Distinct Origins of Neocortical Projection Neurons and Interneurons *In Vivo*

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Recent studies in rodents have suggested that some cortical GABAergic interneurons arise within the neuroepithelium of the subcortical telencephalon then migrate dorsally into the cerebral cortex. These studies have relied heavily on short-term organotypic culture methods and on the analysis of mutant mice that die during the neonatal period. The purpose of this study is to ascertain directly whether cells labeled in the subcortical telencephalon *in vivo* differentiate into mature cortical interneurons and whether any cortical interneurons arise from the dorsal, cortical neuroepithelium. Mitotic cells within the neonatal cortex or subcortical telencephalon were labeled by focal injections of [^{3}H]thymidine into the brains of neonatal ferrets. The fates of labeled cells were assessed in mature animals 6 weeks later. Our results suggest that many cortical interneurons, but not cortical projection neurons, derive from the subcortical telencephalon. Conversely, cortical projection neurons, but few if any interneurons, are generated within the proliferative zones of the neocortex.

Introduction

There are two general classes of neocortical neurons: excitatory projection neurons and inhibitory local circuit neurons (interneurons). Recent evidence suggests that these two cell classes are generated from distinct origins. Studies of cultured brain slices have revealed that cells containing GABA migrate tangentially from the ventral telencephalon into the neocortex (Anderson *et al*., 1997a, 2001; Chapouton *et al*., 1999; Lavdas *et al*., 1999; Wichterle *et al*., 1999). These findings *in vitro* are consistent with lineage experiments indicating that separate progenitors give rise to cortical projection neurons and interneurons (Parnavelas *et al*., 1991; Luskin *et al*., 1993; Mione *et al*., 1994, 1997) and that clones of GABAergic neurons are tangentially dispersed, whereas radially arranged clones are comprised primarily of projection neurons (Tan *et al*., 1998). In addition, several lines of knockout mice that display abnormalities in proliferative tissues of the ventral telencephalon show marked reductions in the numbers of cortical GABA-immunoreactive cells present in the neocortex at birth. These include mutants for the transcription factors *Dlx1* and *Dlx2* – 75% decrease in neocortical GABA cells at P0 (Anderson *et al*., 1997); *Nkx2.1* – 50% decrease in neocortical GABA cells at E18.5 (Sussel *et al*., 1999); and *MASH1* – 50% decrease in neocortical GABA cells at E18.5 (Casarosa *et al*., 1999). Recently, the sources of cortical interneurons have been tracked by dissociating cells from the embryonic telencephalon of genetically altered 'marker' mice and injecting the labeled cells homotopically into wild-type embryonic hosts. Many MGE-derived cells transplanted in this way migrated into the neocortex *in vivo* and differentiated into interneurons (Wichterle *et al*., 2001). Finally, immunohistochemical analysis has revealed that GABA-expressing cells within the intermediate zone and lower marginal zone have a morphology and orientation consistent with a lateral to medial migration, from the ganglionic eminences into the neocortex (Van Eden *et al*., 1989; DeDiego *et al*., 1994). Collectively, these studies have provided compelling evidence that GABA-expressing cells derived from the ganglionic eminences of the ventral telencephalon migrate tangentially into and disperse themselves within the developing rodent neocortex.

Although these studies lend strong support to the notion that some cortical interneurons derive from the ventral telencephalon, their interpretation has been complicated by a dependence on cultured slices and by the assumption that GABA-expressing cells in the cortex of neonatal-lethal mutants would become interneurons in the mature cortex. These studies also leave open the question of whether and to what extent some interneurons might originate locally within the proliferative zone (PZ, the ventricular and subventricular zones) of the neocortex itself. Two lines of evidence suggest that some cortical interneurons may be cortically derived. First, cortical progenitor cells are able to generate GABA- expressing neurons *in vitro* (Gotz *et al*., 1995; Dooley *et al*., 1997; Pappas and Parnavelas, 1997; He *et al*., 2001). Second, disruptions of ganglionic eminence development results in only partial losses of cortical GABAergic cells (Anderson *et al*., 1997, 2001; Casarosa *et al*., 1999; Sussel *et al*., 1999; Sarkisian *et al*., 2001). Indeed, despite the nearly complete absence of migration from the GE to cortex in slice cultures of *Dlx1* and *Dlx2* mutants, the reduction of neocortical GABA-expressing cells is only 75% (Anderson *et al*., 1997, 2001).

To examine whether interneurons in the mature neocortex derive from the ventral telencephalon, and whether the cortical proliferative zone also gives rise to neocortical interneurons, we have studied the spatial origin of cells within the ferret telencephalon *in vivo*. Neurons destined for neocortical layers II and III continue to be generated during early postnatal life in the ferret (McConnell, 1988; Jackson *et al*., 1989; Noctor *et al*., 1997). Using postnatal day (P)1 kits, we placed focal injections of [^{3}H]thymidine into either the neocortical PZ, or into the ganglionic eminence that gives rise to the basal ganglia. The fates of labeled cells were examined in the adult cortex, 6 weeks later. We found that injections of [^{3}H]thymidine placed in the neocortical PZ labeled projection neurons [GluR2/3- and calcium/calmodulin-dependent protein kinase II (CaMKII)], but generally few interneurons (labeled with antibodies to GABA, calbindin, calretinin, parvalbumin, or somatostatin). In contrast, injections of [^{3}H]thymidine into the striatal PZ labeled neocortical interneurons, but not projection neurons. These results suggest that, for the cohort of neocortical interneurons born on P1, very few seem to derive from the underlying cortical PZ, while many mature neocortical interneurons do indeed originate within the subcortical (ventral) telencephalon.

