Origins of Cortical GABAergic Neurons in the Cynomolgus Monkey

In human most cortical γ-aminobutyric acidergic (GABAergic) neurons are produced in the proliferative zones of the dorsal telencephalon in contrast to rodents. We report that in cynomolgus monkey fetuses cortical GABAergic neurons are generated in the proliferative zones of the dorsal telencephalon, in addition to the proliferative region of the ventral telencephalon, the ganglionic eminence (GE), however, with a temporal delay. GABAergic neuron progenitors labeled for Mash1 and GAD65 were present mainly in the GE at embryonic days (E) 47–55, and in the entire dorsal telencephalon at E64–75. These progenitors within the dorsal telencephalon are generated locally rather than in the GE. The ventral and dorsal lineages of cortical GABAergic neurons display different laminar distribution. Early generated GABAergic neurons from the GE mostly populate the marginal zone and subplate, whereas cortical plate GABAergic neurons originate from both ventral and dorsal telencephalon. A differential regulation of the two GABA synthesizing enzymes (GAD65 and GAD67) parallels GABAergic neuron differentiation. GAD65 is preferentially expressed in GABAergic progenitors and migrating neurons, GAD67 in morphologically differentiated neurons. Therefore, the dorsal telencephalic origin of cortical GABAergic neurons is not human-specific but appears as a former event in the ascent of evolution that could provide GABAergic neurons to an expending neocortex.

Keywords: ganglionic eminence, glutamic acid decarboxylase, interneurons, migration, neurogenesis, ventricular zone

Introduction

γ-Aminobutyric acidergic (GABAergic) neurons that represent roughly 15–30% of cortical neurons in human (Houser et al. 1984; Hendry et al. 1987; Jones 1993; Gabbott and Bacon 1996; DeFelipe 2002), play a crucial role in regulating the activity of neuronal networks and the complex interactions among principal neurons, including the emergence of spatio-temporal synchrony of the pyramidal cells (Somogyi and Klausberger 2005; Skaggs et al. 2007). Therefore, the coordination and integration of cortical function depends on the number and diversity of GABAergic neurons. In fact, dysfunction or cell death of several specific types of GABA neurons is a hallmark of various psychiatric and neurological diseases such as schizophrenia (Lewis 2000; Lewis et al. 2005) and epilepsies (Houser 1999; DeFelipe 2004) to which defect in neurogenesis or migration of these neurons can be a predisposing factor (Rakic 2000; Powell et al. 2003; Levitt et al. 2004; Levitt 2005a, 2005b).

Interestingly, increased structural complexity of the brain during mammalian evolution (Uylings and van Eden 1990; Rakic 1995; Kornack 2000) parallels an increased number and diversity of GABAergic neurons (Jones 1993; Gabbott and Bacon 1996; Gabbott et al. 1997; Letinic and Rakic 2001; Zecevic and Rakic 2001; DeFelipe 2002; Wang et al. 2004). For instance, whereas in rat and mouse GABAergic neurons are absent in most dorsal thalamic nuclei except the reticular nucleus (Houser et al. 1980; Esclapez et al. 1993), they are present in all thalamic nuclei in human and nonhuman primates (Smith et al. 1987; Arcelli et al. 1997). In cortex, despite many interneurones appear to be common to all species, some like in layer 1 display more elaborate features in primates that may represent evolutionary specializations (Zecevic and Rakic 2001; DeFelipe 2002; Rakic and Zecevic 2003a).

One could question whether evolution uses the same or different developmental rules to provide that increased population of GABA neurons? Rakic and colleagues have proposed species-specific programs for generation of these neurons in human brain (Letinic et al. 2002). This hypothesis is mostly based on the demonstration of a migratory pathway (previously suggested, Sidman and Rakic 1973; Letinic and Kostovic 1997) of GABAergic neurons from the ganglionic eminence (GE) of the human telencephalon to the thalamus (Letinic and Rakic 2001) that is not observed in macaque and mouse (Letinic and Rakic 2001). More recently the authors suggested that the source of cortical GABAergic neurons might also be human-specific (Letinic et al. 2002). Indeed, in human most neocortical GABA neurons are produced in the proliferative region of the dorsal telencephalon, prospective neocortex (Letinic et al. 2002; Rakic and Zecevic 2003a), whereas in rodents, cortical GABAergic neurons are generated in the subcortical proliferative zone of the ventral telencephalon, the GE, and migrate tangentially to the dorsal telencephalon (Van Eden et al. 1989; Del Rio et al. 1992; DeDiego et al. 1994; De Carlos et al. 1996; Anderson, Eisenstat, et al. 1997; Tamamaki et al. 1997, 1999; Anderson et al. 1999; Lavdas et al. 1999; Parnavelas 2000, 2002; Parnavelas et al. 2000, 2002; Pleasure et al. 2000; Anderson et al. 2001, 2002; Corbin et al. 2001; Marin and Rubenstein 2001, 2003; Jimenez et al. 2002; Nadarajah and Parnavelas 2002; Nadarajah et al. 2002, 2003; Nery et al. 2002; Polleux et al. 2002; Xu et al. 2003, 2004; Wonders and Anderson 2006).

