

# Transcriptional Mechanisms of EphA7 Gene Expression in the Developing Cerebral Cortex

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**The patterning of cortical areas is controlled by a combination of intrinsic factors that are expressed in the cortex and external signals such as inputs from the thalamus. EphA7 is a guidance receptor that is involved in key aspects of cortical development and is expressed in gradients within developing cortical areas. Here, we identified a regulatory element of the EphA7 promoter, named pA7, that can recapitulate salient features of the pattern of expression of EphA7, including cortical gradients. Using a pA7-Green fluorescent Protein (GFP) mouse reporter line, we isolated cortical neuron populations displaying different levels of EphA7/GFP expression. Transcriptome analysis of these populations enabled to identify many differentially expressed genes, including 26 transcription factors with putative binding sites in the pA7 element. Among these, Pbx1 was found to bind directly to the EphA7 promoter in the developing cortex. All genes validated further were confirmed to be expressed differentially in the developing cortex, similarly to EphA7. Their expression was unchanged in mutant mice defective for thalamocortical projections, indicating a transcriptional control largely intrinsic to the cortex. Our study identifies a novel repertoire of cortical neuron genes that may act upstream of, or together with EphA7, to control the patterning of cortical areas.**

**Keywords:** cortex development, ephrin, gradient

## Introduction

The cerebral cortex is organized into numerous distinct areas, each characterized by a unique pattern of connectivity. For instance, specific cortical areas display reciprocal projections with specific nuclei of the thalamus, and cortical neurons from distinct areas send projections to distinct subcerebral targets, such as the spinal cord for motor cortex or superior colliculus for visual cortex.

In addition, cortical areas are connected with individual thalamic nuclei or other subcerebral structures in a topographic fashion, thereby allowing a faithful point to point representation between neurons of each area and connected subcortical structures.

The generation of cortical areas and their topographic organization depends on patterning signals that are intrinsic to the developing cortex, as well as from extrinsic sources. While grafting experiments have shown that thalamic afferents can influence cortical patterning, genetic evidence indicate that the cortex primordium contains intrinsic determinants of the patterning of cortical areas, including graded transcription factors

and morphogens (reviewed in Grove and Fukuchi-Shimogori 2003; Lopez-Bendito and Molnar 2003; Vanderhaeghen and Polleux 2004; Sur and Rubenstein 2005; O'Leary and Sahara 2008; Rakic et al. 2009).

Downstream of these early patterning events, it is thought that axon guidance cues, displayed in regional patterns in the cortex, control the area specific and topographic targeting of cortical input and output. Among these, ephrin-A5 and its receptor EphA7 are expressed in matching complementary gradients in several cortical areas and corresponding thalamic nuclei (Vanderhaeghen et al. 2000; Dufour et al. 2003; Yun et al. 2003; Depaepe et al. 2005; Torii and Levitt 2005). In vitro and in vivo analyses have confirmed the role of these genes in the patterning of the mouse cortex (Depaepe et al. 2005; Miller et al. 2006; Torii et al. 2009) and of thalamocortical connections (Vanderhaeghen et al. 2000; Dufour et al. 2003; Cang et al. 2005), as well as in the reciprocal mapping of cortico-thalamic output (Torii and Levitt 2005).

Interestingly, the selective gradients of expression of ephrin-A5 and EphA7 in the late embryonic cortex appear much later than the previously identified patterning morphogens and transcription factors and are essentially restricted to post-migratory neurons. This raises the question of the upstream mechanisms that control their emergence, which may provide a link between early gradients of expression in the cortical anlage and the later appearance of area-specific patterns of gene expression and topographic connectivity of cortical neurons.

Here, we have focused on the transcriptional regulation of EphA7 in the mouse embryonic cortex. We identified a specific *cis* regulatory element of EphA7 that enabled to generate a transgenic reporter mouse line, where GFP is selectively expressed along gradients of expression within the embryonic cortex, similarly to EphA7 expression. Using this model, we isolated prospectively cortical neurons on the basis of their level of GFP expression and characterized their transcriptome. This enabled to identify an array of candidate genes with differential expression within the late embryonic cortex. This patterned expression was shown to be independent from thalamic input for all genes tested. Our data point to a specific repertoire of genes that may act upstream or together with EphA7 to control the intrinsic patterning of cortical areas.

## Materials and Methods

### pA7-GFP Construct

The pSEAP2-basic plasmid (GenBank U89937) was modified into pEGFP-basic by replacement of SEAP2 cDNA by enhanced GFP (eGFP) cDNA. A

recombinant bacterial artificial chromosome (BAC) EA7-A5-GFP (Depaepe et al. 2005) was digested by BamHI and a fragment containing the 6-kb regulatory sequences, the 5' UTR of EphA7 and the rabbit Beta-globin intron was subcloned into the vector pEGFP-Basic between the transcription blocker and the eGFP cDNA. This construct was named pA7-GFP. Truncated forms of pA7 were generated by further restriction digestions to yield D1 and D2, containing 3.6 and 0.6 kb of EphA7 regulatory sequences, respectively (Fig. 1).

#### Mice

Timed-pregnant mice were obtained from Harlan and local colonies. The plug date was defined as embryonic day E0.5, and the day of birth as P0. Animal care and procedures were in compliance with local ethical committees.

#### Focal Electroporation and Organotypic Cultures

Embryonic brains were embedded in a 3% low melting point agarose in L15 medium supplemented with 30 mM glucose. Coronal sections (300  $\mu$ m) were generated using a vibratome (Leica VT1000S) and collected in an ice-cold L15/glucose medium. For electroporation, each vibrosection was put on a membrane and placed onto a 1% agarose block (in L15 medium) within a setup of 2 horizontally oriented platinum electrodes (Napagene). A small agarose column was made using a 1 mL plastic needle (Plastipak) containing 1% agarose in L15 medium and was stuck to the mobile upper electrode with a drop of 3% agarose in L15 medium. Plasmidic solutions were used at 0.5  $\mu$ g/ $\mu$ L for control plasmids (pCIG-GFP and pCIG-RFP (DsRed2) [Hand et al. 2005]) and 1  $\mu$ g/ $\mu$ L for pA7-GFP, diluted in phosphate-buffered saline (PBS) containing 5% blue trypan. Focal injections in brain slices were performed using micropipettes hooked to a Picospritzer. One drop of L15/glucose medium was put on the slice before electroporation (5 pulses at 150 V, 5 ms length, every 100 ms). Electroporated slices were transferred into fresh ice-cold L15/glucose medium and then placed in a 6-well plate onto precoated poly-L-lysine/laminin inserts, followed by culture using an air-interface protocol (Dufour et al. 2003). Slices were immunostained as previously described (Dufour et al. 2003) using primary antibodies against RFP (rabbit, 1:1000, Chemicon) and GFP (mouse, 1:500, Roche), and Cyanine 3 donkey anti-rabbit (1:500, Jackson Immunoresearch) and Alexa-488 donkey anti-mouse (1:1000, Molecular probes) secondary antibodies.

