

Preferential Origin and Layer Destination of GAD65-GFP Cortical Interneurons

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To identify the origin and track the migratory pathway of specific subpopulations of GABAergic interneurons, we studied tangential migration in a recently developed GAD65-GFP transgenic mouse strain. First, we used immunohistochemical methods to characterize the expression of specific neurochemical markers in the GAD65-GFP neurons. Then, organotypic cultures were used in combination with birth-dating studies to determine the time of generation, place of origin and migratory route of these cells. From E14 to E15, the highest density of GAD65-GFP cells was seen in the lower intermediate zone; however, at later stages more GAD65-GFP cells were observed in the subventricular zone. Migratory GAD65-GFP cells express GAD65, but not calretinin or reelin. Surprisingly, only 4% were calbindin immunopositive. At P21, GAD65-GFP cells were found predominantly in layers II–III and expressed calretinin and neuropeptide Y. Remarkably, almost all cholecystokinin-positive but very few parvalbumin-positive neurons expressed GFP. *In vitro* studies demonstrated that the caudal ganglionic eminence gives rise to a large proportion of GAD65-GFP interneurons and *in vivo* birth-dating experiments showed that GAD65-GFP interneurons in supragranular layers are born at late embryonic development. Taken together these results support the idea that the destination layer of GABAergic interneurons is closely linked to their place of origin and time of generation.

Keywords: cortex, development, GABA, immunohistochemistry, ganglionic eminence, neuronal migration

Introduction

GABAergic interneurons, which represent ~20% of the total number of neurons in the cerebral cortex, play important roles in the control of neuronal activity in the brain (for review see McBain and Fisahn, 2001). They are subclassified according to their morphological and electrophysiological characteristics, their synaptic organization (Kawaguchi and Kubota, 1997; Gupta *et al.*, 2000; for review see Somogyi *et al.*, 1998) or by the expression of different calcium-binding proteins, including calbindin, parvalbumin and calretinin, neuropeptides and neuromodulators such as somatostatin or neuropeptide Y (Fairen *et al.*, 1984; DeFelipe, 1993; Kubota *et al.*, 1994; Gonchar and Burkhalter, 1997; Benes and Berretta, 2001). The synthesis of GABA from glutamate in the brain is dependent upon the enzyme glutamate decarboxylase (GAD). Two distinct isoforms of GAD have been described, a 67 kDa form (GAD67) and a 65 kDa form (GAD65), both of which are capable of synthesizing GABA. While it is generally believed that most GABAergic neurons express both forms of GAD (Esclapez *et al.*, 1994; Fukuda *et al.*, 1997), this hypothesis has never been systematically tested during early development.

Extensive studies have demonstrated that most of the cortical GABAergic interneurons in rodents originate in

subpallial areas of the telencephalon and reach their destination in the cerebral cortex via distinct tangential migratory pathways (O'Rourke *et al.*, 1995, 1997; Anderson *et al.*, 1997, 2001; Tamamaki *et al.*, 1997; Lavdas *et al.*, 1999; for reviews see Parnavelas, 2000; Marín and Rubenstein, 2001, 2003). A recent study of human cortex has shown that a subpopulation of GABAergic interneurons originates from dorsal telencephalic areas (Letinic *et al.*, 2002).

Cell tracing and cell transplantation experiments *in vitro* (Lavdas *et al.*, 1999; Sussel *et al.*, 1999; Anderson *et al.*, 2001; Polleux *et al.*, 2002) and *in vivo* (Wichterle *et al.*, 1999, 2001; Valcanis and Tan, 2003) suggest that the medial ganglionic eminence (MGE) is the primary source of cortical interneurons in rodents. A recent study has also implicated the caudal ganglionic eminence (CGE) as a source of interneurons to the cortex (Nery *et al.*, 2002). The lateral ganglionic eminence (LGE) is believed to generate most of the striatal neurons (Olsson *et al.*, 1998); however, a contribution of interneurons from the LGE to the dorsal telencephalon has also been reported (De Carlos *et al.*, 1996; Anderson *et al.*, 1997).

These studies demonstrate that cortical interneurons are derived from ventral telencephalic areas; however, little is known about whether commitment of an interneuron subclass is linked to its birth-date in a particular ganglionic eminence. We addressed these issues and examined whether tangentially migrating interneurons represent a homogenous population. We characterized them spatially and temporally using immunohistochemistry, organotypic cultures and *in vivo* birth-dating studies. As a tool for these studies we used a recently developed GAD65-GFP transgenic mouse strain, in which green fluorescent protein (GFP) is expressed under the control of the GAD65 promoter. Using this model we found that tangentially migrating interneurons represent a heterogeneous population of neurons. GAD65-GFP cells are mainly generated in the CGE in late stages of embryonic development. In the adult neocortex, the highest density of GAD65-GFP cells was found in supragranular layers. Nearly all of the cholecystokinin (CCK)-positive and half of the calretinin (CR)-positive subpopulations of GABAergic interneuron express GFP. In contrast, very few of calbindin (CB)- or parvalbumin (PV)-positive cells express this marker.

Materials and Methods

Forty-two pregnant GAD65-GFP female mice, three pups at postnatal day (P) 6, and four GAD65-GFP P21 males, housed in the Animal House Facilities of the Department of Human Anatomy and Genetics, University Laboratory of Physiology and the Wellcome Trust Centre for Human Genetics in Oxford, were used in the present study. The care and handling of the animals prior to and during the experimental procedures followed European Union and UK Home Office regulations, and were approved and supervised by the Animal Care and Use

Committee of the institution. We have endeavoured to minimize both the suffering and the number of animals used in this study.

GAD65-GFP Transgenic Mouse Line

Generation and analysis of transgenic mice expressing GFP under the control of the GAD65 promoter will be described in detail elsewhere (F. Erdélyi *et al.*, in preparation; see Erdélyi *et al.*, 2002). Briefly, a genomic clone containing 5.5 kb upstream region and the first six exons of the mouse *GAD65* gene was isolated from a mouse λ phage genomic library, and the GFP marker gene without its own translation start site was fused in frame to the first or third exon of the GAD65 gene. Transgenic mice were derived by standard pronuclear injection of CBA/C57Bl6F2 fertilized eggs. For this study, heterozygous transgenic mice on C57Bl6 background were used from line GAD65_3e/gfp5.5 30. In this line, a 6.5 kb segment of the GAD65 gene that includes 5.5 kb of the 5'-upstream region, the first two exons and a portion of the third exon and the introns in between drives the expression of GFP almost exclusively to the GABAergic neurons in many brain regions including the neocortex and hippocampus. DNA based genotyping is not needed since the brains of neonates fluoresce bright green under appropriate illumination (Fig. 1).

This mouse was originally developed at the Department of Functional Neuroanatomy at the Institute of Experimental Medicine in Budapest, Hungary.

Immunohistochemistry

To characterize the population of tangentially migrating neurons expressing GAD65-GFP, 37 embryos from embryonic day (E) 14 ($n = 9$), E15 ($n = 9$), E16 ($n = 10$) and E18 ($n = 9$), and 7 animals at P6 ($n = 3$) and P21 ($n = 4$) were used for immunohistochemical analysis. Fetuses at each developmental stage were collected by caesarean section after cervical dislocation of the dam. Following decapitation, the heads were placed in cold freshly prepared 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4, overnight. The post-natal GAD65-GFP mice were either deeply anaesthetized with halothane (Halothane-Vet, Merial, Essex, UK) for P6 pups or with an i.p. injection of a Rompun/Imalgene mixture (0.1 ml/kg body wt) for the P21 mice. They were then perfused with 4% PFA in PB saline (0.9% NaCl) through the heart (López-Bendito *et al.*, 2001, 2002). After perfusion, brains were postfixed for 2 h in the same fixative. Tissue blocks containing the neocortex were dissected and washed thoroughly in PB. Using a dissecting microscope with a UV illumination (Leica, MZFLIII, Nussloch, Germany) the GFP-positive brains were selected. GFP-positive brains were sectioned coronally at 60 μ m with a microtome (Leica VT1000S) and collected in 0.1 M PB.

For the immunofluorescence, sections containing the cerebral cortex were incubated in 10% normal goat serum (NGS) in 50 mM Tris buffer (pH 7.4) containing 0.9% NaCl (TBS), with 0.2% Triton X-100, for 1 h. Sections were then incubated at 4°C for 48 h with the following antibodies: rabbit anti-GAD65 (1:500, Chemicon), rabbit anti-CB (1:5000, Swant), rabbit anti-CR (1:2000, Swant), mouse anti-Reelin (1:4000, Chemicon); rabbit anti-PV (1:5000, Chemicon), rabbit anti-Somatostatin (1:2000, Chemicon), mouse anti-CCK (1:2000, Antibody Core Laboratory), rabbit anti-GABA (1:5000, Sigma) or rabbit anti-Neuropeptide Y (1:1000, Chemicon) in TBS containing 1% NGS. After washing in TBS, sections were incubated with Cy3-conjugated goat anti-rabbit or anti-mouse antibodies diluted 1:500 in TBS for 2 h. Immunolabeled sections were mounted in PBS/glycerol and observed with a fluorescent microscope (Leica, DMR) or a laser-scanning confocal microscope (Leica TCS SP1). Images were stored and analyzed using appropriate software supplied by the microscope manufacturer (Leica). Brightness and contrast were adjusted for the whole frame using Adobe Photoshop 6.0 software.