A puzzling question is whether the dorsal telencephalic origin of neocortical GABAergic neurons is human-specific or reflects a boosting of preexisting developmental mechanisms? In favor of the later hypothesis, several lines of mice, knockout for genes expressed specifically in the ventral telencephalon, show a striking, but however not total decrease in the number of cortical GABAergic neurons at birth. These include mutants for the transcription factors Dlx1 and Dlx2 that show 75% decrease in neocortical GABAergic cells at postnatal day.
0 (Anderson, Qiu, et al. 1997; Anderson et al. 1999); Nkx2.1—50% decrease in neocortical GABAergic cells at embryonic day (E) 18.5 (Sussel et al. 1999) and Mash1 that show 50% decrease in neocortical GABAergic cells at E18.5 (Casarosa et al. 1999; Horton et al. 1999). These data suggest that in lower mammals some GABA neurons could also be generated in the proliferative zones of dorsal telencephalon.

On the other hand, it is still debated following the work of Letinic et al. (2002) whether in human, progenitors of GABAergic neurons are produced directly by the proliferative zones of the prospective neocortex or whether some of them might migrate at early stages from the GE to the proliferative zones of the dorsal telencephalon where they would continue to divide locally, giving rise to postmitotic GABA neurons. This later hypothesis was supported by data obtained in the mouse, showing that some progenitors appear to migrate from the GE directly into the subventricular zone (SVZ) of the prospective neocortex after E14.5, but in fact they do not proliferate in their new position (Anderson et al. 2001).

In order to address these questions, we have studied the sources and migratory pathways of neocortical GABAergic neurons in a nonhuman primate, the cynomolgus monkey. For this purpose, we performed in fetuses from E47 to E90 (neurogenesis period, term 165 days), immunohistochemical (IHC) experiments for the following proteins: 1) the basic helix-loop-helix (bHLH) transcription factor Mash1, a marker of GABAergic neuron progenitors (Casarosa et al. 1999; Schuurmans and Guillemot 2002); 2) The two forms of glutamic acid decarboxylase (GAD, GAD65, and GAD67), two synthesizing enzymes for GABA which are coexpressed in almost all GABAergic neurons of the rat, monkey and human adult brain (Kaufman et al. 1991; Esclapez et al. 1994; Hendrickson et al. 1994; Sloviter et al. 1996). In order to further characterize the developmental stage and neural lineages of the different populations of Mash1- and GAD-containing cells, double IHC labelings were performed for 1) the nuclear antigen Ki67 expressed in all proliferating cells and 2) the platelet-derived growth factor receptor-α (PDGFR-α) a marker of oligodendrocyte progenitors (Lu et al. 2000) since it is known that Mash1 promotes also the generation of oligodendrocytes (Parras et al. 2007) that occurs in the ventral and dorsal telencephalon in rodents (Kessaris et al. 2006) as in humans (Ullig et al. 2002; Rakic and Zecevic 2003b; Jakovecviski and Zecevic 2005).

We demonstrate that in the monkey as in human, cortical GABAergic neurons are generated in the proliferative cortical zones of the dorsal telencephalon, in addition to the proliferative subcortical regions of the ventral telencephalon, the GE. Our data further suggest that the first morphologically differentiated GABAergic neurons observed in the marginal zone (MZ) and the subplate (SP) of the dorsal telencephalon are likely generated in the GE, whereas GABA neuron progenitors in the dorsal telencephalon likely contribute to provide neurons of the cortical plate (CP).

