Gene Expression in Cortical Interneuron Precursors is Prescient of their Mature Function

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At present little is known about the developmental mechanisms that give rise to inhibitory γ-aminobutyric acidergic interneurons of the neocortex or the timing of their subtype specification. As such, we performed a gene expression microarray analysis on cortical interneuron precursors isolated through their expression of a *Dlx5/6[Cre-IRES-EGFP]* transgene. We purified these precursors from the embryonic mouse neocortex at E13.5 and E15.5 by sorting of enhanced green fluorescent protein-expressing cells. We identified novel transcription factors, neuropeptides, and cell surface genes whose expression is highly enriched in embryonic cortical interneuron precursors. Our identification of many of the genes known to be selectively enriched within cortical interneurons validated the efficacy of our approach. Surprisingly, we find that subpopulations of migrating cortical interneurons express genes encoding for proteins characteristic of mature interneuron subtypes as early as E13.5. These results provide support for the idea that many of the genes characteristic of specific cortical interneuron subtypes are evident prior to their functional integration into cortical microcircuitry. They suggest interneurons are already relegated to specific genetic subtypes shortly after they become postmitotic. Moreover, our work has revealed that many of the genes expressed in cortical interneuron precursors have been independently linked to neurological disorders in both mice and humans

Keywords: cortical interneurons, subtype specification, transcriptional code

Introduction

The mammalian neocortex consists of 2 basic neuronal cell types: excitatory projection neurons, and inhibitory interneurons. The latter are typically γ-aminobutyric acidergic (GABAergic) and mediate local inhibition of other cortical neurons (Houser et al. 1983; DeFelipe and Farinas 1992). Although inhibitory interneurons comprise only about 20% of the total neuronal population in the cortex, they are crucial components of cortical circuits and their dysfunction is thought to contribute to a variety of neurological disorders such as epilepsy, autism, and schizophrenia (Baraban and Tallent 2004; Levitt et al. 2004; Cossart et al. 2005; Woo and Lu 2006). The exceptional heterogeneity in their morphological, biochemical, and functional properties (DeFelipe 1997; Kawaguchi and Kondo 2002; Markram et al. 2004; Somogyi and Klausberger 2005) likely reflects the complexity of regulating excitation in the cerebral cortex. The timing and molecular basis for the emergence of this diversity is largely unknown.

Two extreme theories have been posited to explain the emergence of cortical pyramidal cells specification and area-lization. The tabula rasa view of this process is that the mature characteristics of cortical subtypes are imposed upon them by their neocortical environment, through cues such as neurotrophins and their synaptic partners (Marty et al. 1997; Kalisman et al. 2005; Sugino et al. 2006). The alternative point of view was invoked by the "protomap hypothesis" (Rakic 1988), which has lead to the idea that combinatorial gene expression during neurogenesis (Jessell 2000) initiates an intrinsic program that ultimately results in the emergence of specific neuronal subtypes. Within the neocortex, evidence for the latter point of view came from landmark studies where the fate of pyramidal cells was challenged through heterotopic transplantation. Through this work, it was found that the laminar fate of cortical pyramidal cells was determined shortly after these cells underwent their final round of DNA replication (McConnell 1988; McConnell and Kaznowski 1991). More recently genetic studies have discovered some of the molecular effectors that mediate these events; including the identification of specific transcription factors that control pyramidal cell fate and areal identity (Hamasaki et al. 2004; Chen, Schaevitz, et al. 2005; Chen, Rasin, et al. 2005; Molyneaux et al. 2005; Cholfin and Rubenstein 2007). The "protomap" and "tabula rasa" theories have been traditionally confined to pyramidal cells. We, however, feel that given the large contribution that cortical interneurons make to this structure the time has come to intergrate this critical class of cells into the existing framework. Our present study takes the first steps in initiating such an effort by attempting to understand how developmental events shape the generation of cortical interneuron diversity. Moreover, as with studies on pyramidal cells, this effort holds the promise of elucidating the genetic basis of developmental neurological disorders whose etiology we suggest results from a failure in this specification process.

To tackle this problem, it was necessary to devise a means to prospectively isolate cortical interneurons prior to their integration into the cortical plate. Cortical interneurons originate embryonically from the ganglionic eminences in the ventral telencephalon and migrate tangentially to populate the neocortex (Anderson, Eisenstat, et al. 1997; Wichterle et al. 2001; Nery et al. 2002). The ventral eminences are marked by the expression of specific transcription factors that are known to be essential for interneuron development. In particular, members of the *Dlx* gene family, which are expressed throughout the subpallial subventricular zone, have been shown to be critical for interneuron specification (Anderson, Qiu, et al. 1997; Pleasure et al. 2000; Petryniak et al. 2007). Mice containing compound *Dlx1/Dlx2* mutations have a severe reduction in tangential migration of interneurons from the ventral eminences to the neocortex, resulting in a massive loss of neocortical GABAergic cells at birth (Anderson, Eisenstat,

et al. 1997). Similarly, null mutations in *Mash1*, a proneural gene expressed in the ventral eminences (Casarosa et al. 1999), and *Nkx2.1* (Sussel et al. 1999), a transcription factor expressed in the medial ganglionic eminence show a pronounced reduction of cortical interneurons and a concomitant reduction in *Dlx5* expression.

These previous results suggest that the *Dlx5* and *Dlx6* genes provide an attractive means for the identification of precursors (i.e., immature but postmitotic cells) destined to give rise to cortical interneurons. To this end, we chose to utilize a *Dlx5/6^{Cre-IRES-EGFP}* transgene (Stenman et al. 2003) to label and purify cortical interneuron precursors at embryonic ages. With this approach, we have identified genes that are enriched and/or highly expressed in embryonic interneuron precursors, many of which are involved in diverse biological functions such as transcription, cellular interaction, neurotransmission and network communication. These results suggest that similar to excitatory neurons (McConnell 1988; Rakic 1988; Chen, Schaevitz, et al. 2005; Chen, Rasin, et al. 2005; Molyneaux et al. 2005; Cholfin and Rubenstein 2007), interneuron specification is initiated prior to their integration into cortical circuitry. Moreover, we find that many of the genes expressed in cortical interneuron precursors are linked to specific neurological disorders.

Materials and Methods

Mouse Lines and Genotyping

All animal handling and maintenance were performed according to the regulations of the Institutional Animal Care and Use Committee of the NYU School of Medicine. The *Dlx5/6^{Cre-IRES-EGFP}* (Stenman et al. 2003) and Z/EG (Novak et al. 2000) transgenic lines were maintained in the Swiss Webster background, and genotyped as previously described (Stenman et al. 2003; Novak et al. 2000).

Cortex Dissection and Fluorescent Activated Cell Sorting

The cortex was identified by its anatomical position and morphology. The cortex of E13.5 and E15.5 Dlx5/6^{Cre-IRES-EGFP} embryos were dissected in cold Dulbecco's Modified Eagle's Medium (DMEM) and treated with 0.25% trypsin (Worthington, Lakewood, NJ) and DNase I (0.1%; Sigma, St. Louis, MO) at 37 °C for 5 min. Dissociated cells from 6 to 8 pooled embryos were used for fluorescent activated cell sorting (FACS) according to the respective brightness of enhanced green fluorescent protein (EGFP). For each sorting, we collected cells not expressing EGFP (EGFP– cells) and cells expressing EGFP (EGFP+ cells).

