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Prospective separation and transcriptome analyses of cortical projection neurons and interneurons based on lineage tracing by Tbr2 (Eomes)-GFP/Dcx-mRFP reporters

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Abstract

In the cerebral cortex, projection neurons and interneurons work coordinately to establish neural networks for normal cortical functions. While the specific mechanisms that control productions of projection neurons and interneurons are beginning to be revealed, a global characterization of the molecular differences between these two neuron types is crucial for a more comprehensive understanding of their developmental specifications and functions. In this study, using lineage tracing power of combining Tbr2(Eomes)-GFP and Dcx-mRFP reporter mice, we prospectively separated intermediate progenitor cell (IPC)-derived neurons (IPNs) from non-IPC-derived neurons (non-IPNs) of the embryonic cerebral cortex. Molecular characterizations revealed that IPNs and non-IPNs were enriched with projection neurons and interneurons, respectively. Expression profiling documented cell-specific genes including differentially expressed transcriptional regulators that might be involved in cellular specifications, for instance, our data found that SOX1 and SOX2, which were known for important functions in neural stem/progenitor cells, continued to be expressed by interneurons but not by projection neurons. Transcriptome analyses of cortical neurons isolated at different stages of neurogenesis revealed distinct temporal patterns of expression of genes involved in early-born or late-born neuron specification. These data present a resource useful for further investigation of the molecular regulations and functions of projection neurons and interneurons.

Keywords

Cerebral cortex; projection neurons; interneurons; transcriptome

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Data Access

RNA-seq data can be accessed at Gene Expression Omnibus (GEO) with accession number GSE62042.

Introduction

In the cerebral cortex, excitatory projection neurons and inhibitory interneurons jointly contribute to the formation and maintenance of functional neural circuits. During development, however, projection neurons and interneurons are generated at two discrete regions of the brain and require distinct molecular programs for cell lineage specification, maturation and/or function. Projection neurons are produced by radial glial cells (RGCs) in the ventricular zone (VZ) of the cortex and they migrate out of the VZ to take residence in distinct cortical layers (Sidman and Rakic, 1973; Mochida and Walsh, 2004; Leone et al., 2008; Kriegstein and Alvarez-Buylla, 2009; Greig et al., 2013; Taverna et al., 2014; Lodato et al., 2015). Interneurons, on the other hand, are generated by progenitor cells located in the ganglionic eminence, and upon birth they travel along a tangential route migrating into the cortex (Metin et al., 2006; Gelman et al., 2012; Sultan et al., 2013; Kepecs and Fishell, 2014). While the molecular mechanisms of specification of projection neurons and interneurons are beginning to be unraveled, direct molecular comparison of these two groups of neurons, particularly at the genome level, is lacking. It is conceivable that characterization of distinct molecular signatures of these neuron types would provide useful insight on their specific regulations and functions pertinent to the maintenance of a balance between excitation and inhibition in the cortex.

The majority of projection neurons in the cortex are generated from RGCs through intermediate progenitor cells (IPCs) or basal progenitor cells (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004), which was shown to contribute to all layers of the cortex (Kowalczyk et al., 2009; Vasistha et al., 2014). IPCs are specifically marked by the expression of transcription factor TBR2(EOMES) (Englund et al., 2005). Interestingly, analyses of TBR2(EOMES) expression by both RNA *in situ* hybridization (Diez-Roux et al., 2011) and immunostaining (Englund et al., 2005) revealed that TBR2(EOMES) was highly expressed in the germinal zone of the cerebral cortex but barely detectable in that of the ganglionic eminence. This prompted us to postulate that if we could separate IPC-derived neurons (IPNs) from non-IPC-derived neurons (non-IPNs) in the cortex, we might be able to achieve simultaneous enrichment of projection neurons and interneurons. In a previous study, we observed that expression of a reporter protein in progenitor cells could inevitably lead to persistent expression of the reporter in differentiated progeny (Wang et al., 2011). While this “carryover” phenomenon presents a problem for identification and purification of progenitor cells, it provides an opportunity for lineage tracing of IPNs and may thus be used for separation of IPNs from non-IPNs.