Organotypic Cultures and CMTMR Tracing Injections

Organotypic slice cultures of embryonic mouse telencephalon were prepared as previously described (López-Bendito *et al.*, 2003). Briefly, five GAD65-GFP pregnant mice at E13 ($n = 2$, $n = 12$ embryos), E14 ($n = 1$, $n = 4$ embryos), E15 ($n = 1$, $n = 7$ embryos) and E16 ($n = 1$, $n = 4$ embryos) stages of gestation (where E0 = day vaginal plug was found) were killed by cervical dislocation. Embryos ($n = 27$) were collected by caesarean section of the dam and rapidly placed in artificial cere-

brospinal fluid (ACSF), in which NaCl was substituted with equiosmolar sucrose, at 4°C. The presence of GFP in each embryo was verified under a dissecting microscope with UV illumination (Leica MZFLIII); only positive embryos were kept for the study. Brains were removed, embedded in 3% low-melting point agarose (Sigma) in GBSS (Gey's Balanced Salt Solution) and 300 μ m thick coronal sections were cut on a vibrating blade microtome (Leica VT1000S). Slices were then transferred to polycarbonate culture membranes (13 mm diameter, 8 μ m pore size; Corning Costar, Cambridge, MA) in organ tissue dishes containing 1 ml of DMEM/F12 (Life Technologies) with 2.4 mg/ml D-glucose, 5 μ l/ml N₂ supplement (Gibco), 0.1 mM glutamine, 50 mg/ml penicillin/streptomycin, and 10% fetal calf serum (FCS, Gibco) at 37°C with 5% CO₂. The procedures described above were performed under sterile conditions.

To determine the contribution of each ganglionic eminence to the GAD65-GFP population of migrating interneurons, we placed 0.7 μ m diameter tungsten-M10 particles (Bio-Rad) coated with 1 mM 5-(and-6)-((4-chloromethyl)benzoyl)amino tetramethylrhodamine (Cell-Tracker Orange CMTMR; Molecular Probes) using a glass micropipette in the LGE (E13, $n = 64$; E14, $n = 20$; E15, $n = 24$; E16, $n = 10$), MGE (E13, $n = 52$; E14, $n = 14$; E15, $n = 26$; E16, $n = 12$) or CGE (E13, $n = 30$; E14, $n = 12$; E15, $n = 16$; E16, $n = 12$) in both hemispheres and at different developmental ages.

Birth-dating In Vivo Studies

Three pregnant GAD65-GFP heterozygote females at E13 ($n = 1$) and E15 ($n = 2$) gestational day received i.p. injections of BrdU. Each female received a total of two BrdU injections separated by 2 h. 5-Bromo 2-deoxyuridine (BrdU, Sigma) was dissolved (40 mg/kg) in PB saline (0.9% NaCl). Pups were left until birth, checked for GFP reactivity and only positive pups allowed to reach P21 days (E13 BrdU injection: $n = 3$ P21, E15 BrdU injection: $n = 5$ P21). After this period, brains were removed as described before and 60 μ m coronal sections were obtained. Prior to immunohistochemistry to reveal BrdU labeling, sections were treated with 2 N HCl for 45 min at 37°C, and then rinsed in 0.1 M PB, pH 7.4. Slices were then incubated in 1:100 mouse anti-BrdU (Progen Biotechnik GmbH, Germany) in 50 mM TBS pH 7.4 overnight at 4°C. After washing in TBS, sections were incubated with Cy3-conjugated goat anti-mouse antibody diluted 1:500 in TBS for 2 h. Immunolabeled sections were mounted in PBS/glycerol and observed with a fluorescent microscope (Leica DMR) or a laser-scanning confocal microscope (Leica TCS SP1).

Statistical Analysis

Quantitative Analysis of GAD65-GFP Cell Density

To establish the density of GAD65-GFP cells and the percentage of double-labeled cells at every developmental stage, a quantitative analysis was conducted in different cortical compartments. First, in order to define those compartments anatomically, freshly fixed slices were counterstained with the chromatin stain bis-benzimide (7 min in 2.5 μ g/ml solution in 0.1 M PB; Sigma). Quantification was carried out under a fluorescence microscope (Leica DMR) using a 270 000 μ m² area under a 20 \times objective lens or a 66 000 μ m² area under a 40 \times objective lens. The fields for quantification were always selected at corresponding places across preparations, perpendicular to the pial surface and adjacent to the pallial-subpallial boundary or at the level of the putative somatosensory cortex. The total number of GAD65-GFP, immunolabeled, and double-labeled cells identified in each of the selected cortical compartments, in all focal planes, were counted. The estimate of the immunopositive proportion of GFP cells is expressed as mean \pm SEM of the proportion found in each of between three and seven preparations, and n indicates the total number of cells counted for each marker. The number of GAD65-GFP cells in each cortical compartment was expressed as density of cells per mm³.

Quantitative Analysis of CMTMR/GAD65-GFP Labeled Cells

For the estimation of the percentage of the GAD65-GFP cells that originate in the different ganglionic eminences, we quantified the percentage of GAD65-GFP cells that contained the cell-tracker after 2 days *in vitro* (DIV). Quantification was carried out under a 20 \times objective lens using a confocal microscope (Leica TCS SP1). Stacks of

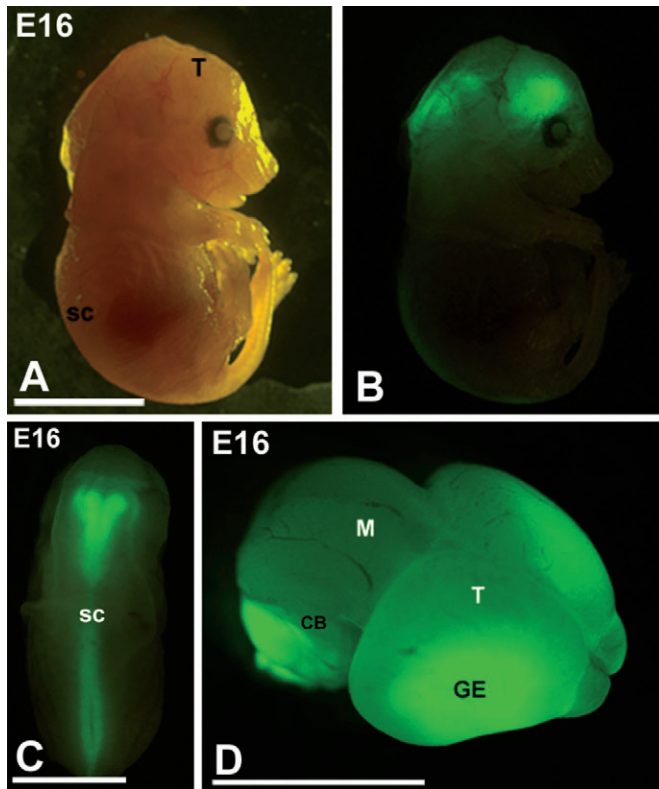


Figure 1. Overall expression pattern of GFP in the GAD65-GFP transgenic mouse. (A, B) Embryonic (E) 16 GAD65-GFP embryo viewed under normal and fluorescence illumination showing the pattern of expression of the GFP protein (B). Note the high expression of GFP in the brain and spinal cord (sc). (C) Dorsal view of an E16 GAD65-GFP embryo showing the expression in the spinal cord. (D) Brain from E16 GAD65-GFP embryo showing the expression of GFP in the telencephalon (T) and cerebellum (CB). Note the high level of expression in the ganglionic eminences (GE). Abbreviation: M, mesencephalon. Scale bars 5 mm.

images were collected at the dorsomedial cortex. Each stack of images, consisting of a number of optical sections collected in the z-plane through a depth of $\sim 70 \mu\text{m}$ tissue thickness, were then collapsed into single images using Leica imaging software. A montage of the neocortex was subsequently assembled from the series of collapsed images and the double-labeled cells were counted. Statistical comparisons were performed using Student's *t*-test. The differences were considered significant at the level of $P < 0.05$. Data are presented as mean \pm SEM.

Results

Distribution of the GAD65-GFP Neurons in the Cerebral Cortex During Development and in the Adult

Previous studies have shown that the vast majority of tangentially migrating interneurons, characterized by an irregular or elongated cell body and a long thick leading process that is often branched, migrate from the ganglionic eminences at very early stages of development (Anderson *et al.*, 1997, 2001; Tamamaki *et al.*, 1997; Lavdas *et al.*, 1999; Wichterle *et al.*, 1999; Marín *et al.*, 2000). In this study we characterized the spatio-temporal distribution of GAD65-GFP interneurons during embryonic and postnatal development.