#### Transgenic Mice

Transgenic mice for the pA7-GFP construct were generated at the transgenic facility of the Universit  catholique de Louvain by

microinjection in fertilized eggs. One founder was identified to be positive by polymerase chain reaction (PCR) and Southern blot screening (data not shown), which was crossed to an outbred CD1 background to generate the pA7-GFP line. PCR primers were targeted on the eGFP transgene (Depaepe et al. 2005), and genotype was confirmed in Southern blot experiments using the eGFP cDNA probe (data not shown). Conditional mutant mice for *Clsr3* were crossed with *Dlx5-6-cre* mice to obtain selective deletion of the gene in the ventral telencephalon (VT), as previously described (Zhou et al. 2008, 2010).

#### Immunostaining of Mouse Embryos

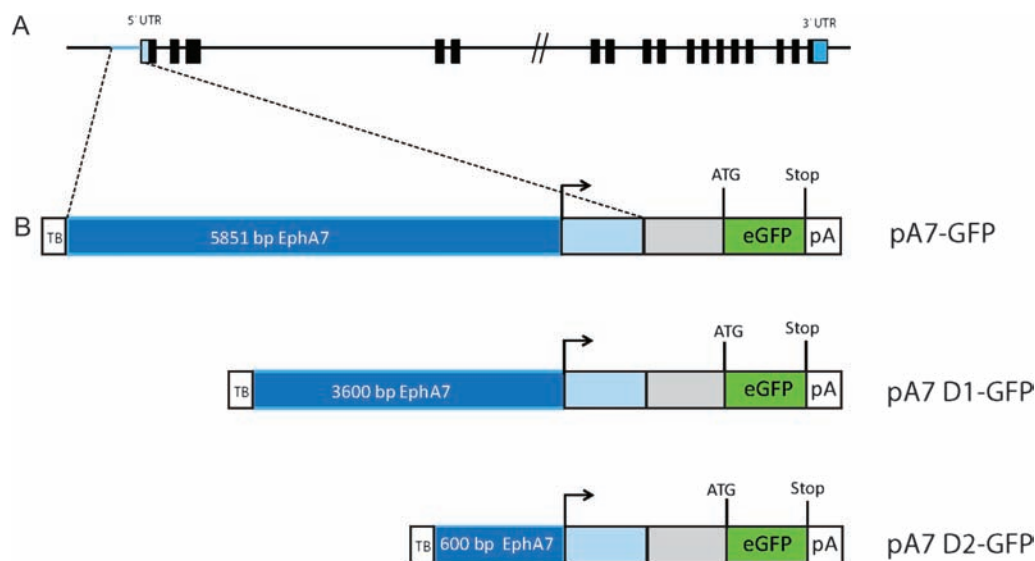
Transgenic embryos were selected under an inverted fluorescent microscope then perfused with 4% Paraformaldehyde (PFA) in PBS. Brains were directly dissected and kept in PFA at 4  C overnight. Vibratome slices (80  $\mu$ m) were collected into PBS and used for immunofluorescence. Slices were transferred into PBS/0.3% TritonX100 (PBST) blocked with PBST/3% BSA during 1 h and incubated overnight at 4  C with the following primary antibodies: chicken anti-GFP (1:2000, Abcam), rat anti-CTIP2 (1:1000, Abcam), or rabbit anti-Tbr1 (1:20000, kind gift from R. Hevner). After 3 washes, slices were incubated in PBST/3% BSA/5% Horse serum during 1 h at RT and incubated 2 h with the secondary antibody: Alexa-488 goat anti-chicken (1:1000, Molecular Probes), Cyanine 3 donkey anti-rat (1:500, Jackson Immunoresearch), and Cyanine 3 donkey anti-rabbit (1:500, Jackson Immunoresearch). Slices were then treated as previously described (Dufour et al. 2003).

#### Dissection and Dissociation of Cortical Cells

For the prospective isolation of cortical neurons displaying different levels of eGFP from pA7-GFP mice, 3 independent experiments were performed on 6 L each. Fresh brain coronal slices (300  $\mu$ m on Leica vibroslicer VT1000S) were isolated from pA7-GFP embryos at E17.5 and collected into ice-cold L-15 medium supplemented with 20 mM glucose. Coronal slices were dissected under inverted fluorescent dissecting microscope (Leica), and the cortex tissue expressing GFP in a latero-medial gradient was further dissected and enzymatically dissociated as previously described (Polleux and Ghosh 2002) with a few modifications. Dissociated cells were gently resuspended using a 3-mL plastic transfer pipette into L-15 medium without phenol red (Gibco) supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 4 mM pH 7.4 and 20 mM glucose.

#### Cell Sorting by Flow Cytometry

Cells were passed through a 40- $\mu$ m cell strainer (BD Falcon) and immediately sorted using a FACS Aria I cell sorter supported by the BD



**Figure 1.** (A) Schematic representation of the EphA7 locus. Exons are represented by boxes, coding exons are in black, and noncoding parts of exons are in blue. (B) Representation of pA7-GFP and D1/D2 deletion constructs containing, from 5' to 3': a transcription blocker site (TB), EphA7 regulatory sequences (dark blue) and its 5' UTR (light blue), rabbit  $\beta$ -globin intron (in gray), eGFP CDS (in green), and a transcriptional stop composed of multiple poly adenylation signal (pA).