RNA Isolation and Microarray Hybridization

Total RNAs from FACS purified cells was prepared by the TRIzol method (Invitrogen, Eugene, OR). Purified RNA (200 ng) was amplified and biotinylated using MessageAmp II-Biotin Kit (Ambion, Austin, TX), and hybridized to microarrays MOE430A (Affymetrix, Santa Clara, CA). This procedure was repeated in triplicate for each sample to produce 3 independent data sets per RNA sample.

Microarray Expression Analysis

The success of the amplification and hybridization was assessed by all the parameters recommended by Affymetrix. We performed array triplicates for each one of our 4 populations (E13.5 EGFP+, E13.5 EGFP–, E15.5 EGFP+, and E15.5 EGFP–). In order to select for the genes that were specifically enriched in cortical interneuron precursors, we performed comparative analysis of the interneuron (EGFP+) and noninterneuron populations (EGFP–) for each of the time points (E13.5 and E15.5). Within each pairwise comparison, our statistical analysis and validation of the candidates is based on analysis of variance

($P \le 0.001$), *t*-test ($P \le 0.001$), and template matching within at least 2 of the arrays. Data processing was performed using MAS 5.0 software (Affymetrix) (Genomics Core Facility, MSKCC, NY). Signal value assigns a relative measure of abundance to the transcript. The detection algorithm uses probe pair intensities to generate a detection *P* value and assign a Present (P), or Absent (A) call. To determine the fold change, EGFP+ versus EGFP– GeneChip probe arrays were compared against each other in order to detect and quantify changes in gene expression. The analysis compares the difference of values of each probe pair in the baseline array (EGFP–) to its matching probe pair on the experiment array (EGFP+). The fold enrichment number is computed using a 1-step Tukey's Biweight method. We select the genes that have a Fold Enrichment ≥ 2 and $P \le 0.001$ for all of the triplicates.

Immunohistochemistry

Brains were fixed by transcardiac perfusion followed by 1 hour of postfixation on ice with 4% formaldehyde/phosphate buffered saline (PBS) solution. Brains were rinsed with PBS and cryoprotected by using 25% sucrose/PBS solution overnight at 4 °C. Cryosections were prepared at 12 μm (for E13.5 and E15.5) or 20 μm (for P30) thickness. Cryostat tissue sections were stained with the following primary antibodies: rabbit anti-green fluorescent protein (GFP) (1:2000; Chemicon), rat anti-GFP (1:1000; Nacalai Tesque, Kyoto, Japan), rat anti-somatostatin (SST) (1:500; Chemicon), rabbit anti-NPY (1:500; Immunostar), mouse anti-parvalbumin (PV) (1:1000; Sigma), rabbit anti-vasointestinal polypeptide (VIP) (1:500; Incstar), and mouse anti-calretinin (CR) (1:1500; Chemicon), rabbit α-GABA (1:1000; Sigma) rat anti-platelet-derived growth factor receptor (PDGFRα) (1:500; BD PharMingen, Franklin Lakes, NJ). Secondary antibodies, raised in donkey, used at 1:1000 (Alexa 594 and Alexa 488) were obtained from Invitrogen. Standard immunohistochemical staining procedures were used. Nuclear counterstaining was performed with 100 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) solution in PBS for 20 min. Fluorescent images were captured using a cooled CCD camera (Princeton Scientific Instruments, Trenton, NJ) using MetaMorph software (Universal Imaging, Downingtown, PA). Molecular expression profiles of interneurons were characterized at postnatal day 30 (P30) in somatosensory barrel cortex. The basic strategy is as described previously (Butt et al. 2005). One brain was used to quantify the percentage each one of the interneuron subtypes PV ($n = 325$), SST ($n = 309$), CR ($n = 362$), NPY ($n = 312$), and VIP ($n = 293$).

In Situ Hybridization

Messenger RNA (mRNA) in situ hybridizations was performed as described previously (Wilkinson and Nieto 1993). RNA antisense probes were prepared by PCR and labeled with digoxigenin. Most RNA probes were generated by linearization of IMAGE clones from Open Biosystems (OB) with their respective enzymes, and RNA polymerases transcription was used to obtain antisense probe. *Ssbp2* (OB clone ID: 3486988, linearization: *Eco*RV, transcription: T7 RNA polymerase); *Carhsp1* (OB clone ID: 4166353, *Kpn*I, T7 RNA polymerases); *Nxph1* (OB clone ID: 7930348, *Nco*I, T7 RNA polymerase); *Viaat* (OB clone ID: 5686843, *Xma*I, T3 RNA polymerase); *Gria1* (OB clone ID: 6842391, *Eco*RI, T3 RNA polymerase); *Kcc2* (OB clone ID: 6838880, *Eco*R1, T3 RNA polymerase); *Nkcc1* (OB clone ID: 4824556, *Hin*cIII , T3 RNA polymerase); *Cacnb4* (OB clone ID: 4501980, *Kpn*I, T7 RNA polymerase); *Cck* (OB clone ID: 1400830, *Xho*I, T3 RNA polymerase); *Npy* (Phage image clone: 482891, *Eco*RI, T3 RNA polymerase); *Sst* (Phage EST clone: 480070, *Eco*RI, T3 RNA polymerase). Some RNA probes were produced by PCR using specific primers: *Satb1* (5′ primer: ACACAGCTC TGCTGCCCAAGCC; 3′ primer GACCAGCT-GAGGACTG ATCGG); *Pbx3* (5′ primer: CGCGG ATCCAAGCAGGA-CATCGGCGACATCC; 3′ primer CCCAAGCTTTGCAGCATAGA GATTGGCCTCC); *Pou3f4* (5′ primer: CGCGGATCCAC TGG GTGAC-CAGTCTTAGCG; 3′ primer CCCAAGCTTATCTCCTGCGCTG-CAGGCTTGG); *Neud4* (5′ primer: TCTGGATGG AGAAGACCCACCG; 3′ primer TCGGGACAC CCAGTCTTCTTGG), *Cux2* (5′ primer: TCCTCCAGCTACTCCGGACAGC; 3′ primer GGAGTATGTGTC-CAGCTCTGG). Images were obtained by bright-field photography on a Zeiss Axioskop using Spot Advanced software.