In this study, we combined Tbr2(Eomes)-GFP and Dcx-mRFP transgenic lines to generate double reporter mice. We could visualize two populations of RFP⁺ cortical neurons in the brains of these reporter mice: one population marked by both RFP and GFP and the other marked by RFP alone, reflecting IPNs and non-IPNs, respectively. We will further show prospective purification and molecular characterization of these neurons from the embryonic mouse cortex and present evidence of prospective separation and enrichment of cortical projection neurons and interneurons.

Materials and Methods

Animals

Tbr2(Eomes)-GFP transgenic reporter mice were obtained from Mutant Mouse Regional Resource Centers (MMRRC) originally produced by the GENSAT consortium (Gong et al., 2003). Dcx-mRFP transgenic reporter mice were previously described (Wang et al., 2011; Hahn et al., 2013) and can be obtained from The Jackson Laboratory under C57BL/6J-Tg(Dcx-mRFP)15Qlu/J. Animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and were carried out in accordance with NIH guideline and the Guide for the Care and Use of Laboratory Animals.

Immunohistochemistry

The immunohistochemistry staining was done as described previously (Wang et al., 2011; Hahn et al., 2013). Each antibody staining was performed on multiple brain sections and the experiment was repeated one or more times. Primary antibodies are as follows: anti-DLX2 (Kuwajima et al., 2006), anti-SATB2 (abcam, ab51502, 1:200 dilution), anti-SOX2 (Santa Cruz Biotechnology, sc-17320, 1:200 dilution), anti-SOX1 (R&D Systems, AF3369, 1:100 dilution), anti-SOX5 (Santa Cruz Biotechnology, sc-20091, 1:200 dilution), anti-CNTN2/TAG-1 (Developmental Studies Hybridoma Bank, 4D7/TAG1, 1:10 dilution), anti-WNT-7b (Novus, NBP1-59564, 1:200 dilution). Secondary antibodies with minimal cross reactivity to other species were obtained from Jackson ImmunoResearch. Images were taken using a confocal microscope (Zeiss LSM510 META NLO Axiovert 200M Inverted) or a fluorescence microscope (Olympus IX81 Automated Inverted). LSM Image Browser and Image-Pro Premier were used for image process and quantification.

FACS-mediated purification of cortical cells

Purification of E15.5 cortical cells using a double reporter strategy was done as previously described (Wang et al., 2011; Hahn et al., 2013). Briefly, we bred heterozygous Tbr2(Eomes)-GFP mice with homozygous Dcx-mRFP mice to yield GFP/RFP double positive embryos. The cortices derived from the double positive embryos were dissociated by trituration in HBSS (Mediatech) with 5mM of EDTA (Invitrogen) and 25µg/ml of DNase I (Roche). Cells were washed once with HBSS and re-suspended in DMEM/F12. FACS was performed with a 4-laser BD FACSAria™ III System (BD Biosciences). Negative cells, cell debris or cell doublets were excluded with gating of side scatter, forward scatter and pulse width. BD FACS Diva software was used for acquisition and data analysis.

RNA-seq

For conventional RNA-seq of E15.5 cortical cells, total RNAs were made from accumulated IPNs and non-IPNs (approximately one million cells) isolated using Trizol reagent (Invitrogen). For RNA-seq of 10,000 purified IPNs and non-IPNs of different stages, total RNAs were prepared using a NuGEN lysis and amplification system. RNA-seq was done by the Integrative Genomics Core at City of Hope. Single End libraries were prepared, size selected, gel purified and sequenced using Illumina HiSeq2000 system following manufacturer's protocols (Illumina). The 40-bp long single-ended sequence reads were

mapped to mouse genome (mm9) using TopHat and the frequency of Refseq genes was counted with customized R scripts. The raw counts were then normalized using trimmed mean of M values (TMM) method and compared using Bioconductor package “edgeR” (Robinson et al., 2010). The average coverage for each gene was calculated using the normalized read counts from “edgeR”. P value was generated using Bioconductor package “edgeR” using statistical model of negative binomial distribution with common dispersion of 0.1. False discovery rate (FDR) was also calculated using “edgeR”.