Materials and Methods

Focal Injections of [^{3}H]Thymidine

Experiments were performed on P1 ferrets (Marshall Farms, NY), an age

at which neurons destined for the superficial cortical layers are being born (McConnell, 1988; Jackson *et al*., 1989). P1 kits were anaesthetized with halothane (1–4% in O_2). A midline incision was made over the scalp and a small hole made through the cranium and dura using forceps. A glass micropipette filled with [^{3}H]thymidine (75 μCi/μl) and fluorodextran (D1280, 50 mg/ml; Molecular Probes) was lowered into the brain using a hydraulic microdrive (Kopf). Injections into the ganglionic eminence were made ~2 mm lateral and rostral to bregma at a depth of 4–5 mm from the pial surface. Injections into the rostral neocortex were placed ~2 mm lateral and rostral to bregma at a depth of 1500–1800 μm and caudal cortical injections were placed ~1.5 mm rostral and lateral to lambda at a depth of 1100–1200 μm. [^{3}H]Thymidine/fluorodextran (~20 nl) was injected using a picospritzer (General Valve). The micropipette was withdrawn and the skin covering the skull sutured closed, then animals were allowed to recovered and to survive for either 2–24 h (to view injection sites) or 6 weeks (to view neurons in their final positions).

As a control for the focal injections, two P1 animals received an i.p. injection of [^{3}H]thymidine (0.5 mCi) to label the progeny of cells regardless of their spatial origin within the brain. The fates of labeled cells were assessed at 6 weeks of age.

Perfusions, Histology and Autoradiography

Animals were deeply anesthetized with nembutal and perfused through the heart with 4% paraformaldehyde, postfixed for 4–6 h and sunk in fixative containing 20% sucrose. Sections were cut at 12 μm on a cryostat, collected onto glass slides (Superfrost Plus) and subjected to immunohistochemistry using the following antibodies: rabbit anti-GABA (1:2000, Sigma); rabbit anti-calbindin (1:2000, Swiss Antibodies); rabbit anti-calretinin (1:1000, Chemicon); rabbit anti-parvalbumin (1:2000, Swiss Antibodies); rat anti-somatostatin (1:200, Chemicon); rabbit anti-GluR2/3 (1:2000, Chemicon); mouse anti-CaMKII (1:1000, Roche; 1:1000, Sigma); and rabbit anti-GFAP (1:1000, Chemicon). Immunoreactivity was revealed using biotinylated secondary antibodies (Vector) and the ABC elite kit (Vector). Sections were then dehydrated through graded alcohols and xylene, dried and subjected to autoradiography for 40 days under NTB2 photographic emulsion (Kodak) to visualize [^{3}H]thymidine-labeled cells.

Microscopy and Quantification

Sections were examined with a Nikon E400 microscope fitted with a Darklite Illuminator (Micro Video Instruments). [^{3}H]Thymidine-labeled cells were identified under darkfield by the aggregations of silver grains and then examined for double-labeling in brightfield. For experiments in which the ganglionic eminence was injected, the positions and phenotypes of [^{3}H]thymidine-labeled cells were determined in four different animals. Three animals received injections into the rostral cortical proliferative zone and four animals received injections into the caudal (visual) cortical proliferative zone. For each animal injected, double-labeling for [^{3}H]thymidine and various immunohistochemical markers was assessed in at least two and generally three sections. Statistical comparisons (for differences in the percentage of cortical [^{3}H]thymidine-labeled cells that also labeled for GABA or for GluR2/3) between experimental conditions (GE, cortex or i.p. injections), were made using unpaired *t*-tests, with the average value for each animal serving as the base unit.

It was crucial to ascertain that injections of [^{3}H]thymidine were targeted correctly to the appropriate brain region and that the injection sites did not involve additional brain structures. Fortunately, [^{3}H]thymidine injections label not only cells that generate young neurons, which disperse away from the injection site through active migration, but also cells that remain in the PZ and are found later in the adult ependymal zone. We believe that these latter cells migrate little, if at all, after [^{3}H]thymidine incorporation, since they remain clustered in a small region after a focal injection (Fig. 2*A*). In contrast, when [^{3}H]thymidine was injected deliberately into the lateral ventricle, [^{3}H]thymidine-labeled cells were found scattered throughout the entire extent of the dorsal and ventral ependymal zones and in the choroid plexus (data not shown). We therefore developed the following criteria for inclusion in the analysis of focal injection sites. In ganglionic eminence experiments, animals included for analysis had [^{3}H]thymidine-labeled cells in the ependymal zone of the ventral third of the lateral ventricle and no labeled cells in the choroid plexus or in the dorsal (cortical) region of the lateral ventricle ependyma. For experiments in which [^{3}H]thymidine was injected into the rostral neocortical PZ, animals were included if labeled cells were found in the ependyma dorsal to the lateral ventricle, but not in the ependyma that lies lateral to the lateral ventricle or in the choroid plexus. Finally, for injections into the PZ of visual (occipital) cortex, animals were included if labeled cells were present in the dorsal wall of the lateral ventricle, but not in the ventral ependymal zone or choroid plexus. A total of four animals met the inclusion criteria for ganglionic eminence injections, three for rostral neocortical injections and three for visual cortical injections.

Within each brain that met the criteria for inclusion, the positions and phenotypes of cells labeled with [^{3}H]thymidine were assessed in brain sections after processing for autoradiography and immunohistochemistry. For ganglionic eminence injections, [^{3}H]thymidine-labeled cells were counted in the cortical grey matter of coronal sections, from the cingulate gyrus, beginning immediately dorsal to the corpus callosum, to the gyrus on the brain's lateral surface that lies at the same dorso- ventral level as the corpus callosum. Sections were collected ~3 mm on either side of the injection site, the approximate location of which could be identified by the hole created in the skull during the injection. For rostral cortical injections, [^{3}H]thymidine-labeled cells were counted in the cortical grey matter of coronal sections from the cingulate sulcus laterally until 100 cells were counted (generally a distance of ~2 mm). For caudal cortical injections, [^{3}H]thymidine-labeled cells were counted in the dorsal regions of cortex from parasagittal sections. Finally, for i.p. injections of [^{3}H]thymidine, labeled cells were counted in coronal sections through the rostral cortex, as described above for the rostral cortical focal injection experiments.

