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## Diversity of Cortical Interneurons in Primates: The Role of the Dorsal Proliferative Niche

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### Summary

Evolutionary elaboration of tissues starts with changes in the genome and location of the stem cells. For example, GABAergic interneurons of the mammalian neocortex are generated in the ventral telencephalon and migrate tangentially to the neocortex, in contrast to the projection neurons originating in the ventricular/subventricular zone (VZ/SVZ) of the dorsal telencephalon. In human and nonhuman primates, evidence suggests that an additional subset of neocortical GABAergic interneurons is generated in the cortical VZ and a proliferative niche, the outer SVZ. The origin, magnitude, and significance of this species-specific difference are not known. We use a battery of assays applicable to the human, monkey, and mouse organotypic cultures and supravital tissue to identify neuronal progenitors in the cortical VZ/SVZ niche that produce a

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**Author Contributions:** N.V.R. performed cell culture, transfection, immunolabeling, and western blot experiments. A.E.A. and X.Y. performed electroporation in animals and immunolabeling experiments. F.M. performed in situ hybridization experiments and immunolabeling. N.V.R., A.E.A., and F.M. analyzed data, performed confocal microscopy, made figures, and contributed to the writing of the manuscript. A.M. and I.J. performed electroporation of human slices and were involved in writing the results. S.A.A., P.R., and N.Z. came up with the idea and developed the experimental strategy, oversaw all aspects of this study, and cowrote the manuscript.

subset of GABAergic interneurons. Our findings suggest that these progenitors constitute an evolutionary novelty contributing to the elaboration of higher cognitive functions in primates.

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## Introduction

Evolution of all multicellular organisms and their organs proceeds by heritable changes in individual cells and cytoarchitectonic composition, which, at some point, transforms into new species-specific traits (Mayr, 2001; Spradling et al., 2008). The human cerebral cortex is not an exception (Rakic, 2009; Zecevic et al., 1999). The basic principle of cortical organization in all mammalian species is similar, although humans have certain distinct features (Bystron et al., 2008; Clowry et al., 2010; Gadisseux et al., 1992; Geschwind and Rakic, 2013; Jones, 2009; Kang et al., 2011; Miller et al., 2014; Preuss, 2001). Several evolutionary advances are attributed to the elaboration of the outer subventricular zone (oSVZ) (Bayatti et al., 2008; Smart et al., 2002; Zecevic et al., 2005). The introduction of a new proliferative niche during evolution provides the necessary environment for the emergence of new progenitor subtypes (Betizeau et al., 2013; Fietz and Huttner, 2011; Hansen et al., 2010; Lui et al., 2011) that generate the enlarged upper cortical layers or the increased number and diversity of corticocortical connections in the human cerebrum (Hill and Walsh, 2005; Rakic, 2009).

During evolution, the diversity of cortical interneurons is increased by introduction of new subtypes (Cajal, 1899; DeFelipe et al., 2002; Gabbott et al., 1997; Jones, 2009). In contrast to the cortical projection neurons generated exclusively in the ventricular zone (VZ) and subventricular zone (SVZ) of the dorsal telencephalon (Rakic, 1972; Sidman and Rakic, 1973), GABAergic interneurons originate from the ganglionic eminence (GE) of the ventral pallidum (Anderson et al., 1997, 2001; De Carlos et al., 1996; Lavdas et al., 1999; Marin et al., 2000). However, a growing body of evidence indicates that in humans and nonhuman primates, progenitor cells located in the cortical VZ/SVZ serve as an additional source of cortical GABAergic interneurons (Al-Jaberi et al., 2013; Clowry, 2014; Cunningham et al., 2013; Fertuzinhos et al., 2009; Jakovcevski et al., 2011; Letinic and Rakic, 2001; Letinic et al., 2002; Petanjek et al., 2009; Rakic and Zecevic, 2003). The magnitude as well as the genetic, molecular, cellular, and evolutionary mechanisms underlying genesis of this phenotypic diversity are still debated (Hansen et al., 2013; Ma et al., 2013).

To address this conceptually and biomedically important issue, we examined the differences in origin, location, and capacity of interneuronal progenitors among humans, macaque monkeys, and mice using a variety of methods and approaches. In particular, we have explored whether the introduction of certain classes of interneurons may represent an evolutionary novelty induced by changes in the location of the mitotic divisions at the time of cell commitment that is regulated by differential expression of transcription factors (TFs) such as Nkx2.1 (TTF1, thyroid transcription factor-1). Nkx2.1 is important in the specification of cortical interneurons in rodents (Anderson et al., 2001; Sussel et al., 1999; Xu et al., 2005).

## Results

### Nkx2.1<sup>+</sup> Cells in the Cortical VZ/SVZ of Primates' Fetal Brain

To analyze *Nkx2.1* gene expression in the cerebral cortex, we studied human and macaque monkey fetal forebrain tissue at the comparable midgestational stages. We found out that *Nkx2.1* was expressed in a decreasing gradient, from high in the medial ganglionic eminence (MGE) to low in the cortical wall, on coronal sections cut through the middle of the telencephalon (Figures 1A–1C). We quantified immunolabeled Nkx2.1<sup>+</sup> cells (15–22 gestational weeks [GW]; n = 6). In particular, a higher percentage of Nkx2.1<sup>+</sup> cells was observed in the cortical SVZ compared to the VZ (Figure 1G). At 15 GW, 4.2% and 9% of cells were Nkx2.1<sup>+</sup> in the VZ and SVZ, respectively; at 18–19 GW, 4.9% and 7.8%; and at 22 GW, 2.6% and 6.4% (Figure 1G). These values probably underestimate the real number of cortical Nkx2.1<sup>+</sup> cells, since we applied restrictive counting criteria including only cells with the brightest signal.

Our analysis shows that the distribution of *Nkx2.1* mRNA transcripts is present throughout the developing human cerebral cortex (Figure 1C). Signal for *Nkx2.1* transcript was observed in all cortical zones, with rare cells also seen in the cortical plate (CP) at midgestation (n = 6; Figure 1C). By combining in situ hybridization and immunohistochemistry on the same sections, we confirmed that cortical cells expressing *Nkx2.1* transcript also produce the appropriate Nkx2.1 protein (Figure 1D). Notably, the majority of cells that show signal for *Nkx2.1* mRNA were GABAergic interneurons, as indicated by their double labeling with Gad67 antibody (Figure 1E). This result was further confirmed by the opposite experiment, where signals for *Gad67* mRNA and immunoreaction for Nkx2.1 were colocalized in the same cortical cells (Figures 1F and S1). As a next step, we assessed mRNA expression of *Nkx2.1* and *Lhx6*, another TF, downstream of Nkx2.1 in the generation of parvalbumin (PV)<sup>+</sup> and Sst<sup>+</sup> interneurons in the human cortical tissue using RT-PCR. We observed a progressive increase of these transcripts over development (Figure 1H).

These findings were corroborated by western blot analysis where protein levels of a number of TFs, important for the generation of GABAergic cells, were assayed in human fore-brain tissue (18 GW). Higher levels of the analyzed TFs were demonstrated in the ganglionic eminence (GE) of the ventral telencephalon, but, importantly, they were also present in the cerebral cortex (Figure 1I). Altogether, these results demonstrated that *Nkx2.1* is expressed in a subpopulation of cortical GABAergic cells in the whole width of the human cortex at midterm.

