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Complementary Expressions of Transcripts Encoding GAD_{67} and $GABA_A$ Receptor α4, β1, and γ1 Subunits in the Proliferative Zone of the Embryonic Rat Central Nervous System

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The developmental stage at which nerve cells initially express specific neurotransmitters and their corresponding receptors remains elusive. In the present study, the distribution patterns of transcripts for the GABA-synthesizing enzyme, glutamate decarboxylase (GAD_{67}), and specific $GABA_A$ receptor subunits were examined in the proliferative zone of the rat central nervous system using *in situ* hybridization. In order to define the DNA synthetic zone of the germinal matrix, tissue sections were taken from embryos whose mothers had been injected with 5-bromo-2′-deoxyuridine (BrdU) and had survived for 1 hr. BrdU immunocytochemistry was used to locate the relative position of BrdU-immunoreactive nuclei within the ventricular zone (VZ). At embryonic day (E) 15 in the alar plate of the lumbar spinal cord, and at E17 and E20 in the dorsomedial sector of the neocortex, densely packed BrdU-immunoreactive nuclei were consistently detected in lateral portions of the inner half of the germinal matrix, indicating that the inner half of the germinal matrix corresponded to the VZ, while the outer half corresponded to the transitional (TZ) or subventricular zone (SV). *In situ* hybridization in tissue sections adjacent to BrdU-immunoreacted ones showed that the transcripts for $GABA_A$ receptor α3, β3, and γ2 subunits were found exclusively in the mantle zone, while those for α4, β1, and γ1 subunits were predominantly detected in the inner half of the germinal matrix (i.e., VZ). Furthermore, in the E15 germinal matrix of the lumbar spinal cord, cells exhibiting α4 subunit mRNA were much more abundant in the receding intermediate plate, which contains mostly postmitotic cells, than in the alar plate comprised of many DNA-synthesizing cells, strongly suggesting that only those cells completing final cell division expressed the subunit mRNAs. In clear contrast, GAD_{67} mRNA was abundant in the outer half of the germinal matrix (i.e., TZ or SV), and in the intermediate zone as well. Immunocytochemical staining of E17 neocortex with anti-GABA antibody revealed a well defined band of GABA-immunoreactive cells and processes in the SV and occasional positive cells in the VZ. It appears that cells in the proliferative zone may express GABA at the migratory stage, whereas cells in the VZ may express mRNAs for $GABA_A$ receptor α4, β1, and γ1 subunits at the premigratory stage, just after completing cell division. The present study suggests that both GABA and $GABA_A$ receptors may be expressed in cells before they migrate to their final positions, and that GABAergic interaction may regulate neuronal migration and differentiation.

[Key words: development, germinal matrix, BrdU immunocytochemistry, cell cycle, glutamate decarboxylase, $GABA_A$ receptor subunits,* in situ *hybridization]

Neurons originate for the most part in the ventricular zone (VZ), a sheet of neuroepithelial cells lining the embryonic ventricular system throughout the brain and spinal cord. An interesting but unresolved issue concerns the developmental stage at which embryonic cells synthesize neurotransmitters and receptors. This is particularly important in the developing central nervous system (CNS), since increasing evidence indicates that classical, fast-acting neurotransmitters may play morphogenic roles in forming the CNS by affecting neuronal proliferation, migration, and differentiation (for reviews, Redburn and Schousboe, 1987; Lauder, 1988, 1993; Meier et al., 1991). GABA is one of the earliest transmitters detected in the developing CNS (Lauder et al., 1986; Ma et al., 1991, 1992a), and has been implicated in playing a role in neuronal development (Hansen et al., 1987). Our previous studies in the spinal cord reported that one of the GABA-synthesizing enzymes, glutamate decarboxylase (GAD_{67}) (Ma et al., 1992b), and its mRNA, particularly its alternatively spliced form (Behar et al., 1994a), are expressed by neuroepithelial cells and postmitotic neuroblasts during embryogenesis. Furthermore, GABA-immunoreactive cells have been detected at different depths of the VZ of rat neocortex (Van Eden et al., 1989; Cobas et al., 1991).

In addition, mRNAs encoding the subunit family of $GABA_A$ receptors have been found throughout the embryonic CNS (Laurie et al., 1992; Poulter et al., 1992, 1993; Ma et al., 1993a,b). In the spinal cord, α2,3,5, β2,3, and γ2,3 subunit mRNAs were detectable as early as embryonic day (E) 12–13 in the mantle zone, where cells differentiate, while the trio of α4 (according to Seeburg's nomenclature, see Wisden et al., 1991), β1, and γ1 transcripts emerged at E13 exclusively in the VZ, where cells proliferate (Ma et al., 1993b). The anatomic localization of both GAD_{67} and $GABA_A$ receptor subunit mRNAs in the proliferative zone suggests a possible role for GABA in the earliest period of neuronal development. However, it is not yet clear when during proliferation cells express GAD and/or $GABA_A$ receptor sub-

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unit mRNAs. This is particularly important in determining when and where putative GABAergic circuits might function and for what purposes.

It is known that neuroepithelial cell nuclei undergo a to-and-fro movement in the VZ during the cell generation cycle (Sauer 1935a,b). Neuroepithelial cells replicate their DNA mainly while their nuclei are distant from the lumen of the neural tube in the synthetic zone (sz). The nuclei with replicated DNA move toward the ventricular surface where cell division occurs in the mitotic zone (mz). After mitosis, the daughter cells may either reenter the cell cycle or migrate from the proliferative zone to form a mantle layer near the external surface of the neural tube, where further differentiation occurs. The classical stages of cell cycle characteristics of proliferative cells are related to the relative position of the proliferative cell nucleus in the VZ; nuclei at the same stage are spatially aligned with each other at the same depth of the VZ. Therefore, the spatial location of aligned nuclei within the VZ is a useful indicator of the cell cycle stage (Takahashi and Caviness, 1993). Cell nucleus location within the VZ can be followed autoradiographically with ^{3}H-thymidine. It has been shown that a single injection of ^{3}H-thymidine is available for DNA synthesis for less than 1 hr in mammals; only a single generation of cells takes up the label (Sidman et al., 1959; Atlas and Bond, 1965). Nuclei in the DNA synthetic phase are situated in the lateral portion of the VZ; this location may help identify the DNA-synthesizing region in the VZ (Sidman et al., 1959). 5-Bromo-2′-deoxyuridine (BrdU) is a thymidine analogue that is also incorporated in the synthetic phase and can be detected with immunocytochemistry (Miller and Nowakowski, 1988); BrdU-labeled cells are now considered the equivalent of thymidine-positive elements (Miller and Nowakowski, 1988). The location of BrdU-positive cells after a 1 hr pulse of BrdU may thus be a useful reference for determining the locations of cells expressing GAD or $GABA_A$ receptor subunit mRNAs with relation to S-phase cells in the proliferative zone.

Here, we demonstrate that there are two anatomically separate subsets of cells in the proliferative zone at spinal and neocortical levels; cells in the mz and sz express $GABA_A$ receptor α4, β1, and γ1 subunit mRNAs, while cells in the transitional zone (TZ) of the spinal cord or subventricular zone (SV) of the neocortex express the GAD_{67} transcript. Cells expressing the receptor subunit mRNAs appear to be premigratory neuroblasts, whereas cells expressing GAD_{67} mRNA may be predominantly postmitotic cells migrating to the mantle zone.

Materials and Methods

Animals and tissue preparation. Timed pregnant Sprague–Dawley rats (Taconic Farms, Germantown, NY) at gestational days 15, 17, and 20 were pulse labeled with BrdU (Sigma Chemical Co., St. Louis, MO; 50 mg/kg body weight, i.p.). Animals were anesthetized with sodium pentobarbital (40 mg/kg body weight, i.p.) 1 hr after BrdU injection. The embryos were immediately removed from the uterus and frozen on dry ice. The embryonic body part containing the lumbar spinal cord or the middle forebrain was cut serially in 12 μm thick coronal sections on a cryostat, thaw-mounted onto gelatin-coated slides, and stored at −70°C until used. For each age, sections were collected from at least three embryos removed from three different mothers. The sections were treated for *in situ* hybridization histochemistry, and alternate sections were prepared for BrdU immunocytochemistry.

After removal from the mothers, some E17 embryos were perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) following phosphate-buffered saline (PBS) for GABA immunocytochemistry. The embryos were postfixed for 4 hr in the same fixative at 4°C, and then soaked in a 30% sucrose phosphate buffer solution at 4°C for 2 d. The middle forebrain part of E17 brain was blocked and cut in 12 μm thick sections on a cryostat, and mounted onto poly-L-lysine-coated slides before immunostaining for GABA.

BrdU immunocytochemistry. Some sections were stained with a monoclonal antibody against BrdU (Becton-Dickinson, Mountain View, CA). The sections were fixed in 70% ethanol for 30 min and then rinsed in PBS (pH 7.3). The sections were immersed in 0.07 N NaOH for 2 min and then in 0.1 M PBS, pH 8.5, for 30 sec, and rinsed again in PBS (pH 7.3). Subsequently, the spinal cord was incubated with a fluorescein isothiocyanate–conjugated anti-BrdU antiserum (diluted to 1:5 in PBS) for 30 min at room temperature. The sections were rinsed three times in PBS and coverslipped with a mixture of glycerol and PBS (3:1).

GABA immunocytochemistry. The GABA immunostaining procedure used in the present study has been previously described (Ma et al., 1992a). Briefly, sections were rinsed in three changes of PBS and incubated in polyclonal antisera to GABA (1:300) (raised in guinea pigs; Eugene Tech Inc., Allendale, NY) for 48 hr at 4°C. Following three rinses in PBS, the sections were incubated with rhodamine-conjugated secondary antibodies at a final dilution of 1:30 at room temperature for 45 min. The sections were rinsed again in PBS, coverslipped with a mixture of glycerol and PBS (3:1), and examined under a Leitz epifluorescence microscope. In order to identify the cytoarchitectural features of immunostained sections, coverslips were removed from some slides after fluorescence signals were photographed, and stained with 2% cresyl violet.