FACSDiva software (Becton Dickinson). Maximum event rate was conservatively limited to 8000/s. High- and low-GFP fluorescent cells were identified in clearly separated gates, using non-GFP expressing cells as negative control, and were sorted directly into 1.5-mL tubes containing cell lysis buffer (Absolutely RNA extraction Kit, Stratagene) for subsequent RNA extraction and microarray analysis or into L-15 medium with 4 mM HEPES, pH 7.4, 20 mM glucose for mRNA amplification step using MessageBOOSTER cDNA Synthesis from Cell Lysates Kit (Epicentre) followed by quantitative real-time PCR (qRT-PCR). The temperature of both the sample and the collection tubes was kept at 4 °C during the entire sorting procedure. A typical sort with 36 transgenic embryos at E17.5 yielded 15 000 high-GFP cells and 90 000 low-GFP cells. After sorting, tubes containing lysis buffer were vigorously vortexed to ensure complete cell lysis and snap frozen into liquid nitrogen, while tubes with L15 medium were first centrifuged and pellets were then snap frozen into liquid nitrogen.

### Microarray

Total RNA was isolated using Absolutely RNA extraction Kit (Stratagene). RNA of high- or low-GFP cells obtained from 3 independent experiments were pooled together, and a double-step amplification (Affymetrix GeneChip Two-Cycle Target Labeling) starting with 8 ng of total mRNA was performed for each group before microarray hybridization using an Affymetrix Mouse 430 2.0 array (VIB MAF MicroArrayFacility, <http://www.microarrays.be/>). Standard quality assessment procedures were performed for all hybridizations according to Affymetrix recommendations. Present/absent calls for each probe set were calculated with Affymetrix MAS 5.0 statistical algorithm, CEL files from 2 chips were normalized using the Robust MultiChip Averaging algorithm.

### Quantitative Real-Time PCR

MessageBOOSTER cDNA Synthesis from Cell Lysates Kit (Epicentre) was used on pellet-sorted cells (1000 cells per reaction) coming from 2 independent sorting experiment (High1, Low1 and High2, Low2). qRT-PCR was performed using Power SybrGreen Mix and a 7500 Real-Time PCR System (Applied Biosystems). PCR primers were designed using primerBlast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). qRT-PCR of each gene of interest were done in duplicate from each sample, and *Actb* was used as housekeeping control gene. All genes were tested on pooled and amplified cDNA coming from different sorting experiment. Quantification was performed using the  $2^{-\Delta\Delta C_T}$  method relative quantification method (Livak and Schmittgen 2001).

### In Situ RNA Hybridization

In situ hybridization using digoxigenin-labeled RNA probes was performed as described previously (Lambot et al. 2005) using PCR amplified riboprobe templates (Thisse C and Thisse B 2008) or plasmids for EphA7 (Depaepe et al. 2005) and Pbx1 (gift from F. Rijli). Sense probe was used as a negative control for each gene tested and revealed no specific staining (data not shown). At least 3 embryos of each genotype were tested for each gene, and for each embryo and each gene on at least 5 different sections at the relevant level of the embryonic cortex. For analyses of *Celsr3* mutants, E17 brains of wild type and mutants (6 for each genotype, from 3 independent litters) were analyzed.

### Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously (Rustighi et al. 2009) on dissociated cortex at E16, using rabbit anti-Pbx1-2-3 antibody (sc-888; Santa Cruz Biotechnology) (Lampe et al. 2008) or control antibodies rabbit anti-HA (sc-805; Santa Cruz Biotechnology) and rabbit anti-RFP (ab62341; Abcam). Quantitative PCR (qPCR) analysis was performed using primers targeting the 11 putative binding sites for Pbx1 (Supplementary Table 4) and actin gene primers as a control. For each primer set, qPCR was performed in duplicate on 3 independent ChIP preparations.

## Results

### Identification of a Regulatory Region Driving EphA7 Expression in the Developing Cortex Using Brain Slice Electroporation Assays

We previously identified a BAC containing part of the EphA7 gene, which enables to recapitulate partially the pattern of expression of EphA7 in vivo (Depaepe et al. 2005). In order to dissect further the putative regulatory elements responsible for this patterned expression, we generated several constructs containing putative regulatory sequences of the EphA7 gene, followed by the reporter gene eGFP (Fig. 1). To test these constructs in a versatile fashion, we developed a system to study transcriptional regulation using focal coelectroporation of reporter constructs in organotypic slices prepared from E13 embryonic mouse forebrain.

To validate the method of focal electroporation to study transcriptional regulatory elements, we first performed coelectroporation of 2 control ubiquitous expression plasmids, pCIG-GFP and pCIG-RFP, which showed a similar pattern of expression of GFP and RFP after 3 days in vitro in all forebrain regions electroporated, with >90% of coelectroporated cells (Fig. 2A-C, for VT and dorsal telencephalon [DT] and data not shown for thalamus and midbrain).