Preliminary reports of some of the findings have been published previously in abstract form (Esclapez et al. 2004).
horse serum. After these steps, sections were rinsed for 30 min in KPBS and incubated for 1 h in biotinylated goat anti-rabbit immunoglobulin G (IgG) or biotinylated horse anti-mouse IgG diluted 1:200 in KPBS containing 3% normal goat serum or normal horse serum. Sections were then rinsed in KPBS, incubated for 1 h with an avidin-biotin-peroxidase solution prepared according to manufacturer’s recommendations (Vector Laboratories). After several rinses in KPBS, sections were processed for the same time (10 min) in 0.04% 3,3'-diaminobenzidine HCl and 0.006% hydrogen peroxide diluted in KPBS. The sections were then rinsed in KPBS, mounted on gelatin-coated slides, dried, dehydrated, and coverslipped with Permount (Fisher scientific, Electron Microscopy Sciences, Washington, PA).

**IHC controls:** No labeling was detected when primary monoclonal antibodies were replaced with normal mouse IgG, or when primary antibodies were omitted. When Ki2 polyclonal antiserum was replaced with normal rabbit IgG, vessels, pia and microglia-like cells were labeled. Antibodies were replaced with normal mouse IgG, or when primary antibodies were replaced with normal horse serum, vessels, pia and microglia-like cells were labeled. With normal rabbit IgG, vessels, pia and microglia-like cells were labeled.

Double IHC labelings for GAD65 and Mash1 (Figs 1A, 2A, 3A), GAD65 and Ki67 (Figs 1A, 3A), and PDGFR-α and Mash1 were performed by sequential immunodetection at 4 °C. Sections were rinsed for 30 min in KPBS, incubated for 1 h in KPBS containing 3% normal horse serum and first processed for detection of GAD65 or PDGFR-α. They were incubated overnight in GAD-6 (1:50) or PDGFR-α (1:50) mouse monoclonal antibody diluted in KPBS containing 1% normal horse serum (Vector Laboratories). Following several rinses in KPBS, sections were incubated for 1 h in biotinylated horse anti-mouse IgG (1:200), rinsed in KPBS and incubated for 2 h in Alexa 488-conjugated Streptavidin (1:200, Jackson Immunoresearch). After intensive rinses in KPBS (3 × 30 min), the sections were processed for detection of Mash1 or Ki67.

For Mash1 immunodetection the mouse on mouse (MOM) kit (Vector Laboratories) was used according to manufacturer’s instruction. Sections were incubated for 1 h in Mouse IgG blocking reagent and overnight in Mash1 mouse monoclonal antibody diluted 1:100 in MOM diluent. Then they were rinsed 3 × 30 min in KPBS and incubated for 2 h in Cy3-conjugated goat anti-mouse IgG (1:200, Jackson Immunoresearch) diluted in KPBS. For Ki67 immunodetection, sections were incubated for 1 h in KPBS containing 3% normal donkey serum (Jackson Immunoresearch), and overnight in Ki67 rabbit monoclonal antibody diluted 1:100 in KPBS containing 1% normal donkey serum (Jackson Immunoresearch). They were rinsed 3 × 30 min in KPBS and then incubated for 2 h in Cy3-conjugated donkey anti-rabbit IgG (1:200, Jackson Immunoresearch) diluted in KPBS. After being rinsed in KPBS all sections were then mounted on gelatin-coated slides, dried and coverslipped with GelMount (Biomedia, Foster City, CA). The specimens were analyzed with Olympus fluoview-500 laser scanning microscope.

**IHC controls:** In all cases, no cross reactivity was detected when at the second step, the primary antibody was omitted. In addition, the antibodies used for double labeling targeted distinct compartments of the cell body (nuclear for Mash1 and Ki67 versus cytoplasmic for GAD65 and membrane for PDGFR-α), and the cell was confirmed as double labeled only if distinct compartments of the same cell were labeled.

**Results**

**E47–E55**

**Mash1-Containing Cells**

From E47 to E55, the bHLH transcription factor Mash1 was expressed mainly in the proliferative zones of the ventral telencephalon including the GE and the septal ventricular zone (VZ) (Fig. 1A,C), and at a lower extent in the most dorsal part of the dorsal telencephalon, on the roof of the lateral ventricle (Fig. 1A).

In the GE, almost all cells displayed a nuclear immunolabeling for Mash1 (Figs 1A,C, 2A, 3A). However, the distribution of these Mash1-containing cells was different in the ventral and dorsal parts of the GE, presumed to correspond respectively to the medial and lateral GEis (Fig. 1C). These cells were organized in two bands in the VZ of the ventral GE (Figs 1C, 2A), whereas they formed a homogenous band in the dorsal part of the GE (Fig. 1C). In addition, the cells located in the proliferative VZ of the GE were strongly labeled, whereas the majority of the cells in the SVZ were less intensively labeled (Figs 1C, 2A, 3A). A similar difference in labeling intensity was observed among the numerous Mash1-containing cells of the prospective septal region. These cells were highly labeled in the VZ, whereas they were moderately labeled in the SVZ (Figs 1C, 2B).