In situ *hybridization.* Sections adjacent to those treated for BrdU immunocytochemistry were processed for *in situ* hybridization employing ^{35}S-labeled oligonucleotide probes as described previously (Ma et al., 1993b). Briefly, the sections were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.3) for 5 min, rinsed twice in PBS, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine HCl/0.9% NaCl for 10 min. They were then dehydrated through a graded series of ethanol solutions, delipidated in chloroform, rehydrated, and air dried. Complementary DNA probes to the $GABA_A$ receptor α3 subunit (Malherbe et al., 1990) 5′-CAC TGT TGG AGT TGA AGA AGC ACT GGG AGC AGC AGC CTT GGA GAT-3′; α4 subunit (according to Seeburg's nomenclature, Wisden et al., 1991) 5′-CAA GTC GCC AGG CAC AGG ACG TGC AGG AGG GCG AGG CTG ACC CCG-3′; β1 (Ymer et al., 1989b) 5′-GAC TTT GTT CAT CTC CAG TTT GTT CTT TTC ATT GGC ACT CTG GTC TTG-3′; β3 (Lolait et al., 1989) 5′-CCC GTG AGC ATC CAC CCG GTT GAT TTC ACT CTT GGA TCG ATC ATT CTT-3′; γ1 (Ymer et al., 1990) 5′-GCC CTC CAA GCA CTG GTA ACC ATA ATC ATC TTC CCC TTG AGG CAT AGA-3′; γ2 (Shivers et al., 1989) 5′-ATA TTC TTC ATC CCT CTC TTG AAG GTG GGT GGC ATT GTT CAT TTG GAT-3′; and GAD_{67} (Kaufman et al., 1986; Erlander et al., 1991) 5′-TAG TAT TAG GAT CCG CTC CCG CGT TCG AGG AGG TTG CAG GCG AAG GCG-3′ were manufactured on an Applied Biosystems 380A DNA synthesizer (Foster City, CA). Probes were 3′-end-labeled with ^{35}S-dATP (Dupont/New England Nuclear, Boston, MA) using deoxynucleotide terminal transferase (Boehringer Mannheim Biochemica, Indianapolis, IN). The sections were hybridized overnight at 37°C with one of the labeled probes dissolved in hybridization buffer at a concentration of 1–2 × 10^6 dpm/50 μl hybridization buffer containing 4× standard saline solution (SSC), 50% formamide, 10% dextran sulfate, 250 μg yeast tRNA, and 1× Denhardt's. The sections were washed four times in 1× SSC for 15 min at 55°C, and then washed twice in 1× SSC for 1 hr at room temperature. The sections were rinsed in distilled water and dried. Slides were apposed to film (hyperfilm βmax, Amersham, Arlington Heights, IL), stored with desiccant at 4°C for 2–3 weeks, developed with Kodak Dektol (1:1 diluted in water), and lightly counterstained with 0.4% cresyl violet.

The specificity of hybridization was assessed by (1) hybridizing some sections with sense probes complementary to the α4 subunit mRNA of $GABA_A$ receptors probe (5′-CGG GGT CAG CCT CGC CCT CCT GCA CGT CCT GTG CCT GGC GAC TTG-3′) and to the GAD_{67} mRNA probe (5′-CGC CTT CGC CTG CAA CCT CCT CGA ACG CGG GAG CGG ATC CTA ATA CTA-3′), which led to no detectable signals; and (2) incubating some sections with ribonuclease (RNase A, 20 μg/ml in 0.5 M NaCl/10 mM Tris-HCl, pH 8, 1 mM EDTA) for 30 min at 37°C prior to hybridization, which led to background levels of scattered grains. In addition, all probes employed in the present study had been previously used, and each probe yielded distinct hybridization signal patterns in rat brain and spinal cord (Ma et al., 1993a,b; Kang et al., 1993).

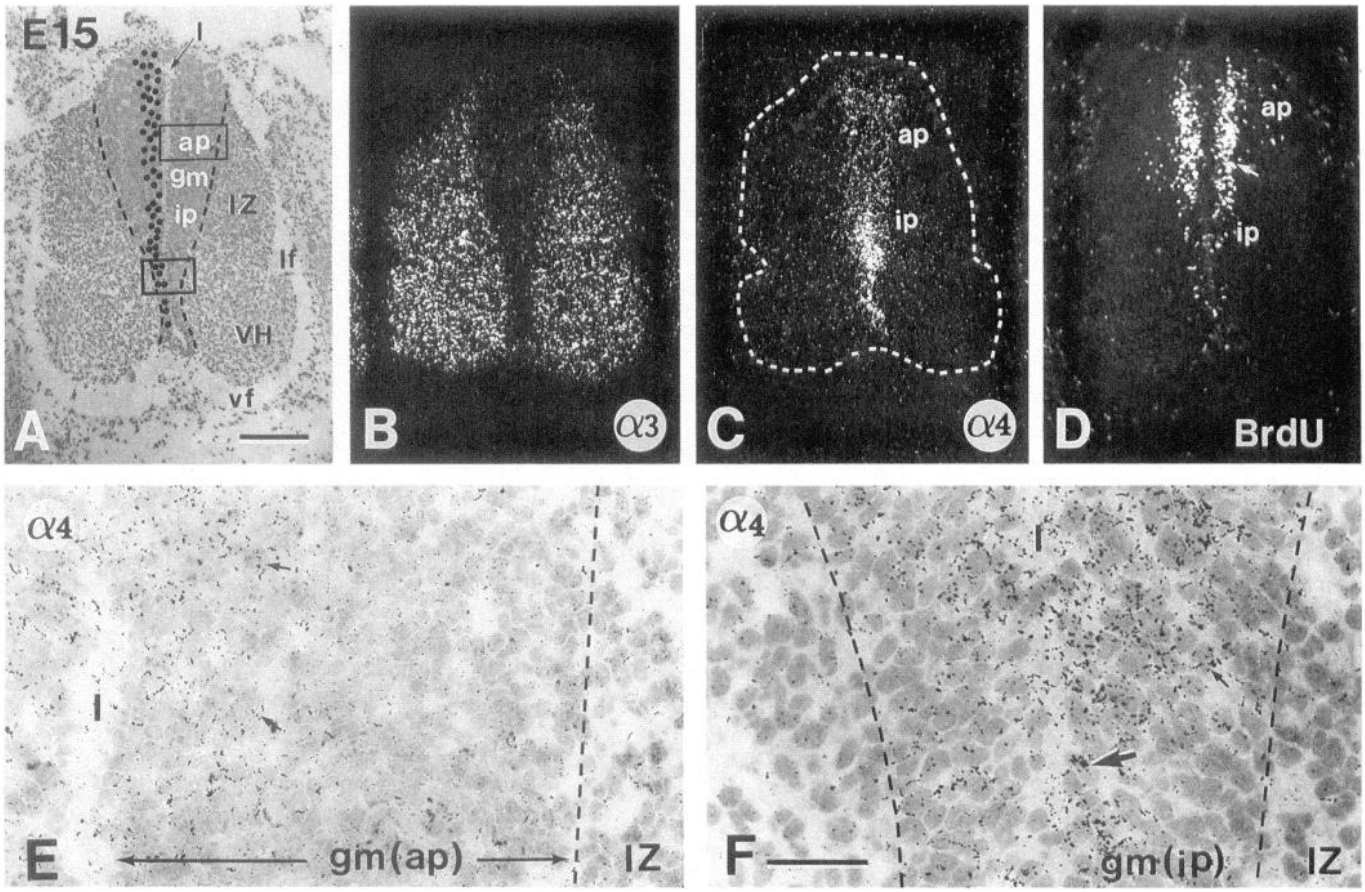

Figure 1. Complementary distributions of $GABA_A$ receptor α3 and α4 subunit mRNAs in the lumbar spinal cord of the embryonic (E) day 15 rat. *A, B,* Paired bright- (*A*) and dark-field (*B*) photomicrographs of a coronal section hybridized with an oligonucleotide probe against $GABA_A$ receptor α3 subunit mRNA. The *black dashed lines* in *A* show the outline of the germinal matrix (*gm*), and the *solid circles* indicate the distribution of α4 subunit mRNA signals. *C,* Dark-field photomicrograph of an adjacent spinal cord section hybridized with a probe for $GABA_A$ receptor α4 subunit mRNA. The hybridization signals are detected in the medial portions of the intermediate (*ip*) and alar (*ap*) plates. *White dashed line* delineates the outline of the cord. *D,* Fluorescence photomicrograph of an adjacent spinal cord section illustrating the distribution of BrdU-labeled cells (*arrow*). The partial overlap of α4 subunit mRNA and BrdU immunoreactivity is seen in the gm. *E,* Higher magnification of the alar plate corresponding to *boxed region ap* in *A* showing α4 subunit mRNA signals in the inner half of the germinal matrix (*arrows*). *F,* Higher magnification of the intermediate plate corresponding to the *lower boxed region* in *A* showing restricted distribution of dense α4 subunit mRNA signals. An *arrow* identifies a labeled cell at the ventricular surface. *IZ,* intermediate zone; *l,* lumen of the neural tube; *lf,* lateral funiculus; *vf,* ventral funiculus. Bar in *A* = 270 μm and applies to *B–D.* Scale bar: *E,* 75 μm for *E* and *F.*

In order to quantify the distribution of BrdU-labeled nuclei and hybridization signals in the embryonic spinal cord, the number of BrdU-labeled nuclei and optical density of hybridization signals in two rectangular regions (each 100 × 400 μm) across the spinal cord wall were outlined for analysis. The two regions were chosen at levels of the alar (ap) and intermediate (ip) plates of the E15 spinal cord, and each region measured was subdivided into 20 bins (I–XX) parallel to the ventricular surface and each was 20 μm long (see Fig. 8). BrdU-labeled nuclei were examined with a Leitz epifluorescence microscope and scored with respect to their bin location. The optical density was converted to digitized images that were analyzed with the program NIH IMAGE 1.49 (Wayne Rasband, NIH, Bethesda, MD). A value of the number of BrdU-labeled nuclei or optical density for each bin was expressed as a percentage over the maximum level.

Results

BrdU-immunoreactive nuclei are restricted in the germinal matrix

In order to define the approximate DNA-synthesizing zone of the germinal matrix in the embryonic spinal cord and neocortex, we pulsed embryos with BrdU for 1 hr. BrdU immunocytochemistry was carried out on coronal sections collected from the caudal neural tube, lumbar spinal cord, and cerebral wall of the middle forebrain. The germinal matrix, or proliferative zone, was readily identified in cresyl violet–counterstained sections, which contained intensely staining, densely packed cells surrounding the lumen of the neural tube. The matrix was distinguished from the intermediate zone (IZ), which consisted of variably oriented, lightly staining cells and fibers, and outlined with dashed lines in Figures 1*A* and 2*A*. At E15, the dorsal horn of the spinal cord is not yet recognizable at the lumbar level, but the generation of dorsal horn neurons reaches a peak in the alar plate (Nornes and Das, 1974; Altman and Bayer, 1984). Consistent with this, we found that a majority of the BrdU-immunoreactive elements were present in the alar plate, the dorsal region of the VZ (Fig. 1*D*). BrdU immunoreactivity was restricted to the nuclei of cells predominantly located in the inner half of the germinal matrix (bins III–V in Fig. 9) at a clearly detectable distance from the lumen of the neural tube (Figs. 1*D*, 3*C*). This region was actually located at the outer aspect of the VZ and corresponded approximately to the sz (Boulder Committee, 1970). Labeling intensity appeared relatively constant among the stained nuclei. Almost no labeled nuclei were visible