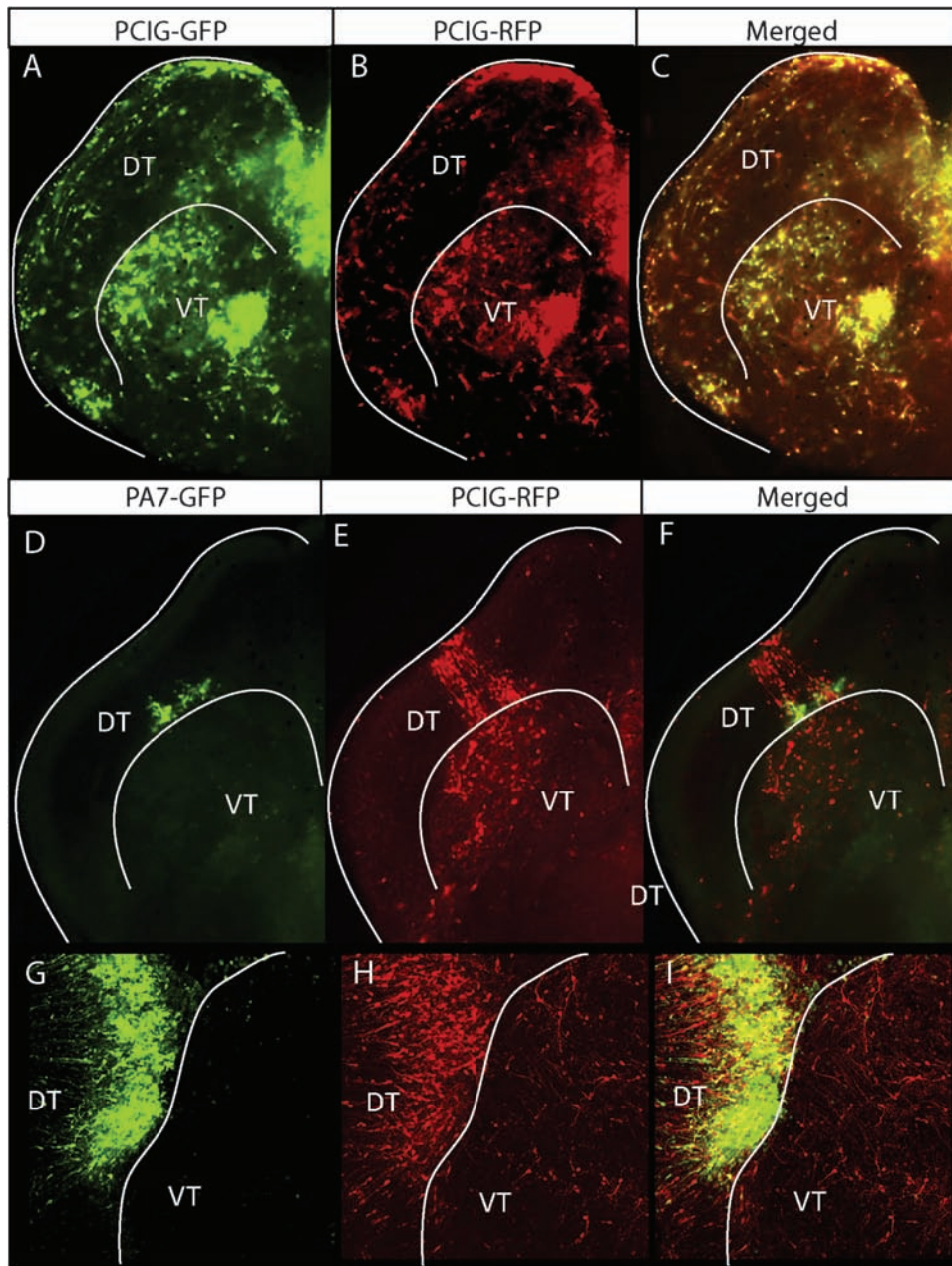
We next tested several constructs containing fragments of the putative regulatory sequences upstream of EphA7 gene transcriptional start (Fig. 1B), using coelectroporation of pCIG-RFP as an internal control. Electroporation of the largest construct, pA7-GFP, showed an expression pattern restricted to the DT, with no expression detectable in the VT (Fig. 2D,F,G,I) ( $N = 42/43$  sections tested). In contrast, RFP driven by pCIG was found to be expressed in both DT and VT (Fig. 2E,F,H,I). Similarly, the electroporation of shorter constructs (pA7D1-GFP and pA7D2-GFP), containing 3.6 kb and 0.6 kb of the regulatory sequences of EphA7, did not show any spatial specificity, with wide expression in all forebrain regions tested ( $N = 9/12$  sections for D1; 19/19 for D2; data not shown).

Overall, these data provide a first indication that the pA7 construct contains *cis* elements sufficient to drive transcription in the early DT, in a pattern reminiscent of EphA7 expression, which is expressed in the DT but not the VT at E12-13 (Depaepe et al. 2005).

### pA7-GFP Transgenic Mice Enable to Recapitulate Cortical EphA7 Expression

In order to examine in more details the transcriptional properties of the pA7GFP transgene, in particular at later stages that are not easily studied using in vitro electroporation, we turned to an in vivo setting and generated a transgenic mouse line using the same construct, which was named pA7GFP.

Embryos derived from the pA7GFP line were then examined for GFP expression at different stages. At early stages (E12-14), GFP was detected only in the DT (Supplementary Fig. 1A-C and data not shown), with no detectable expression in the VT. This is in accordance with the in vitro results and with the endogenous expression of EphA7 (Supplementary Fig. 1D,E and [Depaepe et al. 2005]), although the expression of the transgene was more prominent in post-mitotic neurons in vivo. At this stage, GFP was mainly coexpressed with Tbr1 and/or CTIP2 (Supplementary Fig. 1A-C and data not shown), which are specific markers for deep layer neurons of the cortex (respectively, of layers VI and V [Molyneaux et al. 2007]).