Results

Genetic Fate Mapping of Dlx5/6 Expressing Cells

To confirm that the $Dlx5/6^{Cre\text{-}IRES\text{-}EGFP}$ driver we used (Stenman et al. 2003) is efficiently and selectively expressed within cortical interneuron precursors, we genetically fate mapped Dlx5/6 expressing cells within the adult cortex. We accomplished this by crossing the $Dlx5/6^{Cre\text{-}IRES\text{-}EGFP}$ transgenic mouse line (Stenman et al. 2003) to the Z/EG reporter line (Novak et al. 2000), whereby CRE activity removes a floxed-STOP cassette at the Z/EG locus, resulting in permanent EGFP expression. Previous work has suggested that *Dlx* genes are expressed in both interneuron and oligodendrocyte precursors (He et al. 2001; Marshall and Goldman, 2002), however colocalization immunological analysis of the cortex of $Dlx5/6^{Cre\text{-}IRES\text{-}EGFP}$; Z/EG animals at P30 demonstrated that in this context we exclusively label cortical GABAergic interneurons (Fig. 1*A,B,I*), and not PDGFRα expressing oligodendrocytes

(Fig. 1*H*). Moreover, with this strategy, we observed all the immunological characterized interneuron subtypes at the previously described frequencies, namely PV (47%), SST (35%), and CR (14%) (Wonders and Anderson 2006). We also quantified the percentage of 2 other interneuron markers, observing that 26% of the interneurons express NPY and 12% express VIP (Fig. 1*C-I*) (Martinez-Guijarro et al. 1998).

Isolation of Embryonic Cortical Interneuron Precursors Using the Dlx5/6^Cre-IRES-EGFP Transgenic Mouse Line

In order to identify novel genes that contribute to the embryonic generation and maturation of cortical interneuron subtypes, we have undertaken a genome-wide gene expression profile of interneuron precursors isolated from 2 different embryonic ages (E13.5 and E15.5). These ages span the early stages of interneuron precursor migration from the ventral telencephalon into the neocortex. We took advantage of a $Dlx5/6^{Cre\text{-}IRES\text{-}EGFP}$ transgenic mouse line, whose expression
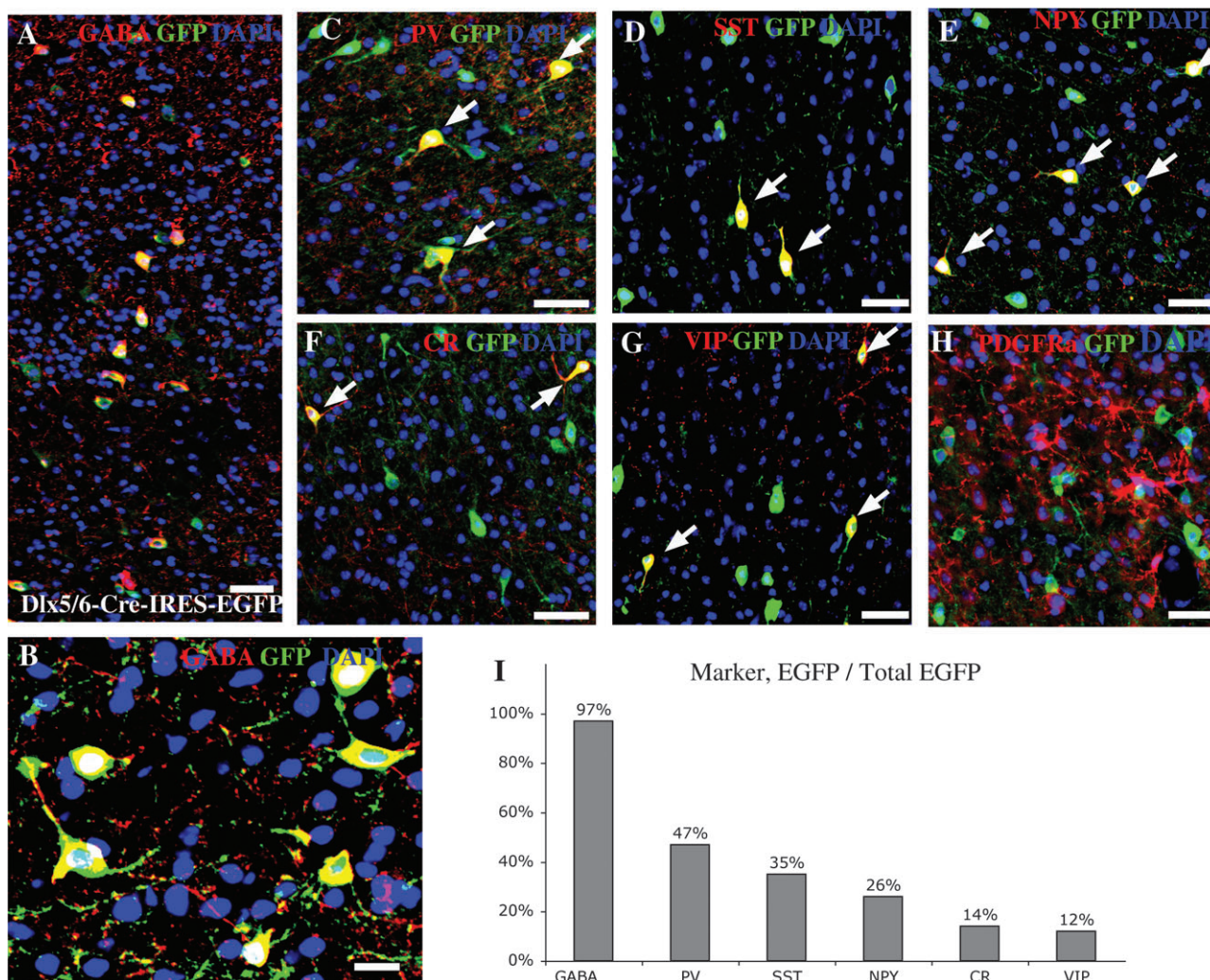

Figure 1. Genetic fate mapping of *Dlx5/6* expressing cells. Representative examples of sections for *Dlx5/6* fate mapping. EGFP, DAPI, and various immunohistochemical markers on coronal sections of P30 brains. (*A*, *B*) show colocalization of GABA and EGFP in the vast majority of fate mapped cells. (*C–G*) Partial colocalization of EGFP and the interneuron subtype markers PV, SST, NPY, CR, and VIP signals. (*H*) No colocalization of GFP and PDGFRα signals was never observed. The frequency of cortical interneuron markers at P30 in mouse somatosensory barrel cortex PV, SST, NPY, CR, and VIP are calculated by comparing the number of cells that are double positive for EGFP and the markers examined as a ratio of the total number of EGFP cells (*I*). The histogram (*I*) summarizes the cell fate mapping in the Dlx5/6^Cre-IRES-EGFP mice. Virtually all cells seen in the cortex are GABAergic. Notably the frequency and the distribution of various cell expressing immunomarkers shown is precisely consistent with that reported previously. Scale bars: (*A*) 35 µm, (*B*) 5 µm, (*C–H*) 35 µm.