GFP expression in the GAD65-GFP transgenic mouse is precisely regulated both spatially and temporally. At E16, GFP expression was found not only in the brain but also in the

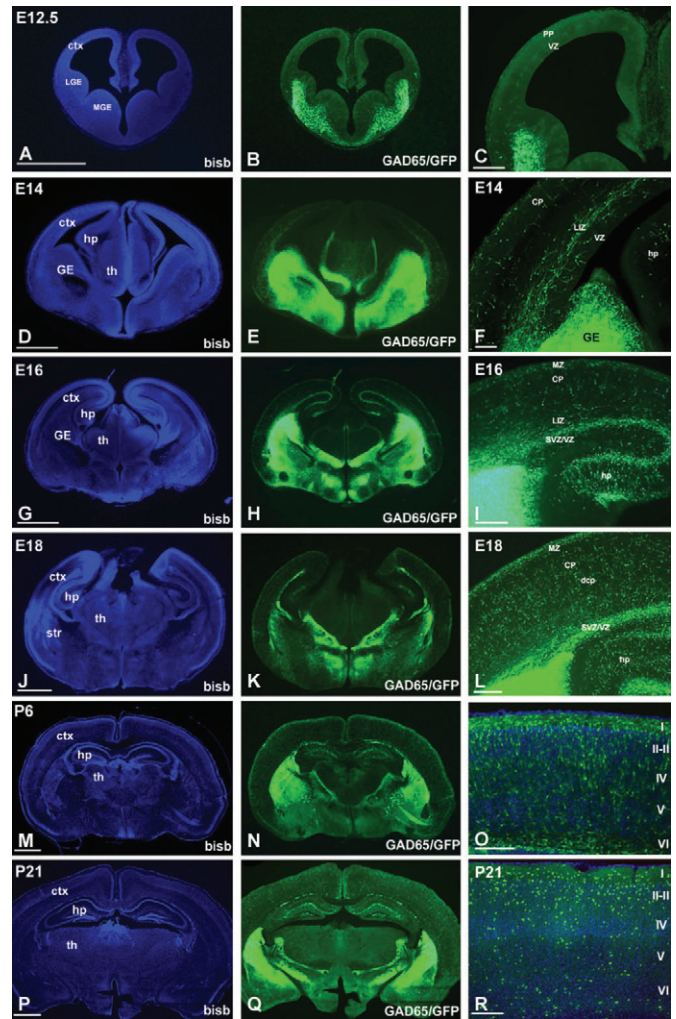


Figure 2. Expression of GAD65-GFP cells in the cerebral cortex during development. (A–L) Coronal sections of the developing embryonic cortex (ctx) showing cytoarchitectonic areas revealed by bis-benzimide (blue, left column) and the same sections viewed under fluorescence microscopy to demonstrate the GAD65-GFP expression (green, middle). High-power views from each age are shown to the right. GAD65-GFP cells were found localized in the postmitotic areas of the ganglionic eminences at E12.5 (GE, B and C). Later in embryonic development, GAD65-GFP cells were found in all cortical compartments: marginal zone (MZ), cortical plate (CP), in the pathway of migratory interneurons, the lower intermediate zone (LIZ), as well as in the subventricular/ventricular zones (SVZ/VZ). In the LIZ, GAD65-GFP cells had the characteristic morphology of migratory interneurons with one or two leading processes pointing in the direction of the migration. Other brain areas including the striatum (str) and thalamus (th) also highly expressed GAD65-GFP (H, I, L). (M–R) Corresponding coronal sections at two postnatal ages (left and middle columns). Higher-power images of the neocortical layers counterstained with bis-benzimide are shown to the right. GAD65-GFP cells were found in all cortical layers but preferentially localised at supragranular layers (D, R). Dcp: deep cortical plate. Scale bars: A, B, D, E, G, H, J, K, M, N, P, Q, 1 mm; C, I, L, O, R, 200 μm ; F, 100 μm .

spinal cord (Fig. 1A–C) and ectodermal placodes of the whiskers (data not shown; see also Tamamaki *et al.*, 2003). In the brain, high expression of the GFP protein was detected in the telencephalon, especially in the ganglionic eminences (Fig. 1D).

The earliest embryonic age studied was E12.5. At this age, GAD65-GFP cells were localized outside the proliferative zones at the level of the LGE and MGE (Fig. 2B,C). At this age, the developing dorsal telencephalon was devoid of GAD65-GFP

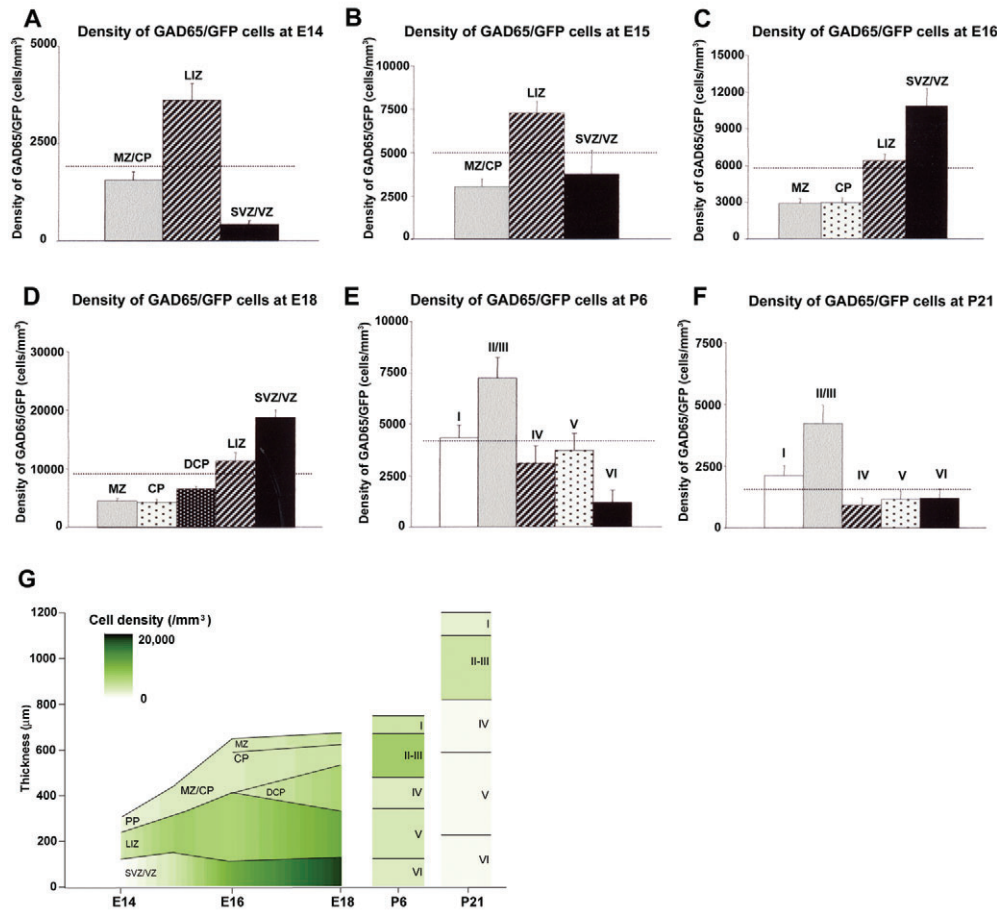


Figure 3. Density of GAD65-GFP cells in the cerebral cortex during development and in adult. (A, B) At E14 and E15, the majority of the GAD65-GFP cells were found in the LIZ; however, a 5-fold increase in the density of GAD65-GFP cells was observed in the subventricular zone from E14 to E15 (SVZ at E14: ~420 cells/mm³; SVZ at E15: ~3700 cells/mm³). (C, D) At later stages of embryonic development, quantitatively more GAD65-GFP cells were distributed in the SVZ and an overall increase of GAD65-GFP cells was observed throughout the cortex compared to early embryonic stages (E14: ~1900 cells/mm³; E15: ~4800 cells/mm³; E16: ~5800 cells/mm³; E18: ~9800 cells/mm³). (E, F) At postnatal day (P) 6 and P21 cortex, GAD65-GFP cells were distributed throughout all cortical layers but preferentially in layers II and III (layers II/III at P6: ~7200 cells/mm³, layers II/III at P21: ~4200 cells/mm³). Horizontal dotted lines in each figure indicate the average density of GAD65-GFP cells in the cortex at each age. Data are presented as mean \pm SEM. (G) Colour-code diagram using different tones of green (light to dark) showing the density of GAD65-GFP cells in different cortical compartments and layers through development. Dark green indicates a high density of cells.

cells. However, later in development, at E14 and E15, GFP-positive cells were found densely distributed throughout the ventral pallidum, striatum and neocortex (for E14 see Fig. 2E,F). In the neocortex, GFP-positive cells were distributed throughout the lower intermediate zone (LIZ), marginal zone (MZ) and cortical plate (CP). Quantitative studies (E14, $n = 24$ and E15, $n = 19$) showed that the highest density of GAD65-GFP cells in the cortical wall was found in the LIZ at these ages (~600 cells/mm³ at E14 and ~7200 cells/mm³ at E15; Fig. 3A,B).