In the dorsal telencephalon, some Mash1-labeled cells were also observed in the VZ and SVZ (Figs 1A, 2F) but their number and labeling intensity were much lower than in the proliferative zones of the GE and septal region (compare Fig. 2F with Fig. 2A,B). Furthermore, in these prospective cortical regions, a clear decreasing gradient of Mash1 labeling intensity was evident in all sections processed at E47 and E55 (Fig. 1A). The moderate level of labeling observed in the most dorsal part of the dorsal telencephalon, on the roof of the lateral ventricle (Figs 1A, 2F), decreased in the lateral and medial wall (Fig. 1A), and a striking feature was the absence of Mash1 immunoreactivity in the most ventral part of the dorsal telencephalon close to the GE (Figs 1A, 3A). No labeling was observed in the intermediate zone (IZ), SP, CP, and MZ.

**GAD65-Containing Cells**

Numerous GAD65-labeled cells were present in the telencephalon. The vast majority of these GAD65-labeled cells were observed in the GE and septal proliferative zones and likely corresponded to neuronal progenitors (Figs 1D, 2C,D). They displayed a round-shape, a thin cytoplasmic ring, a rather large nucleus and were closely apposed to each other (Fig. 2D, insert). Their pattern of distribution corresponded mainly to that of the moderately Mash1-labeled cells (compare Fig. 2C with A, D with H). Thus, in the GE as well as in the septal proliferative zone, the GAD65-labeled cells were mainly located in the SVZ, only few labeled cells being present in the VZ (Fig. 2C,D).

In contrast to GE and septal region, no GAD65-containing progenitor-like cells were observed in the VZ and SVZ of the dorsal telencephalon (Fig. 2G). Instead, numerous GAD65-labeled cells exhibiting the characteristic morphology of early migrating neurons, including either a leading or both leading and lagging processes were present in the lateral and medial wall, (Figs 2G, 3B–E). These migrating-like neurons likely originated from the GE, since a dense band of migrating cells leaving the GE was also observed in all sections. The density of these migrating GAD65-containing neurons varied within the different zones. A large stream of tangentially migrating neurons was located in the lower IZ/upper SVZ of the dorsal telencephalon, on all coronal sections from rostral to caudal levels (Fig. 3B–D). Smaller streams of migration were present in the MZ, whereas a few tangentially oriented migrating GAD65-labeled neurons were also observed in the SP (Fig. 3E). Finally, none of these tangentially migrating GAD65-containing neurons were observed in the proliferative zones of the dorsal telencephalon (VZ and deep SVZ) (Fig. 3B).

**Double Labeling Experiments for GAD65 and Mash1; GAD65 and Ki67**

These experiments clearly showed that at E47–E55, most GAD65-containing cells in the proliferative zones of the GE and
septal region were labeled for Mash1 (Fig. 5A) and Ki67, a marker of proliferative cells (Fig. 5E,F) and therefore confirm that these cells likely correspond to progenitors of GABAergic neurons. In contrast, migrating-like GAD65-labeled cells leaving the GE or tangentially oriented in the upper SVZ/lower IZ of the dorsal telencephalon did not contain detectable levels of Mash1 (Fig. 5A) and the vast majority of these cells were not labeled for Ki67 (Fig. 5E,F). However, these migrating-like GAD65-containing neurons were surrounded by many mitotic cells labeled for Ki67. These mitotic cells were particularly numerous in the IZ and the VZ of the dorsal telencephalon (Fig. 5E).

Double Labeling Experiments for Mash1 and PDGFR-α
At this developmental stage some PDGFR-α-containing cells were present in the GE and all these presumed oligodendrocyte progenitors were clearly colabeled for Mash1 (Fig. 6A). These double-labeled cells for PDGFR-α and Mash1 in the GE were clearly less numerous than double-labeled cells for GAD65 and Mash1 (compare Fig. 5B with Fig. 6A). Many