Results

Neuronal lineage tracing of IPNs and non-IPNs by the *Tbr2*(*Eomes*)-GFP transgene

We used a line of BAC-based *Tbr2*(*Eomes*)-GFP transgenic reporter mice (Gong et al., 2003; Kwon and Hadjantonakis, 2007) for tagging IPCs and a line of *Dcx*-mRFP reporter mice (Wang et al., 2011) for labeling neurons. When these two lines are combined for generating double reporter mice, as expected we could detect cortical cells that were positive for both GFP and RFP reporters as well as a group of cells solely positive for RFP (Figure 1A). These cells would represent IPNs and non-IPNs, respectively. To test separation of these two populations of cortical neurons, we performed fluorescence-activated cell sorting (FACS) of cortical cells derived from the embryonic cortices of the *Tbr2*-GFP/*Dcx*-mRFP double reporter embryos. The sorting profiles (Figure 1B and Figure S1) indicated that GFP⁺RFP⁺ (IPNs) and GFP⁺RFP⁻ (non-IPNs) cells displayed distinct distribution patterns that could be gated for isolation through multiple stages of cortical development. GFP expression in the cortex of the double reporter mice appeared to persist into postnatal development (Figure S1).

Selective expression of projection neuron-specific and interneuron-specific genes in IPNs and non-IPNs respectively

To study IPNs and non-IPNs, we first used FACS-based cell sorting to accumulate purified cells from the cortices at E15.5, a peak stage of cortical neurogenesis, and then performed next-generation RNA sequencing (RNA-seq) to characterize their gene expression profiles (Table S1). The sequencing results showed that IPNs and non-IPNs are two distinct groups of neurons that display differential patterns of expression of a large number of genes. IPNs showed prominent expression of many genes with important functions in projection neurons, such as transcription factors *Fezf2*, *Satb2*, *Sox5*, and *Tbr1* (Table 1A), whereas non-IPNs were characterized with high expression of *Gad1*, *Gad2*, *Dlx2*, *Dlx5*, and *Lhx6* (Table 1B), genes specifically expressed by interneurons.

To further analyze gene expressions of IPNs and non-IPNs, we next looked at these cells obtained from the E13.5 and E16.5 cortices, an early stage when interneurons newly reached the cortex from the ganglionic eminence (E13.5) and a stage when late-born projection neurons were being generated (E16.5). As non-IPN cells from the E13.5 cortices were very limited after sorting, we performed gene expression profiling of both E13.5 and E16.5 stages using an amplified RNA-seq method with fewer purified cells (10,000 cells). We found that the amplified method could yield a transcriptome overall comparable to that obtained by conventional RNA-seq (without amplification) (Figure S2). Importantly, the E13.5 and

E16.5 transcriptomes (Table S2) similarly showed that IPNs and non-IPNs were distinct groups of neurons enriched with specific factors typical of projection neurons (Table 1A) and interneurons (Table 1B), respectively.

To validate the above gene expression profiles, we performed immunostaining to characterize protein expression patterns. We looked at protein expression of SATB2, a marker for upper layer projection neurons, and DLX2, a marker for interneurons. *Satb2* and *Dlx2* genes were shown differentially expressed in IPNs and non-IPNs, respectively (Table 1). In the brains of the double reporter embryos, SATB2 (Figure 2A) was selectively expressed by the GFP⁺RFP⁺ (IPN) cells but not in the GFP⁺RFP⁻ (non-IPN) cells, whereas DLX2 (Figure 2B) could be seen more specifically expressed in non-IPN cells (95% non-IPNs were positive for DLX2, N>200). Co-staining of DLX2 and SATB2 confirmed little overlap of their expression patterns in the developing cortex (Figure 2C). These protein expression patterns are thus consistent with the observed gene expression profiles obtained from isolated IPNs and non-IPNs.