By E16 ($n = 19$), GAD65-GFP cells occupied the full dorso-ventral extent of the cortex, and the hippocampal anlage (Fig. 2H,D). This progression in a ventral to dorsal direction has been described previously (Jimenez *et al.*, 2002), as ganglionic eminence-derived interneurons populate the hippocampus (Pleasure *et al.*, 2000). GAD65-GFP cells were also observed in the ventral thalamus, hypothalamus and striatum. At E16 and E18 ($n = 20$), a higher density of GAD65-GFP cells were observed in the subventricular zone (SVZ) of the cerebral

cortex, than in any other cortical compartment (Fig. 2I,L, Fig. 3C,D). A 5-fold increase in the density of GAD65-GFP cells in the SVZ/ventricular zone (VZ) was found at E18 compared to E15 (~3800 cells/mm³ at E15; ~10800 cells/mm³ at E16; ~18750 cells/mm³ at E18; Fig. 3G).

In order to analyze the distribution of this population of cells during postnatal stages of development we performed quantitative studies at P6, i.e. after the migration of neurons has finished, and at P21. At both ages, GAD65-GFP cells were distributed in all layers of the neocortex. However, significantly more GAD65-GFP cells were seen localized in layers II and III (~7200 cells/mm³ at P6; ~4200 cells/mm³ at P21) than in any other layer (Fig. 2O,R; Fig. 3E,F). This distribution suggests a preferential targeting of the GAD65-GFP cells to the supragranular layers of the cortex. The average number of GAD65-GFP cells in the cerebral cortex increased through embryonic development. However, the overall density of GAD65-GFP decreased by two-thirds in P21 cortex compared to late embryonic stages (see Fig. 3, dotted horizontal lines).

GAD65-GFP Cells Are GAD65 and GABA Positive but Do Not Express Calbindin, Calretinin or Reelin at Embryonic Stages

To characterize the GAD65-GFP migratory interneurons neurochemically, immunohistochemical studies were performed at different developmental stages using common markers for early interneuronal populations. In all cortical compartments, ~99% of the GAD65-GFP cells (643/651) expressed the GAD65 protein through development (Fig. 4A–I). Conversely, >75% of GAD65-immunopositive cells expressed GFP (E14, 76%; E15, 78%; E16, 89%; E18, 95%). The expression of the neurotransmitter GABA by this population of cells was also determined. GABA was detected in ~22% (48/214) of the GAD65-GFP cells at E16 and in ~48% (142/297) at E18. In adulthood, the percentage of GABA-immunopositive GAD65-GFP cells increased considerably (see below).

Previous studies have described a high level of expression of the calcium-binding protein CB in GABAergic tangentially migrating interneurons. Overall, in all cortical compartments and throughout embryonic development, only 4% of GAD65-

GFP cells (79/2098) were observed to express CB (Fig. 5A,B). This result suggests that the CB-positive migratory neurons described in previous studies are only a small fraction of the total GAD65-expressing migratory interneurons.

Another population of migratory neurons that appears early in development are the Cajal-Retzius cells (Meyer *et al.*, 1999; for review see Frotscher, 1998). These cells express the calcium-binding protein CR and the extracellular matrix protein, reelin (Ogawa *et al.*, 1995). Virtually no GAD65-GFP cells expressed CR (7/1178 GAD65-GFP cells) and none expressed reelin (0/715 GAD65-GFP cells) during embryonic development (Fig. 5D–G). These results demonstrate that GAD65-GFP cells are not Cajal-Retzius cells. However, reelin has been shown to be expressed in some GABAergic neurons in the adult neocortex (Alcantara *et al.*, 1998; Pesold *et al.*, 1998). Interestingly, at P21, as many as ~65% of GAD65-GFP cells in layer I expressed reelin and ~41% of the reelin-positive cells in layer I were GFP positive. Fewer GAD65-GFP cells were reelin-immunopositive in the other cortical layers (layer II/III, 26%; layer IV, 29%; layer V, 18%; layer VI, 9%).

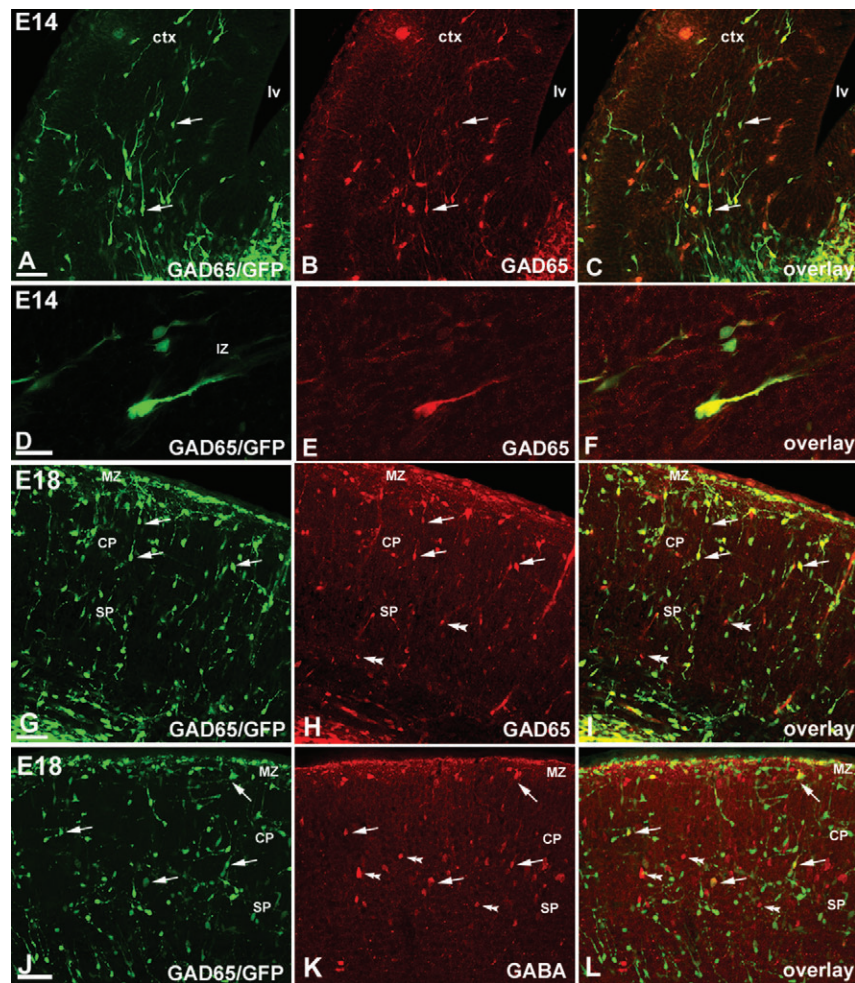


Figure 4. Double labeling of GFP expression (green) and GAD65 or GABA immunoreactivity (red) in embryonic cortex. (A–I) GFP expression (left column) and immunohistochemistry for GAD65 (middle) with overlay (right) confirmed that the GAD65-GFP cells are indeed expressing this protein. The colocalization between GAD65-GFP cells and GAD65-positive neurons was very high (~98%) in all cortical compartments studied (arrows). However, a small fraction of GAD65 positive cells at E18 were not GFP positive (double arrowheads; H, I). (J–L) GFP expression (left) and immunolabeling for the neurotransmitter GABA (middle) with overlay (right) showing colocalization of GFP with GABA at E18 (arrows). Numerous GABA positive cells were not positive for GFP (double-arrowheads) and vice versa. Abbreviations: ctx, cortex; lv, lateral ventricle; CP, cortical plate; IZ, intermediate zone; MZ, marginal zone; SP, subplate. Scale bars: A–C and G–L, 100 μ m; D–F, 20 μ m.