For quantification of the laminar distribution of [^{3}H]thymidine-labeled cells, the positions of labeled cells were reconstructed using darkfield microscopy and a camera lucida drawing tube (Nikon). Sections were counterstained with cresyl violet to enable the identification of laminar borders using brightfield optics. The positions of labeled cells were then tabulated into histograms for each type of injection. One section from one animal was used for the rostral cortex and for the i.p. injection. To obtain an adequate number of cells, two sections from one animal were used for the GE injection. Because the total numbers of labeled cells varied greatly among types of injections, it was not possible to count precisely the same number of labeled cells in each experimental condition; however, the same regions of the cortex were surveyed in the GE and rostral cortex cases to ensure that the areal distributions of labeled cells were comparable.

Results

Placement of Focal Injections in the Developing Striatum or Neocortex

To track the adult fates of neurons generated in either the ganglionic eminence of the developing striatum or the PZ of the neocortex, focal injections of [^{3}H]thymidine were placed into either region in neonatal (P1) ferrets. We determined the approximate size and location of these injections by killing several animals 1 h after injection and processing their brains for autoradiography. Figure 1*A* and *B* show an example of an acute injection into the ventral ganglionic eminence. Although cells were labeled in the proliferative zone of the GE, no labeled cells were present in the cortical VZ or in the choroid plexus. Animals that received an injection of [^{3}H]thymidine into the neocortical PZ and were killed 1 h later showed a similarly clustered distribution of labeled cells, only in these animals the labeled cells were found in the PZ underlying the neocortex and no labeled cells were present in the subcortical PZ or the choroid plexus (Fig. 1*C*,*D*). In contrast to these focal injections, when [^{3}H]thymidine was injected into the lateral ventricle, labeled cells were found throughout the entire PZ of that hemisphere and within the choroid plexus (data not shown).

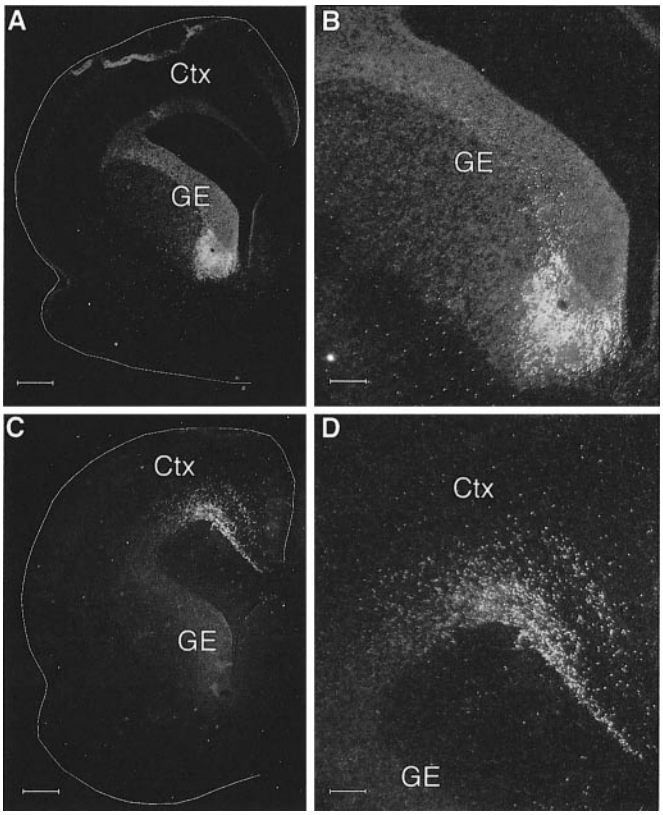

Figure 1. Distribution of labeled cells 1 h after injection of [^{3}H]thymidine on P1. P1 kits received a focal [^{3}H]thymidine injection into either the GE (*A*, *B*), or into the cerebral cortex (*C*, *D*), followed by 1 h survival. Shown are darkfield autoradiographs of coronal brain sections; dorsal is up, medial is to the right. The pial surface of the brain is outlined in white in (*A*, *C*). Scale bar = 500 μm in (*A*, *C*) and 200 μm in (*B*, *D*).

We then studied the distribution of the [^{3}H]thymidine-labeled cells several weeks following the injection on P1. [^{3}H]Thymidine injections label not only the neurons that are being generated on P1, but they also label a small set of cells that remain in the ependymal zone, which develops from the remnants of the PZ (McConnell, 1988). By determining the location of labeled ependymal cells, we were able to estimate the site of each original injection. An example of the distribution of [^{3}H]thymidine-labeled cells in a 6-week-old animal that received a ventral GE injection at P1 is shown in Figure 2*A*. Note that labeled cells are present in the ependymal region at the base of the lateral ventricle, but not in the ependyma dorsal to the lateral ventricle. Labeled cells are also scattered throughout the ventral pallidum, striatum and cortex; the distributions and phenotypes of the latter will be discussed below. Analysis of sections from mature animals that received a focal injection of [^{3}H]thymidine into the neocortical PZ also revealed cases in which labeled ependymal and subependymal cells were confined to the dorsal domain of the lateral ventricle (cf. the case shown in Fig. 2*B*; ependymal cells were present in other sections). Brains that showed similar restrictions in the distribution of labeled ependymal cells were included for further analysis (see Materials and Methods). In contrast to these examples of focal injections, brains from control animals that received an i.p. injection of [^{3}H]thymidine on P1 contained labeled ependymal cells along the entire lateral ventricle, as expected (Fig. 2*C*).