Figure 1. Mash1 (A,C) and GAD65 (D) labeling patterns in coronal sections of monkey fetal telencephalon at E47 (A) and E55 (B–D). (A) At E47 Mash1 was highly expressed in the proliferative zone of the ventral telencephalon, the GE. Mash1 immunoreactivity was also observed in the proliferative zones of the dorsal telencephalon and followed a clear dorso-ventral decreasing gradient in the labeling intensity within the entire cortical wall. Whereas moderate level of labeling was observed in the most dorsal part of the telencephalon, on the roof of the lateral ventricle (vent) (indicated by square and magnified in Fig. 2E), the intensity of staining decreased in the lateral and medial wall. No Mash1 immunoreactivity was observed close to the GE, nor in the most ventral part of the medial telencephalic wall that corresponds at this level to dorsal hippocampus (dhipp). No Mash1 immunoreactivity could be seen in the thalamic nuclei (th). (B–D) Adjacent sections from E55 fetal brain at a rostral level in which the lateral ventricle displayed its rostral dilatation (paleocortical ventricle indicated by a star). (B) Cresyl violet labeled section, in which the following regions were specified: corpus callosum (CC), septum (sept), caudate nucleus (caud), putamen (put), capsula interna (CI), and primordium of the dorsal hippocampus (dhipp). (C) Strong intensity of labeling for Mash1 was present in the GE (box magnified in Fig. 2A) and septal proliferative zone (box magnified in Fig. 2B). (D) GAD65-immunolabeling was observed in the same regions of the ventral telencephalon as Mash1, the GE and the septal eminence (boxes enlarged in Fig. 2C,D). The levels of sections (A, B, C, D) are indicated by dotted lines on the schematic drawing of monkey fetal brain. Crossed arrows indicated the orientation of sections (A, B, C, D). Scale bar = 1 mm (same for all the figures).
Figure 2. Comparison of labeling for Mash1 (A, B, E) and GAD65 (C, D, F) in the proliferative zones of GE (A, C, septum (B, D) and dorsal telencephalon (E, F) in coronal sections of monkey fetal telencephalon at E47 (E, F) and E55 (A–D). Figures correspond to magnified regions indicated by boxes in Figure 1A, C, D. (A) E55. In the GE many cells were labeled for Mash1 in the VZ and the SVZ, the intensity of labeling being much higher in the VZ than in the SVZ. Moreover, the cells highly labeled for Mash1 in the VZ of the ventral part of GE were organized in two bands in contrast to the dorsal part where the highly labeled cells for Mash1 were distributed in a homogeneous band (see Fig. 1C). (B) E55. In the prospective septal region (Sept) numerous cells highly labeled for Mash1 were observed in the VZ whereas the cells in the SVZ displayed a moderate level of labeling. (C) E55. Many cells labeled for GAD65 were observed in the GE. These cells were mainly located in the SVZ, only a few cells being present in the VZ. (D) E55. Many cells labeled for GAD65 were present in the SVZ of the septal region. Box: magnified region. (E) E47. Cresyl violet labeled section, in which the different cellular zones of the most dorsal part of the dorsal telencephalon are delineated. (F) E47. Some cells moderately labeled for Mash1 were observed in the proliferative zones of the most dorsal part of the dorsal telencephalon on the roof of the lateral ventricle. Most of these Mash1 positive cells were present in the upper part of the SVZ, on the border to the IZ. Some cells were also observed in the deepest part of the VZ. (G) E47. In contrast to Mash1 no GAD65-containing cells were observed in the VZ and SVZ of the most dorsal part of the dorsal telencephalon. However in this region, some GAD65-containing cells with a characteristic morphology of early postmitotic migrating neurons, including a leading process, and tangentially oriented, were observed in the IZ and the CP (arrows). Scale bars = 50 μm (A–D), 10 μm (box in D).
PDGFR-α containing cells were also observed in the SP (Fig. 6B) but in contrast to those present in the GE they displayed very low (Fig. 6D) or no detectable levels of Mash1 (Fig. 6C). No PDGFR-α containing cells were observed in the VZ and SVZ of the dorsal telencephalon.

**E64–E75**

**Mash1-Containing Cells**

In GE and septal region of the ventral telencephalon, the pattern of labeling for Mash1 displayed strong similarities with that observed at E47 and E55. On the contrary, in the dorsal telencephalon it was very different from that observed at earlier stages. A massive expression of Mash1 was observed now in the VZ and SVZ, which extended to the entire dorsal telencephalon including its most ventral part close to the GE (compare Fig. 4A–C with Fig. 3A). These Mash1-labeled cells present in the VZ and SVZ of the whole dorsal telencephalon, displayed a strong intensity of labeling (Fig. 4A–C) similar to that observed in the proliferative zones of the GE (Fig. 4A) and septal region. In addition numerous cells lightly labeled for Mash1 were present in the lower part of the IZ and upper part of the SVZ (Fig. 4A) whereas Mash1-labeled cells were still not observed in the SP, CP and MZ (Fig. 4B).