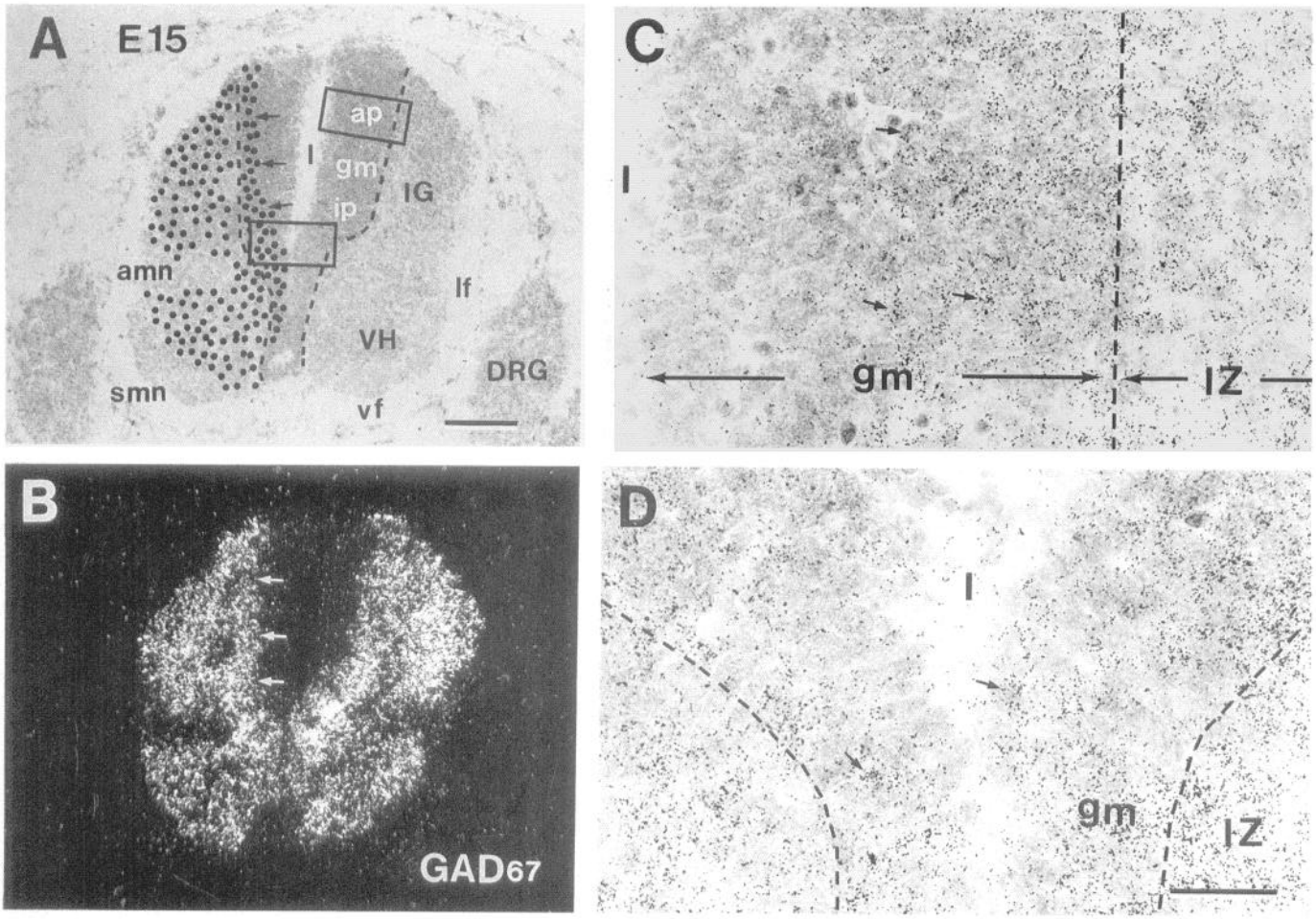

Figure 2. Localization of GAD_{67} mRNA signals in the E15 lumbar spinal cord. *A* and *B,* Bright-field (*A*) and dark-field (*B*) photomicrographs of a coronal section hybridized with the GAD_{67} mRNA probe. *Solid circles* in *A* indicate distribution of GAD_{67} transcripts in the lateral portions of the intermediate (*ip*) and alar plates (*ap*), as well as in the ventral horn (*VH*) and intermediate gray (*IG*). *Arrows* in *B* indicate the edge of GAD_{67} signals. *C,* Higher magnification of *boxed region ap* in *A* showing abundant grains (*arrows*) distributed in the outer half of the germinal matrix. *D,* Higher magnification of the *lower boxed region* in *A* showing dense grains (*arrows*) in the receding intermediate plate. *amn,* autonomic motoneurons; *IZ,* intermediate zone; *l,* lumen of the neural tube; *lf,* lateral funiculus; *smn,* somatic motoneurons; *vf,* ventral funiculus; *VZ,* ventricular zone. Scale bar: *A*, 270 for *A* and *B.*; *D*, 35 μm for *C* and *D*.

at the ventricular surface, corresponding to the mz (Fig. 3*C*). In addition, a few BrdU-labeled nuclei (arrow in Fig. 3*C*) were present in the outer half of the germinal matrix (bins VI–X in Fig. 9), which seemed to correspond to the SV in the developing neocortex (Bayer and Altman, 1991). This has not yet been described in the developing spinal cord, and we refer to this region as the *transitional zone* (TZ). In the receding intermediate plate (ip), very few labeled nuclei were detected. No labeled elements were found in the IZ (Figs. 1*D,* 3*C*).

BrdU immunoreactivity was also examined in the cerebral wall of the dorsomedial neocortex at E17 and E20. It is known that the VZ is most prominent in the E17 cerebral wall, and declines at E20, but there is a growing thickness of the SV and an increase in the SV mitotic cells from E17 to E20 (Bayer and Altman, 1991). A band of BrdU-labeled nuclei was seen exclusively in the germinal matrix of the E17 (Fig. 5*E*) and E20 (Fig. 7*C*) cortex. The band of densely packed BrdU nuclei was located in the inner half of the germinal matrix that was distant from the ventricular surface. This heavily labeled band corresponded to the sz (Bayer and Altman, 1991), and was thicker in E17 than in E20. Quite a few labeled nuclei were scattered in the outer half of the germinal matrix (arrows in Fig. 7*C*), corresponding to the SV (Bayer and Altman, 1991).

$GABA_A$ receptor α4, β1, and γ1 subunit mRNA signals predominate in the inner half of the germinal matrix

Coronal spinal and neocortical sections adjacent to the BrdU-immunostained ones were hybridized separately with α3, α4, β1, β3, γ1, and γ2 subunit mRNA probes of the $GABA_A$ receptor. The cresyl violet–counterstained sections hybridized with the probes were used to delineate the borders of the germinal matrix and the IZ, and then to carefully define the location of hybridization signals in different subregions within the germinal matrix (see the BrdU immunocytochemical data described above). In the E15 spinal cord, α3 subunit mRNA hybridization signals were widespread throughout the IZ, but absent in the germinal matrix (Fig. 1*B*). In contrast, α4 subunit mRNA signals were largely restricted to cells surrounding the lumen (Fig. 1*C*), and present in the inner half of the germinal matrix (Fig. 3*B*; bins I–V in Fig. 9). The α4 signals were not detected in the mantle zone. When compared to an adjacent section immunoreacted with anti-BrdU, the distribution of α4 transcripts par-

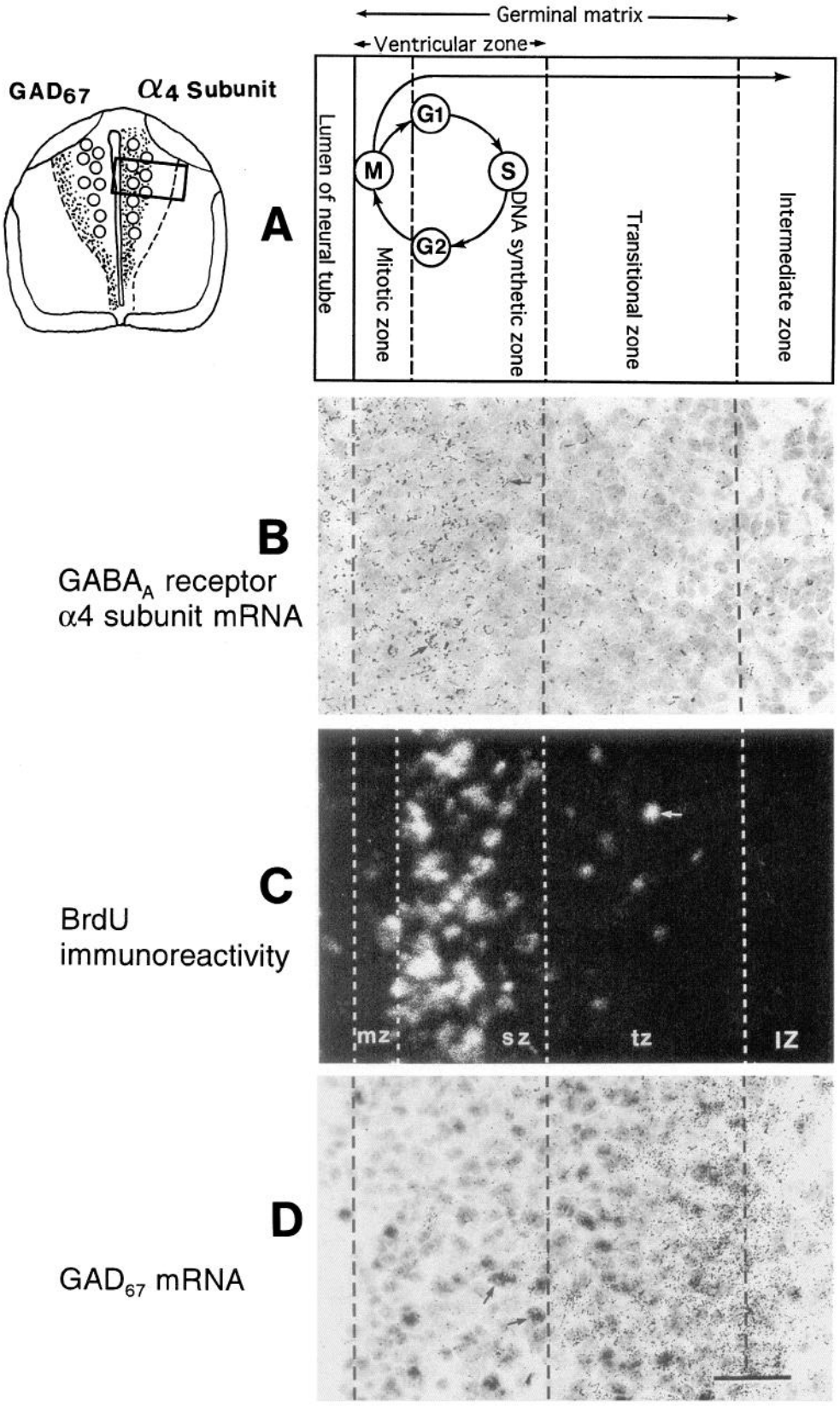

Figure 3. Complementary distributions of transcripts encoding $GABA_A$ receptor α4 subunit and GAD_{67} proteins in the germinal matrix. Schematic diagram at *upper left* shows distribution patterns of transcripts (*dots*) encoding GAD_{67} (*left*) and α4 subunit (*right*) in relation to the location of BrdU-immunoreactive cells (*open circles*). *A,* Schematic representation of the germinal matrix subdivisions as seen in adjacent sections (*B–D*) which correspond to *boxed region ap* in Figure 1*A* and *boxed region* in the schematic diagram at *upper left. Circled letters* represent phases in the cell cycle: *S,* DNA synthetic phase; *G2,* imminently premitotic phase; *M,* mitotic phase; *G1,* quiescent or preparatory state for S-phase. *B,* Bright-field photomicrograph showing that the autoradiographic signals of α4 transcripts are primarily concentrated within the inner half of the germinal matrix, including the mitotic and synthetic zones. *C,* Fluorescence photomicrograph showing that BrdU-immunoreactive cells are abundant in the region distant from the lumen, which corresponds to the synthetic zone (*sz*). A few labeled cells are also seen in the transitional zone (*arrow, tz*) and mitotic zone. *D,* Bright-field photomicrograph showing intense hybridization signals for GAD_{67} mRNA within the outer half of the germinal matrix corresponding primarily to the tz. A few labeled cells are seen in the DNA synthetic zone (*small arrows*). *Dashed lines* delineate the zones in each section corresponding to the schematic representation in *A*. Scale bar: *D*, 30 μm for *B–D*.