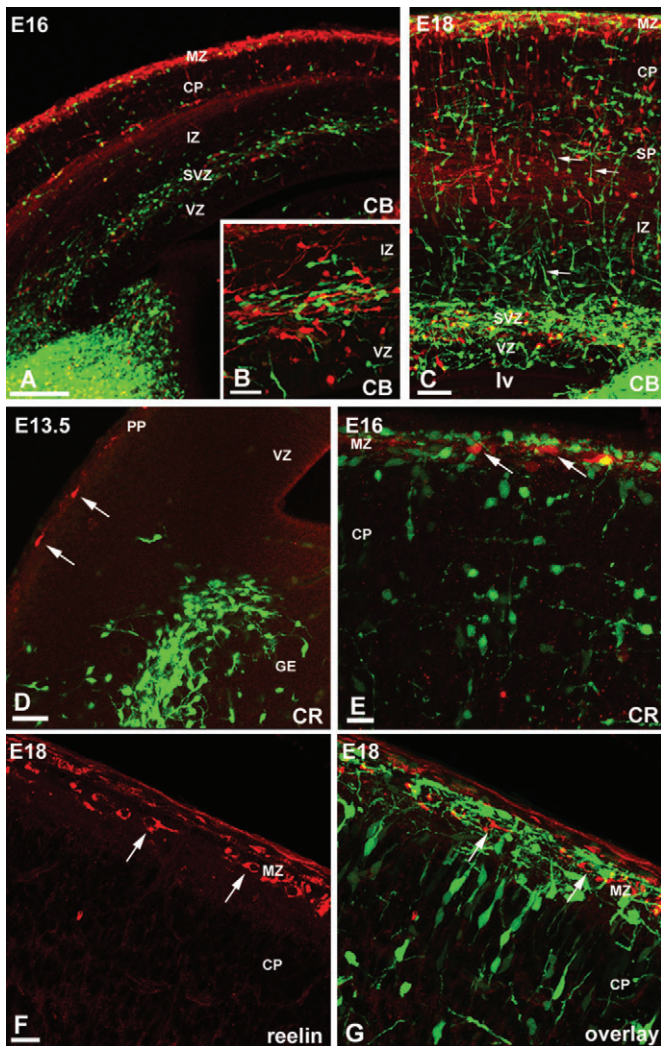


Figure 5. Lack of colocalization of calbindin, calretinin and reelin with the GAD65-GFP neurons. (A–C) Calbindin immunoreactivity (red) in E16 (A, B) and E18 (C) GAD65-GFP (green) cortex. The calcium binding protein calbindin is expressed in cells of the cortical plate (CP), marginal zone (MZ), lower intermediate zone (LIZ) and subplate (SP) at both embryonic ages. Only ~10% of the GAD65-GFP cells in the MZ showed calbindin immunoreactivity, the percentage of colocalization in the rest of the cortical layers was even lower (~3%). Note the radial orientation of the GAD65-GFP cells in E18 cortex (C, arrows). (D, E) Calretinin immunoreactivity (red) in embryonic GAD65-GFP (green) cortex. Immunopositive cells were localized mainly in the preplate (PP) at E13.5 (D) and in the MZ at E16 (E) (arrows); some scattered cells were also seen in other cortical compartments. Very few of the GAD65-GFP cells showed calretinin labeling at embryonic stages. (F, G) Reelin immunoreactivity (red) in E18 GAD65-GFP (green) cortex. Immunopositive cells were localized at the MZ of the cortex. No reelin-positive GAD65-GFP neurons were observed. Abbreviations: SVZ, subventricular zone; VZ, ventricular zone; IZ, intermediate zone; lv, lateral ventricle; GE, ganglionic eminence. Scale bars: A, 200 μ m; B, 40 μ m; C, D, 100 μ m; E–G, 20 μ m.

Layer-dependent Neurochemical Expression by GAD65-GFP Cells in the Young Adult Cortex

To confirm that the GAD65-GFP cells express GAD65 and GABA at adult stages, immunohistochemical studies were performed at P21. In all layers of the neocortex, ~98% of the GFP-positive cells expressed immunodetectable GAD65 (Fig. 6A,C,D; 493/502 GAD65-GFP cells). The distribution of GAD65-immunoreactive cells in P21 neocortex was similar to the GAD65-GFP labeling (Fig. 6B), and an intense neuropilar immunoreactivity was observed throughout all layers. Approx-

imately 93% of the GAD65-immunopositive cells expressed GFP at this age in the cerebral cortex (411/440 GAD65-positive cells). In order to identify the percentage of the GAD65-GFP cells that contain GABA and vice versa in P21 neocortex, immunohistochemistry and quantitative analysis was performed (Fig. 6E–G). GABA was detected in only ~71% of the GAD65-GFP expressing cells in cortex overall (334/470 GAD65-GFP cells). Moreover, only ~51% of the GABA-immunopositive cells were GAD65-GFP positive in the neocortex (334/659 GABA-positive cells).

To determine whether the GAD65-GFP phenotype is associated with specific neurochemical compositions of interneurons, we used specific antibodies against markers of different subpopulations such as CB, CCK, CR, PV, somatostatin (SOM) and neuropeptide Y (NPY). The distribution and features of the different populations of interneurons have been described before (DeFelipe, 1993; Kubota *et al.*, 1994; Gonchar and Burkhalter, 1997). The colocalization of GFP with these different markers was layer dependent. The proportions of cells in which expression of GFP colocalized with different markers in each cortical layer are illustrated in Figure 7.

In layer I, GAD65-GFP cells most commonly co-expressed CR and NPY. Approximately 14% of the GFP cells expressed CR ($n = 107$ in three preparations) and 19.8% NPY ($n = 100$). In layers II/III, GAD65-GFP cells mainly expressed CR (~22%, $n = 206$; see Fig. 6H–J), NPY (~19%, $n = 228$) and CCK (~10%, $n = 269$). In layer IV, CR (~50%, $n = 42$), CCK (~21%, $n = 41$) and SOM (~16%, $n = 55$) were the markers most commonly co-expressed by the GAD65-GFP cells. In layer V, GAD65-GFP cells mainly co-expressed CR (~31%, $n = 42$) and SOM (~30%, $n = 84$). Finally, in layer VI, GAD65-GFP cells colocalised mostly with CR (~15%, 8/51), NPY (~18%, $n = 64$) and CCK (~35%, $n = 112$).

Of all cells counted in the neocortex, the proportion of GAD65-GFP cells among CCK-positive cells was ~94% ($n = 52$) and among CR-positive cells ~43% ($n = 229$). The corresponding number for SOM-positive cells was ~5% ($n = 783$; see Fig. 6K–M), PV-positive cells ~2% ($n = 253$; see Fig. 6N–P) and CB-positive cells ~1% ($n = 970$).

Preferential Origin of GAD65-GFP Cells from the CGE: Migration via the SVZ

It has been demonstrated that the ganglionic eminences (medial, lateral and caudal) are the main sites of origin of interneurons of the cerebral cortex (Tamamaki *et al.*, 1997; Lavdas *et al.*, 1999; Nery *et al.*, 2002; for review see Marín and Rubenstein, 2001). In order to determine the contribution of each ganglionic eminence to the cortical population of GAD65-GFP cells, we performed *in vitro* organotypic culture studies. Migratory cells from each ganglionic eminence in the GAD65-GFP mice were labeled with a cell-tracker (CMTMR) at different ages and the percentage of double-labeled cells was quantified. The migration of interneurons takes place from E13 to E16 in mice; therefore we used brain slices taken from E13–E16 embryos and maintained them for 2DIV.

Colocalization of GAD65-GFP and CMTMR was observed 2 days after dye injection in LGE, MGE or CGE at all developmental stages investigated. Both CMTMR-positive cells, GFP-positive cells and double-labeled cells were seen extending along the ventrodorsal extent of the cortex (Fig. 8C,H,M), migrating along the SVZ/VZ of the cortex (Fig. 8D,I,N). Other GFP- and CMTMR-positive cells were located in the marginal zone and intermediate zone. These results suggest that GAD65-