Mitotic Cells in the Ganglionic Eminence can Become Cortical Interneurons In Vivo

To determine the fates of cortical cells born within the ventral GE on P1, animals were allowed to survive for 6 weeks and their brains were processed for [^{3}H]thymidine autoradiography and for immunohistochemistry using a variety of markers. Four animals met the criteria for focal injection into the ventral GE

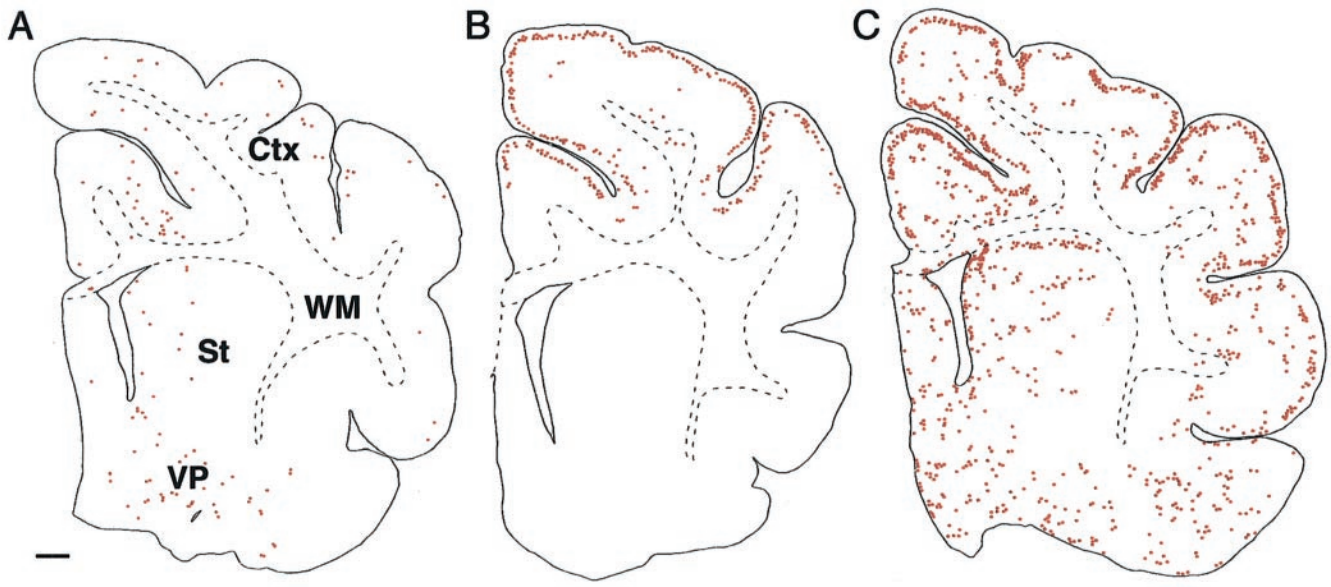

Figure 2. Distribution of [^{3}H]thymidine-labeled cells at 6 weeks. P1 kits received a focal [^{3}H]thymidine injection into either the GE (*A*), the cerebral cortex (*B*), or an i.p. injection (*C*) and the positions of [^{3}H]thymidine-labeled cells (red dots) were reconstructed from coronal brain sections (dorsal is up, medial is to the left). (*A*) Following injections into the ventral GE, more cells are found in the ventral pallidum than in the striatum, as expected. Focal GE injections also labeled cells that were scattered sparsely across a wide domain of the neocortex. (*B*) Cells labeled by focal cortical injections were restricted to the neocortex and occupied a more limited medial–lateral extent compared to GE injections. (*C*) Cells labeled after an i.p. injection of [^{3}H]thymidine were found throughout the brain. Abbreviations: Ctx, cortex; St, striatum; VP,ventral pallidum; WM, white matter. Scale bar = 2 mm.

(labeled cells present in the ependyma of the lower third of the lateral ventricle) and the positions and phenotypes of labeled cells were assessed in three sections each from each animal. Figure 2*A* shows an example of the distribution of [^{3}H]thymidine-labeled cells in one animal. In concordance with the migration pattern of ventral GE cells *in vitro* (de Carlos *et al.*, 1996; Anderson *et al.*, 1997, 2001; Tamamaki *et al.*, 1997; Chapouton *et al.*, 1999; Lavdas *et al.*, 1999; Wichterle *et al.*, 1999; Marin *et al.*, 2000; Letinic and Rakic, 2001), labeled cells were scattered throughout the ventral pallidum, striatum and neocortex. Within the cortex, cells were present across the full medial–lateral extent of cortex (see, for example, Fig. 2*A*) and across at least 6 mm of cortex on the rostral–caudal axis.

Within the neocortex, many labeled cells expressed markers characteristic of cortical interneurons. Figure 3 shows examples of [^{3}H]thymidine-labeled cells that were immunoreactive for the neurotransmitter GABA and the GABA synthesizing enzyme GAD67. Results from all four animals are shown in Table 1. Despite a variable number of [^{3}H]thymidine-labeled cortical cells per section and per animal (averaging 7.7, 0.7, 1.3 and 38 cells/section), many [^{3}H]thymidine-labeled cortical cells were double-labeled for GABA (68, 40%, 40 and 75%, respectively). Double-labeling for GAD67 in sections from the same four animals produced similar results (56, 75, 67 and 66%). These results indicate that some cortical interneurons originate in the subcortical telencephalon *in vivo* and that more than half of the labeled cells that migrated into the cortex from the basal telencephalon express markers of cortical interneurons.

To determine whether subtypes of cortical interneurons are differentially labeled after focal GE injections, we ascertained whether [^{3}H]thymidine-labeled cells also expressed calretinin (not shown), parvalbumin, calbindin, or somatostatin (Fig. 3). When data from all four animals were combined, 23% of [^{3}H]thymidine-labeled cortical cells were also labeled for parvalbumin. However, the proportion of cortical cells double-labeled for parvalbumin was highly variable (1/26 from animal 1; 3/11 from animal 2; 2/10 from animal 3; 25/86 for animal 4). A similar pattern emerged for the calcium binding protein calbindin: 17% of [^{3}H]thymidine-labeled cells in the cortex were also calbindin positive (1/26, 2/16, 1/16 and 29/168 for each animal, respectively). Somatostatin was expressed by 6 of the 99 [^{3}H]thymidine-labeled cells from the four animals. Interestingly, calretinin, which is readily detectable in a substantial subpopulation of cortical interneurons, was present in none of 221 cells counted. The absence of calretinin positive cells among the population generated in the P1 GE is consistent with the possibility that different subtypes of cortical interneurons may have distinct spatial or temporal origins.