We asked what distinct features of IPN-specific and non-IPN-specific genes would display. Analyses using the DAVID (Database for Annotation, Visualization, and Integrated Discovery, <http://david.abcc.ncifcrf.gov>) bioinformatics resource showed that the top biological processes in genes differentially expressed in IPNs (Table S3) include neuron projection morphogenesis, axonogenesis, and cell projection organization (Figure 3A), while those of non-IPN cell-specific genes (Table S3) include behavior, cell-cell signaling and transmission of neuron impulse (Figure 3B). Classification of molecular function in differentially expressed genes revealed enrichment of activities of calcium ion binding, PDZ domain binding, protein kinases and sulfotransferases in IPN cell-specific genes (Figure 3A) and enrichment of activities of various types of channels and transcription factors in non-IPN cell-specific genes (Figure 3B). Gene family or pathway analyses showed that axon guidance and tight junction are among the top pathways enriched in IPNs, and lysosome and glycosaminoglycan degradation are among the top pathways enriched in non-IPNs (data not shown). These data thus collectively suggest that IPNs, as expected, were populated with cortical projection neurons, and non-IPNs were highly enriched with cells with interneuron characteristics.

Temporal patterns of gene expression during cortical neurogenesis

Our comparative transcriptome analyses between IPNs and non-IPNs at different stages of cortical neurogenesis allowed us to look at temporal patterns of gene expression during cortical neurogenesis. Figure 4A (based on Table S4) summarized differentially expressed genes encoding proteins involved in transcriptional regulation. The list included many known transcription factors important for projection neuron or interneuron functions, as well as revealed factors that were not yet fully characterized in these cells. Amongst these transcription regulators, two main types of temporal patterns of gene expression in projection neurons could be seen. One group of genes (e.g. *Fezf2*) displayed a peak expression at E15.5 (Figure 4B). Another group of genes (e.g. *Satb2*) showed increasingly stronger levels of expression during the progression of neurogenesis (Figure 4C). Expressions of several generally known housekeeping genes (Figure 4D) expressed with

relatively more uniform levels at different stages. The two patterns of expression were overall consistent with the temporal RNA *in situ* hybridization data of the Allen Brain Atlas (Figure S3). Importantly, the two temporal profiles appeared to be consistent with the functions of FEZF2 and SATB2 in early-born and late-born neuron specifications (Chen et al., 2005; Chen et al., 2005; Molyneaux et al., 2005; Alcamo et al., 2008; Britanova et al., 2008), respectively. Therefore, distinct temporal patterns of gene expression documented in the IPN transcriptome may help uncover novel regulators of projection neuron specifications and development.