### Nkx2.1<sup>+</sup> Cells Proliferate in the Cortical VZ/SVZ Cells and Retain Radial Glia Cell Properties

Examination of the cortical VZ/SVZ in developing human and monkey cortex revealed numerous Nkx2.1<sup>+</sup> cells in various stages of the mitotic cycle or as just completed cell division (Figures 2A–2C). This finding has been confirmed by their colabeling with Ki67, a marker of S-to-M cell-cycle phases (Figures 2B and 2C). Proliferating Nkx2.1<sup>+</sup> cells were distributed throughout the cortical VZ and oSVZ, including even the upper cortical regions, such as the intermediate zone and subplate. In five specimens ranging from 20 to 24 GW,

we estimated that more than half of all Nkx2.1<sup>+</sup> cells (59% ± 5%) were proliferating in the cortical VZ/SVZ (Figure 2G) with a gradient from MGE to neocortex (Figure S2). These results were confirmed by fluorescent in situ hybridization (FISH) for *Nkx2.1* and anti-Ki67 antibody on the same sections, which demonstrated *Nkx2.1* mRNA signal in 4%–5% of all SVZ cells, a fraction of which colabeled with the proliferation marker, confirming our results from double-labeling immunohistochemistry.

The oSVZ is an evolutionary new niche, especially well developed in primates, that contains complex lineages and a mosaic of precursors immunolabeled with markers typical of apical and basal radial glia cells (RGCs) (Betizeau et al., 2013; Hansen et al., 2013; Lui et al., 2011). Here, we demonstrated that numerous cells double labeled with pVim/Nkx2.1 or Sox2/Nkx2.1 are apical radial glia progenitors proliferating on the VZ surface (Figures 2D and 2E). Double-labeled pVim/Nkx2.1 cells display mainly unipolar morphology when found in the VZ and bipolar morphology in the SVZ (Figure 2D). Other Nkx2.1-expressing cells in the SVZ are probably basal progenitors, as they were double labeled with Pax6 (Figure 2F). The number of double-labeled Nkx2.1/Pax6 cells estimated in eight cases ranging in age from 17 to 24 GW (n = 8) revealed that 17.5% ± 2.5% of all Pax6<sup>+</sup> cells coexpressed Nkx2.1 (Figure 2). In contrast, 80% ± 3.5% of all Nkx2.1<sup>+</sup> cells in the VZ/SVZ coexpressed Pax6, whereas 20% were only Nkx2.1<sup>+</sup>. These results demonstrated that distinct subpopulations of RGC progenitors in the cortical VZ/SVZ of the human fetal brain express Nkx2.1 transcription factor.

### **Nkx2.1 Expression and Proliferation in Nonhuman Primates**

In both human and nonhuman primates, Nkx2.1<sup>+</sup> cells were localized in multiple levels of the developing dorsal cerebral wall, from VZ/SVZ to the cortical plate (CP; Figure 3A). Their number increased from embryonic day 70 (E70) to E92 and E121 (Figure 3B). Moreover, a percentage of these Nkx2.1<sup>+</sup> cells were double labeled with the proliferative marker Ki67 (Figure 3C). Another subpopulation of Nkx2.1<sup>+</sup> cells is GABAergic and colabeled with both *Gad67* mRNA and anti-GABA antibody (Figure 3D). In E121 specimens, we estimated that in the CP, Nkx2.1<sup>+</sup> cells constitute up to 3% of the total number of cells; approximately 30% of the Nkx2.1<sup>+</sup> cells are GABAergic, suggesting that they are still not fully mature. On the other hand, 20% of the GABAergic cells in the CP are expressing Nkx2.1, and only sparse Nkx2.1-expressing cells were colabeled with a marker of early oligodendrocyte progenitors, platelet-derived growth factor (PDGF) receptor  $\alpha$  (Figure 3E). These results demonstrate that in both human and nonhuman primates, a subpopulation of cycling cortical progenitor cells express Nkx2.1 gene and protein and differentiate into GABAergic cells.

### **Subpopulations of Cortical Neurons Express Nkx2.1 in Humans and Nonhuman Primates**

Next, we observed colocalization of Nkx2.1 with either Lhx6 or Sst protein at midgestation (Figures S3A and S3B), which is congruent with the known lineage progression of Nkx2.1<sup>+</sup> to Sst and PV cells in the mouse cortex (Butt et al., 2005; Xu et al., 2004). Expression of Nkx2.1 in human cortical GABAergic cells represents an important distinction compared to mice, where *Nkx2.1* is downregulated before cells migrate from GE to the cortex (Nóbrega-Pereira et al., 2008). We extended our previous findings obtained from human fetal brain at

midgestation (Jakovcevski et al., 2011) to a newborn, where a distinct subpopulation of CalR/Nkx2.1 and PV/Nkx2.1 neurons were observed in all cortical layers (Figures S3D and S3E). These subtypes of cortical neurons have not been previously described and may represent human- or primate-specific cortical interneuron subtypes. Furthermore, their localization in the upper cortical layers (I–III) makes it very unlikely that they migrate to the olfactory bulb. In addition, anti-PDGF receptor  $\alpha$  antibody, which labels oligodendrocyte progenitor cells, only occasionally colocalized with Nkx2.1, suggesting that most Nkx2.1<sup>+</sup> cells are interneurons (Figure S3C).

### Electroporation of mNkx2.1 into Human Slice Culture

We used organotypic slice cultures of the human fetal VZ/SVZ for electroporation of the full-length murine *Nkx2.1* (*mNkx2.1*) gene at 16 and 21 GW (Figure 4A). Immunostaining with Nkx2.1 antibody confirmed that all the transfected cells express Nkx2.1 at high levels (Figure 4B). Electroporated mNkx2.1<sup>+</sup> cells in the human cortical slices 5 days after transfection were colabeled with Lhx6 (Figure 4C), Sox6 (Figure 4D), and GABA (Figure 4E). This is consistent with the idea that overexpression of the *Nkx2.1* gene induces interneuron-like precursors (Lhx6<sup>+</sup>, Sox6<sup>+</sup>) with a potential to generate GABAergic neurons. This conclusion is also supported by the fact that, at least after 5 days, all electroporated cells in human cortical slice were GABAergic.

### In Utero Electroporation of Human and Mouse Nkx2.1 into E14.5 Mouse Brain

To further pursue the idea of primate-specific GABAergic interneurons, we performed a phylogenetic analysis of the Nkx2.1 coding sequences among eight species (Figure S4). Although *Nkx2.1* was found to be highly conserved, it accrued noticeable changes in nucleotide sequence since the rodent-primate ancestor (2.32% in rodents; 3.42% in primates), which may affect its interactions with other transcription factors and downstream targets. Furthermore, the human transcript of *Nkx2.1* contains a different 5' UTR and exon 1 that gives rise to a longer protein (by 30 amino acids [aa]) with a distinct N terminus.

In order to test whether ectopic expression of the *Nkx2.1* genes in the mouse cortical VZ/SVZ zone is sufficient to induce generation of interneurons, and to shed light to the possible functional differences between the mouse and human Nkx2.1 protein, due to the nucleotide changes, we ectopically introduced *hNkx2.1* or *mNKX2.1* by in utero electroporation (IUE) into lateral ventricle of mouse embryos at E14.5 and followed the fate of the electroporated cells in later developmental stages (E16.5, postnatal day 1 [P1], and adult [P21]).

Two days after IUE (E16.5), Nkx2.1 protein was found to be expressed only in *hNkx2.1/EGFP*<sup>+</sup> and *mNkx2.1/EGFP*<sup>+</sup> cells, but not in the control *EGFP* electroporated cells, indicating that appropriate protein can be produced only after transfection of *mNkx2.1* or *hNkx2.1*. In contrast to controls (Figure 5C), most of the *hNkx2.1* and *mNkx2.1* electroporated cortical progenitors remained in the VZ 2 days after electroporation (Figure 5E) and displayed a radial morphology with long basal processes characteristic of VZ RGCs. We found that only a minority of *hNkx2.1*<sup>+</sup> and *mNkx2.1*<sup>+</sup> cells in the VZ expressed the pallial progenitor markers Pax6 (n = 6; 8.1% and 5.8%) and Tbr2 (12.3%, 10.1%) versus

controls (Pax6 = 22.7% and Tbr2 = 24.1%; Figure 5). On the other hand, in the SVZ, more IUE cells with either *hNkx2.1* or *mNkx2.1* took on multipolar morphologies and expressed Pax6 (15.9% and 12.1%) versus controls (2.6%; Figures 5F and 5I). In contrast, in the SVZ region, few *hNkx2.1* cells (0.6%) and *mNkx2.1* electroporated cells (0.5%) were positive for Tbr2 versus controls (13.3%; Figures 5G, 5H, and 5J). Because IUE captures VZ cells passing through the S phase of the cell cycle (Stancik et al., 2010), the outcome of symmetric divisions (generating two Pax6<sup>+</sup> progenitors) and asymmetric divisions (generating one Pax6<sup>+</sup> progenitor and one Tbr2<sup>+</sup> intermediate progenitor) is influenced by the expression of genes involved in fate determination. Therefore, ectopic expression of either *hNkx2.1* or *mNkx2.1* disrupts the default (Pax6 > Tbr2) pathway in the dorsal VZ (Figure 5). However, neither *mNkx2.1* nor *hNkx2.1* electroporated cells expressed detectable levels of several proteins typically associated with cortical interneurons, including CalR or GABA. These results indicate that after a short period of differentiation (2 days), the IUE of either the human or mouse *Nkx2.1* gene at the E14.5 cortical stem cells does not induce the generation of cortical interneurons.