To ascertain whether the GE also generates excitatory projection neurons destined for the neocortex, cells labeled with [^{3}H]thymidine by focal GE injections were also immunostained

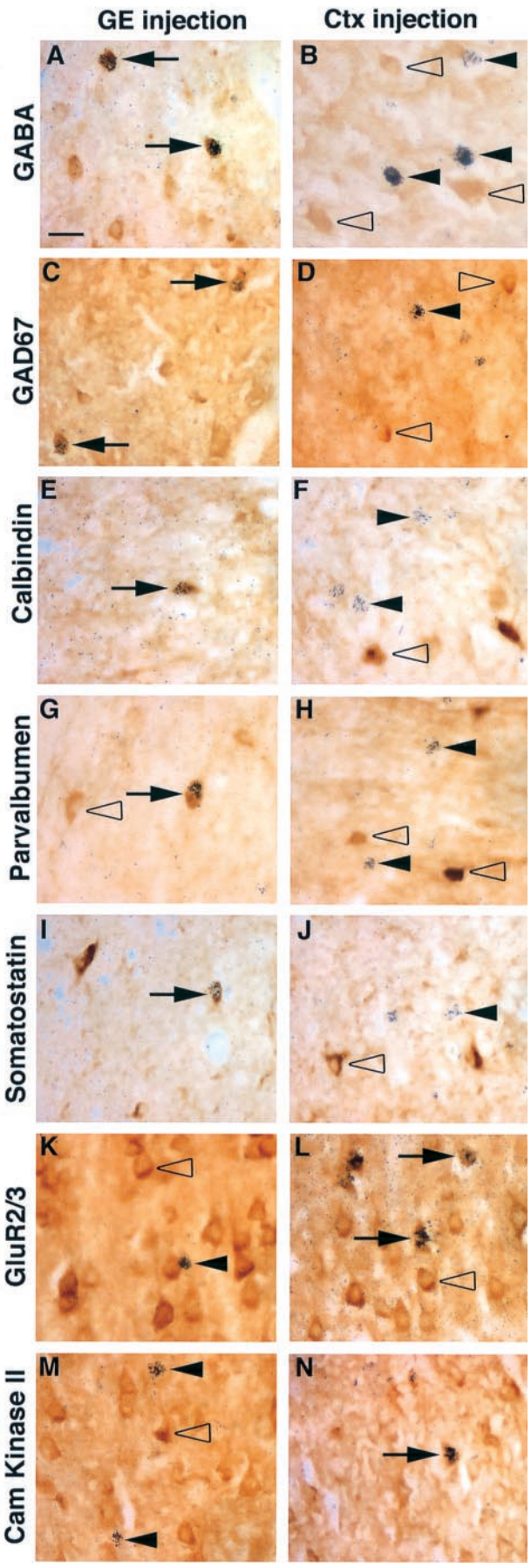

Figure 3. Immunohistochemical labeling of [^{3}H]thymidine-labeled cells in the neocortex at 6 weeks. The [^{3}H]thymidine-labeling appears as black dots overlying the cells in these brightfield autoradiographs. Antibody labeling produced a brown reaction product. Filled arrowheads indicate examples of cells labeled by [^{3}H]thymidine only, open arrowheads indicate cells labeled by immunohistochemistry only and filled arrows indicate double-labeled cells. In the left column, neurons were labeled by a focal injection of [^{3}H]thymidine into the ganglionic eminence (GE) of P1 kits. In the right column, neurons were labeled by injection into the neocortex. Markers for interneurons (A–J) label cortical neurons from the GE (A, C, E, G, I), but rarely label cortical neurons from the cortical proliferative zone (PZ; B, D, F, H, J). In contrast, markers of cortical projection neurons (K–N) label cortical neurons from the cortical PZ (L, N), but rarely label cortical cells from the GE (K, M). Scale bar = 20 μm.

Table 1

Tabulation of the immunohistochemical profiles of cortical [^{3}H]thymidine-labeled cells at 6 weeks in animals injected with [^{3}H]thymidine on P1, using double-labeling of interneuron or projection neuron markers and [^{3}H]thymidine (dbl./total, %)

	GE	Rostral Ctx	Caudal Ctx	Intraperitoneal
GABA	147/202 (73%)	30/763 (4%)	89/1200 (7%)	119/600 (20%)
GAD67	111/168 (66%)	4/782 (1%)	60/1200 (5%)	136/600 (23%)
Parvalbumin	31/133 (23%)	1/720 (0%)	1/1174 (0%)	
Calbindin	29/168 (17%)	1/810 (0%)	5/1100 (0%)	
Calretinin	0/221 (0%)	1/828 (0%)	0/900 (0%)	
Somatostatin	6/99 (6%)	0/778 (0%)	0/300 (0%)	
CaMIIK	0/181 (0%)	196/775 (25%)	786/1200 (66%)	222/600 (37%)
GluR2/3	13/188 (7%)	485/882 (55%)	916/1200 (76%)	343/619 (55%)

Ganglionic eminence, $n = 4$; rostral cortex, $n = 3$; caudal cortex, $n = 4$; i.p.,, $n = 2$). For each antibody, data were pooled from three sections from each experimental animal and are expressed as the number of double-labeled cells for the corresponding antibody, the total number of [^{3}H]thymidine cells counted and the percentage of cells that showed double-labeling.
Abbreviations: GE, ganglionic eminence; Ctx, cortex.

with markers for projection neurons. Figure 3 shows examples of sections stained with antibodies against the GluR2/3 subunit of the AMPA glutamate receptor and for CaMKII; the data for all four animals that received focal GE injections are summarized in Table 1. Of 181 [^{3}H]thymidine-labeled cortical cells, none expressed CaMKII. GluR2/3, which is also primarily present in projection neurons, was expressed in only 13 of 188 cortical cells that originated in the GE, most (8/13) of the double-labeled GluR2/3 cells were located in layer I, where they probably represent a subtype of interneuron (Vickers *et al.*, 1993).