**GAD65-Containing Cells**

The labeling pattern for GAD65 at E64–E75 (Fig. 4E,F) was also very different from that described at earlier stages (Figs 3B–E, 2C,D,F, 1D) especially in the dorsal telencephalon. GAD65-containing cells were not only numerous in the GE and septal region (Fig. 4E–H), as at earlier stages (Figs 1D, 3B), but they were also present in great number in the VZ and SVZ in all regions of the dorsal telencephalon (Fig. 4E), in contrast to the earliest stages (compare Fig. 4E with Fig. 3B). In these
Proliferative zones of the prospective cortex, two main populations of GAD65-containing cells were observed. The first one, corresponded to round-shaped cells without any evident processes, highly labeled for GAD65 like the cells in the GE. They were often distributed by groups of 2 or 3 closely juxtaposed cells and likely corresponded to GAD65-containing progenitors (Fig. 4G). The second population corresponded to migrating-like GAD65-containing neurons. However the orientation of these postmitotic migrating neurons also differed strikingly from that observed at E47–E55. Instead of being tangentially oriented, they displayed multidirectional orientations at E64–E75 (Fig. 4H). These nontangentially oriented GAD65-containing neurons were observed in all layers of the dorsal telencephalon including IZ, SP, CP, as well as the SVZ and VZ.

**Double Labeling for Mash1 and GAD65; GAD65 and Ki67**

At E64–E75, GAD65-containing cells that coexpressed Mash1 were still numerous in the GE and septal region of the ventral telencephalon (Fig. 5B), but now also in the VZ and SVZ of the dorsal telencephalon. These colabeled cells in the cortical wall, often formed groups of 2 or 3 closely associated cells (Fig. 5D) suggesting ongoing divisions further supported by the presence of many double-labeled cells.
for GAD65 and Ki67 in these cortical proliferative zones (Fig. 5G). In contrast to these progenitor-like cells, migrating-like GAD65-containing neurons did not express Mash1 or Ki67 (Fig. 5H,I).

**Double Labeling Experiments for Mash1 and PDGFR-α**

At this developmental stage, PDGFR-α-containing cells were present in the GE but also now in the SVZ and IZ of the dorsal telencephalon and all presumed oligodendrocyte progenitors were clearly colabeled for Mash1 (Fig. 6E). Many PDGFR-α containing cells were also observed in the SP (Fig. 6F) and some in the CP (Fig. 6G), these cells displayed morphological feature of oligodendrocytes and did not display detectable levels of Mash1 (Fig. 6F,G).

**E83–E90**

A clear decrease of Mash1 immunolabeling in the proliferative zones of the telencephalon was observed at E88 (Fig. 4D) as compared to E64–E75 (Fig. 4B,C). On the other hand, the morphological types (progenitors and postmitotic neurons), distribution and orientation profiles of GAD65-containing cells were similar to those described at E64–E75.
GAD67 IHC Labeling

Many cell bodies and processes were labeled with GAD67-specific antiserum K2, however the pattern of labeling differed from that obtained with GAD65-specific antibody. The vast majority of GAD67-containing cells corresponded to more differentiated neurons, that is, neurons with strongly labeled cell bodies and proximal dendrites (Fig. 7a', b, c, c'). Interestingly, a few labeled neurons were observed already in the cortical wall from E47 on, then their number increased considerably with different time courses according to the different layers (Fig. 7A-C). They were located in the MZ and just under the CP, in the developing SP, at E47–E55 (Fig. 7a'). It is mainly in that layer that their number increased from E55 to E75 (Fig. 7A-C). In contrast in the developing CP, very few GAD67-containing neurons were observed before E75. Their number increased significantly at E75–E88 and they displayed morphological features of more differentiated maturing neurons (Fig. 7c').

A few migrating-like GAD67-containing neurons were also clearly observed from E47 to E88 in the IZ, SP, MZ, and CP (Fig. 7A, a, b), but in a much lower number than migrating-like GAD65-labeled neurons at all developmental stages studied.

Finally, progenitor-like GAD67-labeled cells were rarely detected in the proliferative region of the entire telencephalon including the GE, septal region and VZ and SVZ of the cortical wall at all developmental stages studied.