Differential gene expression between projection neurons and interneurons

Differentially expressed genes between IPNs and non-IPNs might be informative for better understanding the distinct mechanisms of development of projection neurons and interneurons. Table 2 listed several examples of such differentially expressed genes based on the transcriptome analyses. *Cntn2/Tag-1*, a known axonal marker, appeared to be more strongly expressed in IPNs but not non-IPNs. *Wnt7a* and *Wnt7b* were better known for stem/progenitor cell functions; however, the transcriptome data indicated that they displayed complementary expression levels in non-IPNs and IPNs. *Sox1* and *Sox2* genes were also known associated with neural progenitor cells in the embryonic and adult brains. Surprisingly, the transcriptome data revealed that they were highly expressed in non-IPNs but little in IPNs. To validate these observations, we performed immunostaining on embryonic brain sections using available antibody combinations. Co-staining of CNTN2 with SOX5 or DLX2 (Figure 5A) in the E15.5 cortex showed that CNTN2 was expressed in many SOX5 positive projection neurons, but not in DLX2 positive interneurons, consistent with the gene profiling data. WNT7B staining signal was stronger in the cortical plate (CP) than in the ventricular zone (VZ), similar to what was observed in the RNA *in situ* hybridization data (genepaint.org; Figure 5B). Many WNT7B positive cells also expressed SATB2 (Figure 5B). On the other hand, a previous study reported that transcript of *Wnt7a* was expressed in the migratory stream of interneurons in the cortex as well as in the progenitor cells of the VZ (Faux et al., 2010) (also see Figure 5B). Co-staining of DLX2 with SOX2 showed that most DLX2⁺ cells in the marginal zone (MZ) and the intermediate zone (IZ) were positive for SOX2 (Figure 6A), whereas SOX2 and SATB2 did not seem to co-express (Figure 6B). We also looked at expression of DLX2 and SOX2 in several other stages of cortical development. Immunostaining of DLX2 and SOX2 in the E13.5 and postnatal stage cortical sections similarly revealed co-expression of the two transcription factors (Figure 6C and 6D). Furthermore, co-expression of DLX2 with SOX1 (Figure 6E) could be seen in multiple cells. Interestingly, SOX6 (also displaying enriched expression in non-IPNs in our transcriptome data) was previously shown to be expressed by interneurons and could regulate their development (Azim et al., 2009; Batista-Brito et al., 2009). Thus, multiple SOX proteins selectively continue their expression from neural progenitor cells into the interneuron lineage. These protein expression patterns collectively lend support for the differential gene expression patterns revealed by transcriptomes of IPNs and non-IPNs (Tables S1 and S3), which can serve as a resource useful for further characterizing projection neuron- or interneuron-specific genes.

Discussion

Consistent with our previous report (Wang et al., 2011), this study further demonstrated that regardless of specific promoter/enhancers used (Nestin promoter of previous study vs. Eomes promoter of this study), genetic labeling of a primitive cell type with one reporter is not sufficient. However, as we presented in this study, the longer turnover time of a reporter protein produced in primitive cells could be utilized for the purpose of lineage tracing, and when combined with a second reporter specific for daughter cells, could be used for identification and/or separation of subtypes of daughter cells. In our case, we found that combining Tbr2(Eomes)-GFP reporter with a neuronal reporter could achieve separation of IPNs and non-IPNs from the embryonic cortex.

Our data suggest that through separation of IPNs and non-IPNs, we achieved simultaneous enrichment of embryonic cortical projection neurons and interneurons. This was supported by the following observations. First, gene profiling of IPNs and non-IPNs obtained from different developmental stages revealed highly specific expression of most known markers of projection neurons and interneurons, respectively. Second, protein expression analysis by immunostaining further verified the selective expression patterns of these markers in IPNs and non-IPNs. In addition, gene profiling data also identified multiple genes, such as *Cntn2*, *Wnt7a*, *Wnt7b*, *Sox1*, and *Sox2*, with specific expression in one cell type that was not previously known but was validated by our additional expression analyses. Therefore, while IPNs and non-IPNs isolated based on double reporter expression might include other neuronal subtypes in addition to projection neurons or interneurons, for example the non-IPN fraction might also contain TBR2⁻DCX⁺ neurons that were generated from RGCs via direct differentiation (Noctor et al., 2004), our data collectively suggested that our approach of cell separation had yielded significant enrichment of projection neurons and interneurons, respectively.