At P1, the pattern of Nkx2.1 staining in the mouse control brain (Figure S5) was similar to that previously described (Marin et al., 2000). Nkx2.1 expression was restricted to the basal ganglia, while in the cortex only sparse (isolated) Nkx2.1<sup>+</sup> cells could be identified, similar to previous reports in neonatal mice (Ohira et al., 2010). In control animals electroporated with (*pCAGG-EGFP*), transfected cells were not colabeled with the anti-Nkx2.1 antibody and were predominantly located in the upper cortical layers II/III of the cortex and less often in the VZ/SVZ (Figure S5B). In contrast, *hNkx2.1/EGFP*<sup>+</sup> transfected cells were mainly located in the VZ, although individual cells with migratory, bipolar morphology were also demonstrated throughout the cerebral wall, from the ventricular to pial surface (Figure S5C). Immunohistochemical analyses showed that the majority of electroporated (*hNkx2.1/EGFP*<sup>+</sup>) cells were colabeled with the antibody to Nkx2.1 (Figure S5C) or Lhx6<sup>+</sup> and were distributed in the VZ/SVZ, but rarely in the cortical plate (Figure 6A). In addition, a number of electroporated cells expressed GABA (Figure 6B) and were occasionally labeled with CalR, whereas other interneuron markers, such as PV or Sst, did not colabel cortical cells at P1. This finding indicates that after 7 days of differentiation, ectopic expression of *hNkx2.1* gene is sufficient to impart GABAergic-like properties in neurons generated in the mouse dorsal pallium.

To determine if *hNkx2.1/EGFP*<sup>+</sup> transfected progenitor cells develop the phenotype of mature interneurons, we harvested embryonically (E14.5) electroporated brains at P21. The majority of enhanced GFP (*EGFP*)<sup>+</sup> cells expressed Nkx2.1 protein and became cells colabeled with the general neuronal markers NeuN and SMI31 (Figure 7). At the same time, these cells did not express the glutamatergic marker Tbr1 (Figure 7), but they were immunoreactive to Cux2, a marker of excitatory and inhibitory cells of the upper cortical layers (Cubelos et al., 2008; Nieto et al., 2004), and only occasionally to CalR (Figure S6). Although immunoreaction to the other interneuron markers, such as Sox6, PV, Sst, GABA, or Gad65/67, was present in these sections, there was no colocalization with Nkx2.1 or EGFP staining. These results show that ectopic *hNkx2.1* expression into embryonic murine



cortical stem cells can initiate GABAergic fate, but in the absence of proper supportive transcription factors, mature cortical interneuron fate cannot be achieved.

### Transfection of *hNkx2.1* and *mNkx2.1* in Mouse Embryonic Progenitor Cell Culture

To verify that no *Nkx2.1*<sup>+</sup> cells from the GE were present in our dorsal pallium cultures, we initially (4 hr postisolation) immunolabeled them with the anti-*Nkx2.1* antibody. At this time point, no *Nkx2.1*<sup>+</sup> cells were observed; however, 45% of isolated cells expressed GABA. Three days after isolation (3 days in vitro), cells were transfected with either *hNkx2.1* or *mNkx2.1*, and in the next 3 and 7 days, the expression of cell-type-specific markers was assessed. Numerous transfected cells at both time points were  $\beta$ -III-tubulin<sup>+</sup> young neurons (Figure S7). Furthermore, some of the transfected cells were CalR<sup>+</sup>, suggesting that in vitro, both *mNkx2.1* and *hNkx2.1* overexpression in mouse dorsally derived progenitor cells can induce generation of CalR<sup>+</sup> neurons, similar to what has been observed after IUE at P1 (Figure S7).

### Discussion

In the present study, we provide evidence that, in addition to the origin of cortical interneurons in the human GE of the ventral telencephalon, *Nkx2.1*<sup>+</sup> progenitors reside in the cortical VZ/oSVZ of both human and nonhuman primates at midgestation. Furthermore, we also show that both *hNkx2.1* and *mNkx2.1* proteins have the capacity to initiate, but not to sustain, GABAergic phenotype in the embryonic murine cortex. Our experiments indicated that *Nkx2.1*<sup>+</sup> cells generated in the dorsal telencephalon might constitute an evolutionary novelty that was introduced in the primate's phylogenetic tree together with other species-specific molecular differences (Geschwind and Rakic, 2013; Preuss, 2001). Even among primates, human-specific and ape-specific nucleotide substitutions have been noted in the regulatory non-coding region of *Nkx2.1* that may precipitate a change in tissue expression and molecular interactions (Prabhakar et al., 2008).

Due to the pronounced differences in the distribution, number, and composition of interneuron subtypes in primates (Jones, 2009), it is essential to examine multiple cortical regions at different ages. At midgestation, we found that *Nkx2.1*<sup>+</sup> cells represent between 4% and 9% of all oSVZ cells; the respective number of the mRNA *Nkx2.1*<sup>+</sup> cells was 2%–3%, suggesting a faster turnover of mRNA compared to protein. Notably, approximately 50% of the immunolabeled *Nkx2.1*<sup>+</sup> cells are proliferating. In the light of the substantial genetic differences in gene expression between primates and rodents (Miller et al., 2014), it would be important to assess the magnitude of cortically derived interneurons in the circuitry of evolutionary new areas in humans, such as Broca and Wernicke (Geschwind and Rakic, 2013; Johnson et al., 2009). Our study indicates that neural stem cells in the proliferative zones of the dorsal telencephalon in human and nonhuman primates have a capacity to generate a subset of GABAergic interneurons that may contribute to their higher diversity compared to rodents (Al-Jaberi et al., 2013; Betizeau et al., 2013; Clowry et al., 2010; Clowry, 2014; Hansen et al., 2010; Jakovcevski et al., 2011; Lui et al., 2011). In fact, the *Nkx2.1* transcription factor was seen in both apical and basal radial glia progenitors labeled with Sox2, p-Vimentin, or Pax6, further supporting their cortical origin. In rodents,

Pax6 and Nkx2.1 transcription factors were not reported to be coexpressed (Sussel et al., 1999), suggesting that two cortical cell populations, Nkx2.1<sup>+</sup>/Pax6<sup>+</sup> and Nkx2.1<sup>+</sup>/Pax6<sup>-</sup>, might have origins in the cortical SVZ and GE, respectively. Alternatively, these cells may represent differentiation stages of the same lineage, where the absence of Pax6 would indicate the exit of Nkx2.1<sup>+</sup> cells from the progenitor stage.