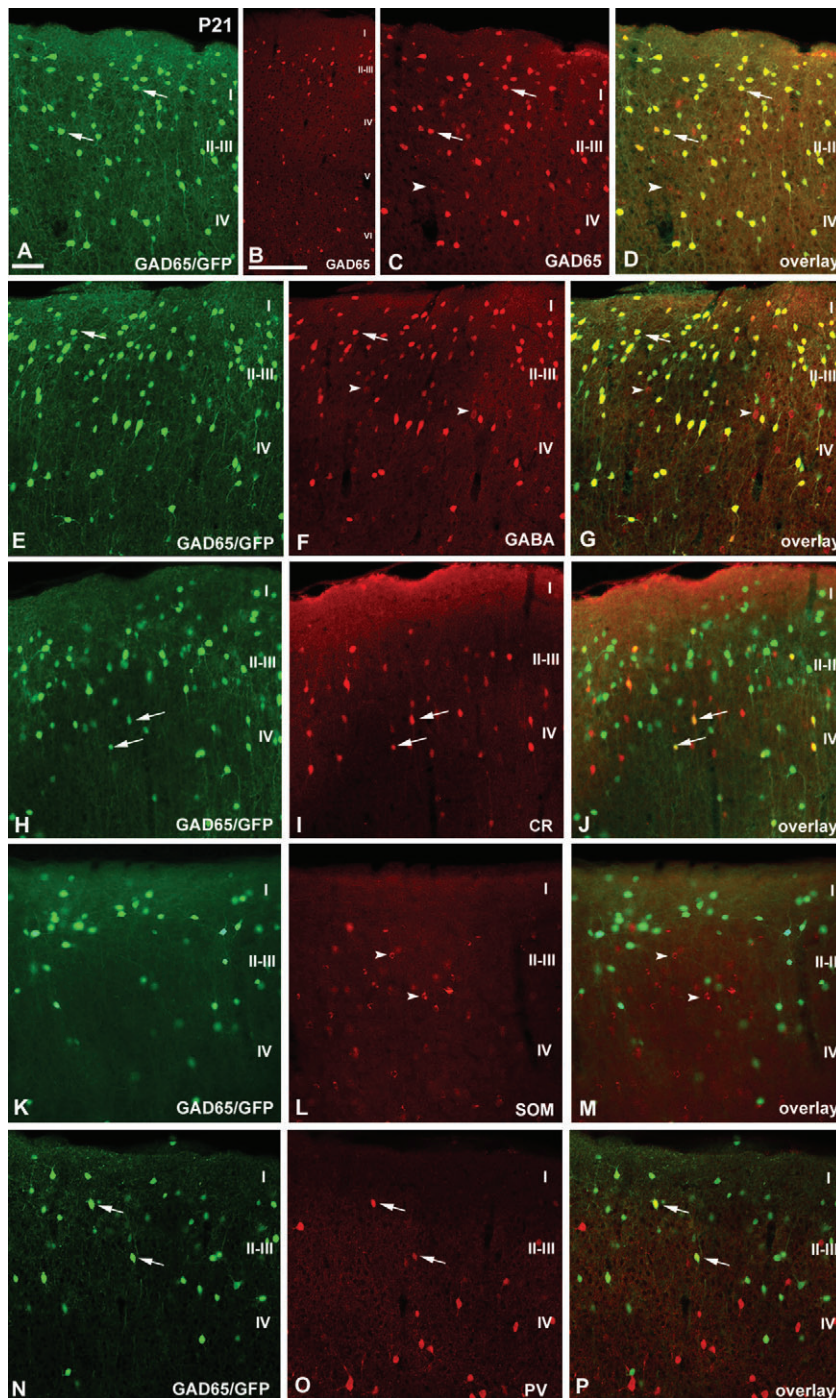


Figure 6. Colocalization of GFP with GAD65, GABA, calretinin, somatostatin and parvalbumin immunoreactivity in the neocortex at P21. (A–G) Colocalization of GFP (left) with GAD65 and GABA immunoreactivity (middle) in supragranular layers. Overlay is shown to the right. Most of the GFP neurons were immunopositive for GAD65 and GABA (arrows). This colocalization was observed throughout all cortical layers. Some GAD65- or GABA-immunopositive cells do not colocalise with GFP (arrowheads). (B) shows a low-power view of GAD65 immunoreactivity throughout the cortical layers. (H–M) Examples of the expression and colocalization of GFP with specific interneuronal markers. Around 20% of GAD65-GFP neurons in supragranular layers were immunopositive for the calcium binding protein calretinin (CR) (arrows). Fewer GFP cells were immunopositive for the markers somatostatin (SOM) or parvalbumin (PV). Arrowheads indicate examples of immunoreactive cells that do not colocalize the GFP. Cortical layers are indicated with roman numerals. Scale bars: A, C–P, 100 μ m; B, 400 μ m.

GFP cells arise throughout embryonic development and from all three ganglionic eminences. However, labeling of the CGE resulted in more GFP-positive CMTMR-migrating interneurons. This pattern was not significant until E15 (E15: LGE, $16.5 \pm 3.4\%$ CMTMR/GAD65-GFP positive cells; MGE, $18.4 \pm 3.3\%$; CGE, $30.3 \pm 3.9\%$; E16: LGE, $20.8 \pm 2.9\%$; MGE, $22.4 \pm 4.2\%$;

CGE, $36.1 \pm 1.6\%$), when considerably more migratory neurons were positive for GFP (Fig. 8P,Q; Fig. 9).

Moreover, the overall contribution of the ganglionic eminences to the GAD65-GFP population of neurons seems to increase during embryonic development (Fig. 9). Thus, $16.8 \pm 2.0\%$ of GAD65-GFP cells were CMTMR-positive at E13 + 2DIV

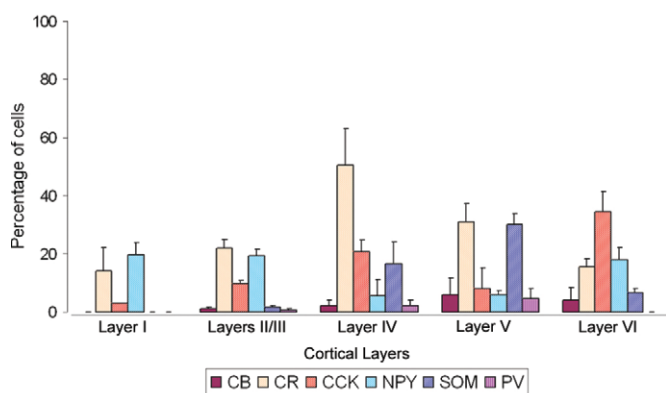


Figure 7. Histogram showing the percentage of GFP cells that express different interneuronal markers in the neocortex at P21. The colocalization between GFP and different markers is layer-dependent. In layer I, the markers most commonly expressed by GFP cells were calretinin (CR, 14%) and neuropeptide Y (NPY, 19.8%). In layers II/III, the most commonly expressed markers colocalizing with GFP were CR (~22%), NPY (~19%) and cholecystokinin (CCK, ~10%). In layer IV, CR (~50%), CCK (~21%) and somatostatin (SOM, ~16%) were the markers most frequently expressed by the GAD65-GFP cells. In layer V, GFP cells expressed mainly CR (~31%) and SOM (~30%). Finally, in layer VI GAD65-GFP cells colocalized mostly with CR (~15%), NPY (~18%) and CCK (~35%). The parvalbumin (PV) expression was very low among GFP cells overall (~2%). Data are presented as mean \pm SEM.

versus $27.3 \pm 2.6\%$ of them at E16 + 2DIV. Therefore, the GAD65-GFP population of interneurons appears to migrate preferentially at late stages of embryonic development and then largely from CGE.

When GAD65-GFP slices from late stages of development were analysed after 2DIV, large numbers of radially oriented GAD65-GFP cells with their leading processes oriented towards the pial surface were observed within the upper IZ and CP, some of which were CMTMR positive (Fig. 8). Radially oriented GAD65-GFP cells were also observed in fixed tissue at late stages of embryonic development (see Fig. 5C,G) and during the first postnatal week. These results suggest that GAD65-GFP cells migrate tangentially towards the cortex and then acquire a substrate for migration based on radial glia interactions utilized by pyramidal neurons to reach their appropriate layer.

GAD65-GFP Cells in Layers II–III Are Born at Late Stages of Embryonic Development

Birth-dating studies that combine GABA immunocytochemistry with [3 H]thymidine autoradiography have demonstrated that interneurons, as well as pyramidal neurons, follow the inside-out pattern of corticogenesis (Miller, 1986; Peduzzi, 1988). Our studies using the cell-tracker CMTMR in slice cultures have shown that more GAD65-GFP cells migrate towards the cortex at late stages of embryonic development. This raised the question of whether the preferential destination of late-born GAD65-GFP cells might be the upper cortical layers. To address this question, we performed *in vivo* birth-dating studies in the GAD65-GFP mouse. BrdU injections were given to pregnant females at two distinct periods of gestation; early (E13) and late (E15) stages based on the time of interneuronal migration. Our analysis of P21 brains showed that administration of BrdU at early stages resulted in the labeling of cells in the infragranular layers of the cortex (Fig. 8T). Double-labelling experiments showed that a large number of GAD65-GFP cells in the infra-

granular layers were also stained for BrdU (Fig. 8U) indicating that those cells were born at the time of BrdU administration. By contrast, when BrdU pulses were given at E15, BrdU-positive cells were mainly located in layers II and III of the neocortex (Fig. 8R). In those layers, numerous BrdU and GFP-positive cells were observed (Fig. 8R,S) indicating that this cohort of GAD65-GFP cells was generated at E15. These results suggest that during cortical layer formation GAD65-GFP cells follow a deep-layer-first, superficial-layer-last spatio-temporal sequence similar to their pyramidal cell counterparts.

Discussion

Using GAD65-GFP transgenic mice as a tool to study migration of interneurons we demonstrated here that: (i) tangentially migratory interneurons represent a heterogeneous population of neurons from very early stages of development; (ii) GAD65-GFP cells are born throughout embryonic development, but mostly at late stages and preferentially in the CGE; (iii) many of them express CR and NPY in the adult neocortex and conversely; (iv) almost all of the neocortical CCK-positive interneurons, and half of the CR-positive population express GFP; and finally (v) the GAD65-GFP population of cells mostly target supragranular layers most likely according to an inside-out neurogenetic gradient.