We anticipate that future studies of the differentially expressed genes between IPNs and non-IPNs would help advance our understanding on the progression of projection neuron and interneuron development. As one example, the transcriptome analysis revealed selective expression of *Wnt7a* and *Wnt7b* in interneurons and projection neurons, respectively. This complementary pattern of expression suggested distinct canonical or non-canonical mechanisms of Wnt pathway operating in these two populations of neurons, which might be associated with distinct functions. As another example, the gene profiling data revealed selective expression of *Sox1* and *Sox2* genes in cortical interneurons but not projection neurons. SOX2 is known to be expressed and function in many stem/progenitor cells, including embryonic stem cells and neural stem/progenitor cells. Previous gene expression analyses using Sox2-GFP reporter mice, for example in the studies by D'Amour et al. (D'Amour and Gage, 2003) and Ellis et al. (Ellis et al., 2004), did not report expression of SOX2 in neurons. This was likely due to the very small population of interneurons relative to a much bigger population of neural progenitor cells that could be derived from the embryonic brains (e.g. Figure 2B and 6A) during cell purification. In contrast, sorting of non-IPNs in this study achieved enrichment of interneurons by means of their separation from IPNs as well as from neural progenitor cells. The continued expression of SOX2 into interneurons suggests that it may be important for interneuron specification, maturation

and/or function. Consistent with this thought, a recent study showed that SOX2 could convert astrocytes into neurons resembling interneurons (Su et al., 2014). It is worth noting that WNT7A and SOX2 display similar expression profile/pattern from progenitor cells to interneurons. This raises an interesting question as whether these two molecules may function in a linear pathway during interneuron development.

We have also documented transcriptomes of cortical neurons at several different stages of cortical neurogenesis. These temporal profiles of gene expression would be potentially useful for understanding molecular specifications of early-born and late-born projection neurons. For instance, our data revealed that transcription factors important for early-born neuron production, such as *Fezf2*, *Sox5*, and *Tbr1*, showed increased expression from E13.5 to E15.5 but their expression levels started to decrease at E16.5. On the other hand, *Satb2*, which is important for late-born neuron generation, displayed increasingly higher expression into E16.5. The distinct expression patterns of these factors were in agreement with their known functions during temporal cell fate specification in the cortex. These distinct temporal patterns are in general also consistent with the neuronal fate specification by sequential expression/activation of transcription factors previously observed in the *Drosophila* nervous system (Bayraktar and Doe, 2013; Li et al., 2013). We anticipate that the temporal transcriptomes documented in this report would help uncover novel regulators of early-born and late-born projection neuron specifications. Finally, it is conceivable that use of combinations of transcription factors selectively expressed in projection neurons or interneurons would facilitate more efficient production of projection neuron subtypes and interneuron subtypes, respectively, in therapeutic reprogramming. The differentially expressed transcriptional regulators identified in this study would be expected to aid such efforts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Use of double reporter labeling for separation of IPNs and non-IPNs

A. In the embryonic cortices of the double reporter mice, two populations of neurons could be visualized. The majority population displayed both GFP and RFP, whereas a minority population showed RFP alone (arrow indicated). White bars indicate 100 μ m.

B. FACS profile of dissociated E13.5, E15.5 and E16.5 cortical cells derived from the double reporter embryos showed that GFP/RFP double positive cells (IPNs) and RFP singly positive cells (non-IPNs) could be gated for simultaneous isolation. Sorting procedures were discussed more in details in Materials and Methods.

Figure 2. Expression of projection neuron and interneuron markers in IPNs and non-IPNs

A. IPNs but not non-IPNs showed expression of SATB2. Arrows indicated singly RFP positive cells that were not stained with SATB2. White bars indicate 100 μm.

B. Non-IPNs were associated with DLX2 expression. Arrows indicated singly RFP positive cells co-expressing DLX2. 95% non-IPNs (N>200) were positive for DLX2 expression. White bars indicate 100 μm.