The forced expression of Nkx2.1 in the MGE deriving cells in the mouse prevents migration of interneurons in the cerebral cortex, hence promoting a striatal over cortical interneuron fate (Nóbrega-Pereira et al., 2008). Here, we have taken a different approach, forcing human or mouse Nkx2.1 expression in the mouse cortical progenitors. Although Nkx2.1 is believed to be a key transcription factor governing interneuronal fate specification, we demonstrated that neither hNkx2.1 nor mNkx2.1 expression in mice is sufficient for cells to develop a mature phenotype of cortical interneurons when introduced at E14.5. In the short period following transfection, the autonomous regulation of cell fate is predominant. In the long run, however, these neurons could downregulate both GFP and Nkx2.1, or they do not have essential molecular signaling to accomplish differentiation into mature GABAergic interneurons. This stands in contrast to reports in rodents that at E12.5, ectopic expression of the Dlx gene in the cerebral cortex induces GABAergic phenotype (Anderson et al., 1999; Stühmer et al., 2002), whereas the ectopic expression of Mash1/Ascl1 in cortical neurons upregulates the expression of Dlx1/2 (Fode et al., 2000) and GABAergic differentiation (Roybon et al., 2010). Although our findings, based on overexpression, cannot be taken as a proof of fate determination, they suggest that Nkx2.1 itself might not be the only determinant of cortically derived interneurons demonstrated in primates.

We suggest that the existence of interneuronal progenitors in the neocortex of primates should be considered as an evolutionary novelty based on the results from a battery of methodological approaches used in the developing brain of rodents and human and nonhuman primates. We show that cortical Nkx2.1 expression, in both apical as well as basal RGC progenitors, is present in the human and monkey oSVZ at midgestation. The possibility that interneuronal progenitors initially come from the GE and migrate to the neocortical VZ/SVZ, where they continue to proliferate as a new class of intermediate progenitors, cannot be excluded (Taniguchi et al., 2013; Wu et al., 2011). However, such a scenario does not diminish the significance of this event as evolutionary innovation (Spradling et al., 2008). It is analogous to the development of the new progenitor types in the oSVZ, even though these cells also initially originate from the VZ. Furthermore, it is likely that Nkx2.1<sup>+</sup> progenitors have a dual origin (both GE and cortical VZ/SVZ). The precedent exists in dual origin of oligodendrocytes (OL) in mice, where early-generated OLs in embryos have ventral origin, whereas in the postnatal telencephalon, OLs are generated dorsally (Kessaris et al., 2006). The changed location of mitotic progenitors to different niches during evolution often results in a change in cell fate (Rompolas et al., 2013; Inan et al., 2012).

Due to technical limitations, it is difficult to precisely estimate the exact size of the proliferative interneuronal progenitor pool in the human cortical SVZ. For example, the presence of cortical Nkx2.1<sup>+</sup> cells was recently shown in the human fetal VZ/SVZ (Hansen et al., 2013; Ma et al., 2013), but it was considered to be a smaller population than in our



study. This discrepancy might be due to differences in ages studied or experimental conditions, including antibodies used. For example, cortical *Nkx2.1*<sup>+</sup> cells in our study are mainly present at midgestation (20–24 GW), when upper cortical layers are formed. Furthermore, the functional significance of even a small proportion of cortically generated GABAergic interneurons should not be underestimated, as exemplified in the comparison of the cellular and molecular differences between humans and chimpanzees (reviewed in Geschwind and Rakic, 2013). A small, cellular change may contribute to evolutionary processes, just as a few nucleotide substitutions in conserved noncoding sequences in the mammalian genome do.

Numerous examples of interspecies differences have been reported, including von Economo neurons, interstitial neurons, or GE-generated interneurons for the thalamus in primates (Clowry et al., 2010; Clowry, 2014; Gal et al., 2006; Geschwind and Rakic, 2013; Hsieh-Li et al., 1995; Inan et al., 2012; Marin et al., 2000; Zecevic et al., 1999, Zecevic et al., 2011). However, more directly relevant to the present findings is the fact that diverse progenitors such as *Gsx2*<sup>+</sup>, *CalR*<sup>+</sup>, *Mash1/Ascl1*<sup>+</sup>, and *COUP TFII*<sup>+</sup> cells proliferate in the human and nonhuman primate VZ/oSVZ at midgestation (Jakovcevski et al., 2011; Letinic et al., 2002; Petanjek et al., 2009; Radonji et al., 2014a; Reinchisi et al., 2012). Furthermore, human RGCs express growth factor *PDGFD* and its receptor, which is not observed in mice (Lui et al., 2014). Supporting evidence is also obtained in vitro from modeling human cortical development using induced pluripotent stem cells (Mariani et al., 2012), human embryonic stem cells (Reinchisi et al., 2013), or enriched RGCs (Radonji et al., 2014b; Yu and Zecevic, 2011). Recently, large-scale studies of genes involved in cortical development have shown considerable species-specific differences between humans and mice (Kang et al., 2011; Miller et al., 2014).

In conclusion, the present results support the often-neglected notion, started with Ramon y Cajal (1899), that human cortical GABAergic neurons are not only more numerous but also more diverse. If any part of our brain should be different from any other species, it is the cerebral cortex; and within it, interneurons (stellate cells) are traditionally considered to contribute to human mental abilities (Rakic, 1975; Cajal, 1899). Moreover, cortical GABAergic interneurons are considered to be involved in the pathogenesis of psychiatric disorders (Levitt et al., 2004; Lewis et al., 2012). For example, a subpopulation of *CalR*<sup>+</sup> and *PV*<sup>+</sup> cells that in neonatal human brain retains *Nkx2.1* expression could be more vulnerable and selectively targeted in human-specific neuropsychiatric disorders.

## Experimental Procedures

### Animals and Human Tissue

We used CD1 pregnant mice in accordance with protocols approved by the Animal Care Committee of the University of Connecticut Health Center (Farmington, CT) and the Yale Institutional Care and Use Committee. Handling of the human material was done following all necessary requirements and regulations set by the Institutional Ethics Committee.

## Tissue Processing and Immunostaining

Tissue was processed as previously described (Radonji et al., 2014b). For detailed protocol and a list of antibodies, see the Supplemental Experimental Procedures and Table S2.

## In Situ Hybridization Procedure

Brain sections were postfixed in 4% paraformaldehyde-PBS and then incubated overnight at 70°C with digoxigenin-labeled cRNA probes corresponding to human Nkx2.1 nucleotides 1,331–2,020 (BC006221.2) or Gad67 (a kind gift from Drs. William Andrews and John Parnavelas). See the Supplemental Experimental Procedures for more information.

## Technical Note

The quality of the collected human brain tissue can vary, affecting results obtained with immunohistochemistry and in situ hybridization in human fetal brains compared with rodent brains obtained in a controlled laboratory setting. Therefore, an occasional negative result, especially for in situ hybridization, could not be taken as factual. To address this problem, we increased the number of cases.

## Cell Counting and Statistical Analysis

Ten adjacent optical fields of view were selected in each culture and examined at 40× magnification and the percentage of immunolabeled cells of total bisbenzimidazole positive cells was calculated (see the Supplemental Experimental Procedures). The data were expressed as means ± SEMs and analyzed using Student's *t* tests. The criterion for significance was set at  $p < 0.05$ .

## RT-PCR and Western Blot Analysis

RT-PCR was performed with a Realplex<sup>2</sup> Mastercycler and western blot as previously described (Radonji et al., 2014a). See the Supplemental Experimental Procedures for more information.

## Organotypic Slice Culture

Tissue blocks containing the VZ/SVZ, the adjacent white matter, and the cortical plate were embedded in 4% low-melted agarose and cut frontally into 250- $\mu$ m-thick slices. Slices were transferred onto Nucleopore polycarbonate track-etched membranes in Neurobasal/B27 medium with N2 and kept at 37°C for 2 hr before electroporation. Electroporated slices were fixed for 30 min in 4% formaldehyde, cryoprotected overnight with 30% sucrose at 4°C, and mounted in the Tissue-Tek (Sakura) on glass slides. Slices were resectioned with a cryostat into 20- $\mu$ m-thick sections and processed for immunohistochemistry.