tially overlapped the band of BrdU-immunoreactive cells in the sz (Fig. 3*A–C*). There was also some distribution of α4 subunit mRNA signals complementary to that of BrdU-labeled cells, although both signals were present in the germinal matrix. BrdU-labeled cells were rarely seen in the receding intermediate plate (ip) in which expression of the α4 subunit mRNA reached a maximum; on the contrary, intensely BrdU-labeled nuclei were present in the alar plate (ap), in which only low-to-moderate

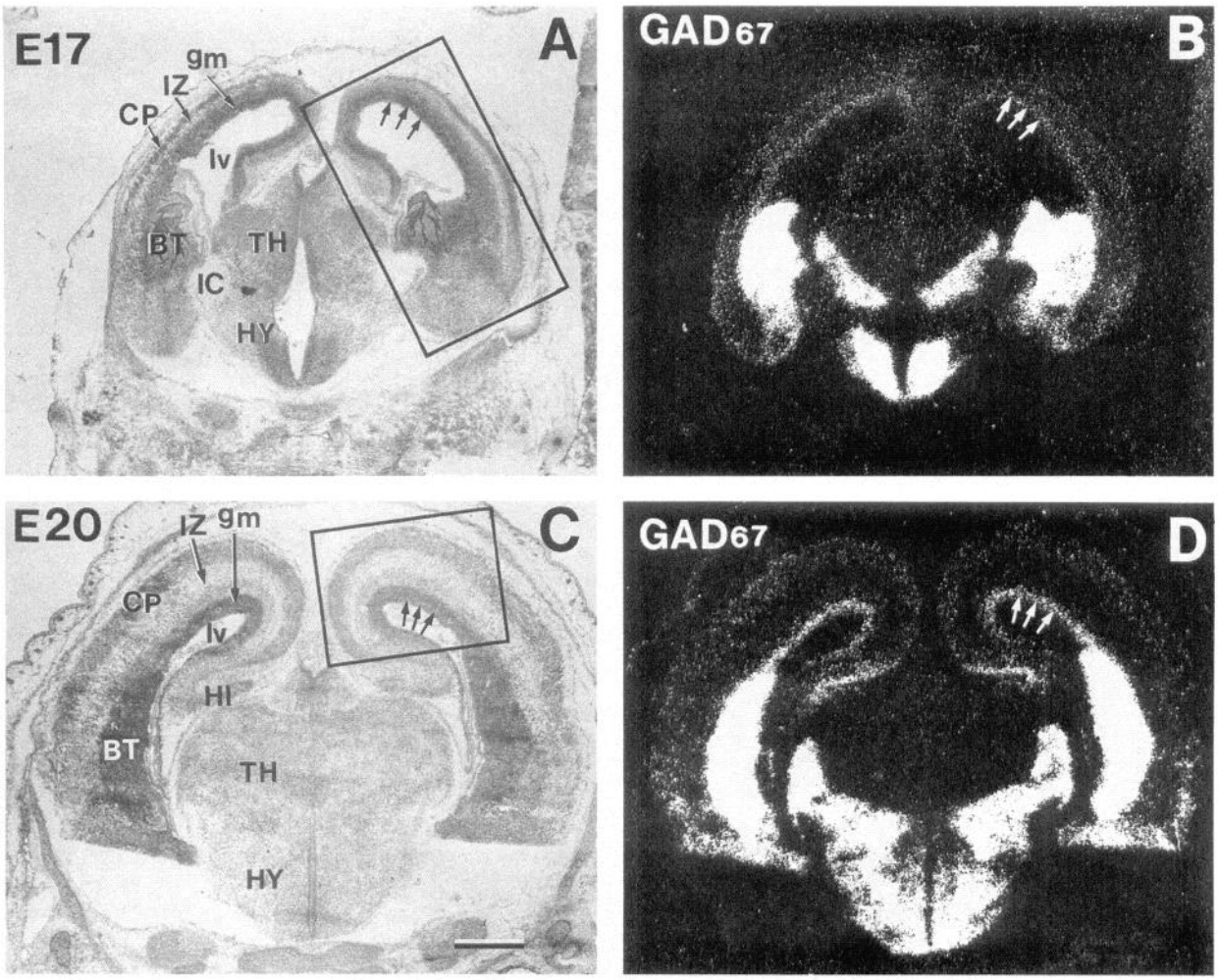

Figure 4. Distribution of mRNA for GAD_{67} in the germinal matrix (*gm*) of rat neocortex. Bright-field (*A, C*) and dark-field (*B, D*) photomicrographs of coronal sections through the middle forebrain of E17 (*A, B*) and E20 (*C, D*) rats showing low (E17, *arrows* in *B*) and moderate (E20, *arrows* in *D*) levels of GAD_{67} transcripts in the compact germinal matrix. *BT,* basal telencephalon; *CP,* cortical plate; *HI,* hippocampus; *HY,* hypothalamus; *IC,* internal capsule; *IZ,* intermediate zone; *lv,* lateral ventricle; *TH,* thalamus. Scale bar: *C*, 850 μm for *A–D*.

levels of α4 subunit mRNA were detected (Fig. 1*C,D*). Taken together, the results indicate that expression of the α4 subunit mRNA appeared to follow rather than precede the appearance of BrdU-labeled cells.

As in the spinal cord, α4 mRNA signals were mainly present in the inner half of the germinal matrix in the neocortex, corresponding to the VZ at E17 (Fig. 5*D*) and E20 (Fig. 6*B*); low levels of α4 signals were found in the outer half of the germinal matrix, corresponding to the SV as well, but the signals were not detectable in the IZ (Fig. 7*B*). In contrast, α3, β3, and γ2 subunit signals were detected only in the cortical (CP) and subcortical (SP) plates (Figs. 5*C*, 6C,*D*).

In both the spinal cord and neocortex, signals for β1 and γ1 subunit mRNAs were present in the inner half of the germinal matrix, similar to the α4 subunit mRNA (not shown). Thus, the trio of α4, β1, and γ1 transcripts emerged in parallel at spinal and cortical levels of the developing neuraxis in the germinal matrix.

GAD_{67} mRNA signals were primarily detected in the outer half of the germinal matrix

Some sections adjacent to those processed for BrdU immunocytochemistry were also hybridized with GAD_{67} mRNA probe. In the alar plate of E15 spinal cord, GAD_{67} mRNA was particularly widespread in the outer half of the germinal matrix, as well as in the IZ (Fig. 2*B–D*; bins VI–XX in Fig. 9). In the germinal matrix, hybridization signals were situated in a band at increasing distances from the lumen in the ventrodorsal axis (Fig. 2*A,B*), which overlapped the TZ (Fig. 3*D*). A few labeled cells were present in the DNA synthetic zone (arrows in Fig. 3*D*). In the intermediate plate, the hybridization signals were abundant in the entire germinal area, even in cells near the ventricular surface (Fig. 2*D*; bins I–III in Fig. 9). In the intermediate gray and ventral horn (Fig. 2*A,B*), hybridization signals covered all portions except motor areas. These unlabeled regions probably included somatic (smn) and autonomic motoneurons (amn); the latter appeared to be sympathetic preganglionic neurons in the developing intermediolateral nucleus (Markham and Vaughn, 1991).

In the neocortex, GAD_{67} mRNA signals were low in abundance at E17 (arrows in Fig. 4*B*) and became moderately well expressed at E20 (arrows in Fig. 4*D*) in the germinal matrix. Higher magnification showed that these signals were present in cells distributed throughout the outer half of the germinal matrix, corresponding to the SV (arrows in Fig. 7*D*).

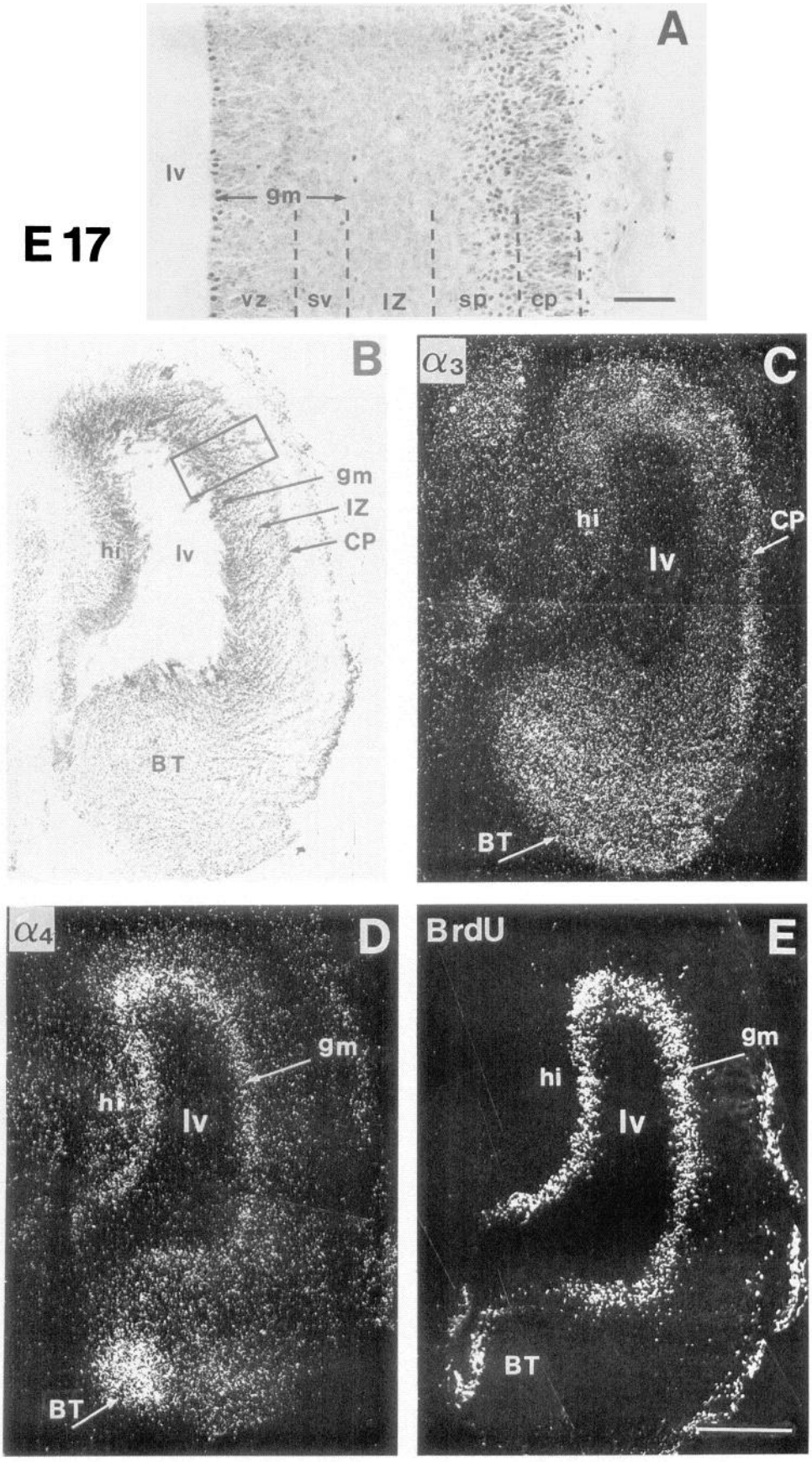

Figure 5. Distributions of mRNA signals for α3 and α4 subunits of $GABA_A$ receptors and BrdU-labeled cells in the neocortex of an E17 rat. *A,* Higher magnification view of the dorsomedial region of the cerebral wall, corresponding to the *boxed area* in *B,* showing all the layers in the E17 neocortex. *B* and *C,* Paired bright-field (*B*) and dark-field (*C*) photomicrographs of a coronal section through the neocortical region corresponding to the *boxed area* in Figure 4*A,* showing abundant α3 signals in the cortical plate (*CP, C*) of the neocortex. *D,* Dark-field micrograph of a cortical section adjacent to *B* hybridized with the α4 subunit probe showing the abundant signals in the germinal matrix (*gm, D*). *E,* Fluorescence photomicrograph of a cortical section adjacent to *D* showing a cluster of BrdU-labeled cells partially overlapping α4 signals in the germinal matrix of the neocortex. *BT,* basal telencephalon; *hi,* hippocampus; *IZ,* intermediate zone; *lv,* lateral ventricle; *sp,* subcortical plate; *sv,* subventricular zone. Scale bar: *A,* 75 μm; *E,* 700 μm for *B–E.*