of EGFP from a minimal promoter activated by the Dlx5/6 enhancer allowed us to purify interneuron precursors that have migrated into the neocortex at embryonic stages. To obtain samples tissues, we dissected the cortices of transgenic embryos using the landmarks indicated in Figure 2A and B. By restricting our analysis to Dlx5/6 precursors in the cortex, we avoided the Dlx5/6 lineages that contribute to ventral structures, such as the striatum and olfactory bulb. Due to the

robust expression of EGFP in interneuron precursors in these transgenic mice, it was possible to FACS cortical interneuron precursors (EGFP+ cells) from noninterneurons (EGFP– cells). Specifically, we collected 2.5% of the isolated EGFP+ E13.5 population, and 6.5% of the E15.5 EGFP+ cells (data not shown). We also collected non-EGFP expressing cells (EGFP– cells) from each time point for comparison. We detected no contamination in either the EGF+ or EGFP– populations (data not shown).
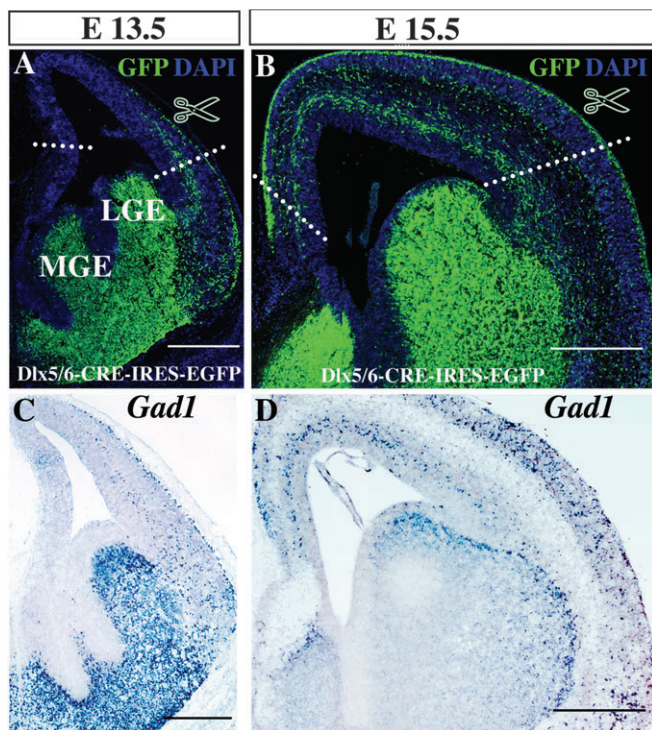
Microarray Analysis of Gene Expression in Cortical Interneuron Precursors

Validating our technical approach, we found that many of the genes known to be expressed in cortical interneurons (i.e., *Gad1*, *Dlx5*, *Dlx1*, *Dlx2*, *Lhx6*) are highly enriched in our data set (Fig. 2; Supplemental Tables 1 and 4). By contrast and as expected, we did not detect the expression of genes restricted to interneuron progenitors (such as *Nkx2.1* and *Nkx6.2*). As these genes shut off very quickly within the ventral progenitor regions, they are no longer present in the interneuron precursors that have migrated into the embryonic neocortex. Similarly, we did not detect the expression of genes expressed in pyramidal cells (such as *Ngn2*, and *Pax6*), nor markers of oligodendrocyte lineages (such as *Sox10* and *Pdgfrx*). In Figure 3, we show a summary of the classes of proteins encoded by the genes whose expression was upregulated by at least 2-fold in the EGFP+ population identified in this screen. With the exception of the cellular interaction and neuropeptide classes, there were not substantial differences between the 2 ages. The total number of genes with at least 2-fold upregulated expression at E13.5 was 416 and at E15.5 was 371.

Transcription Factors: New Candidates for Interneuron Maturation and Subtype Determination

In our analysis, we identified a number of transcription factor mRNAs that were enriched in interneuron precursors (Supplementary Table 1). Some of these genes such as *Mafb* (Cobos et al. 2006; Wonders and Anderson 2006) are known to be expressed in cortical interneuron precursors , whereas others have yet to be characterized. Among the genes encoding for transcription factors identified, the expression of a subset of
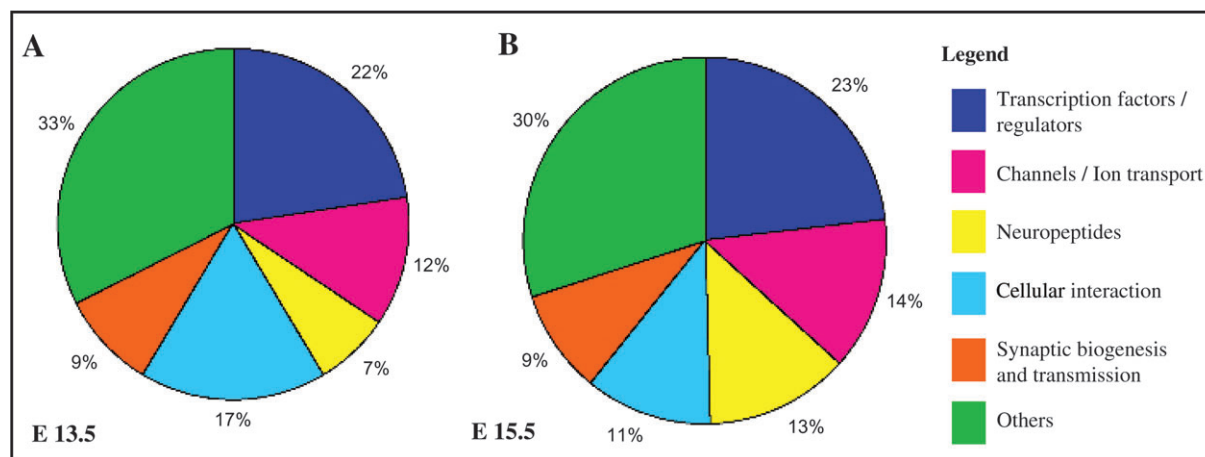

Figure 2. Isolation of cortical interneuron precursors using the transgenic Dlx5/6^Cre-IRES-EGFP mouse. EGFP immunohistochemistry on coronal sections of E13.5 and E15.5 brains. (*A*, *B*) The cortical region indicated was dissected, dissociated and FACS sorted at both ages (*A*) E13.5 and (*B*) E15.5. (*C*, *D*) In situ hybridization on similar cryosections using the *Gad1* antisense probe. Note that within the cortex the distribution of the EGFP precursors shown in (*A*) and (*B*) closely resembles the pattern of *Gad1* expression seen in (*C*) and (*D*). Scale bars: (*A–D*) 250 μm.