In sum, focal injections of [^{3}H]thymidine into the GE of the ventral telencephalon *in vivo* labeled cells that migrated into the neocortex and differentiated into interneurons. Many of these cells expressed GABA, some expressed the interneuron markers parvalbumin or calbindin and a few expressed detectable levels of somatostatin or calretinin. In contrast, none expressed the pyramidal neuron marker CaMKII and most of the few that expressed GluR2/3 are likely to represent a subtype of cortical interneuron that resides in layer I. Thus, we conclude that the majority of GE-derived neurons that migrate into and differentiate within the dorsal telencephalon become interneurons in the mature cerebral cortex.

Mitotic Cells in the Neocortex Primarily Become Cortical Projection Neurons

To follow the fates of cortical cells born within the PZ of the neocortex, P1 ferrets received focal injections of [^{3}H]thymidine into either the caudal (occipital) PZ or the rostral (somatomotor) PZ. The animals survived for 6 weeks and their brains were processed for both [^{3}H]thymidine autoradiography and immunohistochemistry. A total of seven animals met the criteria for focal injection into the cortical PZ (rostral injections, $n = 4$; caudal injections, $n = 3$); this procedure did not result in ependymal labeling of the subcortical telencephalon or choroid plexus. At maturity, the majority of the labeled cells were located in the superficial layers of the neocortex (Fig. 2*B*). No labeled cells were found in the striatum, consistent with previous studies suggesting that cells derived from the cerebral cortex generally do not migrate into subcortical structures (Fishell *et al.*, 1993; Chapouton *et al.*, 1999). Within the neocortex, labeled cells were dispersed over several millimeters of cortical tissue (Fig. 2*B*) and spanned multiple cortical areas. For example, when focal injections were placed caudally in the occipital cortex, labeled neurons occupied areas 17, 18 and 19 and the splenial visual area, and possibly extended into more rostral regions as well (data not shown). Because it is impossible to determine the precise volume or diameter of the original injection site within the PZ, it is not possible to calculate how far the labeled neurons dispersed tangentially relative to their site of origin. However, these results are consistent with previous studies, both *in vivo* (O'Rourke *et al.*, 1995, 1997) and *in vitro* (O'Rourke *et al.*, 1992), showing that young cortical neurons can use both radial and tangential pathways for migration away from the VZ and that tangential migration can occur in all directions (O'Rourke *et al.*, 1997).

The phenotypes of [^{3}H]thymidine-labeled cells were assessed in three sections from each animal for each immunohistochemical marker. Examples of single- and double-labeled cells are shown in Figure 3. In the three animals that received rostral cortex injections at P1, GABA was detected in [^{3}H]thymidine-labeled cells rarely and with considerable variability (range, 1.2–4.5%: 3/247 cells were double-labeled in animal 1, 17/294 cells in animal 2 and 10/222 cells in animal 3). We found higher percentages of double-labeled cells in sections that showed high levels of background staining, raising the possibility that much of the co-labeling may be false. GAD67, one of the synthesizing enzymes for GABA that is also present in most cortical interneurons, was detectable in many fewer cortically derived cells (only 4/782 cells were double-labeled, from the three animals combined). To assess further whether the double-labeling of cortically derived cells with GABA or GAD67 indicates that some interneurons derive from the cortical neuroepithelium, co-labeling for [^{3}H]thymidine and markers of interneuron subtypes was also examined (Fig. 3 and Table 1). In contrast to the GE-derived cortical cells, very low numbers of cortically derived cells co-labeled for parvalbumin (1/720), calbindin (1/810), or somatostatin (0/778).

We also assessed the percentage of GABA and [^{3}H]thymidine co-labeling after injections into the visual cortical neuroepithelium (Table 1). The results were similar to those from rostral cortex injections, ranging from virtually none to ~10% double-labeling, depending on the animal. Again, we observed that a higher fraction of double-labeled cells was found in sections with higher levels of background staining. It thus remains unclear as to whether the dorsal neuroepithelium produces a small number of cortical interneurons, or virtually none (see Discussion).

Intraperitoneal Injections of [^{3}H]thymidine at P1 Labels both Cortical Projection Neurons and Interneurons

To compare the generation of GABAergic cells in the cortex or in the GE to the overall generation of GABAergic cells, regardless of location, two animals received i.p. injections of [^{3}H]thymidine on P1. The fates of [^{3}H]thymidine-labeled cells in the cortex were assessed at 6 weeks of age using immunohistochemistry for GABA, GAD67 and GluR2/3 (Table 1). Approximately 20% of the [^{3}H]thymidine-labeled cortical cells were also labeled for GABA (119/600; combined total for three sections from each of the two animals) and 24% co-labeled for GAD67 (136/600). In contrast, 55% (343/619) of cortical thymidine-labeled cells co-labeled for the pyramidal neuron marker GluR2/3. Statistical analysis revealed significant differences between the percentage of cortical cells that were double-labeled for both GABA and [^{3}H]thymidine in focal cortical versus i.p. injections (d.f. = 3, $P < 0.003$) and between results from focal GE versus i.p. injections (d.f. = 4, $P < 0.02$). These results reveal that the GE produces a higher than expected fraction of interneurons relative to the overall production of GABAergic neurons and that the neocortical PZ produces many fewer interneurons than

would be expected. Significant differences were also found between the percentage of cortical GluR2/3 and [^{3}H]thymidine double-labeled cells in focal GE versus i.p. injections (d.f. = 4, $P < 0.0001$) and between focal GE and focal cortical injections (d.f. = 5, $P < 0.0001$). These results reveal that many fewer cortical projection neurons than expected were produced in the GE. The percentages of cortical [^{3}H]thymidine-labeled cells that also express GABA or GluR2/3, for each of the four injection locations, are shown in Figure 4.

Cortical Cells from the Cortical and Subcortical Telencephalon have Similar Laminar Distributions

To compare the laminar distributions of cortical cells from ventral versus dorsal origins, the laminar locations of [^{3}H]thymidine cells were plotted in coronal sections following either GE or rostral cortical injections. As shown in Figure 5, cells labeled with [^{3}H]thymidine on P1 primarily occupy the superficial cortex (layers II and III), regardless of whether the cells were derived from the dorsal or ventral telencephalon. This result is consistent with previous work revealing that cortical GABA and non-GABA expressing cells have similar birthdates (Peduzzi, 1988).