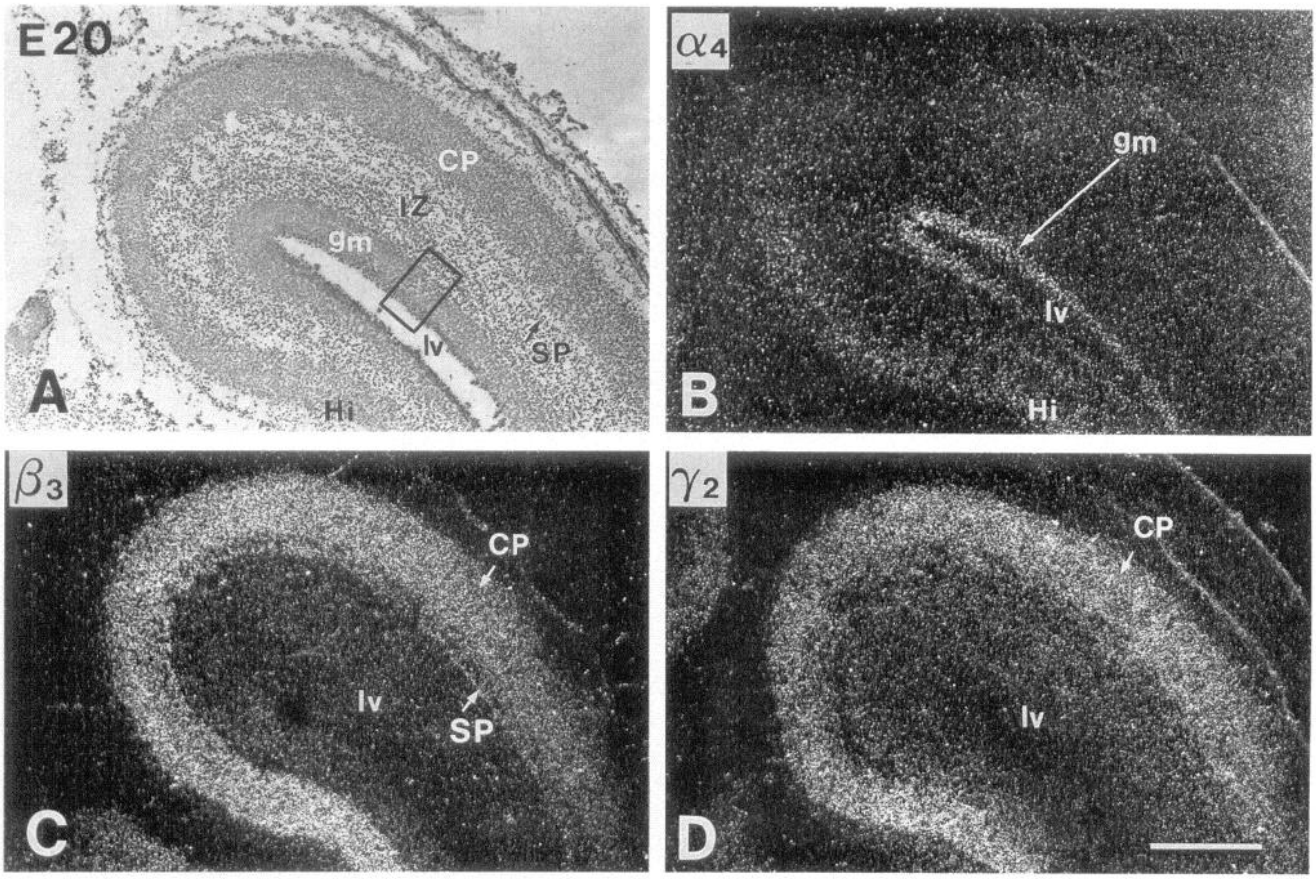

Figure 6. Distributions of mRNA signals for $GABA_A$ receptor α4, β3, and γ2 subunits in the neocortex of the E20 rat. *A* and *B*, Paired bright-field (*A*) and dark-field photomicrographs of a coronal section through the dorsomedial region of the neocortex boxed in Figure 4*C* showing the distinct localization of α4 subunit mRNA in the germinal matrix (*B*). *C* and *D*, Dark-field photomicrographs of adjacent cortical sections hybridized with the β3 or γ2 probes showing the distributions of the two mRNA signals in the cortical (*CP*) and subcortical (*SP*) plates. *Hi*, hippocampus; *IZ*, intermediate zone; *lv*, lateral ventricle. Scale bars: *A*, 350 μm; *D*, 520 μm for *A–D*.

GABA-immunoreactive cells and processes are detected in the subventricular zone and occasionally in the ventricular zone

GABA immunoreactivity was examined in coronal sections through the dorsomedial portions of the E17 neocortex. Three distinct bands of GABA-immunoreactive cells and processes were detected at different depths of the cerebral wall (Fig. 8). The first band contained closely packed GABA-positive cells in the formative layer I. The second band was present within the SP with a low intensity of immunostaining. GABA-immunoreactive cells were mainly clustered in the third band within the SV. They were variably oriented with extensive processes. In the present study, the SV was defined as the space containing mitotic cells (encircled in Fig. 8*D*), in which both GAD_{67} mRNA and GABA-immunoreactive cells were observed. In addition, a few individual positive cells (arrow in Fig. 8*C*) and processes (small arrows in Fig. 8*C*) were scattered within the VZ.

Discussion

Our *in situ* hybridization study reveals consistent and unique distribution patterns of transcripts encoding GAD_{67}, a marker of GABA-synthesizing cells, and $GABA_A$ receptor subunits in the germinal matrix and mantle zone of the embryonic spinal cord and neocortex. DNA-synthesizing cells in the germinal matrix were identified using a 1 hr pulse of BrdU and their distribution carefully and quantitatively compared to that of GAD and $GABA_A$ receptor subunit transcripts.

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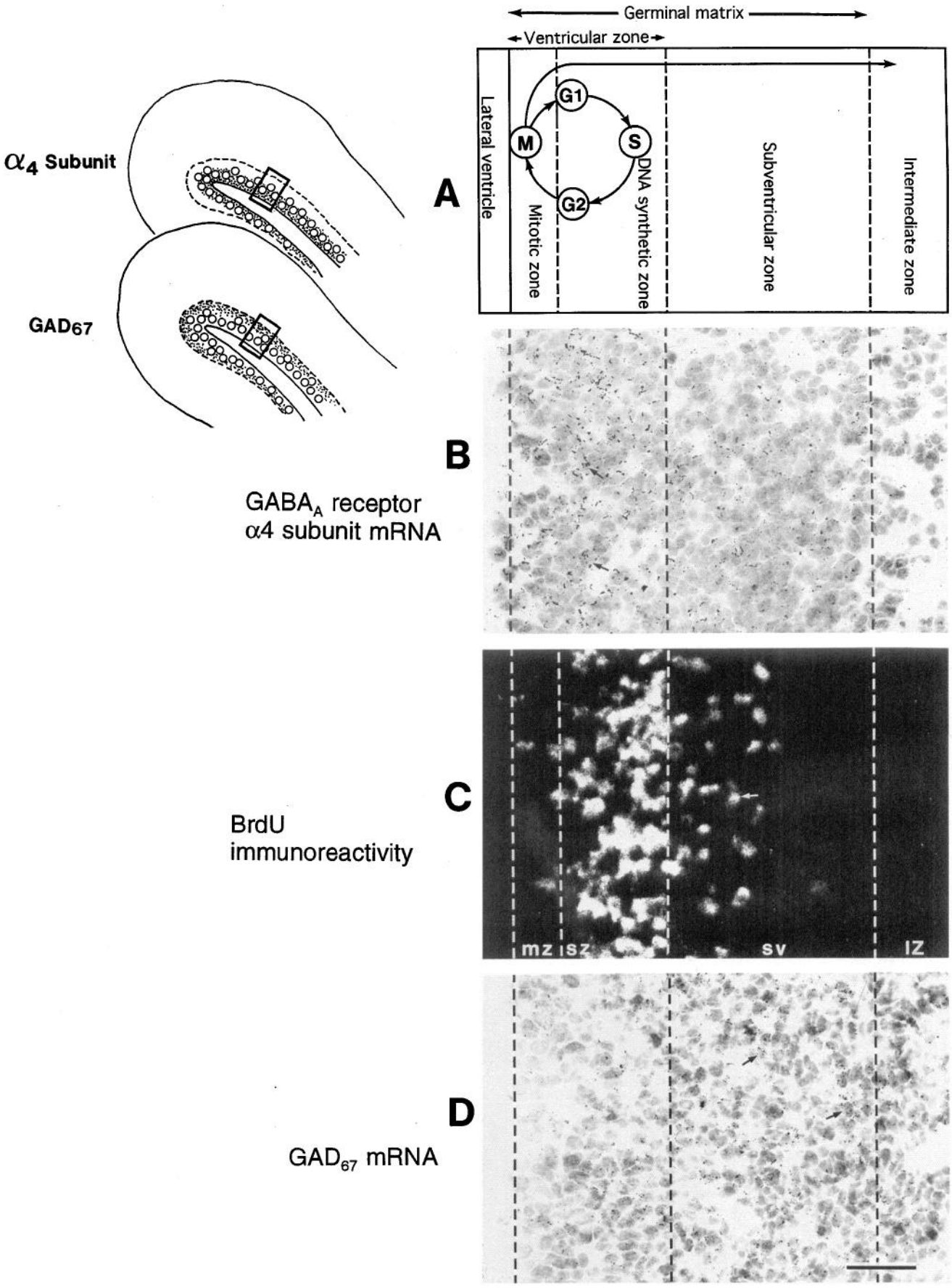
Figure 7. Complementary distributions of α4 subunit mRNA and GAD_{67} mRNA signals in the germinal matrix of the E20 neocortex. Schematic diagram at *upper left* shows distribution patterns of mRNAs (*dots*) for α4 subunit and GAD_{67} relative to the location of BrdU-labeled cells (*open circles*). *A*, Schematic representation of the germinal matrix subdivisions visualized in adjacent sections (*B–D*), which corresponds to the dorsomedial region of the neocortex boxed in Figure 6*A* and in the schematic diagram. *Circled letters* represent cell cycle phases (see Fig. 3). *B*, Bright-field photomicrograph showing that the autoradiographic grains signaling the $GABA_A$ receptor α4 subunit mRNA are concentrated within the mitotic and synthetic zones (*arrows*). *C*, Fluorescence photomicrograph showing abundant BrdU-immunoreactive cells in the region corresponding to the synthetic zone (*sz*), and some labeled cells in the subventricular zone (*arrow, sv*) and occasionally in the mitotic zone (*mz*). *D*, Bright-field photomicrograph showing a moderate number of hybridization signals for GAD_{67} mRNA in cells (*arrows*) within the outer half of the germinal matrix corresponding to the subventricular zone (*sv*). *Dashed lines* delineate the zones in each section corresponding to the schematic representation. Scale bar: *D*, 30 μm for *B–D*.