Figure 3. Classes of genes enriched in the interneuron population. The proportion of different classes of genes with assigned function, which are enriched in the interneuron population by at least 2-fold at E13.5 (total number genes: 416) (*A*) and E15.5 (total number genes: 371) (*B*). In this study we validated the expression of some interneuron enriched genes encoding transcription factors, neuropeptides, channels, and ion transport, and finally genes encoding cellular interaction molecules.
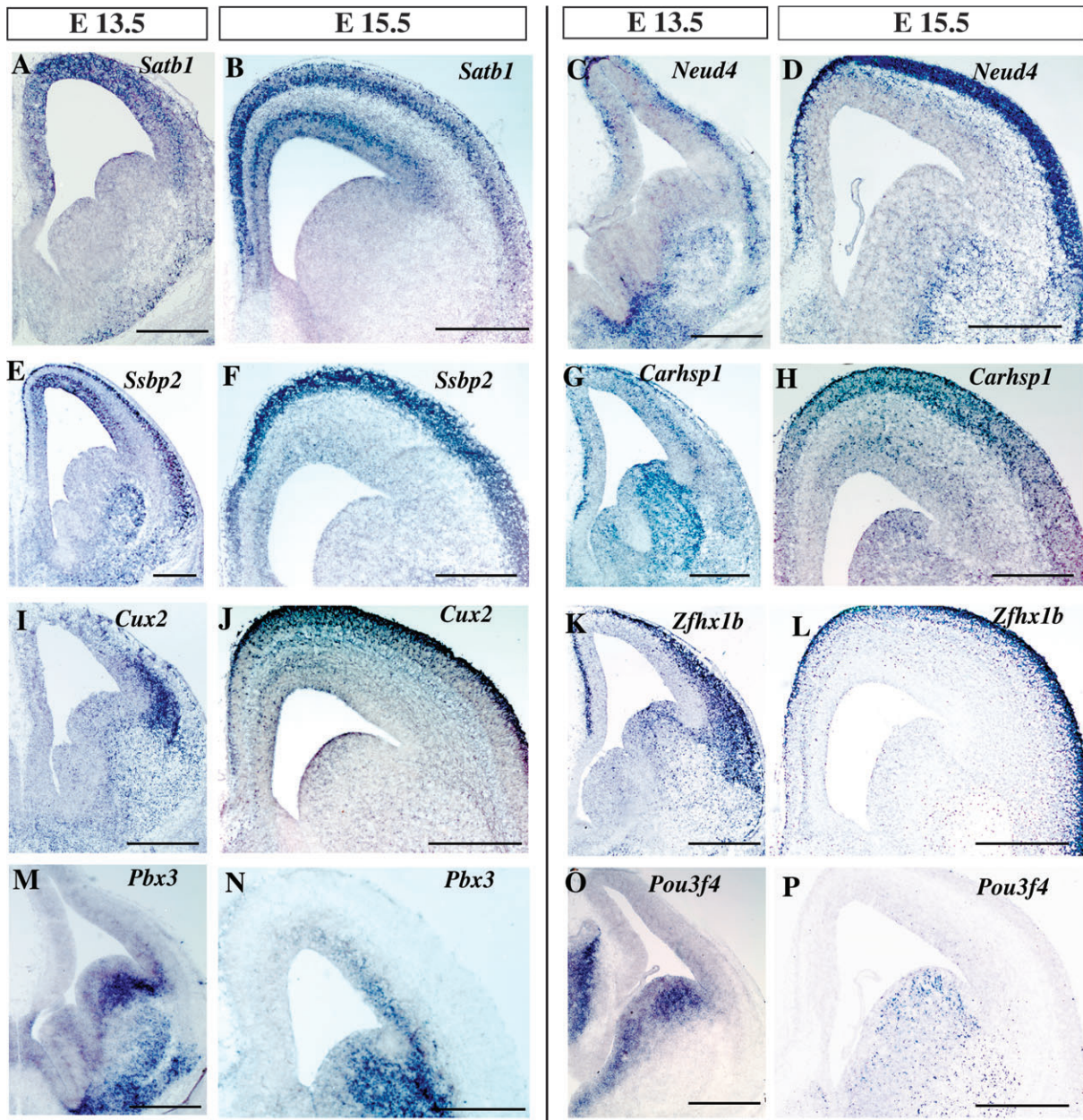
Figure 4. Expression of genes encoding transcription factors. (A–F) Expression patterns of genes encoding transcription factors enriched in interneuron precursors at E13.5 and E15.5 revealed by in situ hybridization. *Satb1*, *Neud4*, and *Ssbp2* were broadly expressed in postmitotic interneurons at both ages. (G–L) *Carhsp1*, *Cux2*, and *Zfxh1* were strongly expressed in migrating interneurons and detected within the ganglionic eminences. (M–P) *Pbx3* and *Pou3f4* were more strongly expressed in the ganglionic eminences and were downregulated in interneurons as they migrated toward the cortex. Scale bars: (A–F) 250 μm.

them was restricted to postmitotic interneurons (*Satb1*, *Neud4*, and *Ssbp2*) (Fig. 4A–F), whereas the expression of others such as *Carhsp1*, *Cux2*, *Zfhx1b*, *Pbx3*, and *Pou3f4* was detected both within the ganglionic eminences, as well as migrating interneurons (Fig. 4G–P).

Migrating Interneurons Express Subtype-Specific Neuropeptides

Neuropeptides and calcium-binding proteins provide some of the most robust markers for mature cortical interneuron subtypes. Surprisingly we found that some of these markers are already expressed in interneuron precursors by E13.5. Our microarray and in situ hybridization data revealed that the

genes encoding the neuropeptides *Sst*, *Npy*, and *Tac1* (Substance P) are exclusively expressed in cortical interneurons at both E13.5 and E15.5 (Fig. 5A–F Supplementary Table 2). We observed that many of the interneuron precursors invading the cortex already express SST and NPY (Fig. 5G,H). Similarly, *Cck* is enriched in interneuron precursors but expressed to a lesser extent in other cortical cells (Fig. 5I, Supplementary Table 2). Interestingly the genes encoding for the SST and CCK receptors (*Sstr2* and *Cckar*, respectively) have a complementary expression pattern in the noninterneuron populations (Fig. 5, Supplementary Table 2), whereas *Npy2r*, the gene encoding the receptor for NPY, is only expressed in interneurons (Fig. 5I, Supplementary Table 2).
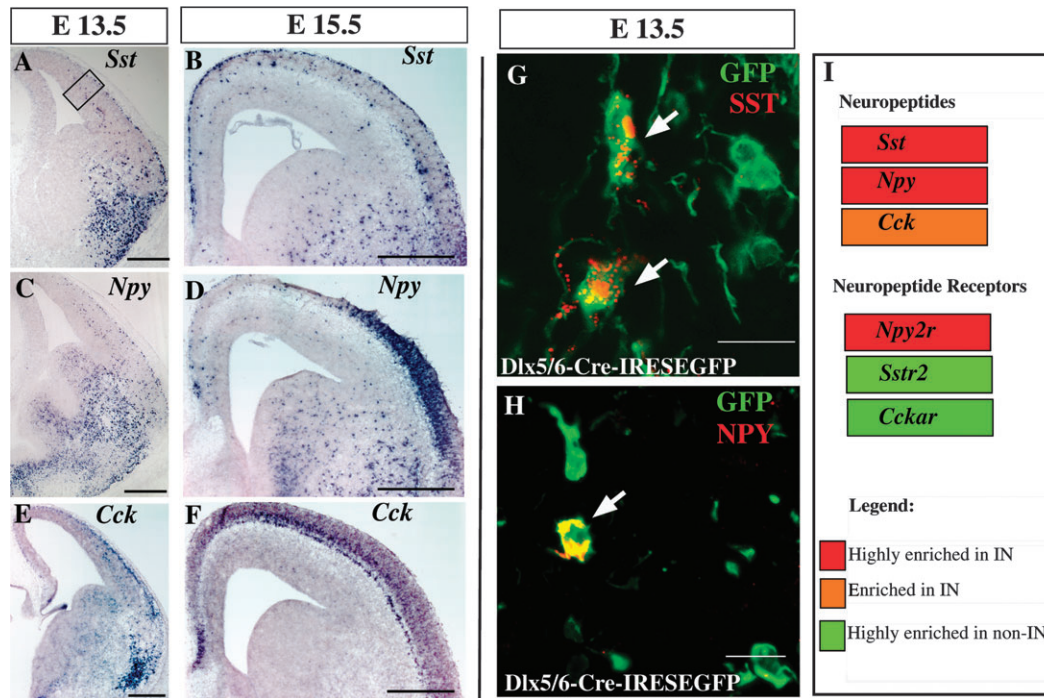
Figure 5. Expression of neuropeptides. (*A–F*) In situ hybridization for *Sst*, *Npy*, and *Cck* and double immunostaining for (*G*) EGFP/ SST, and (*H*) EGFP/ NPY of Dlx5/6[Cre-IRES-EGFP] mouse shows that subpopulations of cortical interneuron precursors express SST and NPY (arrows). Panels (*G*) and (*H*) corresponds to the location of the box in (*A*). (*I*) Microarray fold enrichment (color code) in interneuron precursors for the neuropeptides *Sst*, *Npy*, *Cck*, and neuropeptide receptors *Npy2r*, *Sstr2*, and *Cckar2*. Scale bars: (*A–F*) 250 μm, (*G–H*) 10 μm.