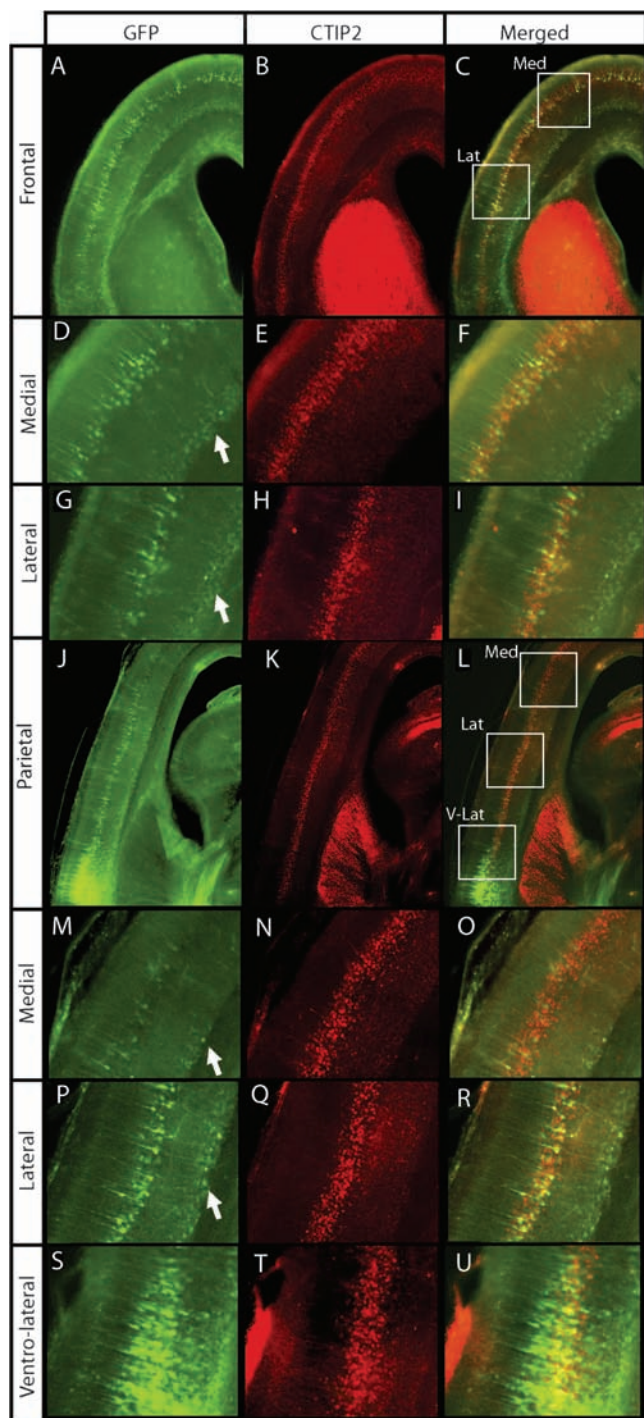


**Figure 2.** Focal electroporation identifies pA7 regulatory element to drive expression selectively in the DT. (A–C) Focal co-electroporation of pCIG-GFP and pCIG-RFP in E13 embryonic forebrain slices reveals wide coexpression of both transgenes in DT and VT. (D–I) Focal co-electroporation of PA7-GFP with pCIG-RFP indicates that PA7 drives selective expression of GFP (in green) in the DT and not the VT (D, F, G, I), while pCIG-RFP allows expression of RFP (in red) in both DT and VT (E, F, H, I). Lateral is left and dorsal up in all panels.

At E17, GFP was found to be expressed in deep layer V/VI and subplate cortical neurons (Supplementary Fig. 1F–I and Fig. 3), similarly to the EphA7 mRNA (Torii and Levitt 2005) and was mostly colocalized with CTIP2 (Fig. 3), thus corresponding mainly to layer V projection neurons.

The GFP reporter gene was expressed in the cytoplasm as well as in the dendrites and axons of the neurons, allowing to visualize the pattern of projections of the cells. GFP-positive axons were found in the internal capsule down to subcerebral targets in thalamus and midbrain, as well as in the anterior commissure (Supplementary Fig. 1G,H and data not shown), further indicating GFP expression in projection neurons of the developing cortex. At these stages, we also observed GFP expression in cell bodies of other forebrain structures expressing EphA7 such as the piriform

cortex and hippocampus (Fig. 3J and Supplementary Fig. 1G–I) and also scattered cellular expression in midbrain and hindbrain (data not shown). In contrast, no GFP expression was detected in the striatum and thalamus (Supplementary Fig. 1 and Fig. 3), which normally expresses EphA7 mRNA at embryonic and early postnatal stages (Depaepe et al. 2005; Dufour et al. 2006; Passante et al. 2008). Overall, these data indicate that the pA7 element allows expression of GFP along a specific pattern that is similar to some of the endogenous pattern of EphA7 in vivo in the cortex. However, in other regions such as the striatum and thalamus, the expression of the transgene does not recapitulate EphA7 transcriptional regulation, suggesting that other regulatory elements situated outside of pA7 are responsible for expression of the gene in these regions.



**Figure 3.** (A–U) Analysis of GFP expression (in green) in the cortex of pA7-GFP mice at E17, in comparison with CTIP2 (in red). (A–I) In the frontal cortex, GFP (in green) is expressed at similar levels throughout the medio-lateral axis of the cortex, including in the CTIP2-positive layer (in red). Boxes in (C), corresponding to lateral (Lat) and medial (Med) frontal cortex, are magnified in (D–I). (J–U) GFP is expressed in a high lateral to low medial gradient in the parietal cortex, particularly within the CTIP2-positive neurons. Boxes in (L), corresponding to ventro-lateral (v-lat), lateral (Lat), and medial (Med) cortex, are magnified in (M–U). Lateral is left and dorsal up in all panels.

A striking aspect of EphA7 expression in the developing cortex is its expression in graded patterns (Depaepe et al. 2005; Torii and Levitt 2005). We observed that from E17 until at least P6, GFP was expressed in the deep cortical layers in shallow gradients along the rostrocaudal axis and the latero-medial

of the DT (Fig. 3). In the most rostral parts of the cortex, GFP was expressed in a high medial, to low lateral, gradient (Supplementary Fig. 1F). In anterior frontal cortex, there was no discernible gradient (Fig. 3A–I), while in more caudal sections within the fronto-parietal cortex, GFP was detected in an opposite latero-medial gradient (Supplementary Fig. 1H,I and Fig. 3J–U). The graded expression of GFP followed the course of cortical layers, thus marking selective subpopulations of neurons within the same layer, with highest expression in the ventrolateral neurons and low but detectable expression more medially (Fig. 3M–U). The graded expression of the transgene in deep layers of the cortex thus strikingly recapitulates the pattern of EphA7 (Depaepe et al. 2005; Torii and Levitt 2005), in particular in the frontal/parietal cortex containing presumptive motor and somatosensory areas.

#### **Prospective Isolation and Transcriptome Analysis of Cortical Neurons Expressing Distinct Levels of GFP/EphA7**

Taking advantage of the observation that the pA7GFP transgene expression *in vivo* allows to recapitulate some of the cortical gradients of EphA7, we isolated prospectively neurons expressing distinct levels of GFP, in order to identify genes that would be coexpressed along similar gradients.