C. DLX2 and SATB2 showed little overlap in expression during cortical neurogenesis. White bars indicate 100 μm.

A	IPN-specific genes	P-Value (-log10)	Gene Ontology_Biological processes	P-Value (-log10)	Gene Ontology_Molecular functions
		17.09	Neuron projection morphogenesis	3.62	Calcium ion binding
		16.13	Axonogenesis	3.53	Protein kinase activity
		16.12	Neuron projection development	3.49	PDZ domain binding
		15.60	Cell morphogenesis involved in differentiation	3.35	Transmembrane receptor protein tyrosine kinase activity
		14.58	Neuron development	3.26	Transferase activity, transferring sulfur-containing groups
		13.79	Neuron differentiation	2.89	Sulfotransferase activity
		13.38	Cell projection organization	2.58	Protein tyrosine kinase activity
		13.06	Cell morphogenesis	2.53	Phosphatase regulator activity
		11.40	Axon guidance	2.51	Protein domain specific binding
B	non-IPN-specific genes	P-Value (-log10)	Gene Ontology_Biological processes	P-Value (-log10)	Gene Ontology_Molecular functions
		5.96	Behavior	4.27	Transcription factor activity
		5.72	Cell-cell signaling	3.72	DNA binding
		4.95	Transmission of nerve impulse	3.70	Channel activity
		4.72	Pallium development	3.57	Lipid binding
		4.24	Sensory organ development	2.87	Cation channel activity
		3.84	Cerebral cortex development	2.65	Voltage-gated channel activity
		3.74	Telencephalon development	2.46	Extracellular ligand-gated ion channel activity
		3.71	Forebrain neuron differentiation	2.40	Calcium channel activity
		3.67	Neuron differentiation	2.25	Metal ion transmembrane transporter activity
		3.58	Phagocytosis, engulfment	2.16	Transcription regulator activity

Figure 3. Characterization of IPN-specific genes and non-IPN-specific genes
A. Top ten Biological Processes and Molecular Functions in IPN-specific genes (Supplemental Table 3) revealed by DAVID Bioinformatics Resources.
B. Top ten Biological Processes and Molecular Functions in non-IPN-specific genes (Supplemental Table 3).

Figure 4. Temporal patterns of gene expression during cortical neurogenesis

A. Differentially expressed regulators of transcription between IPNs and non-IPNs. Transcription regulators were identified using DAVID Bioinformatics Resources (the list of genes and RNA-seq expression data were summarized in Supplemental Table 4). The list was assembled using genes with Log2 fold change (FC) between the two cell populations ≥ 1 , and normalized RNA-seq signal in the more highly expressed cell type ≥ 10 . Colors indicate the expression levels of individual genes. Gene orders were arranged by sorting with

expression in E15.5 IPNs (for IPN-specific genes marked with Red font) and non-IPNs (for non-IPN-specific genes marked with Green font), respectively.

B. *Fezf2*-group of genes showing a peak expression at E15.5.

C. *Satb2*-group of genes showing increasingly higher expression from E13.5 to E16.5.

D. Temporal patterns of general housekeeping genes such as *Actb*, *Gapdh*, and *Tbp*.

Figure 5. Expression of CNTN2 and WNT7B by projection neurons but not interneurons

A. Co-expression of CNTN2 and SOX5 could be seen in the intermediate zone and cortical plate. CNTN2 was not co-expressed with DLX2. Arrows indicated representative neurons co-expressing CNTN2 and SOX5. White bars indicate 100 μ m.

B. WNT7B staining signal was more prominent in the cortical plate than in the ventricular zone and co-expression of WNT7B and SATB2 could be seen in many cells. Arrows indicated representative neurons co-expressing WNT7B and SATB2. Lower panels show RNA *in situ* hybridization patterns of *Wnt7b* and *Wnt7a* in the cortex (from genepaint.org).

Figure 6. Expression of SOX1 and SOX2 by interneurons but not by projection neurons

A. Majority (97%, N>400) of DLX2 positive neurons in the E15.5 cortex also showed expression of SOX2, in the marginal zone (MZ) or the intermediate zone (IZ). Arrows indicated representative neurons co-expressing DLX2 and SOX2. White bars indicate 100 μ m.