## In Vitro Electroporation of Human Slices

Slices were electroporated using a BTX electroporation system (Electro Square Porator T830, BTX) set to low-voltage mode, with a charging voltage 100–150 V, delivering two pulses of 5 ms each. Constructs used for electroporation were cloned into pCAGGS expression vector. Constructs were injected by a nanoinjector (Nanonject II, Drummond) into the ventricular zone. The electroporation was done as described before (Xu et al., 2005).

## Plasmids and In Utero Electroporation

The PCMV6-AC vector containing the human Nkx2.1 cDNA full length (transcript variant 1) was purchased from Origene (NM\_001079668). Mouse Nkx2.1 cDNA full length containing vector was obtained from Stewart Anderson's lab (University of Pennsylvania School of Medicine). Briefly, 1 µl of plasmid mixtures of human-Nkx2.1 or mouse-Nkx2.1 cDNA and pCAGG-EGFP(3:1 ratio, 2 µg/µl total mixture concentration) was injected into the cerebral ventricles followed by IUE as described earlier (Dominguez et al., 2013; Gal et al., 2006).

## Primary Cell Culture of Cortical Progenitors

Medial pallium was dissected from the forebrain in order to ensure presence of only dorsal cortical progenitors. Dissociated cell culture was prepared as described previously (Zecevic et al., 2005). Conucleofection was performed with hNkx2.1 or mNkx2.1 plasmid and CMV-EGFP-N2 plasmid (Clontech) (ratio 1:1) using Amaxa-Nucleofector. The control sample contained only CMV-EGFP plasmid.

More details about the experimental procedures and material are provided in the Supplemental Experimental Procedures.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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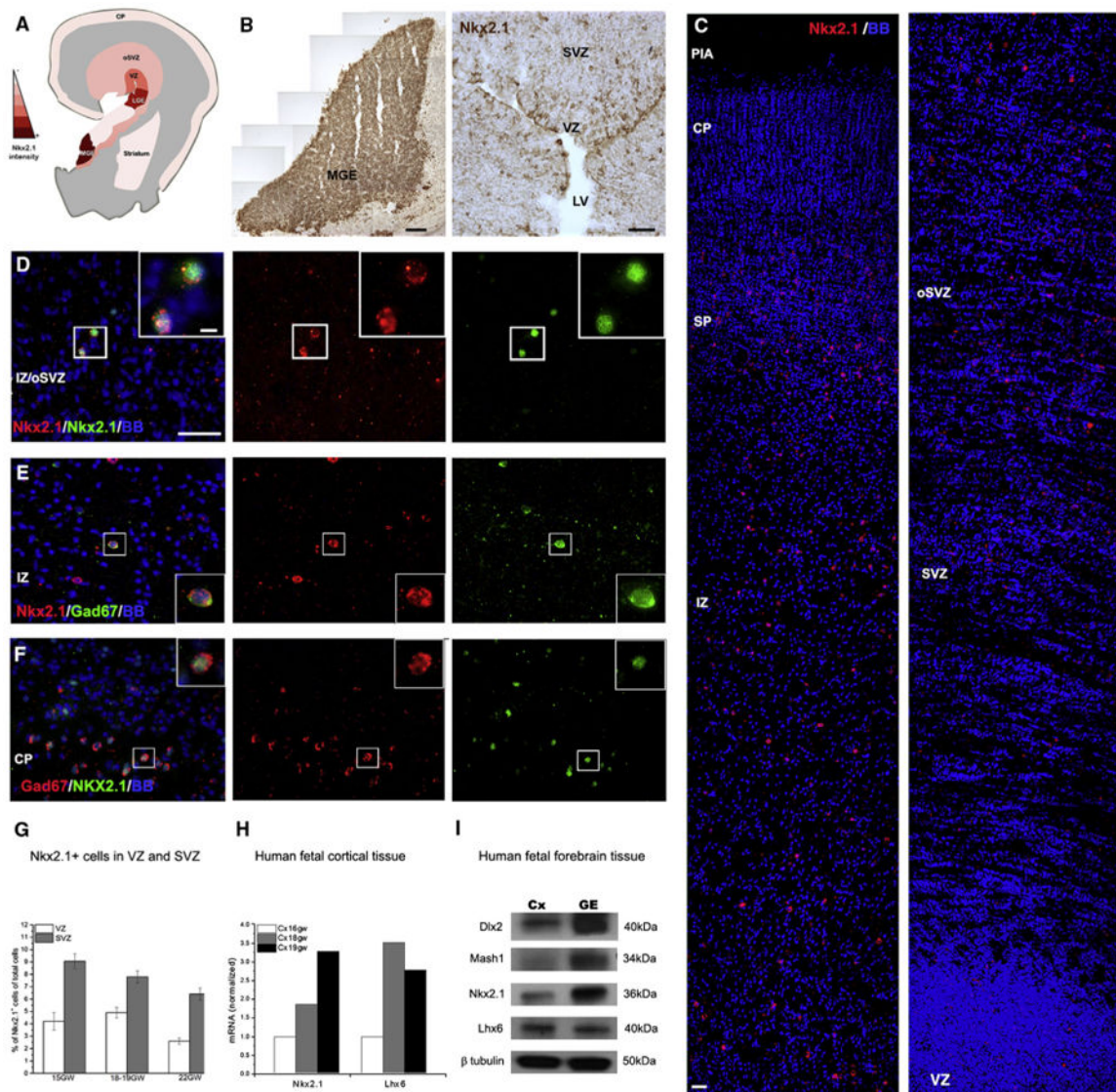
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### Highlights

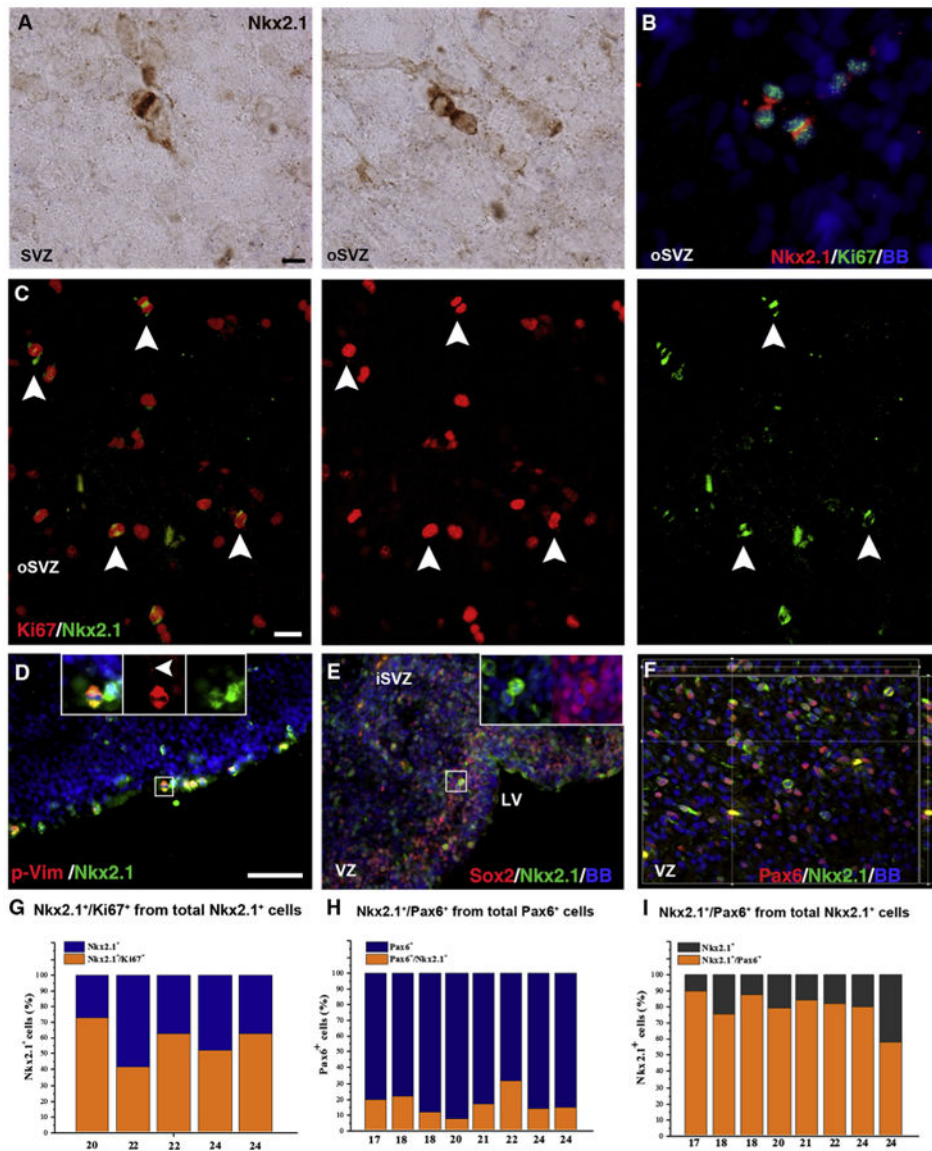
- Heterogeneity of interneuronal progenitors is increased in primate cerebral cortex
- Dorsal VZ/SVZ niche in primates generates a subset of cortical GABAergic interneurons
- Dorsally originating cortical interneurons in primates may be an evolutionary novelty