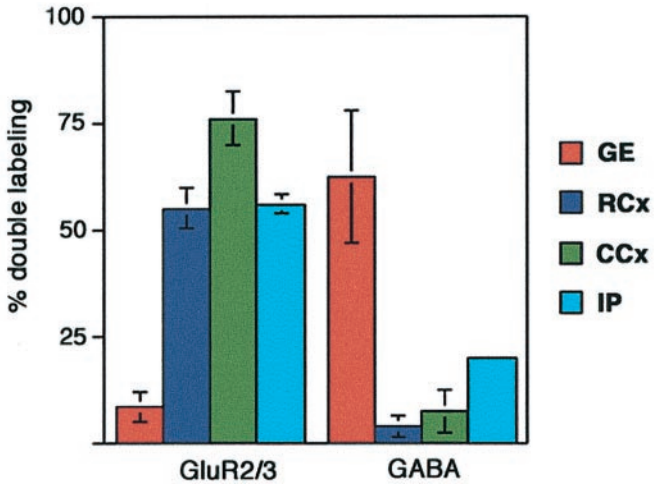

Figure 4. Percentages of [^{3}H]thymidine-labeled cortical cells (± SDs) that also label for GABA or GluR2/3 for each injection location. Abbreviations: GE, ganglionic eminence; RCx, rostral cortex; CCx, caudal cortex; IP,intraperitoneal.

Discussion

By placing small injections of [^{3}H]thymidine into targeted domains of the neuroepithelium *in vivo*, we have tracked the fates of cortical neurons that originated in the subcortical versus the cortical telencephalon. Proliferating cells in the medial ganglionic eminence give rise to mature cortical interneurons, but not to cortical projection neurons. In contrast, the neocortical proliferative zones give rise to cortical projection neurons but to very few interneurons. These results confirm and extend previous work on the generation of cortical interneurons and provide direct evidence *in vivo* that proliferating cells within the subcortical telencephalon give rise to GABAergic interneurons in the mature neocortex.

Mitotic Cells in the Ganglionic Eminence can Become Cortical Interneurons

Although a variety of studies have suggested that GABAergic cells from the subcortical telencephalon populate the neonatal cortex, this report demonstrates survival of many of these interneurons into maturity (Wichterle *et al.*, 2001). Focal injections of [^{3}H]thymidine into the ventral portion of the ganglionic eminence labeled cells scattered throughout the ventral pallidum and striatum, as well as the cerebral cortex. Within the neo- cortex, these cells were distributed across its lateral–medial extent (see Fig. 2*A*) and across at least 6 mm along the rostral–caudal axis. Within the cortex, roughly two-thirds of these cells co-labeled with antibodies that recognize GABA and/or GAD67, and a minority (~20%) were labeled with the interneuron markers parvalbumin or calbindin. In contrast, very few cortical cells that originated in the GE could be labeled with markers of cortical projection neurons. These results indicate that neurons that are generated in the neonatal GE and migrate into the neocortex have fates restricted to cortical interneurons *in vivo*.

Mitotic Cells in the Neocortex Primarily Become Cortical Projection Neurons

Previous studies on the origins of cortical interneurons have left open the question of whether a subpopulation of these cells derives from the cortical proliferative zone. Mice in which GE development was disrupted through targeted genetic manipulations have shown deficits in the production and/or differentiation of cortical interneurons, but typically the reductions in interneuron number have fallen in the range of only 50–75%. Thus it seems possible that the neocortical PZ is also a major source of interneurons. In line with this notion, studies of the

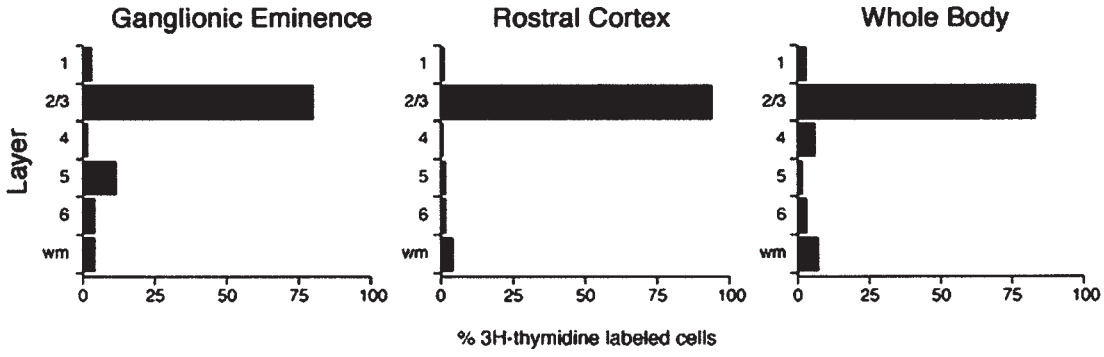

Figure 5. Laminar distribution of cells from GE, rostral neocortex, or whole body (i.p.) injections. Consistent with previous reports that an inside-out gradient of neurogenesis exists for both cortical projection neurons and interneurons (Fairén *et al.*, 1986; Peduzzi, 1988), cells born in either the GE or cortex at P1 primarily occupy the superficial cortical layers. Abbreviation: WM, white matter.

differentiation of cortical progenitors *in vitro* have suggested that these cells generate interneurons in culture and in numbers that bear a close resemblance to the percentage of interneurons seen *in vivo* (Gotz *et al*., 1995). However, an analysis of the phenotypes of lacZ chimeric mice has suggested that neocortical progenitors produce primarily or exclusively projection neurons *in vivo* (Tan *et al*., 1998). We attempted to resolve this issue by making focal injections of [^{3}H]thymidine into the cortical proliferative zone of neonates and assessing the fates of labeled neurons in the mature cortex 6 weeks later. As expected from previous birthdating studies (McConnell, 1988; Jackson *et al*., 1989), cells labeled by these injections migrated primarily into layers II and II. Roughly two-thirds of the [^{3}H]thymidine-labeled cells could be immunostained with antibodies that recognize GluR2/3 or CaMKII, markers of cortical projection neurons. In contrast, very few (<5%) of these cells labeled with the interneuron markers GABA or GAD67. Our control experiments showed that ~20% of the neurons generated on P1 from all spatial origins differentiate into GABAergic neurons; thus, the production of interneurons in the cortex accounts at best for only a small fraction of the interneurons present at maturity.