The mice used in this study have a 6.5 kb segment of the GAD65 gene driving the expression of GFP in GABAergic neurons. We studied the temporal and spatial distribution of these cells during development. An overall increase of GAD65-GFP cell density in the cerebral cortex was observed through embryonic development, followed by a 50% decrease in density at P21 compared to P6 neocortex. Cell-death in the neocortex has been described to occur through embryonic and early postnatal development that may explain this finding (Verney *et al.*, 2000). However, we cannot exclude the possibility that changes in the expression pattern of GAD65 within interneurons during postnatal development may occur or that the increase in cortical volume postnatally effectively reduces the density of GAD65-GFP cells. Nevertheless, GAD65 immunoreactivity was detected in almost all of the GAD65-GFP cells, thus confirming the expression of this protein by GFP cells. Conversely, a large proportion of GAD65-immunopositive cells expressed GFP. This proportion increased with development, reaching 93% at P21. Only 5.5 kb upstream regulatory region and 1.1 kb from the GAD65 structural gene (of the 70 kb GAD65 locus) were incorporated in the DNA construct that was used to create this transgenic line. It is possible that the lack of GFP expression in some GAD65-immunopositive cells could be due to missing regulatory sequences that lie outside the included region of the GAD65 gene, and we cannot exclude the possibility that this lack of expression is interneuron subpopulation specific.

The neurotransmitter GABA was detected in ~50% of the GAD65-GFP cells at embryonic stages. This lack of colocalization is likely to be due to sensitivity problems of the immunohistochemistry method, however we can not rule out the possibility that some GAD65-positive cells at these early stages may not synthesize GABA. Such low percentages of GABA immunoreactivity in migratory interneurons have been reported earlier in both *in vivo* and *in vitro* studies (Anderson *et al.*, 1997, 2002; Wichterle *et al.*, 1999, 2001; Valcanis and Tan, 2003). Conversely, only 51% of GABA-positive cells

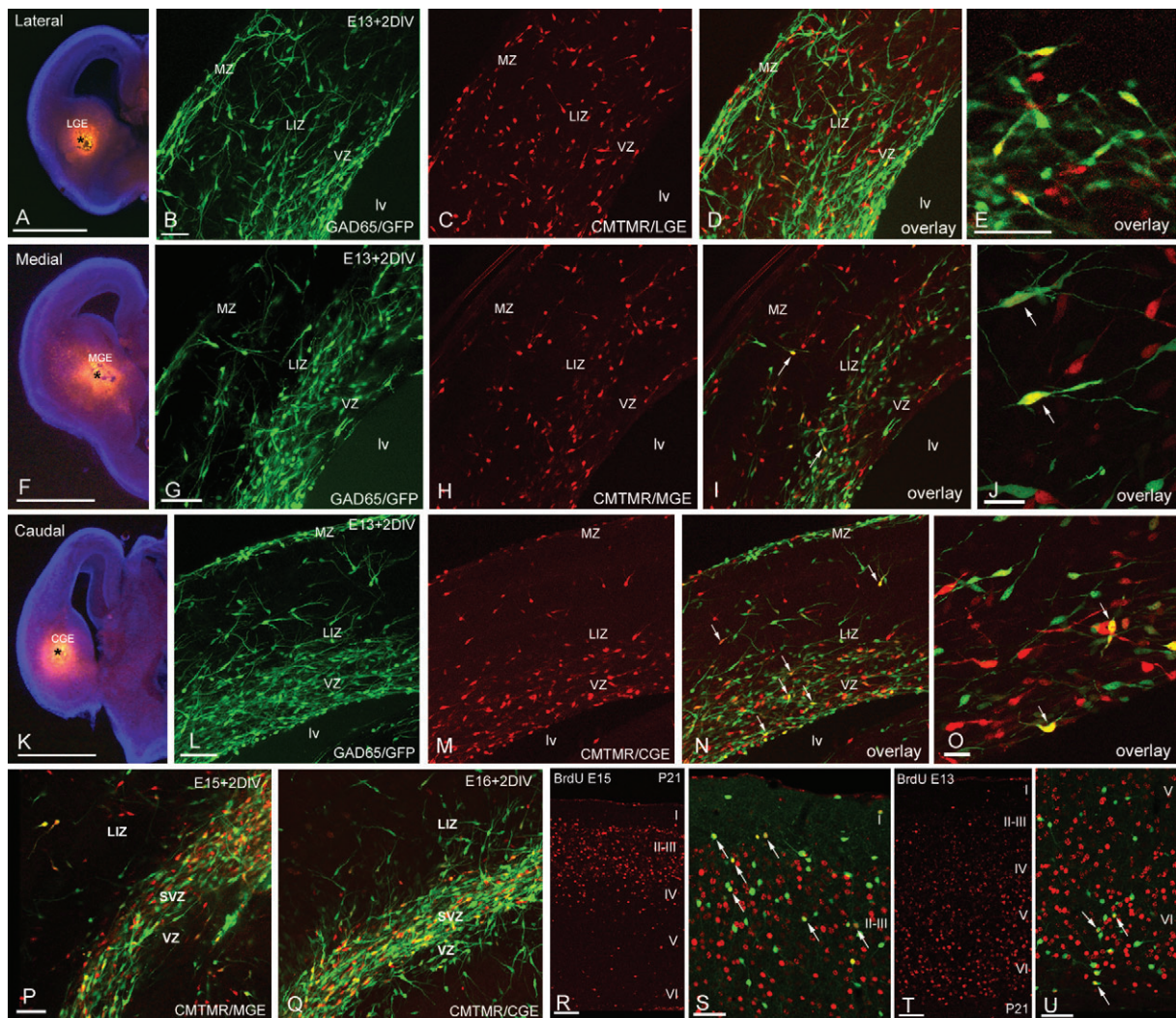


Figure 8. Birth-dating and migration studies of the GAD65-GFP cells. (A–O) Pattern of CMTMR labeling (red) after 2 days *in vitro* (DIV) following injections in each of the three ganglionic eminences in E13 GAD65-GFP culture slices. Examples of the injections in the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE) are shown in A, F and K, respectively, using *bis*-benzimidazole as counterstain (blue). After 2DIV, CMTMR-positive cells were localized along the ventro-dorsal extent of the cortex. Many CMTMR positive cells were GFP positive (green), migrating along the subventricular zone (SVZ), ventricular zone (VZ) and lower intermediate zone (LIZ). The highest frequency of double labeled cells was observed after CMTMR was placed in the CGE (L, M). Arrows indicate double positive cells. (P, Q) Overlay images of the distribution and colocalization of GAD65-GFP cells and CMTMR after 2DIV following tracer injection in the MGE at E15 (P) or in the CGE at E16 (Q). Note more double positive cells migrating through the SVZ and VZ at these later stages. (R) BrdU-labeled cells (red) in the neocortex at P21 after two BrdU pulses at E15. Note that BrdU-labeled cells are preferentially localized in supragranular layers. (S) Overlay image of supragranular layers of the neocortex showing numerous GAD65-GFP cells containing BrdU (arrows). (T) BrdU-labeled cells in the neocortex at P21 after two BrdU pulses at E13. Note that in this case BrdU-labeled cells are preferentially localized in infragranular layers. (U) Many GAD65-GFP cells localized at layers V and VI contained BrdU (arrows). Cortical layers are indicated with roman numerals. Abbreviation: lv, lateral ventricle. Scale bars: A, F, K, 1 mm; B–D, E, G–I, P–U, 100 μ m; J, O, 20 μ m.

expressed detectable levels of GFP. At least three possible explanations could account for this: (i) some of the GABA-positive but GFP-negative cells might belong to a population that expresses very low level of endogenous GAD65; (ii) the GFP expression in some neurons could be below detection limit despite normal GAD65 expression; and (iii) some GABAergic neurons might express GAD67 but not GAD65-GFP. Interestingly, however the apparent lack of GFP expression in some GABAergic neurons was associated with the presence of specific neurochemically-defined markers (see below).

In P21 mice, we found that the proportion of GAD65-GFP cells that were positive for GABA had increased to ~70%; this might reflect a developmental upregulation of GABA synthesis in GAD65-positive interneurons. The remaining 30% of GFP

cells that were GABA-negative might still contain GABA but remain undetected by our immunohistochemical method.