Discussion

A major goal of the present study was to determine whether in nonhuman primates neocortical GABAergic neurons originate from two different sites of origin (i.e., dorsal and ventral telencephalon) as in human (Letinic et al. 2002; Rakic and...
Figure 7. GAD67 labeling patterns in coronal sections of monkey fetal telencephalon at E55 (A, a, a'), E68 (B, b) and E75 (C, c, c'). (A) At E55 as at earlier stage of cortical development, GAD67-containing neurons were mainly present in the MZ and below the CP in the upper part of the subplate (SPu). In the MZ these neurons were surrounded by dense plexus of stained fibers as illustrated at higher magnification (inset a'). Some neurons were also observed in the IZ (arrows). Many of them in this layer displayed a unipolar migratory-like morphology (inset a). (B) At E68 a general increased density of GAD67-labeled neurons was observed but this increase was particularly striking in the lower part of the subplate (SPL) (arrows). Inset b: In this later layer, many GAD67-labeled cells corresponded to morphologically differentiated neurons, that is, neurons with strong labeling of the cell body and proximal dendrites (arrow). These neurons contrast with migratory-like postmitotic neurons lightly labeled for GAD67 (arrowhead). (C) At E75 the thickness of the SP and the density of GAD67-containing neurons further increased. More GAD67-labeled neurons were also observed in the MZ and the IZ. Some GAD67-containing neurons were now present in the CP (arrows). Higher magnification illustrating morphologically differentiated neurons and axon terminals labeled for GAD67 in the SP (inset c) and in the CP (inset c'). (D) At E75 adjacent section stained for cresyl violet showed that the SP is at this age up to 3 times thicker than the CP. Scale bars = 200 µm (A–D), 50 µm (a', b, c, c'), 25 µm (a).
labeled for Mash1, whereas postmitotic migrating GAD65-containing cells are not labeled for Ki67 and therefore detectable level of Mash1. Furthermore, these migrating-like GAD65-containing cells are not expressed in the SVZ, but the vast majority of these progenitors in the VZ and SVZ of the dorsal telencephalon from E64 on, are in keeping with previous studies that report oligodendrocyte genesis in both ventral and dorsal telencephalon in rodents (Kessaris et al. 2006) and in humans (Ulfìg et al. 2002; Rakic and Zecevic 2003b; Jakovcevski and Zecevic 2005). In addition, our results clearly illustrate, that at these developmental stages, Mash1/GAD65-containing progenitors are more numerous than Mash1/PDGFR-α progenitors in the GE as well as in the SVZ/IZ of the dorsal telencephalon. These two populations of progenitors likely provide respectively migrating-like GAD65-containing neurons and PDGFR-α-containing oligodendrocytes.

In the Cynomolgus Monkey Cortical GABA Neurons are Generated in the Proliferative Regions of both Ventral and Dorsal Telencephalon

In macaque from at least E47 on, a contingent of cortical GABAergic neurons is produced in the GE. At E47–E55, in addition to progenitor-like cells containing Mash1 and GAD65 located in the proliferative zone (SVZ and VZ) of GE, our data clearly illustrate a large contingent of GAD65-containing cells with clear morphological features of postmitotic migrating neurons leaving the GE and displaying a main tangential orientation in the upper SVZ and lower IZ of the dorsal telencephalic wall. Such migratory pathways of GABAergic neurons from the GE to the prospective cortical region are similar to those previously reported in rodents (DeDiego et al. 1994; Tamamaki et al. 1997; Anderson et al. 2001; Marin and Rubenstein 2001, 2003; Jimenez et al. 2002; Nadarajah and Parnavelas 2002; Polleux et al. 2002) and were also described in human fetuses (Letinic et al. 2002).

In addition to this GE origin, our data demonstrate that cortical GABAergic neurons are also produced in the proliferative zones of the dorsal telencephalon. Indeed from E64 on, that is, 2 weeks later than in the GE, many Mash1-containing progenitors that do coexpress GAD65 are now present in the SVZ and VZ of the entire dorsal telencephalon. These double-labeled cells display features of proliferative cells and these neuronal progenitors are clearly morphologically different from adjacent postmitotic GAD65-containing migrating neurons. The latter as mentioned before are not labeled for Mash1 and display clear leading processes. Many of these migrating neurons display nontangential orientation as opposed to the tangentially oriented migrating neurons observed at earlier stages (E47–E55). The multidirectional orientations of migrating-like GAD65 neurons at E64–E75 and the presence of GAD65-containing progenitors in the VZ and SVZ of the dorsal telencephalon strongly support that many migrating GAD65-containing neurons originate from precursors of GABAergic neurons in the prospective cortex.