**Figure 1. The Expression of Nkx2.1 Transcription Factor in the Human Forebrain**  
 (A–C) Drawing of a coronal section of a 20 GW forebrain with distribution pattern of immunolabeled Nkx2.1<sup>+</sup> cells. (B) MGE and cortical VZ/SVZ. (C) In situ (FISH) signal for mRNA *Nkx2.1* (red) in the whole cerebral cortex at midterm.  
 (D) In situ hybridization (red) and immunolabeling with Nkx2.1 antibody (green) show colocalization of mRNA and protein.  
 (E and F) (E) mRNA *Nkx2.1* (red) and Gad67 immunolabeling (green) in the same cells; (F) mRNA *Gad67* (red) and Nkx2.1 immunolabeling (green) in the same cells. (G) Graphs demonstrating the percentage of Nkx2.1<sup>+</sup> cells from total number of cells in three gestational ages (15, 18–19, and 22 GW) and two regions: ventricular zone (VZ) and subventricular zone (SVZ).  
 (H) RT-PCR of human cortical tissue for *Nkx2.1* and *Lhx6* in three stages: 16, 18, and 19 GW; y axis, values represent fold increase normalized to 16 GW values.  
 (I) Western blot analysis of Nkx2.1, Mash1, Lhx6, and  $\beta$ -tubulin in Cx and GE.

(I) Western blot for transcription factors at 18 GW in the cortex (Cx) and ganglionic eminence (GE).

CP, cortical plate; VZ, ventricular zone; SVZ, subventricular zone. Scale bars represent 200  $\mu\text{m}$  (B) and 50  $\mu\text{m}$  (C–F) 50 $\mu\text{m}$ ; insets in (D–F) represent 10  $\mu\text{m}$ .



**Figure 2. Cells Expressing Nkx2.1 Are Proliferating Progenitors in the VZ/SVZ**

(A and B) Nkx2.1-immunopositive cells have morphology of dividing cells in the SVZ. (B) The same morphology is seen with double-labeled Nkx2.1 (in red) and Ki67 (green) cells at 18 GW.

(C) Numerous Nkx2.1/Ki67 cells (arrowheads) are proliferating in the SVZ at 20 GW.

(D) Double-labeled p-vimentin (pVim) and Nkx2.1<sup>+</sup> proliferating cells on the ventricular surface. Inset shows different channels of the boxed area; arrowhead points to a radial process.

(E) Double-labeled Sox2 (red) and Nkx2.1 (green) cells in the VZ/SVZ at midgestation. Inset shows different channels of the boxed area.

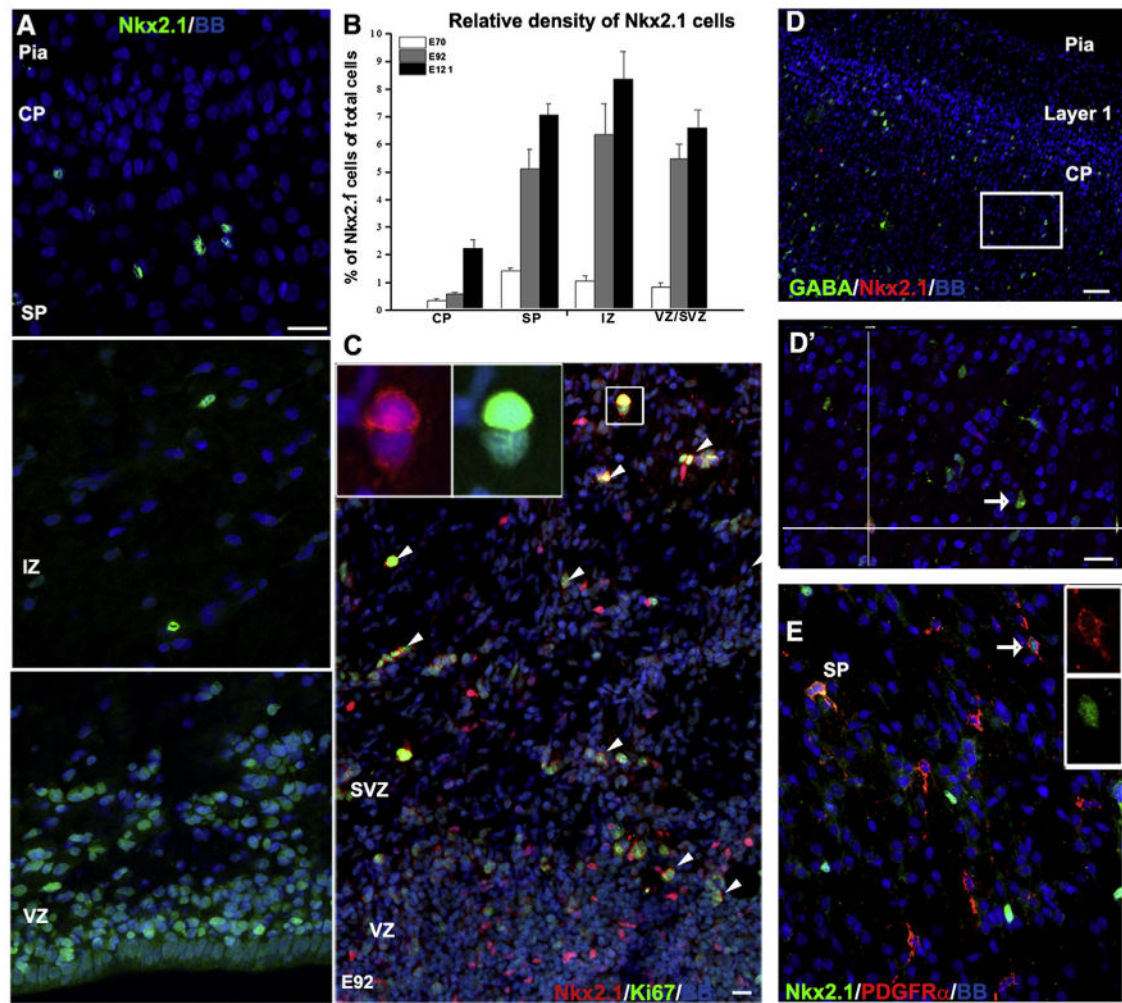
(F) Optical section through the oSVZ shows a Pax6/Nkx2.1 double-labeled cell.

(G) Percentage of dividing cells (Nkx2.1/Ki67) from all Nkx2.1<sup>+</sup> cells in the VZ/SVZ in five fetal brains, from 20 to 24 GW (x axis).