B. SOX2 staining showed little overlap with SATB2 staining.

C. Co-expression of DLX2 and SOX2 could be seen at an earlier stage of neurogenesis (E13.5). Arrows indicated representative neurons co-expressing DLX2 and SOX2. ctx, cortex; ge, ganglionic eminence.

D. Co-expression of DLX2 and SOX2 during postnatal development. Arrows indicated representative neurons co-expressing DLX2 and SOX2.

E. Co-expression of DLX2 and SOX1 was evident in the embryonic cortex.

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Table 1
Representative differentially expressed cortical neuronal markers between IPNs and non-IPNs

A. This table summarizes some of the IPN enriched genes that are known to be important for projection neurons. The numbers indicate normalized RNA-seq signals in IPNs and non-IPNs as well as the Log2 fold change (FC) between the two cell types.

B. This table summarizes some of the non-IPN enriched genes that are known to be important for interneurons. The numbers indicate normalized RNA-seq signals in IPNs and non-IPNs as well as the Log2 fold change (FC) between the two cell types.

A. IPN enriched									
Symbol	IPN	E15.5 Non-IPN	Log2FC	IPN	E13.5 Non-IPN	Log2FC	IPN	E16.5 Non-IPN	Log2FC
Bhlhe22	158.97	16.85	-3.24	175.69	22.42	-2.97	235.33	92.02	-1.35
Foxf2	214.17	11.66	-4.20	36.89	21.37	-0.79	33.50	9.84	-1.77
Foxg1	369.70	121.41	-1.61	174.33	86.69	-1.01	206.77	96.32	-1.10
Neurod1	35.92	5.20	-2.79	98.18	10.79	-3.19	26.65	4.18	-2.67
Neurod2	478.78	34.28	-3.80	52.28	5.26	-3.31	54.35	27.31	-0.99
Neurod6	988.05	68.94	-3.84	262.50	25.75	-3.35	354.96	114.16	-1.64
Sab2	89.76	7.67	-3.55	7.79	1.62	-2.26	170.28	85.55	-0.99
Sox5	165.40	9.63	-4.10	25.30	6.61	-1.94	66.09	28.10	-1.23
Tbr1	254.84	24.53	-3.38	79.28	8.60	-3.20	56.45	28.31	-1.00
B. Non-IPN enriched									
Symbol	IPN	E15.5 Non-IPN	Log2FC	IPN	E13.5 Non-IPN	Log2FC	IPN	E16.5 Non-IPN	Log2FC
Arx	5.76	340.09	5.88	2.06	73.35	5.16	3.31	68.34	4.37
Dlx1	8.95	576.11	6.01	0.29	27.28	6.55	2.23	64.53	4.85
Dlx2	2.75	163.96	5.89	0.09	45.84	9.05	1.81	88.67	5.62
Dlx5	1.56	92.26	5.86	0.00	9.56	13.75	0.24	10.96	5.54
Gad1	2.27	171.16	6.23	1.73	38.61	4.48	0.47	50.91	6.77
Gad2	1.84	125.29	6.08	0.72	42.96	5.90	2.14	88.23	5.36
Lhx6	5.38	399.84	6.21	0.02	5.73	7.89	1.15	45.80	5.31
Npy	9.71	205.07	4.39	1.09	41.42	5.25	13.46	65.01	2.27
Sst	6.64	747.62	6.80	0.92	136.99	7.21	0.37	33.70	6.52

Table 2
Representative genes not previously known for selective expression in projection neurons or interneurons

The numbers indicate normalized RNA-seq signals in IPNs and non-IPNs as well as the Log2 fold change (FC) between the two cell types.

Symbol	E15.5_IPN	E15.5_Non-IPN	Log2FC
Cntn2	429.86	39.46	-3.45
Wnt7b	170.08	20.87	-3.03
Wnt7a	6.22	59.95	3.27
Sox1	2.79	66.80	4.58
Sox2	6.84	142.17	4.37