α4 Subunit
GAD67
A
Germinal matrix
Ventricular zone
Lateral ventricle
Mitotic zone
DNA synthetic zone
Subventricular zone
Intermediate zone
G1
S
G2
M
B
GABA$_A$ receptor
α4 subunit mRNA
C
BrdU
immunoreactivity
mz
sz
sv
IZ
D
GAD$_{67}$ mRNA

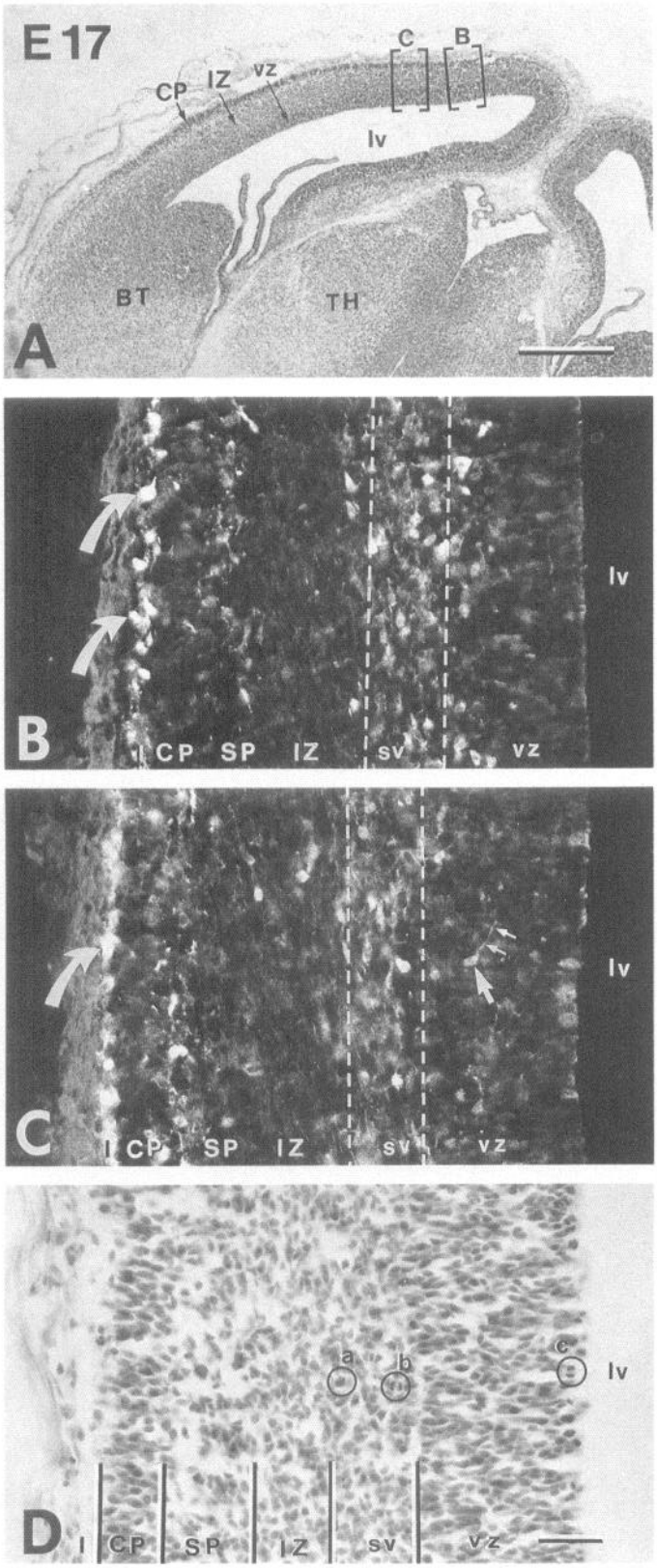


Figure 8. GABA-immunoreactive cells and processes are detected in the subventricular zone (*sv*) of E17 neocortex. *A,* Cresyl violet–counterstained section through the E17 middle forebrain pretreated for GABA immunocytochemistry. *B, C,* Fluorescence photomicrographs taken from two regions of the dorsomedial neocortex and corresponding to *boxed areas B* and *C* in *A*. A well defined band of GABA-IR cells and processes is readily detected in the sv (between *dashed lines*), and a few GABA-positive cells (*arrow* in *C*) and processes (*small arrows* in *C*) are clearly seen in the vz in both *B* and *C*. Densely packed GABA-positive cells with a high intensity of immunostaining are seen within the formative lamina I (*I, large curved arrows*). In addition, a cluster of labeled cells with relatively low staining intensity is also found in the outer part of the subcortical plate (*SP*). *D,* Cresyl violet–counterstained section of *C* showing the layers of the neocortex. Two mitotic cells in *circles a* and *B* are seen within the sv; another mitotic cell marked *c* is within the vz. *BT,* basal telencephalon; *CP,* cortical plate; *IZ,* intermediate zone; *lv,* lateral ventricle; *TH,* thalamus. Scale bar: *A,* 350 μm; *D,* 50 μm for *B–D*.

GAD mRNA appears in migrating postmitotic cells

^{3}H-thymidine autoradiographic studies have shown that there is a rough ventral-to-dorsal gradient in generation of rat spinal cord neurons (Nornes and Das, 1974). On E15, motoneuron generation in the ventral horn and relay neurons in the intermediate gray ends, while dorsal horn neurogenesis peaks (Altman and Bayer, 1984). In the dorsomedial section of the neocortex, the volume of the neuroepithelium peaks at E17. At E20, the thickness of the neuroepithelium decreases, but there is an increase in the depth of the SV and in the proportion of SV mitotic cells (Bayer and Altman, 1991). We focused on these regions actively generating neurons in the spinal cord and cortex to compare the distributions of GAD and $GABA_A$ receptor subunit transcripts and place them in the context of neurogeneration. Our BrdU immunocytochemistry shows that most labeled cells were heavily stained, the fluorescence intensity being nearly equal among these cells. This suggests that a 1 hr pulse of BrdU effectively labels only a single generation of cells (Sidman et al., 1959; Atlas and Bond, 1965), since lightly stained cells are thought to represent the dilution of BrdU in successive generations of germinal cells. The bands of densely packed, BrdU-immunoreactive cells in the E15 spinal cord and in the E17 and E20 neocortex were located at some distance from the lumen of the neural tube, presumably marking the DNA synthetic zone.

It has been suggested that the embryonic CNS consists of four distinct fundamental zones: ventricular, subventricular, intermediate, and marginal (Boulder Committee, 1970). Cells proliferate in both the VZ and SV. However, the SV in the developing spinal cord has received less attention than that in the cerebral cortex. In the embryonic spinal cord, the entire intensely staining region surrounding the lumen has been simply defined as the VZ in the literature. The present study has shown that at E15 there is a region in the alar plate of the lumbar cord located lateral to the zone of cells densely labeled with anti-BrdU, which contains a few heavily labeled, DNA-synthesizing cells, suggesting that the sz is not the only germinal source of the dorsal horn. This region spreads dorsoventrally in the outer half of the germinal matrix. Our Nissl-counterstained sections have shown that cells in the outer half of the alar plate, compared to the inner half, were less packed and variably oriented; this spinal region probably corresponds to the SV in the embryonic neocortex (Bayer and Altman, 1991). This region has also been demonstrated in rats labeled with a single dose of ^{3}H-thymidine on day E15 and killed 2 hr later (see Fig. 112*A* in Altman and Bayer, 1984). Here, most labeled proliferating cells are located in the medial portions of the alar plate, while only a few labeled cells appear in the outer half of the alar plate. The outer half contains many cells labeled in rats injected with ^{3}H-thymidine on day E15 and killed on day E16, indicating that numerous cells in the outer half migrated toward the formative dorsal horn

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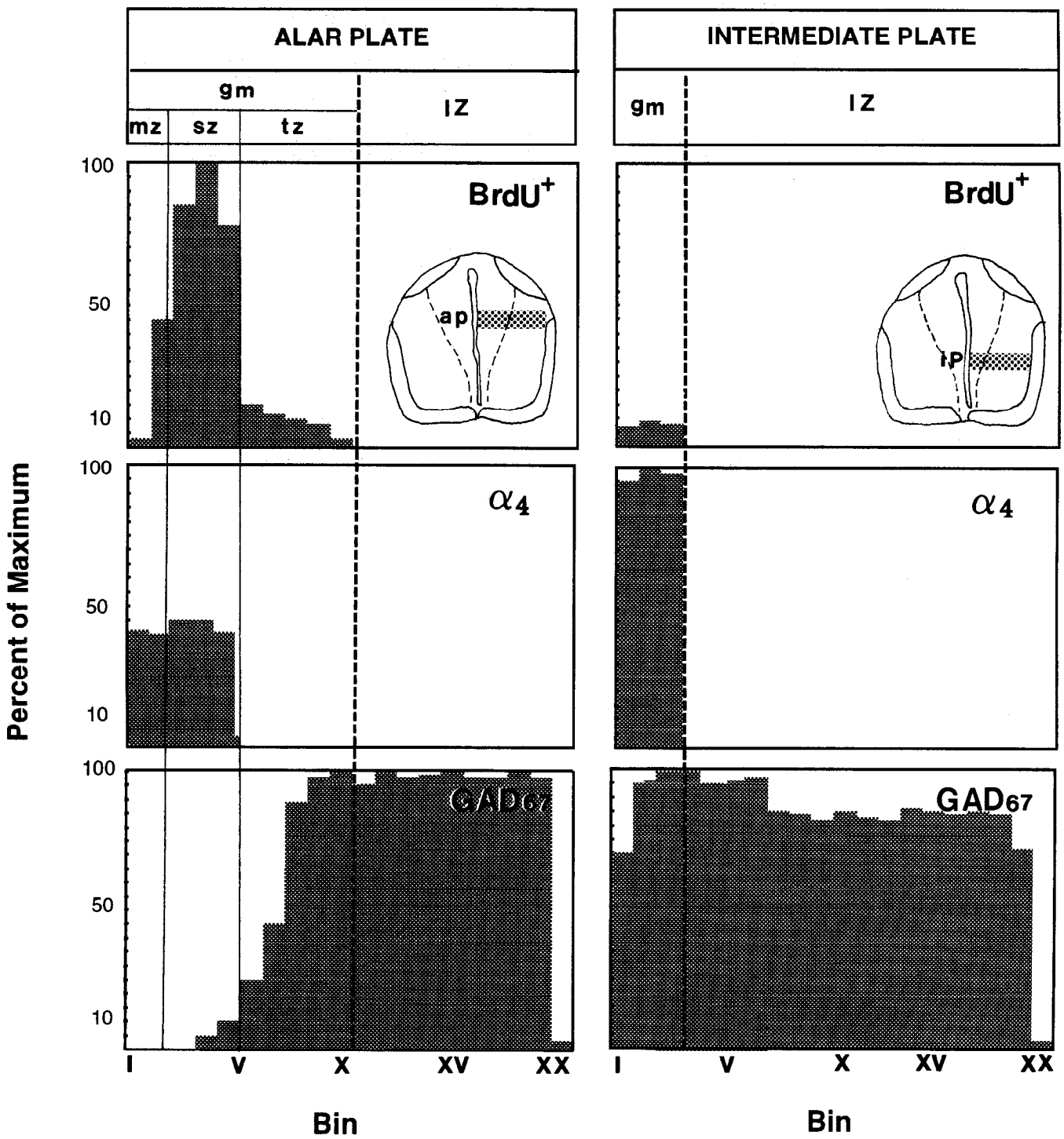