(H and I) Percentage of double-labeled Nkx2.1/Pax6 cells from (H) all Pax6<sup>+</sup> cells in the VZ/SVZ of eight fetal brains ranging from 17 to 24 GW or (I) from all Nkx2.1<sup>+</sup> cells in the VZ/SVZ of the same cases.

Scale bars represent 10  $\mu\text{m}$  (A and B), 20  $\mu\text{m}$  (C), and 50  $\mu\text{m}$  (D–F).





### Figure 3. Nkx2.1<sup>+</sup> Progenitors in the Fetal Monkey Cerebral Cortex

(A) Distribution of Nkx2.1<sup>+</sup> cells (green) throughout the cerebral cortex at E121.

(B) Relative density of Nkx2.1<sup>+</sup> cells from total cells at three embryonic ages in different cortical zones. CP, cortical plate; SP, subplate; IZ, intermediate zone; VZ/SVZ, ventricular/subventricular zone.

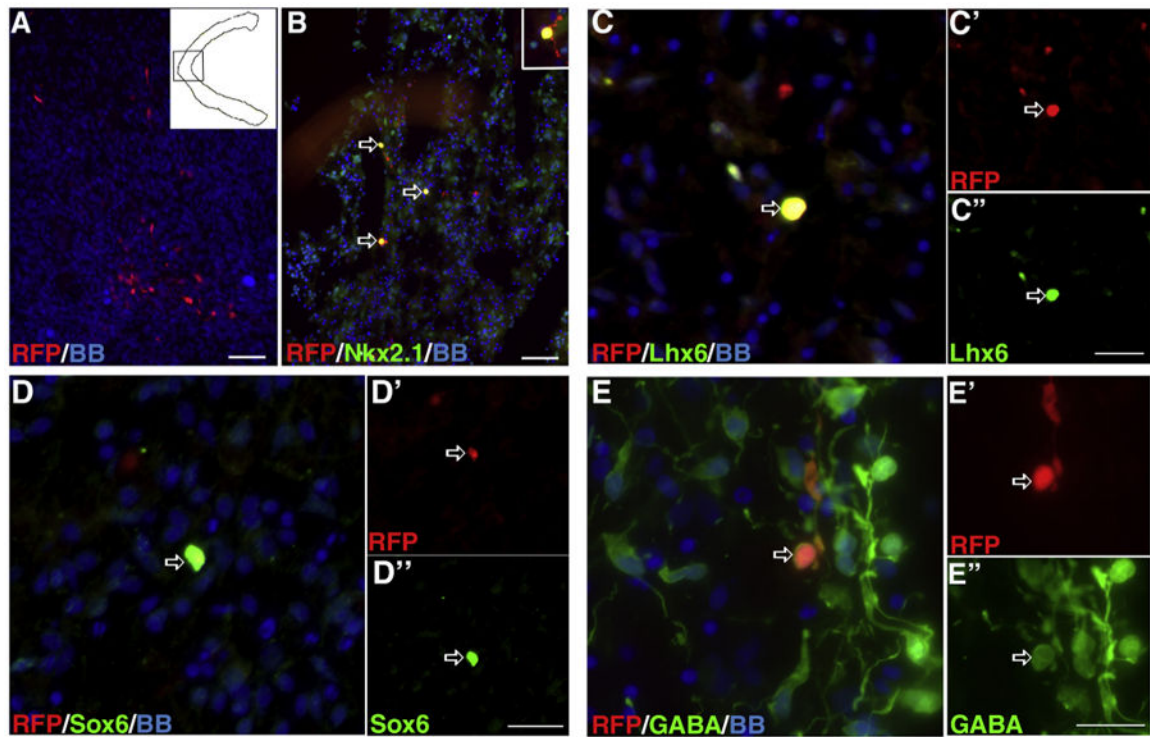
(C) Double labeling of Nkx2.1 (red) and Ki67 (green) show proliferating cells (arrowheads) at E92 monkey VZ/SVZ. Insets: higher magnification of the boxed area shows two different channels of a dividing Nkx2.1<sup>+</sup> cell.

(D) Colabeling with anti-GABA (red) and anti-Nkx2.1 (green) antibodies in the cerebral cortex of E121 monkey.

(D') Higher magnification of double-labeled cell, obtained by optical sectioning; arrows point to single-labeled GABA cells.

(E) Colabeling with PDGF receptor  $\alpha$  (red) and Nkx2.1 (green) shows a single (arrow) double-labeled cell.

Scale bars represent 20  $\mu$ m (A, D', and E) and 50  $\mu$ m (D).



**Figure 4. Mouse *Nkx2.1* Electroporated Cells in Human Slices Generate Interneurons**

(A) mNkx2.1 (red, RFP<sup>+</sup>) cells in the cortical VZ/SVZ of the 16 GW human slices.

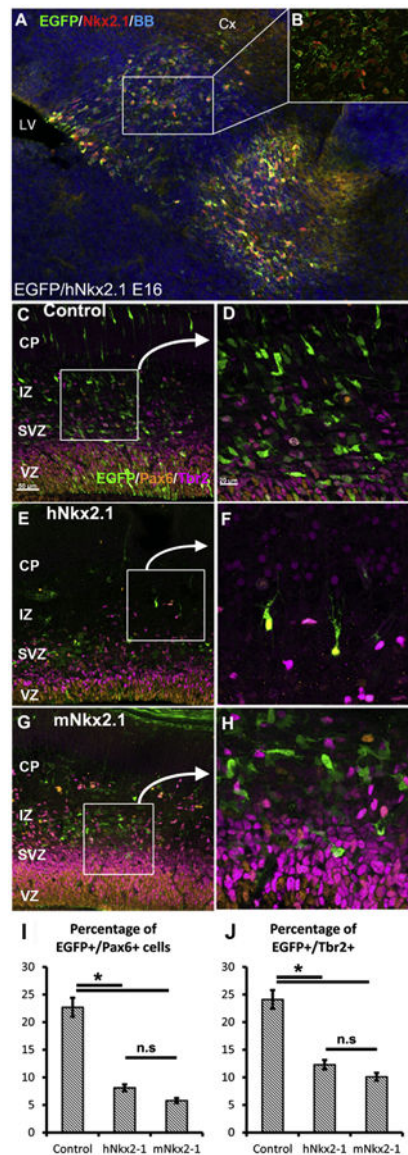
Inset shows schematic drawing of the slice. (B) mNkx2.1-overexpressing cells (red) stained with the anti-Nkx2.1 antibody (green) validating the gene overexpression. Arrows point to colocalized cells (yellow) shown in the inset.

(C) Nkx2.1-overexpressing cells (red) immunostained for Lhx6 (green). The overlay image (panel on the left) and separate channels (panels on the right) are shown. Arrow points to a cell with colocalization of mNkx2.1 and Lhx6.

(D) Nkx2.1-overexpressing cells (red) immunostained for Sox6 (green). The overlay image (panel on the left) and separate channels (panels on the right) are shown. Arrow points to a cell with colocalization of mNkx2.1 and Sox6.

(E) Nkx2.1-overexpressing cells (red) immunostained for GABA (green). The overlay image (panel on the left) and separate channels (panels on the right) are shown. Arrow points to a cell with colocalization of mNkx2.1 and GABA.

BB bisbenzimidazole (blue) was used as nuclear counterstaining on all panels. Scale bars represent 50  $\mu\text{m}$  (A and B) and 20  $\mu\text{m}$  (C–E).



**Figure 5. Coronal Section of E16.5 Mouse after Electroporation with *hNkx2.1*, *mNkx2.1*, or Control *EGFP* at E14.5**

(A) Progenitors in the dorsal telencephalon express EGFP<sup>+</sup> (green) and nuclear Nkx2.1 protein (red) after IUE with hNkx2.1 (BB bisbenzimidazole in blue).