Cellular Interaction Genes Enriched in Migratory Interneurons

Cell surface molecules are involved in essential neurological processes, such as migration and synapse formation. In Supplementary Table 3 we present the genes involved in cellular interactions that are enriched in interneuron precursors. With the exception of *Cxcr4*, the genes we identified have not previously been implicated in cortical interneuron development. One of the highly expressed interneuron genes is *Nxph1* (Fig. 6*A,B*), which encodes a secreted peptide that binds to α-neurexins. Although known to be expressed in the adult murine brain (Petrenko et al. 1996), its specific expression in interneurons during development was not recognized (Fig. 6*A,B*). We also detected that *Neto 1* expression, a gene encoding a member of the neuropilin family is confined to interneurons. In contrast, we have detected that *Nrp1* expression, another member of the same family, while present in interneurons, is more strongly expressed in noninterneuron populations (Supplementary Table 3). In addition, we identified a number of additional genes encoding cell surface proteins, such as *Chl1*, *Dscaml*, and *Nlgn2* with enriched expression within cortical interneuron precursors (Supplementary Table 3).

Migrating Interneurons Express Channels and Synaptic Machinery

Not surprisingly, many of the genes we identified are involved in GABA signaling and are enriched at GABAergic synapses. As expected the expression of *Viaat*, the gene encoding the GABA vesicular transporter (VGAT) was confined to the interneuron population. However we were surprised that its expression was observed as early as E13.5. Similarly, the GABA-A transporter (*Gat1*), and the GABA-A receptor (*Gabrg2*) genes were already expressed in embryonic interneuron precursors (Fig. 6*M*, Supplementary Table 4). Although the expression of the K/Cl cotransporter *Nkcc1* was present at similar levels in both interneuron precursors and other cortical cells, *Kcc2* expression was highly enriched in interneuron precursors (Fig. 6*C,D,G,H,M*, Supplementary Table 4). Conversely, there was an enrichment in the expression of genes involved in glutamate signaling and glutamatergic-synapses in the interneuron population. For instance, we detected the exclusive expression of the glutamate receptor gene *Grik1* (also known as *Glur5*), as well as the enriched expression of both the ionotropic glutamate receptor gene AMPA1 (*Gria1*), and the gene encoding the glutamate receptor interacting protein 1 (*Grip1*) in interneuron precursors (Fig. 6*I,J,M*, Supplementary Table 4). Finally, we also found genes encoding multiple calcium, sodium, and potassium voltage-gated channels to be strongly expressed within interneuron precursor populations (Fig. 6*K,L,M*, Supplementary Table 4).

Migrating Interneurons Express Genes Implicated in Neurological Disorders

A variety of genes with enriched expression in cortical interneuron precursors have been linked to different neurological disorders. In Table 1 we show which of these genes have been implicated in specific human disorders, as well as the consequence of the dysfunction of these genes in mouse models. The expression data for these genes is listed in Supplementary Tables 1, 3, 4, and 5.
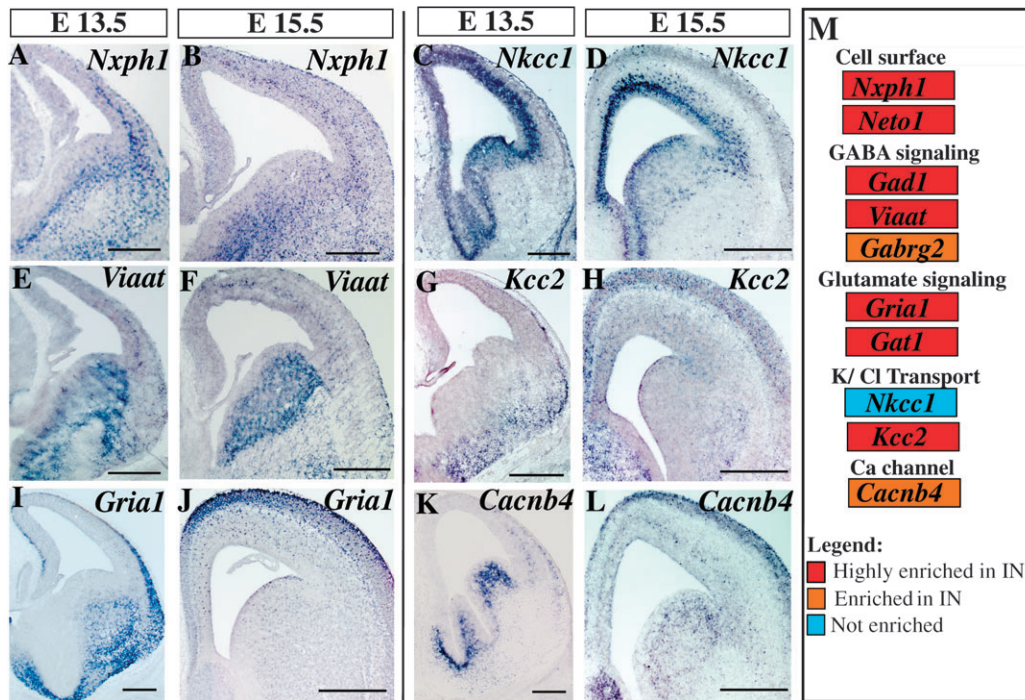
Figure 6. Expression of genes encoding cellular interaction molecules. In situ hybridization for *Nxph1*, *Nkcc1* (*C*, *D*), *Viaat* (*E*, *F*), *Kcc2* (*G*, *H*), *Gria1* (*I*, *J*), and *Cacnb4*. (*l*) Microarray fold enrichment (color code) in the interneuron precursor population for select genes involved in cell surface interactions, GABA signaling, glutamate signaling, and ionic regulation. Scale bars: (*A–L*) 250 μm.