Figure 9. Complementary distributions of mRNAs for α4 subunit and GAD_{67}, and spatial segregation of α4 subunit mRNA and BrdU-labeled nuclei in the germinal matrix (*gm*). Graphic representation of the regional distribution of BrdU-immunoreactive nuclei and transcripts for $GABA_A$ receptor α4 subunit and GAD_{67} across the spinal cord wall of an E15 rat, whose mother had received a single injection of BrdU and survived for 1 hr. The analyses were conducted in two rectangular regions at levels of the alar (*ap*) and intermediate (*ip*) plates (*shaded areas* in the schematic diagrams of E15 spinal section). Each region is 100 μm by 400 μm in the coronal plane, beginning at the ventricular surface, and consists of a germinal zone (*mz, sz, tz*) and an intermediate zone (*IZ*). The region measured is subdivided into 20 bins each 20 μm long (I–XX) parallel to the ventricular surface. A value of the number of BrdU-labeled nuclei or optical density of hybridization signals for each bin is expressed as a percentage of the maximum level. In the ap, the germinal matrix extends over 200 μm (10 bins), but only 60 μm (three bins) in the receding intermediate plate. In the ap, BrdU-labeled nuclei are concentrated in bins III–V, corresponding to the synthetic zone (*sz*); the BrdU-poor area is located in bins VI–X, corresponding to the transitional zone (*tz*). The distribution of GAD_{67} mRNA (bins VI–XX) is complementary to that of α4 subunit mRNA (bins I–V). There are distinct differences in distributions of BrdU-labeled cells and α4 subunit mRNA between the alar and intermediate plates; α4 subunit mRNA in the ip (bins I–III) is much more abundant than in the ap (bins I–V), while BrdU-labeled nuclei are predominant in the ap.

(Altman and Bayer, 1984). Since this region lies at the junction of the VZ and IZ and contains many migrating cells, we have used the term *transitional zone* (TZ) to refer to this part of the germinal matrix in the developing spinal cord. It is noteworthy that GAD_{67} mRNA signals were barely detected in the VZ, but became abundant in the TZ (cord) or SV (cortex) and continued to be present throughout the IZ. After birth, the signals were widespread in all layers of the spinal cord except the motoneuron pools (Ma et al., 1994). From this we conclude that cells in the proliferative zone begin to express *in situ*-detectable levels of GAD_{67} mRNA as they are migrating out of the germinal matrix.

The above conclusion is supported by an immunocytochemical study in which the differentiation of the GABA phenotype in dissociated cell cultures of rat cerebral cortex was examined with a 1 hr pulse of BrdU (Gotz and Bolz, 1994). Cells dissociated during the S-phase failed to express GABA. However, cells dissociated and cultured 24 hr after the S-phase began to express GABA, indicating that GABA synthesis in cell culture becomes functional within 24 hr of terminal DNA synthesis. Our previous immunostaining of embryonic spinal cord with the K2 antibody, which primarily recognizes GAD proteins encoded by the GAD_{67} gene (Kaufman et al., 1986; Erlander et al., 1991), showed that some cells in the VZ, including a small number of mitotic cells, were immunoreactive (Ma et al., 1992b). In the present study, only a few cells in the DNA synthetic zone were labeled with the probe for GAD_{67} mRNA (Fig. 3). This discrepancy may be related to the polyclonal nature of the K2 antibody, which recognizes full-length GAD protein as well as truncated GAD proteins encoded by alternatively spliced variants of GAD_{67}. It is known that during embryonic development, GAD_{67} transcripts are spliced to include an exon that contains an embryonic stop codon (Bond et al., 1990). The alternatively spliced transcript may result in a truncated protein. The probe for GAD_{67} mRNA used in the present study may not hybridize the spliced sequence, but K2 may detect the truncated protein which may synthesize low levels of GABA (Szabo et al., 1994), below detection by immunocytochemistry.

$GABA_A$ receptor α4, β1, and γ1 subunit mRNAs transiently appear in premigratory neuroblasts

We have also demonstrated that, in clear contrast to the GAD_{67} mRNA distribution pattern in the outer aspect of the germinal matrix, $GABA_A$ receptor α4, β1, and γ1 subunit mRNAs were abundant in the inner half of the germinal matrix, corresponding to the VZ, including the sz and mz. They were barely detected in the TZ or SV and not detectable in the IZ. This implies that proliferating cells might express the trio of subunit mRNAs. However, it cannot be determined precisely from the present study whether some DNA-synthesizing cells also express the transcripts; in order to answer this question it will be necessary to use a double-labeling technique combining *in situ* hybridization with BrdU immunocytochemistry in the same section. It is worth mentioning that in the lumbar region of the E15 spinal cord there are complementary distributions of transcripts for trio subunits (α4, β1, and γ1) of $GABA_A$ receptor and BrdU-labeled nuclei in the VZ along the ventrodorsal axis. The subunit transcripts were more abundant in the receding VZ (ventral part) than in the neurogenesis-active VZ (dorsal part), indicating that cells in the VZ may begin to express the mRNAs immediately after their last cell division.

It is interesting to note that α4, β1, and γ1 subunit mRNAs begin appearing in the mantle zone of many brain regions with distinct distributions during the late embryonic/early postnatal period (Laurie et al., 1992; Poulter et al., 1992, 1993). The transcript for α4 subunit is abundant in the thalamus, hippocampus, neocortex, and basal nuclei; β1 mRNA is found in the hippocampus and olfactory bulb, and γ1 mRNA is concentrated in the limbic system, such as the septum, hippocampus, amygdala, and hypothalamus. This late wave of subunit expression in the mantle zone may be involved in GABA's role as a chemical mediator of intercellular communication.

GABA may be involved in neuroblast migration

The functional significance of $GABA_A$ receptor subunit and GAD_{67} mRNA expression in cells of the germinal matrix has not yet been determined. The present study has shown that mRNAs encoding GAD and $GABA_A$ receptor subunits may be expressed by neuroblasts as they are migrating; α4, β1, and γ1 subunit mRNAs whose products are thought to comprise Cl^- ion-selective channels seem to precede GAD_{67} mRNA. GABA immunostaining of E17 neocortex has shown a cluster of positive cells in the SV, and occasionally in the VZ, indicating that GAD_{67} mRNA–containing cells detected in the proliferative zone may synthesize GABA. GABA present in cells and processes within the SV and mantle zone may be released and influence neuroblast motility and migration. In fact, using an *in vitro* microchemotaxis assay, it has been shown that subpopulations of cells acutely dissociated from the embryonic spinal cord (Behar et al., 1994b) and cortex (Behar et al., 1994c) migrate in response to GABA. These chemotropic effects were mimicked by muscimol and baclofen and inhibited to a variable degree by bicuculline, picrotoxin, and saclofen, suggesting that the effects on motility involve GABA receptors whose pharmacology overlaps with both $GABA_A$ and $GABA_B$ receptor types. The majority (>95%) of migrating cells were immunoreacted with anti-neurofilament antibodies and were considered postmitotic neuroblasts. How $GABA_A$ receptor subunits that are putatively colocalized in premigratory neuroblasts are involved, if at all, in the chemotropic effects of GABA is a challenge for future study.

The trio of α4, β1, and γ1 transcripts could encode proteins that assemble into Cl^- channels. The $GABA_A$ receptor/Cl^- ion channel complex is thought to be a hetero-oligomer composed of five subunits (for review see Olsen and Tobin, 1990). There is evidence from recombinant studies that single subunits, for example, the β1 subunit itself, can form functional Cl^- channels (Sigel et al., 1989). In codfish brain, a functional receptor is formed *in vivo* from five identical subunits (Deng et al., 1991); and two mRNAs for α1 and β1 subunits injected together into oocytes created receptors that mimicked many properties of native $GABA_A$ receptor/Cl^- channels (Schofield et al., 1987). Although we do not know if the trio of transcripts results in proteins that assemble into Cl^- channels, patch-clamp recordings of cells in the VZ of rat neocortex reveal functional $GABA_A$-type receptors coupled to Cl^- conductance mechanisms (Lo Turco and Kriegstein, 1992). Furthermore, neuroepithelial cells acutely cultured from the embryonic spinal cord at E13 depolarize in response to muscimol (Walton et al., 1993) and exhibit Cl^- channels gated by GABA (Serafini et al., 1993). Digital videomicroscopic and flow cytometric recordings demonstrate that over 80% of the spinal cord and cortical neuroblasts depolarize to GABA and muscimol during the last week of embryogenesis (Mandler et al., 1990; Maric et al., 1993; Walton et al., 1993).

These results indicate that GABA receptor–coupled Cl^- channels are widespread and functional during embryogenesis of the spinal cord and cortex, where they serve to depolarize cells according to the existing Cl^- ion gradient. Perhaps the trio of α4, β1, and γ1 transcripts encodes proteins that form some of the functional Cl^- channels and these mediate some of the motility signals.

Finally, it is well established that the $GABA_A$ receptor/Cl^- ion channel is the target for a wide variety of clinically important and psychoactive compounds including ethanol (for review see Olsen and Tobin, 1990). Proteins encoded by $GABA_A$ receptor subunit α4, β1, and γ1 mRNAs in the mitotic and synthetic zones may be targets for these drugs and perhaps other teratogens. Prenatal exposure of rats to teratogens such as ethanol causes profound disruptions in neuronal generation and migration within the cortical germinal matrix (Miller, 1988, 1989). Such alterations can lead to microcephaly, craniofacial malformations, behavioral abnormalities, and cognitive or learning deficiencies; all are characteristic of the fetal alcohol syndrome in children (Sulik et al., 1981). Although the molecular basis and cellular mechanisms of the fetal alcohol syndrome have not been elucidated, ^{3}H-thymidine autoradiographic studies indicate that ethanol inhibits cell proliferation and migration in the VZ, whereas it stimulates proliferation of subventricular cells (Miller, 1989). The products of α4, β1, and γ1 transcripts in the proliferative zone may mediate some of the teratogenic effects of ethanol.