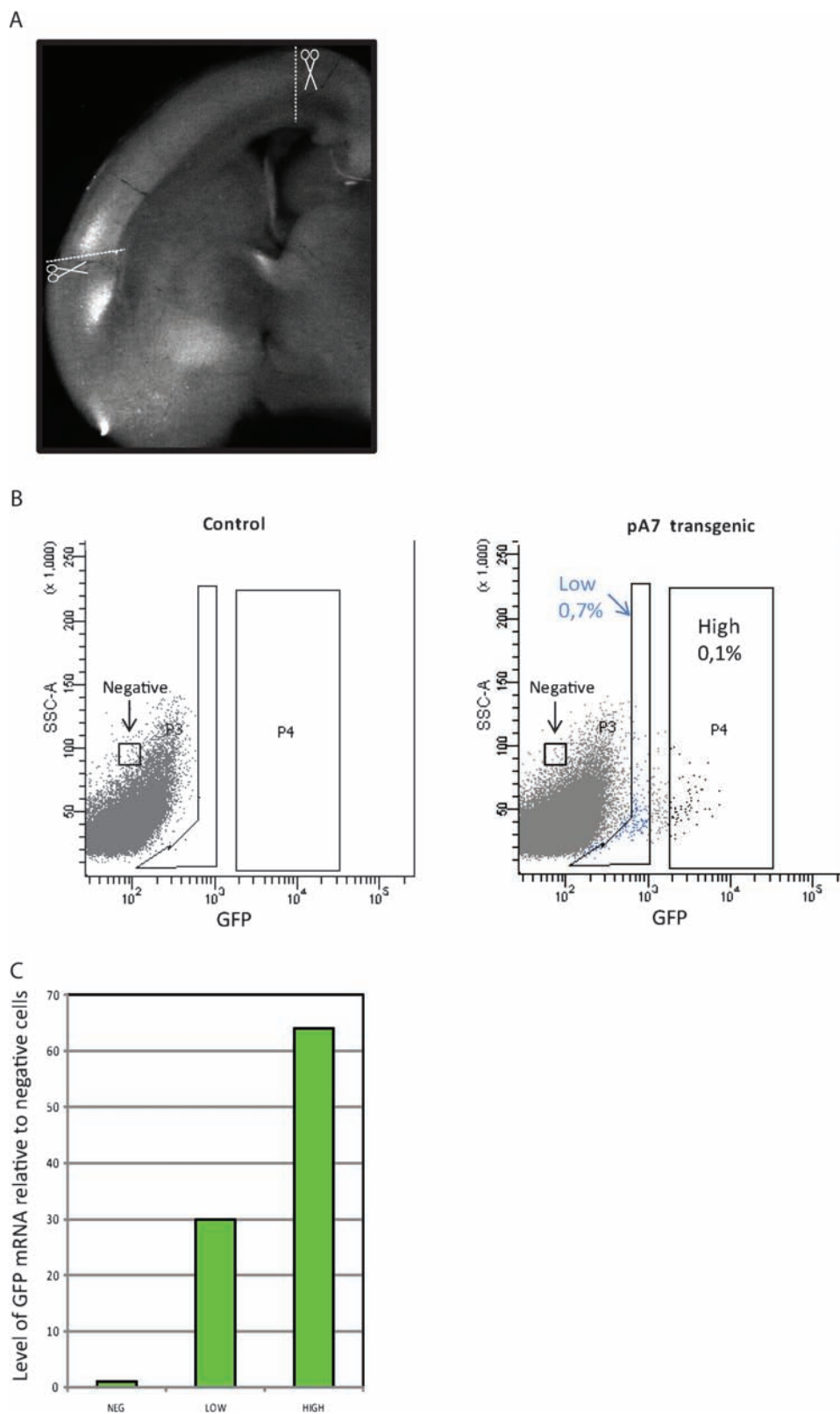
To this end, the parietal cortex of transgenic embryos was dissected and dissociated into a single cell suspension, followed by analysis and sorting by a fluorescence activated cell sorter (FACS) to identify and separate populations of neurons expressing distinct levels of GFP (Fig. 4). Two frames (High and Low) were arbitrarily defined and reused systemically for each sorting experiment, thereby allowing the selective sorting of neuronal cells expressing either “high” or “low” amounts of GFP (Fig. 4B). These were both clearly distinct from each other and from GFP-negative neurons (Fig. 4B), which was further confirmed by performing qRT-PCR for GFP expression in each sorted cell population (Fig. 4C).

In order to compare the transcriptome of these distinct neuronal populations, high and low GFP neurons were then isolated from several FACS experiments, and their RNA extracted and amplified to probe them on Affymetrix transcriptome microarrays.

Analysis of the microarray data, focusing on comparison between high GFP and low GFP neuronal populations enabled to identify a wide array of differentially expressed genes. Using as a cut-off fold-changes equal or superior to 1.5, 2880 probes (corresponding to 2353 genes) were found to be upregulated in high GFP neurons, while 1992 probes (corresponding to 1629 genes) were found to be upregulated in low GFP neurons. Importantly, EphA7 itself was found among the genes enriched in the high GFP population. Gene ontology analyses using David package ([david.abcc.ncifcrf.gov](http://david.abcc.ncifcrf.gov)) and Genomatix suite ([Genomatix.de](http://Genomatix.de)) indicated that most genes upregulated in the high GFP population corresponded to neuronal or developmental genes, including axon guidance and cell signaling genes, as well as transcription factors (Supplementary Tables 1 and 2).

#### **Validation of the Transcriptome Analyses: Identification of Genes Displaying EphA7-Like Patterned Expression in the Developing Cortex**

In order to validate the microarray data, 21 genes upregulated in the “high GFP” (GFP, EphA7, Cdh8, Cpne4, EphA5, Fn1,



**Figure 4.** Prospective isolation of GFP<sup>+</sup> cortical neurons from pA7-GFP mice. (A) The latero-medial gradient present in the parietal neocortex is visualized in vivo on live vibrosections (A). (B) FACS analysis on dissociated cortex from pA7 transgenic (right panel) or control (left panel) mice, showing cells expressing low or high levels of GFP. (C) Expression of GFP RNA in high GFP and low GFP cortical neuron populations and in control cortical neurons expressing no GFP.