Tangential Migratory Interneurons Are a Heterogeneous Population of Cells

Several molecular markers of tangentially migrating subcortical cells have been identified, including Nkx2.1, Lhx6, 7, Dlx 1,2,5,6, GABA and CB (Anderson *et al.*, 1997, 1999; Lavdas *et al.*, 1999; Sussel *et al.*, 1999; Nadarajah *et al.*, 2003). However, it remains unclear whether distinct subtypes of interneurons might express these or other markers during their migration. CB has been extensively used as a marker for migratory interneurons; but only 4% of the GAD65-GFP migratory cells were positive for this protein. This result demonstrates that CB-

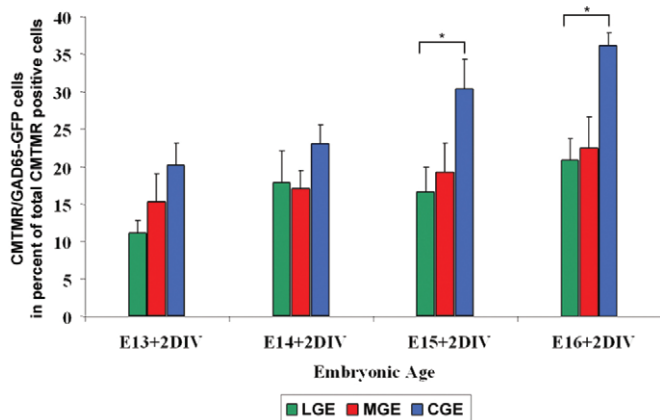


Figure 9. Large proportion of GAD65-GFP cells originate from the caudal ganglionic eminence. Histogram showing the percentage of CMTMR-positive cells that are GAD65-GFP positive after 2DIV. CMTMR was injected in either of the three ganglionic eminences, LGE (green), MGE (red) and CGE (blue) at four embryonic ages E13, E14, E15 and E16. Each ganglionic eminence gives rise to GAD65-GFP cells during development; however, significantly more GAD65-GFP cells are generated in the CGE at E15 and E16 ($*P < 0.05$).

positive cells only represent a subpopulation of the GABAergic migrating cells at embryonic stages. Different origins for the CB-positive interneurons have been recently suggested (Ang *et al.*, 2003).

The calcium-binding protein CR and the extracellular matrix protein reelin have also been used as markers of early neuronal populations (i.e. Cajal-Retzius cells), but virtually no GAD65-GFP interneurons expressed CR and none expressed reelin at embryonic stages. These results confirm previous studies showing that during embryogenesis reelin is not expressed by GABAergic neurons (Alcantara *et al.*, 1998). An origin other than MGE for the CR-expressing cells has been suggested (Meyer *et al.*, 1998; Lavdas *et al.*, 1999; Wichterle *et al.*, 2001; Anderson *et al.*, 2002; for review see Xu *et al.*, 2003). At P21, we found that CR is one of the neurochemical markers expressed by a high proportion of GAD65-GFP cells. This protein is expressed mainly by double bouquet and bipolar cells localized in layers II and III, and also by other non-identified neurons in infragranular layers (Conde *et al.*, 1994; Gabbott and Bacon, 1996a). A similar percentage of CR expression in GAD67-GFP cells in layers II-III of the neocortex has recently been reported (Tamamaki *et al.*, 2003). An upregulation of CR expression by some GAD65-GFP cells may well occur at postnatal stages. Remarkably, almost all CCK-containing interneurons in all layers of the P21 neocortex expressed GFP and very few of the PV-positive cells expressed GFP at detectable levels. As PV cells account for ~50% of neocortical interneurons (Gonchar and Burkhalter, 1997), it is conceivable that a large proportion of the GFP-negative population of GABA-positive neurons are PV cells. This would suggest that subpopulations of GABAergic interneurons characterized by expression of distinct neurochemical markers also have a different embryonic origin.

GAD65-GFP Cells Migrate to the Cortex via the SVZ

The highest density of migrating GAD65-GFP neurons in our tissue culture assay was found in the SVZ (BrdU-labeled proliferative region adjacent to the cortical VZ; Boulder Committee,

1970) of the cortex. This region has been previously shown to contain a large number of MGE-derived Lhx6- (Lavdas *et al.*, 1999) and CB-expressing cells (Del Rio *et al.*, 2000) and to be the main migratory pathway for MGE cells (Wichterle *et al.*, 1999). Interestingly, when GAD65-GFP slices from late embryonic stages were analyzed after 2DIV, many GAD65-GFP cells were observed migrating radially from the SVZ towards upper regions of the cortex. These results were also observed in fixed tissue at late stages of embryonic development and during the first postnatal week. Similar results have been reported from *in utero* studies using MGE-derived cells (Wichterle *et al.*, 2001) and a GAD67-GFP mouse (Tamamaki *et al.*, 2003). Together, these data suggest that tangentially migrating interneurons, independent of their neurochemical nature use the SVZ as a substrate for their migration. Once they arrive in the cortex, they may follow similar cues as pyramidal neurons, through radial-glia interactions (Wichterle *et al.*, 1997), to reach their final destination layer in the cortex. Migration towards the VZ (ventricle-directed migration) by some tangentially migrating interneurons has also been described (Nadarajah *et al.*, 2002), suggesting that some interneurons could require cues from the underlying VZ to migrate to the appropriate layer within the cortex. Using real-time imaging these authors described that after pausing at the VZ, tangentially migrating cells resume their migration radially in the direction of the pial surface to take up their positions at the CP. This feature has also been observed in a GAD67-GFP mouse (Tanaka *et al.*, 2003). This study showed that some GABAergic interneurons migrate radially to the MZ where a multidirectional tangential migration occurs. These radially oriented interneurons may well correspond to the GAD65-GFP cells we describe here.

CGE as a Source of GAD65-GFP Interneurons

Cell-tracing experiments using fluorescent dyes in organotypic cultures, and *in utero* transplantation studies have shown the MGE to be a major source of cortical interneurons (Lavdas *et al.*, 1999; Wichterle *et al.*, 1999; Anderson *et al.*, 2001; Jimenez *et al.*, 2002; Nadarajah *et al.*, 2002; Polleux *et al.*, 2002). LGE-derived cells have been shown to give rise to the projection neurons of the striatum (Olsson *et al.*, 1998; Marín *et al.*, 2001), interneurons of the olfactory bulb (Lois and Alvarez-Buylla, 1994; Wichterle *et al.*, 2001) and some interneurons of the cortex (De Carlos *et al.*, 1996; Tamamaki *et al.*, 1997). Our organotypic culture experiments provide additional evidence that the MGE is an important source of cortical interneurons. However, we have also shown here that the contribution from CGE to the GAD65-GFP population of interneurons is quantitatively at least as important at all embryonic ages studied compared to LGE and MGE. Indeed, after E15 when more GAD65-GFP cells migrate towards the cortex, a significantly larger fraction of these cells were labeled from CGE injections. A recent study has implicated the CGE as a source of distinct cortical and subcortical interneurons (Nery *et al.*, 2002). These authors described that CGE-derived neurons are located in layer V of the neocortex and express 17% CB, 27% SOM and only 3% PV (MGE-derived cells give rise to 30% of PV positive interneurons). In our case, although GAD65-GFP cells preferentially target layers II and III of the neocortex those neurons that were located in layer V expressed similar percentages of SOM (30%) and PV (4%) (but only 6% CB). The experiments performed by Nery and colleagues were done using E13.5 CGE-derived cells, and it is possible that CGE cells at later stages of

development could additionally give rise to supragranular interneurons. Future *in utero* transplantation studies using GAD65-GFP CGE-grafts will help resolve whether it is the GAD65-GFP cells from the CGE that target supragranular layers.

A recent study performed by Tamamaki and colleagues in the motor cortex of a GAD67-GFP mouse (Tamamaki *et al.*, 2003) showed some differences in the percentages of CR, SOM and PV in layers II–III and V of the neocortex compared to our study in the GAD65-GFP mouse. Overall, the proportion of CR-positive cells was smaller among GAD67-GFP cells (~14% compared to ~43% in the GAD65-GFP), whereas PV-positive and SOM-positive occurred at a higher percentage (PV: ~40% compared to ~2%, and SOM: ~23% compared to ~5%). The GABA level in the GAD67-GFP knock-in mouse brains is significantly reduced at birth compared to wild-type mouse (Tamamaki *et al.*, 2003), and it remains a possibility that this reduction in GABA level might affect the anatomical development and alter the total number of GABAergic neurons or the number of specific subpopulations of interneurons in this mouse. However, these GABAergic interneurons might also have a different origin. There is evidence that ~70% of the MGE-derived cortical cells express SOM or PV (for review see Xu *et al.*, 2003), suggesting that at least a proportion of the GAD67-GFP cells originates in the MGE. Also, it remains unknown whether the origin and distribution of different classes of interneurons are the same or vary between neocortical areas.

Layer Specificity for the GAD65-GFP Interneurons

Cell lineage studies have established that specification of the projection neuron phenotype takes place at the level of the progenitor, even before the onset of the neurogenesis (Tan *et al.*, 1998). Thymidine birth-dating studies have shown that the final destination of any given cortical neuron depends on its date of generation: early-born neurons are destined for layers VI, V and IV, while late-born neurons will populate layers II and III. We have shown using BrdU birth-dating studies that late-born GAD65-GFP cells colonize mainly layers II and III while early-born GAD65-GFP cells are localized in infragranular layers of the adult neocortex, strongly supporting the ideas of target-layer specification and commitment also for interneurons. Similar results were obtained by Valcanis and Tan (2003), who showed that early-born MGE cells populate lower cortical layers, principally layer V, whereas late-born progenitors will target layers II and III of the neocortex. However it is not yet known whether such layer-fate specification is a general feature of all three GEs. Another important question that remains to be answered is whether interneurons of upper and deeper cortical layers share the same origin and lineage. Future *in vivo* and *in vitro* experiments using the GAD65-GFP mouse will provide some illuminating answers.

Notes

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