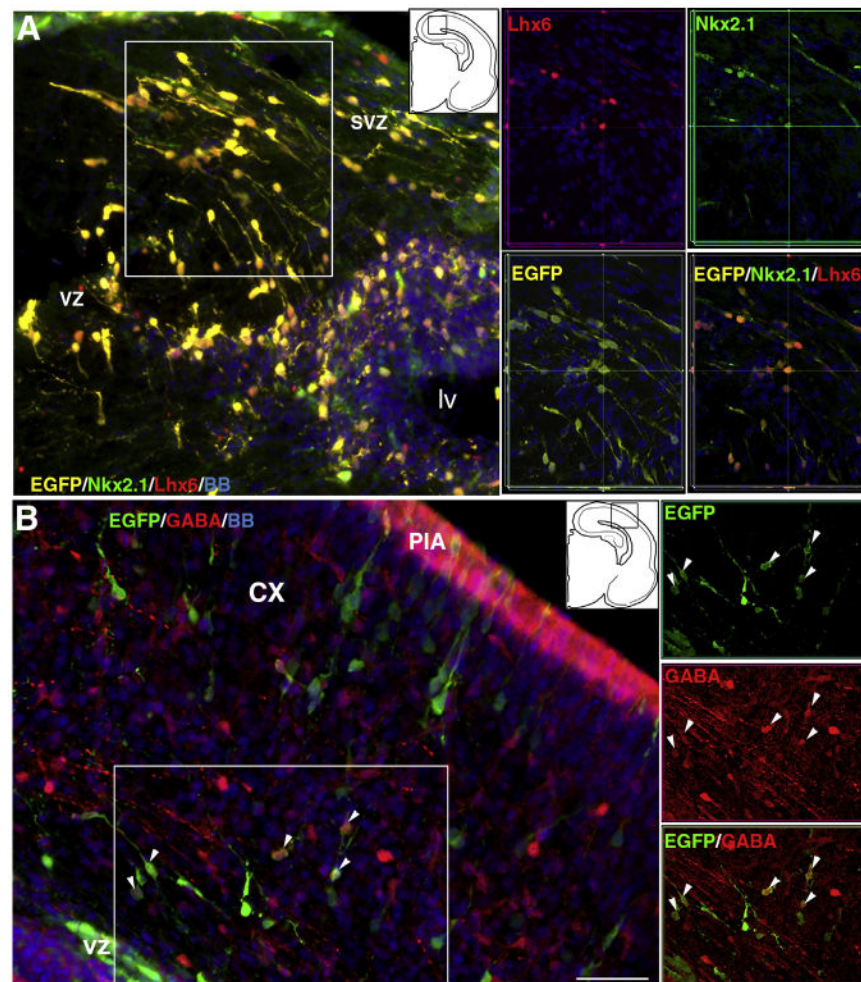
(B) Inset shows colocalization of EGFP (green) and Nkx2.1 (red).

(C–H) Sections from electroporated animals stained for Pax6 and Tbr2 (C) and inset (D) show control EGFP electroporation; (E) electroporation with EGFP/hNkx2.1: labeled cells are mainly in the VZ and SVZ region. Inset in (F) shows colocalization of EGFP/hNkx2.1 with Pax6 (orange), but not Tbr2 (magenta), in a single confocal plane. Electroporation with EGFP/mNkx2.1: labeled cells (G and H, inset) in the VZ/SVZ region are distributed in a similar fashion to EGFP/hNkx2.1.

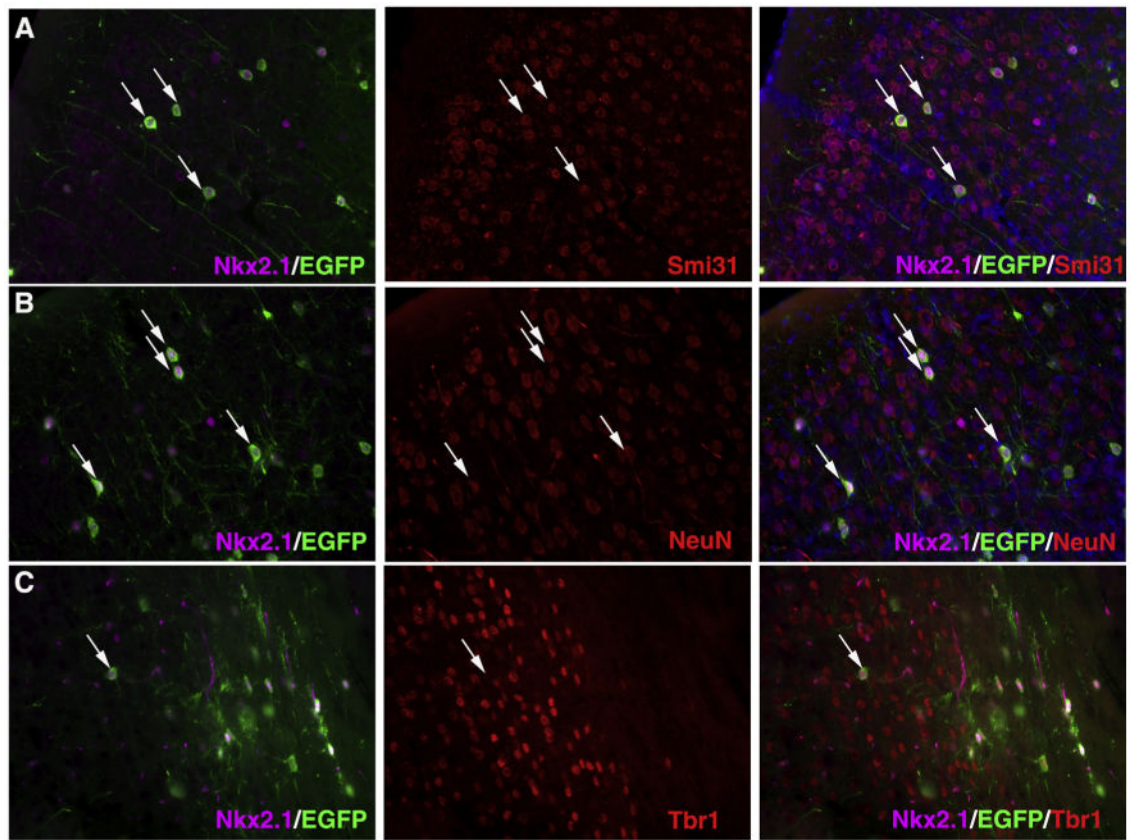
(I and J) Quantification of EGFP<sup>+</sup> cells expressing Pax6 (I) or Tbr2 (J) showed a significant drop in colabeling after IUE with mNkx2.1 or hNkx2.1 when compared to controls (n = 6;

$\pm$ SEM; \* $p < 0.001$ ). The difference between hNkx2.1 and mNkx2.1 on the exclusion of Pax6 and Tbr2 labeling was not significant (n.s., not significant).





**Figure 6. At P1, Cells Electroporated with *hNkx2.1* at E14.5 Express *Lhx6* and GABA**  
 (A) The overlay image (panel on the left) and separate channels (optical sectioning panels) are shown on the right. The cell in the center is transfected with *EGFP*<sup>+</sup> (yellow) and colabeled with *Nkx2.1* (green) and *Lhx6* (red) in the VZ/SVZ.  
 (B) Transfected *EGFP*<sup>+</sup> cells (green) are colabeled (arrowheads) with GABA (red). The overlay image (panel on the left) and separate channels (optical sectioning) are shown on the right. Insets: drawings of sections with boxed areas presented on photographs.  
 Scale bars represent 50  $\mu$ m (A) and 20  $\mu$ m (B).



**Figure 7. Coronal Sections of P21 Animals Electroporated at E14.5 with *hNkx2.1* Construct**  
 Transfected EGFP<sup>+</sup> cells mostly express Nkx2.1 (magenta), while some Nkx2.1<sup>+</sup> cells downregulated EGFP expression.

(A and B), Nkx2.1<sup>+</sup> cells colocalize with (A) SMI31 and (B) NeuN.

(C) Nkx2.1<sup>+</sup> cells do not colocalize with Tbr1. Pia is on top and left.

Scale bar represents 20 $\mu$ m.