Foxp1, Lhx2, Lime1, Lmo1, Myt1l, Neo1, Ntf3, Rbm39, Rorb, Satb1, Sfrs8, Smarca5, Spred2, Tubb3, Zdhhc20, and Zfp53) and 6 genes upregulated in the “low GFP” (Beta2m, Atoh1, Crybb1,

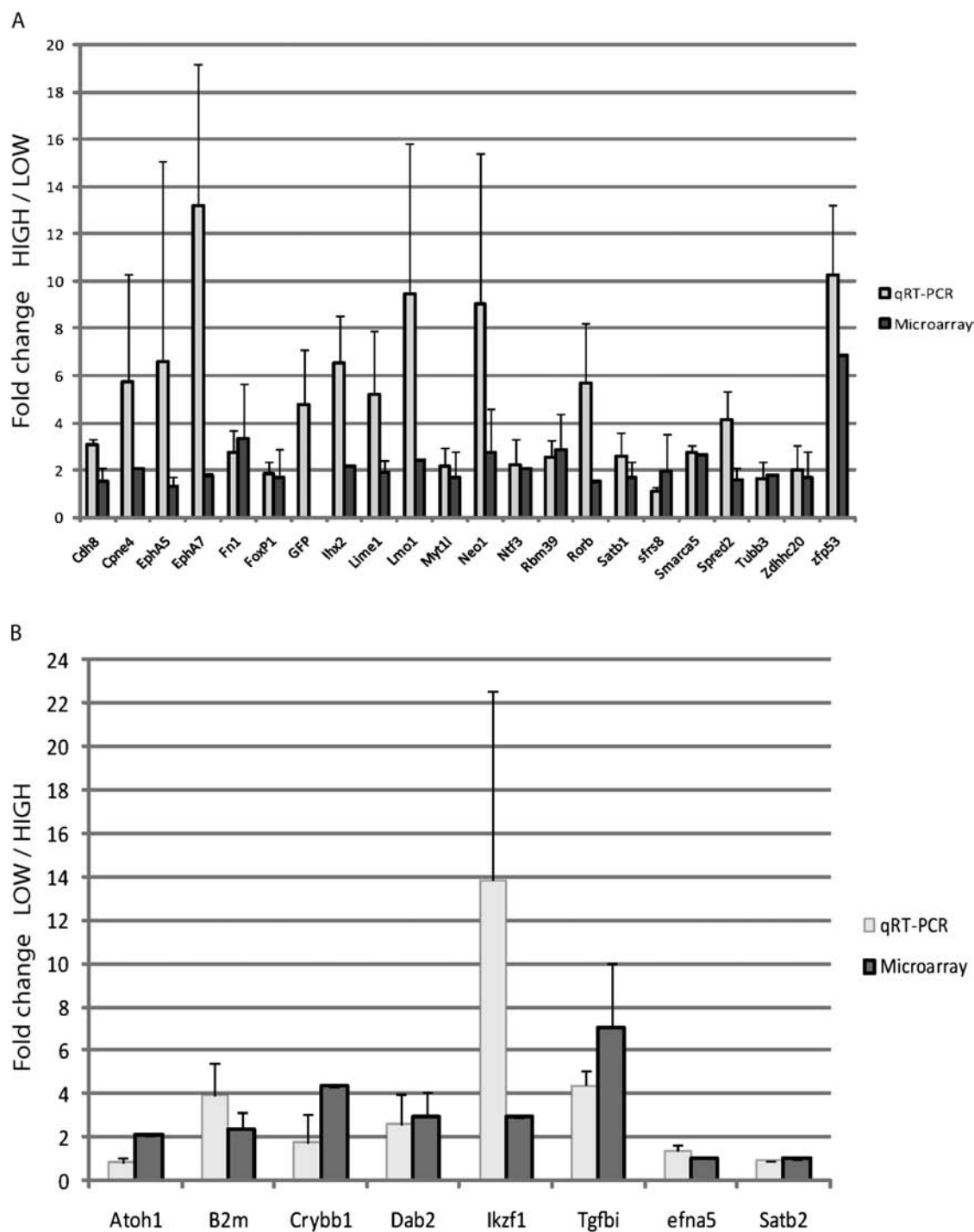
Dab2, Ikzf1, and Tgfb1) neuronal populations were tested by qRT-PCR on samples isolated from additional, independent, FACS sorting experiments. Among these, 26 genes showed

similar differential expression, while only 1 of them (Atoh1) showed no apparent differential expression (Fig. 5A,B).

qRT-PCR also confirmed that genes expressed in a layer-specific pattern but not expected to be expressed differentially along the medial-lateral axis, such as *Satb2* and *CTIP2*, were indeed expressed at the same levels in the high GFP and low GFP populations (Fig. 5B, and data not shown). Importantly, qRT-PCR also confirmed differential expression of *EphA7* to be

enriched in the high GFP population, while *ephrin-A5* (*efna5*) was found to be enriched in the low GFP population (Fig. 5), as predicted from complementary expression of *EphA7* and *ephrin-A5* in vivo (Depaepé et al. 2005; Torii and Levitt 2005).

Collectively, these data thus enabled to identify a specific repertoire of genes displaying differential expression between the 2 neuronal populations, the expression of which was confirmed by qRT-PCR for most genes tested.



**Figure 5.** Comparison of qRT-PCR and microarray data for genes differentially expressed between high GFP and low GFP cortical neurons. (A) Genes upregulated in the high GFP cells. (B) Genes down-regulated in the high GFP cells. Microarray values are represented in dark gray, and qRT-PCR values in light gray. For microarray data, values represent the fold change calculated for the mean of all probes corresponding to the same gene. For qRT-PCR, the values represent the mean of duplicates obtained from 2 independent sorting experiments. Error bars are standard error on the mean.

In order to probe further the *in vivo* significance of the genes identified, we selected a subset of genes, corresponding to transcription factors or cell adhesion/guidance or signaling molecules, for which we analyzed the expression in the developing cortex by *in situ* hybridization, focusing on genes upregulated in the high GFP population (Fig. 6).