Due to high levels of background staining of the tissue with the GABA antibodies, it was difficult to determine whether our results indicate that no cortical interneurons derive from the cortical PZ, or whether a small population in fact does. We believe that the former possibility is more likely, for several reasons. First, experiments in which background staining was low gave rise to very small numbers of cortically derived neurons that co-labeled as interneurons. For example, only 4/782 [^{3}H]thymidine-labeled cells were immunolabeled for GAD67 in the rostral cortex injection (Table 1). Second, recent evidence suggests that most cortical pyramidal neurons also express low levels of GABA (Hill *et al*., 2001), thus raising the possibility that weak GABA labeling detected in some cortically derived cells in this study may be corroborating this result. Third, although a subpopulation of GE-derived cortical neurons labeled with the interneuron markers parvalbumin, calbindin, or somatostatin (23, 17 and 6%, respectively; Table 1), virtually none of these markers labeled cortical neurons derived from either the rostral or cortical injections. However, since putative cortically derived interneurons may comprise a set of subtypes distinct from those from the GE and since the rodent cortical neuroepithelium has the potential to generate GABAergic cells *in vitro* (Gotz *et al*., 1995; He *et al*., 2001), the question of whether a small population of cortical interneurons is cortically derived remains unsettled. In addition, this study covers only a limited period of neurogenesis, thus leaving open the possibility that cortically derived cortical interneurons may be generated at a different time.

Two recent studies using mouse strains that permit fate mapping from distinct progenitor regions also support the notion that few cortical interneurons derive from the cortical PZ. The first of these studies used mice expressing the cre recombinase under control of the cortex-specific *Emx1* promoter (Gulisano *et al*., 1996; Iwasato *et al*, 2000), which were crossed with reporter mice carrying an inactive, 'floxed' form of the *lacZ* gene (Iwasato *et al*., 2000). In these animals, the β-galactosidase-labeled cells expressed the expected markers of pyramidal neurons, but did not express GABA. Similar results have been obtained with another *Emx1-Cre* mouse line (K. Jones, personal communication). The second study utilized mice harboring a transgene containing an enhancer from the *Dlx5/6* locus driving expression of β-galactosidase (Stuhmer *et al*., 2002), which results in β-galactosidase expression by most GABAergic neurons of the forebrain, including embryonic and postnatal local circuit neurons in the neocortex. These results suggest that >95% of cortical GABAergic cells express *Dlx5/6* at some stage of their development and they support the idea that most cortical GABAergic neurons are derived from the subpallium in rodents.

Regulation of Interneuron Phenotype during Cortical Development

Taken together, the results of the present and previous studies strongly suggest that projection neurons and interneurons of the neocortex derive from distinct regions of the telencephalon. While the present results have dealt specifically and exclusively with the generation of interneurons at the end of neurogenesis in the ferret, for those cells that are destined for the upper layers II and III, the results of experiments on brain slices (de Carlos *et al*., 1996; Anderson *et al*., 1997, 2001; Tamamaki *et al*., 1997; Chapouton *et al*., 1999; Lavdas *et al*., 1999; Wichterle *et al*., 1999; Marin *et al*., 2000; Letinic and Rakic, 2001) and those performed *in vivo* with lineage tracers or chimeric mice (Parnavelas *et al*., 1991; Luskin *et al*., 1993; Mione *et al*., 1994, 1997; Tan *et al*., 1998) provide a similar view of telencephalic development. These studies suggest collectively that the most dorsal regions of the telencephalic neuroepithelium give rise to glutamatergic projection neurons. In contrast, the dorsal portion of the ventral telencephalon, the LGE, gives rise to GABAergic projection neurons of the striatum and globus pallidus (Marin *et al*., 2000), as well as to GABAergic interneurons of the olfactory bulb (Anderson *et al*., 1997b; Wichterle *et al*., 2001) and cerebral cortex. Finally, the most ventral regions of the telencephalon may also generate the cholinergic projection neurons of the nucleus basalis and cholinergic interneurons of the striatum (Marin *et al*., 2000).

This division of the developing telencephalon into progenitor domains in which neuronal fate may be restricted to a particular neurotransmitter phenotype suggests that similar mechanisms may control region-specific neurogenesis and neurotransmitter specification (Anderson *et al*., 1999; 2001; Parnavelas, 2000; Marin and Rubenstein, 2001). For example, the basic helix–loop–helix gene *Mash1* plays an important role in neurogenesis of the striatum (Casarosa *et al*., 1999; Horton *et al*., 1999). Ectopic expression of *Mash1* in the cortex induces expression of GAD67 (Fode *et al*., 2000). In addition, the transcription factor *Dlx2* which, together with *Dlx1*, has a crucial function in striatogenesis, induces GAD67 and GABA expression when expressed within cortical cells from *Dlx1/Dlx2* mutant mice (Anderson *et al*., 1999) or in slices of wild-type prenatal neocortex (Stuhmer *et al*., 2002). It remains to be determined whether genes that control neurogenesis within the dorsal telencephalon also function to induce a glutamatergic phenotype.

Evidence supporting the subcortical origin of cortical interneurons has now been obtained in rodents (Marin and Rubenstein, 2001), ferrets (this study), as well as in primates, including humans (Letinic and Rakic, 2001). Future work will be required to elaborate on the signals that generate subdivisions within the telencephalon and on the relationship of these signals to cell type specification. In terms of the development of cortical interneurons, it will be important to determine whether the phenotypic and functional diversity of interneuron subtypes is induced by signals at their origin within the GE, at their location of final differentiation in the cerebral cortex, or both.

Notes

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