Furthermore, our results strongly support the view that the progenitors of GABAergic neurons observed from E64 on in the proliferative zones of the prospective neocortex are generated locally rather than to migrate from the GE to the proliferative...
zones of the dorsal telencephalon. We observed at E47–E55, an expression of Mash1 in the VZ/SVZ of the most dorsal part of the dorsal telencephalon whereas no Mash1-containing cells were observed in the dorsal telencephalic wall close to the GE, arguing against a migration of these progenitors from the GE to the dorsal telencephalon. At these earlier ages, the Mash1-containing cells in the dorsal telencephalon do not contain GAD65 and their GABAergic phenotype could be discussed. We cannot completely exclude that the Mash1-containing cells could generate other types of cells than GABAergic neurons such as oligodendrocytes but these cells do not contain PDGFR-α. It is thus likely that this expression of Mash1 in the dorsal telencephalon at E47–E55 reflects the beginning of GABAergic neurogenesis in this region since at E64 the vast majority of Mash1-containing cells in the VZ/SVZ of the entire dorsal telencephalon express GAD65, whereas only a few Mash1-containing cells express PDGFR-α and these oligodendrocyte progenitors cells are mainly located in the SVZ/IZ.

Temporal Differences in the Generation and Laminar Targets of Cortical GABAergic Neurons

Our data further demonstrate that induction of neurogenesis for GABA neurons occurs earlier in the GE and septal proliferative regions than in the proliferative zones of the prospective neocortex. GABAergic neurons progenitors containing Mash1 and GAD65 are present as early as E47 in the GE and only twenty days later in the proliferative zone of the dorsal telencephalon. In addition, the data obtained also suggest that GABAergic neurons observed in the MZ and SP are generated in the subcortical proliferative region such as the GE whereas those in the CP are likely generated in both subcortical and cortical proliferative zones. Indeed, as early as E47, morphologically differentiated GAD67-containing neurons were observed first in the MZ and the SP just below the CP. A similar pattern of early generated GABAergic neurons was described in human fetuses (Zecevic and Milosevic 1997; Meyer et al. 2000). Their number increases in the SP with the development of this layer between E47 and E68. The fact that GAD67-containing neurons in the MZ and SP began to morphologically differentiate at a period during which GABAergic progenitors are mainly observed in the ventral telencephalon and the routes of migrating GABA neurons are mainly tangential strongly favors their generation in the GE. The role of such early generated GABAergic neurons in the MZ and SP has still to be demonstrated, however it has been shown that SP neurons (Hanganu et al. 2002) as well as early generated neurons in the hippocampal MZ are functional and play a crucial role in early generated network activity (Khazipov et al., 2001). From E68–E75 on GABAergic neurons integrate the CP following an inside-out gradient. Their number increases during a period where progenitors of GABAergic neurons are also massively produced in the proliferative regions of the entire dorsal telencephalon suggesting that in primates, a large proportion of cortical GABA neurons could also originate from the pool generated in proliferative cortical regions.

Preferential Expression of the Two Synthesizing Enzymes for GABA (GAD65 and GAD67) during Neuronal Maturation

The present data suggest that the two isoforms of GAD are differently regulated at the protein level during development of GABAergic neurons. GAD65 is preferentially expressed in progenitors and early postmitotic migrating neurons, whereas GAD67 is preferentially expressed in GABA neurons that start to morphologically differentiate. Thus, Mash1-containing progenitors do contain GAD65 whereas few of these cells are detectable with GAD67 antibodies. In addition many neurons with profile of tangentially migrating neurons were labeled for GAD65 as previously reported in the rat (Del Rio et al. 1992) and human (Meyer et al. 2000) but at a much lower extent for GAD67. In contrast many differentiated GABAergic neurons were labeled for GAD67. All these data suggest the presence of the two isoforms in all prospective and mature GABA neurons. Their differential regulation at the protein level during development could serve different function, but this has to be demonstrated.

Conclusion

In conclusion, in monkeys as in humans, cortical GABAergic neurons are generated in the proliferative cortical regions of the dorsal telencephalon, in addition to the proliferative regions of the ventral telencephalon. This neurogenesis of GABAergic neurons occurs very early during brain development in both ventral and dorsal telencephalon but with distinct temporal profiles. It starts at the beginning of the main neurogenesis period in the ventral telencephalon and only 2 weeks later in the dorsal telencephalon. Strong arguments favor a direct induction of this later event in the prospective neocortex rather than to result from migration of progenitors from the proliferative region of the ventral telencephalon. The dorsal telencephalic origin of neocortical GABAergic neurons is therefore not human-specific and appears as a former event in the ascent of evolution. This mechanism could take place through mammalian evolution in order to provide GABA neurons to an expending neocortex.

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