Discussion

Through a whole genome analysis of mRNAs expressed in embryonic cortical interneuron precursors, we have identified genes that are likely involved in the maturation of this population. Moreover the proteins encoded by these genes likely have functional significance in establishing cortical interneuron diversity. To our surprise, we found that as early as E13.5 cortical interneuron precursors express mature markers, such as neurotransmitters, channels, receptors, and synaptic machinery. This data suggests that as in pyramidal neurons (McConnell 1988; Rakic 1988; Chen, Schaevitz, et al. 2005; Chen, Rasin, et al. 2005; Molyneaux et al. 2005; Cholfin and Rubenstein 2007), interneuron subtype fate is restricted prior to their integration into cortex (Butt et al. 2005; Whichterle et al. 2001; Nery et al. 2001). Moreover, the observation that many of the genes identified are associated with developmental neurological disorders argue that disruption of cortical interneuron development contributes to the underlying etiology for a number of these diseases (Rodier 2000).

Transcription Factors

Acquisition of interneuron subtype identity, similar to the spinal cord, likely relies on the combinatorial expression of specific transcription factors (Flames et al. 2007). Moreover, the assignment of a particular neuron to a given subclass is likely the result of progressive restrictions beginning in the proliferative neuroblasts and subsequently refined (Jessell 2000; Fishell 2008). In our analysis, we have identified novel transcriptional activator/repressor pathways that likely direct the maturation of specific cortical interneuron subtypes. Although some are present in the majority of interneuron precursors, others exhibit a more restricted pattern of expression that may be indicative of a role in establishing subtype or regional specificity.

Of particular interest in cortical interneuron fate determination are global regulators of gene expression, such as *Satb1*, a gene that is known to be involved in chromatin remodeling and which modulates expression at loci (Alvarez et al. 2000; Cai et al. 2003, 2006) including the specific Dlx5/6 region (Horike et al. 2005). Similarly, both *Ssbp2* and *Neud4* appear to be broadly expressed in postmitotic interneurons, and are likely involved in general maturation. In addition, genes such as *Cux2*, *Zfhx1b*, *Carhsp1* show robust expression in postmitotic neurons, but are also minimally expressed within the proliferative ventral eminences. Although *Cux2* has been previously shown to be expressed in some migrating cortical interneuron precursors (Zimmer et al. 2004), the expression of *Zfhx1b* and *Carhsp1* in interneurons was unknown. Our analysis also revealed a group of transcription factor genes including *Pou3f* (de Kok et al. 1995; Phippard et al. 1999) and *Pbx3* that are mainly expressed in the ventral eminences. The *Pbx* genes encode a family of highly conserved homeodomain proteins, and it has been shown that PBX colocalizes in the ventral telencephalon with DLX early in development (Toresson et al. 2000). Given their restricted expression patterns, these genes represent good candidates for regulating the maturation, and diversification of cortical interneurons.

Migrating Interneuron Express Mature Subtype Interneuron Markers

Interneurons are typically subdivided into different classes according to their expression of calcium-binding proteins (PV, CR, and calbindin) and neuropeptides (SST, NPY, VIP, CCK, Substance P). Here we show that cortical interneuron

Table 1
Genes expressed in developing interneurons linked to neurological disorders

Gene function	Gene	Human		Mouse	
		Disease	Ref.	Endophenotype	Ref.
Transcription factors/regulators	*Npas3*	Schizophrenia	(Pickard et al. 2005; Pickard et al. 2006)	Impaired prepulse inhibition; Impaired social recognition.	(Erbel-Sieler et al. 2004)
	Hdac11	Autism	(Szatmari et al. 2007)	NA	
	Cited2	Autism	(Szatmari et al. 2007)	NA	
	Meis1	Autism	(Szatmari et al. 2007)	NA	
	Zfhx1b	Autism; Mental retardation; Epilepsy	(Inlow and Restifo 2004; Dastot-Le Moal et al. 2007; Hoffer et al. 2007)	NA	
	Arx	Epilepsy; mental retardation; lissencephaly	(Inlow and Restifo 2004; Kato et al. 2007)	Deficit in interneuron migration.	(Colombo et al. 2007)
Cell surface	*Ncam1*	Schizophrenia; Bipolar disorder	(Atz et al. 2007)	Abnormal interneurons; Impaired sensory gating and fear conditioning; Deficient prepulse inhibition; Impaired startle response.	(Pillai-Nair et al. 2005) (Plappert et al. 2005, 2006)
	Chl1	Mental retardation	(Frints et al. 2003)	Impaired prepulse inhibition; Impaired acoustic startle response	(Irintchev et al. 2004)
	Cntnap4	Autism	(Sebat et al. 2007)	NA	
	Sema3a	Autism	(Szatmari et al. 2007)	NA	
Channels/neurotransmission	*Cacnb4*	Autism; epilepsy	(Escayg, De Waard, et al. 2000; Szatmari et al. 2007)	Ataxia; lethargy; seizures.	(Fletcher and Frankel 1999) (Burgess and Noebels 1999a, 1999b)
	Cacng2	Schizophrenia; epilepsy	(Sutrala et al. 2007)	Epileptic seizures	(Letts et al. 1998)
	Scn1a	Mental retardation; epilepsy	(Escayg, MacDonald, et al. 2000; Escayg et al. 2001; Inlow and Restifo 2004)	Epileptic seizures	(Ogiwara et al. 2007)
	Abat	Mental retardation; Autism; Schizophrenia	(Inlow and Restifo 2004: Barnby et al. 2005; Zhang et al. 2005)	NA	
	Kcnk2	Autism	(Szatmari et al. 2007)	*Kcnk2* deletion as an antidepression behavioral phenotype	(Heurteaux et al. 2006)
Others	*Dcx*	Epilepsy; Mental retardation	(Reiner et al. 2006)	Deficient interneuron migration.	(Friocourt et al. 2007)
	Man2a1	Autism	(Szatmari et al. 2007)	NA	
	Nr2f2	Autism	(Szatmari et al. 2007)	Defective tangential cell migration	(Tripodi et al. 2004)
	Shank3	Autism	(Durand et al. 2007; Szatmari et al. 2007)	NA	
	Sez6l2	Autism	(Szatmari et al. 2007)	NA	
	Dpp6	Autism	(Szatmari et al. 2007)	NA	
	Centg2	Autism	(Szatmari et al. 2007) (Wassink et al. 2005)	NA	
	Dtna	Autism	(Szatmari et al. 2007)	NA	

precursors express some of these markers during embryonic development. Specifically, we detected the selective expression of genes encoding for *Npy*, *Sst*, *Tac1* (Substance P), and *Cck* in the interneuron precursor population as early as E13.5, earlier than has been previously reported (De Belleroche et al. 1990; Morozov and Freund, 2003). Interestingly, in a complementary fashion, the expression of both the SST receptor gene (*Sstr2*), and the CCK receptor gene (*Cckar*) are enriched in noninterneuron populations. Although both of these receptors are expressed in mature pyramidal cells and are thought to modulate glutamatergic transmission (Gallopin et al. 2006), it was not previously appreciated that the genes encoding these receptors are expressed at embryonic stages. Furthermore, in contrast to CCK and SST, both NPY and the NPY receptor gene (*Npy2r*) were both exclusively expressed in immature interneurons and thus may function specifically within these caudal ganglionic eminence–derived subclasses (Butt et al. 2005). In addition to their role in functional signaling, these signaling peptides, as elsewhere in the central nervous system (Komuro and Rakic 1998), may also be utilized in directing the migration of this population.

