were more intensely concentrated at the inner border of the marginal zone, whereas GABA-labeled axons appeared to be uniformly distributed. One possible explanation for this difference is that the translocation of GAD65 to terminals is completed earlier than that of GABA. Thus, if the GAD65-labeled axons joined the marginal zone along its inner border, axons located next to the pia surface would be older than those adjacent to the intermediate zone and, therefore, may still contain GABA but not GAD65.

Extrinsic marginal zone axons degenerate in vitro

The isolated spinal cord slices provided a paradigm in which we could examine the intrinsic and extrinsic contribution of GABAergic neurons to the future white matter. Many fewer GAD65-containing marginal zone axons were detectable after 4 days in vitro, suggesting that those that were maintained originated from intrinsic GABAergic neurons, whereas those that degenerated were derived from GABAergic neurons at other spinal cord levels or supraspinal sources. Because numerous GAD65- and GABA-positive neurons were detected in vitro, GABAergic neurons most likely represent a previously unrecognized component of the axons that populate the marginal zones at each spinal cord level. As our cultures were prepared from young embryos, most GABAergic marginal zone axons probably originate from other spinal cord levels rather than from supraspinal neurons.

Functional significance of GABAergic spinal cord neurons

Recent studies of patterning genes in mice have postulated that the restricted expression of certain homeodomain transcription factors play an instructive role in determining cell fate in the developing neural tube (Saueressig et al., 1999; Moran-Rivard et al., 2001). One such factor, Engrailed (En1), has been shown to colocalize with a population of GAD65-positive commissural neurons in the spinal cord and, thus, may play a role in specifying GABAergic neuron identity in the ventral spinal cord (Saueressig et al., 1999). In addition, another transcription factor, Evx1, was shown to define a separate population of ventral commissural neurons (Moran-Rivard et al., 2001) that project their axons rostrally along a pathway similar to our GAD65- and GABA-positive ventral commissural neurons. Expression of these homeodomain transcription factors, therefore, may define different populations of neurons that ultimately express the GABAergic phenotype.

Physiological studies have shown GABA to function as an excitatory neurotransmitter in early developing spinal cord, despite the fact that GABA is a major inhibitory neurotransmitter in the adult nervous system (Krnjevic et al., 1977; Cherubini et al., 1991; Wu et al., 1992; Wang et al., 1994). GABA has been shown to induce membrane depolarizations in embryonic spinal cord that then shift to hyperpolarizations during postnatal development (Cherubini et al., 1991; Wu et al., 1992; Wang et al., 1994). Although, the function of GABA-induced depolarizations is unclear, the mechanism by which GABA switches from depolarization to hyperpolarization in late embryonic hippocampal neurons in vitro has been proposed to be controlled by GABA itself (Ganguly et al., 2001). GABA-mediated depolarizations were found to modulate the mRNA levels of KCC2, a K^+-Cl^- cotransporter whose expression correlates with the developmental changes in GABAergic signaling (Lu et al., 1999; Vu et al., 2000; Ganguly et al., 2001). Thus, we suspect that the GABAergic spinal cord neurons detected in this study most likely

initially depolarize their synaptic targets and then at later ages switch to hyperpolarization.

In conclusion, a major finding in this study was the abundance of somal and axonal labeling of developing GABAergic neurons throughout the early embryonic spinal cord. Due to the unique distribution of GAD65 during development, groups of ventrally located contra- and ipsilaterally projecting GABAergic neurons were identified (Phelps et al., 1999; Tran and Phelps, 2000). Although the synaptic targets and functions of these ventral GABAergic neurons currently are unknown and beyond the scope of this study, their number and prominence throughout the spinal cord suggests they constitute large populations of spinal cord neurons that are likely to have important developmental functions.

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