In conclusion, the components for a GABAergic circuit that functions during the embryonic period of spinal cord and cortical development are present. The complementary anatomical distributions of the different components taken together with results on neuroblast motility *in vitro* suggest that one function this putative circuit could serve involves migration out of the neuroepithelium and into the zone of cellular differentiation. The physiological signals mediated by GABA during the embryonic period of morphogenesis remain to be resolved.

References

Altman J, Bayer SA (1984) The development of the rat spinal cord. Berlin: Springer.

Atlas M, Bond VP (1965) The cell generation cycle of the eleven-day mouse embryo. J Cell Biol 26:19–24.

Bayer SA, Altman J (1991) Neocortex development. New York: Raven.

Behar T, Ma W, Hudson L, Barker JL (1994a) Analysis of the anatomical distribution of GAD_{67} mRNA encoding truncated glutamic acid decarboxylase proteins in the embryonic brain. Dev Brain Res 77: 77–87.

Behar T, Schaffner AE, Colton CA, Somogyi R, Olah Z, Lehel C, Barker JL (1994b) GABA-induced chemokinesis and NGF-induced chemotaxis of embryonic spinal cord neurons. J Neurosci 14:29–38.

Behar T, Tran HT, Barker JL (1994c) GABA and NGF induce chemotaxis in cortical neuroblasts. Soc Neurosci Abstr 20:614.3.

Bond RW, Wyborski RJ, Gottlied DI (1990) Developmentally regulated expression of an exon containing a stop codon in the gene for glutamic acid decarboxylase. Proc Natl Acad Sci USA 87:8771–8775.

Boulder Committee (1970) Embryonic vertebrate central nervous system: revised terminology. Anat Rec 166:257–262.

Cobas A, Fairen A, Alvarez-Bolado G, Sanchez MP (1991) Prenatal development of the intrinsic neurons of the rat neocortex: a comparative study of the distribution of GABA-immunoreactive cells and $GABA_A$ receptor. Neuroscience 40:375–397.

Deng L, Nielsen M, Olsen RW (1991) Pharmacological and biochemical properties of the GABA-benzodiazepine receptor protein from codfish brain. J Neurochem 56:968–977.

Erlander MG, Tillakaratne NJK, Feidblum S, Patel N, Tobin AJ (1991) Two genes encode distinct glutamate decarboxylase. Neuron 7:91–100.

Gotz M, Bolz J (1994) Differentiation of transmitter phenotypes in the rat cerebral cortex. Eur J Neurosci 6:18–32.

Hansen GH, Maier E, Abraham J, Schousboe A (1987) Trophic effects of GABA on cerebellar granule cells in culture. In: Neurotrophic activity of GABA during development (Redburn DA, Schousboe A, eds), pp 109–138. New York: Liss.

Kang KM, Ma W, Barker JL (1993) Developmental expression of GAD_{65} and GAD_{67} mRNAs in the reticular nucleus of the rat thalamus. Soc Neurosci Abstr 19:274.15.

Kaufman DL, McGinnis JF, Krieger NR, Tobin AT (1986) Brain glutamate decarboxylase cloned in lambda gt-11: fusion protein produces GABA. Science 232:1138–1140.

Lauder JM (1988) Neurotransmitters as morphogens. Prog Brain Res 73:365–387.

Lauder JM (1993) Neurotransmitters as growth regulatory signals: role of receptors and second messengers. Trends Neurosci 6:233–239.

Lauder JM, Han VKM, Henderson P, Verdoorn T, Towle AC (1986) Prenatal ontogeny of the GABAergic system in the rat brain: an immunocytochemical study. Neuroscience 2:465–493.

Laurie DJ, Wisden W, Seeburg PH (1992) The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci 12:4151–4172.

Lolait SJ, O'Carrol AM, Kusano K, Mahan LC (1989) Pharmacological characterization and region-specific expression in brain of the β2 and β3 subunits of the rat $GABA_A$ receptor. FEBS Lett 258:17–21.

Lo Turco JJ, Kriegstein AR (1992) Amino acid neurotransmitters regulate the proliferation of neocortical progenitors. Soc Neurosci Abstr 18:330.15.

Ma W, Behar T, Shaffner A, Smith S, Barker JL (1991) Parallel expression of $GABA_A$ receptor and GABA in embryonic rat subcortical brain. Soc Neurosci Abstr 17:300.2.

Ma W, Behar T, Barker JL (1992a) Transient expression of GABA immunoreactivity in the developing rat spinal cord. J Comp Neurol 325:271–290.

Ma W, Behar T, Maric D, Maric I, Barker JL (1992b) Neuroepithelial cells in the rat spinal cord express glutamate decarboxylase immunoreactivity *in vivo* and *in vitro*. J Comp Neurol 325:257–270.

Ma W, Liu QY, Maric D, Maric I, Shaffner A, Smith S, Barker JL (1993a) Developmental changes in $GABA_A$ receptor subunit mRNAs correlate with transformation of $GABA_A$ receptor properties in rat dorsal thalamus. Soc Neurosci Abstr 19:274.16.

Ma W, Saunders PA, Somogyi R, Poulter MO, Barker JL (1993b) Ontogeny of $GABA_A$ receptor subunit mRNAs in rat spinal cord and dorsal root ganglia. J Comp Neurol 338:337–359.

Ma W, Behar T, Chang L, Barker JL (1994) Transient increase in expression of GAD_{65} and GAD_{67} mRNAs during postnatal development of rat spinal cord. J Comp Neurol 346:151–160.

Malherbe PE, Sigel E, Baur R, Richards JG, Mohler H (1990) Functional expression and sites of transcription of a novel α-subunit $GABA_A$ receptor in rat brain. FEBS Lett 260:261–265.

Mandler RN, Schaffner AE, Novotny EA, Lange DG, Smith SV, Barker JL (1990) Electrical and chemical excitability appear one week before birth in the embryonic rat spinal cord. Brain Res 522:46–54.

Maric D, Maric I, Smith SV, Barker JL (1993) Attomolar GABA and GABA mimetics induce transient Ca^{2+} release from ryanodine-sensitive stores in embryonic rat cerebral cortical cells. Soc Neurosci Abstr 19:274.13.

Markham JA, Vaughn JE (1991) Migration patterns of sympathetic preganglionic neurons in embryonic rat spinal cord. J Neurosci 8:811–822.

Meier E, Hansen GH, Schousboe A (1985) The trophic effect of GABA on cerebellar granule cells is mediated by GABA receptors. Int J Dev Neurosci 3:401–407.

Meier E, Hertz L, Schousboe A (1991) Neurotransmitters as developmental signals. Neurochem Int 19:1–15.

Miller MW (1988) Effect of prenatal exposure to ethanol on the development of cerebral cortex: I. Neuronal generation. Alcohol Clin Exp Res 12:440–449.

Miller MW (1989) Effects of prenatal exposure to ethanol on the neocortical development: II. Cell proliferation in the ventricular and subventricular zones of the rat. J Comp Neurol 287:326–338.

Miller MW, Nowakowski RS (1988) Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res 457:44–52.

Nornes HO, Das GD (1974) Temporal pattern of neurogenesis in spinal cord of rat. I. An autoradiographic study—time and sites of origin and migration and settling patterns of neuroblasts. Brain Res 73:121–138.

Olsen RW, Tobin AJ (1990) Molecular biology of $GABA_A$ receptors. FASEB J 4:1496–1480.

Poulter MO, Barker JL, O'Carroll A-M, Lolait SJ, Mahan LC (1992) Differential and transient expression of $GABA_A$ receptor α-subunit mRNAs in the developing rat CNS. J Neurosci 12:2888–2900.

Poulter MO, Barker JL, O'Carroll A-M, Mahan LC, Lolait SJ (1993) Coexistent expression of $GABA_A$ receptor β2, β3, and γ2 subunit mRNAs during embryogenesis and early postnatal development of the rat CNS. Neuroscience 53:1019–1033.

Redburn DA, Schousboe A (eds) (1987) Neurotrophic activity of GABA during development. New York: Liss.

Sauer FC (1935a) Mitosis in the neural tube. J Comp Neurol 62:377–405.

Sauer FC (1935b) The cellular structure of the neural tube. J Comp Neurol 63:13–23.

Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA, Seeburg PH, Barnard EA (1987) Sequence and functional expression of the $GABA_A$ receptor superfamily. Nature 328:221–227.

Serafini R, Ma W, Tang MK, Barker JL (1993) GABA receptor–coupled Cl^- channels exhibit heterogeneous electrical properties in early embryonic neuroepithelial cells. Soc Neurosci Abstr 19:472.1.

Shivers BD, Killisch I, Sprengel R, Sontheimer H, Kohler M, Schofield PR, Seeburg PH (1989) Two novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations. Neurons 3:327–337.

Sidman RL, Miale IL, Feder N (1959) Cell proliferation and migration in the primitive ependymal zone: an autoradiographic study of histogenesis in the nervous system. Exp Neurol 1:322–333.

Sigel E, Baur R, Malherbe P, Mohler H (1989) The rat β1-subunit of the $GABA_A$ receptor forms a picrotoxin-sensitive anion channel open in the absence of GABA. FEBS Lett 257:377–379.

Sulik KK, Johnston MC, Webb MA (1981) Fetal alcohol syndrome: embryogenesis in a mouse model. Science 214:936–938.

Szabo G, Katarova Z, Greenspan R (1994) Distinct protein forms are produced from alternatively spliced bicistronic GAD mRNAs during development. Mol Cell Biol 14:7535–7545.

Takahashi T, Caviness VS (1993) PCNA-binding to DNA at the G1/S transition in proliferating cells of the developing cerebral wall. J Neurocytol 22:1096–1102.

Van Eden CG, Mrzljak L, Voorn P, Uylings HBM (1989) Prenatal development of GABA-ergic neurons in the neocortex of the rat. J Comp Neurol 289:213–227.

Walton MK, Schaffner AE, Barker JL (1993) Sodium channels, $GABA_A$ receptors, and glutamate receptors develop sequentially on embryonic rat spinal cord cells. J Neurosci 13:2068–2084.

Wisden W, Herb A, Wieland H, Keinanen K, Luddens H, Seeburg PH (1991) Cloning, pharmacological characteristics and expression pattern of the rat $GABA_A$ receptor α4 subunit. FEBS Lett 289:277–230.

Ymer S, Draguhn A, Werner P, Kohler M, Schofield PR, Seeburg PH (1989a) Sequence and expression of a novel $GABA_A$ receptor α subunit. FEBS Lett 258:119–122.

Ymer S, Schofield PR, Draguhn A, Werner P, Kohler M, Seeburg PH (1989b) $GABA_A$ receptor β subunit heterogeneity: functional expression of cloned cDNAs. EMBO J 8:1665–1670.

Ymer S, Draguhn A, Wisden W, Werner P, Keinanen K, Schofield PR, Sprengel R, Pritchett DB, Seeburg PH (1990) Structural and functional characterization of the γ1 subunit of $GABA_A$/benzodiazepine receptors. EMBO J 9:3261–3267.