Cellular Interactions
Cellular interactions play important roles during development, as well as in the maintenance and modification of synaptic functions in the adult. Cell adhesion molecules are known to play a role in interneuron migration (Metin et al. 2006). In this study, we have observed the expression of a number of genes encoding cell surface proteins, including *Neto1*, *Kitl*, *Chl1*, *Dscaml Ncam*, and *Astn1*, which were not previously known to be expressed in interneuron precursors. *Neto1* shares some homology with the neuropilin family of cell surface receptor genes, and there are indications that like neuropilins, this receptor can bind to class 3 semaphorins (Kolodkin et al. 1997; Michishita et al. 2003). Class 3 semaphorins act as chemo-repulsive signals for neuropilins, and mediate the directed migration of striatal and cortical interneurons, deriving from the ventral eminences (Marin et al. 2001).

Interestingly, our embryonic screen uncovered the expression of genes that are thought to be involved in the wiring of cortical circuits. For instance, we find that *Nxph1*, which encodes a secreted peptide that binds to α-neurexins, is highly expressed as early as E13.5. It has been shown that α-neurexins have a crucial role at both GABAergic and glutamatergic synapses, as α-neurexin knockout mice have reduced neurotransmitter release due to dysfunction of high-voltage activated Ca^{2+} channels (Missler et al. 2003; Beglopoulos et al. 2005). The observation that *Nxph1* is specifically enriched in cortical interneuron precursors suggests a potential role in neurotransmitter release at very early stages of cortical development.

GABAergic Signaling and Synaptic Activity

During development, ambient GABA and glutamate release are known to control neuronal proliferation and migration (Cancedda et al. 2007; Manent and Represa 2007). Previous work on the development of hippocampal circuits has demonstrated that GABA synapses generally become functional before glutamatergic ones (Hennou et al. 2002). Consistent with this, by showing the strong expression of the VGAT gene (*Viaat*) by E13.5, and the preferential expression of genes encoding molecules involved in the synaptic machinery such as Synpr, Scamp, Vamp2, and Sv2a in developing interneurons, our data suggests that cortical interneurons are among the first neurons to participate in synaptogenesis in the neocortex. However, the lower expression of many of these genes in the noninterneuron population is also consistent with the fact that Cajal-Retzius cells (Mienville and Barker 1997), which are glutamatergic, also establish early synapses.

We find that both the GABA$_A$ transporter *Gat1* and receptor gene *Gabrg2* were enriched in interneuron populations by E13.5. In immature pyramidal cells, GABA is excitatory rather than inhibitory due to the higher intracellular concentration of Cl$^-$, resulting from low perinatal expression of KCC2 (Gulyas et al. 2001; Ben-Ari 2002). However, during this same period, the *Kcc2* gene is highly expressed and enriched in cortical interneuron precursors. This suggests that unlike immature pyramidal cells, interneuron precursors may exhibit an inhibitory response to GABA. In fact, this is the case in hippocampal interneurons, where during the first postnatal weeks GABAergic synapses onto interneurons have been shown to be inhibitory (Banke and McBain 2006).

Taken together our study identifies a plethora of novel genes involved in cortical interneuron development. Moreover, our work demonstrates that cortical interneurons express genes that confer physiological function much earlier than presently recognized. These observations suggest the integration of interneurons into cortical circuitry is initiated during embryogenesis. This in turn may provide an explanation for the growing body of evidence supporting a central role for cortical interneurons in the early activity-dependent remodeling of the cortex during the critical period (Hensch and Stryker 2004; Gandhi et al. 2005; Hensch 2005; Katagiri et al. 2007).

Interneuron Development and Neurological Disorders

Many of the genes enriched in migrating cortical interneurons have been implicated in a variety of human developmental and neurological diseases (Table 1). By cross-correlating genes known to be involved in such disorders with our data, we were able to identify disease-related genes encoding all classes of proteins that we found to be enriched in cortical interneuron precursors. These findings potentially implicate cortical interneurons as being causal in a broad swath of neurological disorders. In Table 1, we list some of the genes identified through this analysis.

Six of the genes enriched in cortical interneuronal precursors encoded transcription factors that were found to be to be linked to specific brain dysfunctions. Interestingly, some of these genes resulted in apparent brain abnormalities in genetically engineered mouse knockouts. Specifically, *Npas3* and *Npas1/3* compound mutants have behavioral deficits associated with mental disorders (Erbel-Sieler et al. 2004).

Arx null mice have been implicated in autism and have an impairment in interneuron migration (Colombo et al. 2007).

A number of the genes encoding cell surface molecules were also associated with serious neurological diseases. For example, both *Ncam1* and *Chl1* have been linked to schizophrenia (Irintchev et al. 2004; Atz et al. 2007). Moreover, interneurons in transgenic mice lacking *Ncam1* function have compromised synaptic connectivity and exhibit abnormal behavior (Pillai-Nair et al. 2005). *Chl1* null mice have similar behavioral deficits, as well as axonal guidance (Montag-Sallaz et al. 2002; Irintchev et al. 2004). Similarly, disruption of genes encoding the specific channels that we found to be enriched in cortical interneuron precursors have also been associated with neurological disorders. Indeed, *Cacnb4* mutations have been strongly linked to epilepsy in both mice and humans (Burgess and Noebels 1999a, 1999b; Fletcher and Frankel 1999; Escayg et al. 2000). In addition, a deficiency of 4-aminobutyrate animotransferase in humans, which is essential for catabolism of GABA, was found in our screen to be enriched in cortical interneuron precursors and has been implicated in mental retardation, autism, and schizophrenia (Inlow and Restifo 2004; Barnby et al. 2005; Zhang et al. 2005). Furthermore, neuroligins and their receptors neurexins have been implicated in autism (Ylisaukko-oja et al. 2005; Szatmari et al. 2007). Although not directly linked to this disorder, our microarray results show that both *Nxph1* and *Nlgn2* are enriched in cortical interneuron precursors, making these good candidates for autism related genes.

Although the association between neurological disorders and interneuron function is already substantial, our understanding of this connection is in its nascence. It seems likely that many of the genes identified in our screen will ultimately be causally implicated in the development, establishment and maintenance of interneurons in cortical circuitry. This in turn will no doubt help elucidate the functions of these genes in both normal and abnormal brain function.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

Notes

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