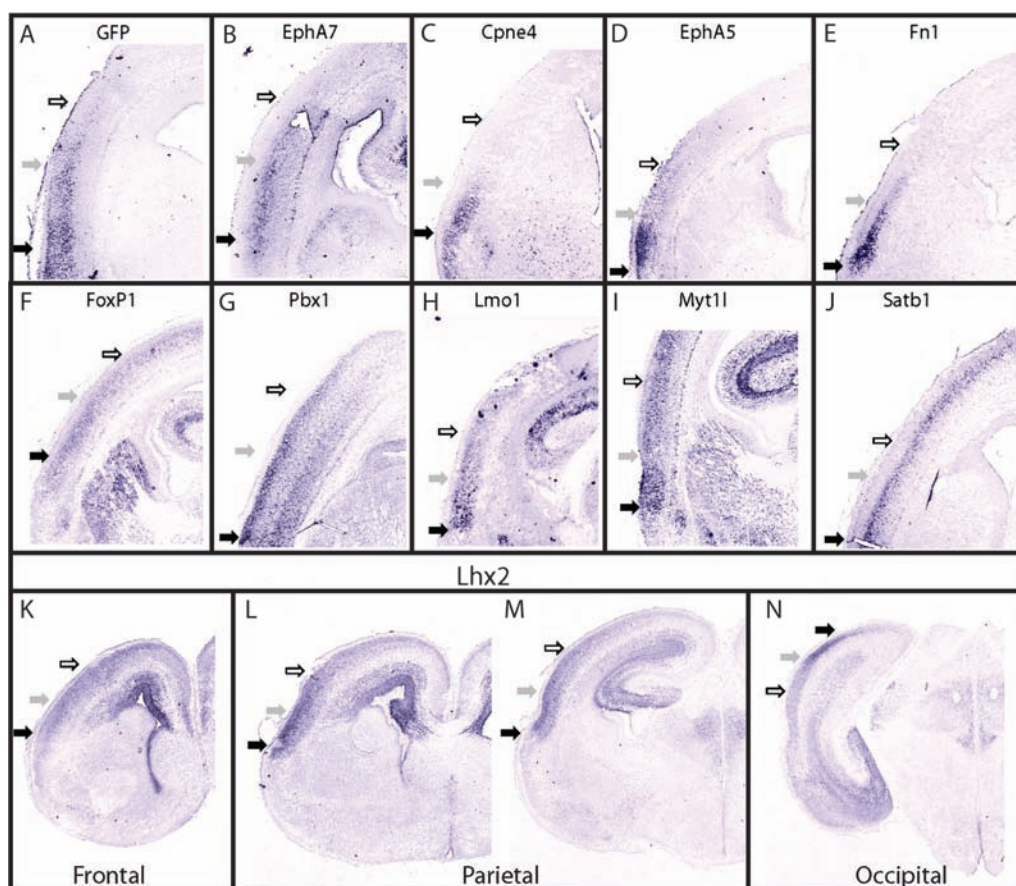
Strikingly, all the genes tested were found to be expressed in the embryonic cortex in a same high lateral to low medial gradient as GFP and EphA7 (Fig. 6*A,B*), thereby confirming the robustness of the transcriptome profiling data. Although every gene examined displayed differential expression in the cortical plate, they also presented specific features. Some genes (Cpne4, EphA5, Fn1) were mostly restricted to the ventrolateral parts of the cortex (Fig. 6*C-E*), while others (FoxP1, Satb1, Myt11) displayed more shallow gradients of expression (Fig. 6*F-J*). Similarly, while some of the genes were expressed in specific layers (Satb1, EphA5-7) (Fig. 6*B,D,J*), most of the others were expressed throughout the cortical plate and some in addition in the subplate (Pbx1, FoxP1, Myt11) (Fig. 6*F,G,D*). Lhx2 displayed a complex pattern, with no obvious lateral-medial pattern in the frontal cortex, a high lateral to low medial gradient in parietal cortex, and an inverted pattern in the occipital cortex (Fig. 6*K-N*).

Overall, our qRT-PCR and *in situ* hybridization analyses thus confirmed the pattern of expression for almost every gene tested in qRT-PCR (26/27) and every gene tested by *in situ* hybridization (10/10), thus indicating that our strategy enabled

the identification of a coherent repertoire of genes displaying robust differential expression in the developing cortex.

#### Identification of Potential Direct Transcriptional Regulators of EphA7

A large number (206) of transcription factors were identified among the differentially expressed genes upregulated in the high GFP/EphA7 population (Supplementary Tables 1 and 2). In order to explore the potential links between these differentially expressed genes and transcriptional mechanisms that lie upstream of EphA7 expression, we performed *in silico* analyses of the pA7 element looking for transcription factor binding sites. This enabled to identify 160 putative binding sites for 26 of the 206 identified transcription factors (Supplementary Table 3). Among these, Pbx1 looked particularly intriguing, since it displays 11 putative binding sites located mainly near the proximal part of the core promoter of EphA7 (Fig. 7*A*). Furthermore, Pbx1 is expressed along a high lateral to low medial gradient throughout the cortical plate at the same stage as EphA7 (Fig. 6*G*). To test if Pbx1 directly binds these putative sites, we performed ChIP on dissociated cerebral cortex at E16 with a Pbx1 antibody. As shown in Figure 7*B*, Pbx1 is specifically immunoprecipitated by the Pbx1 antibody. We thus tested Pbx1 binding to the EphA7/pA7 promoter using a set of primers targeting the 11 Pbx1 putative binding sites (Fig. 7*A*). qRT-PCR analysis of the immunoprecipitated



**Figure 6.** *In situ* hybridization analysis of genes differentially expressed between high GFP and low GFP cortical neurons. (A–J) *In situ* hybridization on coronal sections of E17 embryonic brain for GFP (A), EphA7 (B), Cpne4 (C), EphA5 (D), Fn1 (E), foxP1 (F), Pbx1 (G), Lmo1 (H), Myt11 (I), and Satb1 (J) at the level of parietal cortex. (K–N) *In situ* hybridization for Lhx2 on coronal sections at the level of frontal (K), parietal (L, M), and occipital (N) cortex. Lateral is left and dorsal up in all panels. Black, gray, and white arrows indicate boundaries of higher, intermediate, or lower levels of expression for each gene along the medial-lateral axis.



chromatin revealed a selective enrichment with the primers located at the proximal-most region of pA7, indicating that Pbx1 does indeed bind in vivo to the EphA7 promoter (Fig. 7C).

### Candidate Gene Expression in Mice Defective for Thalamocortical Projections

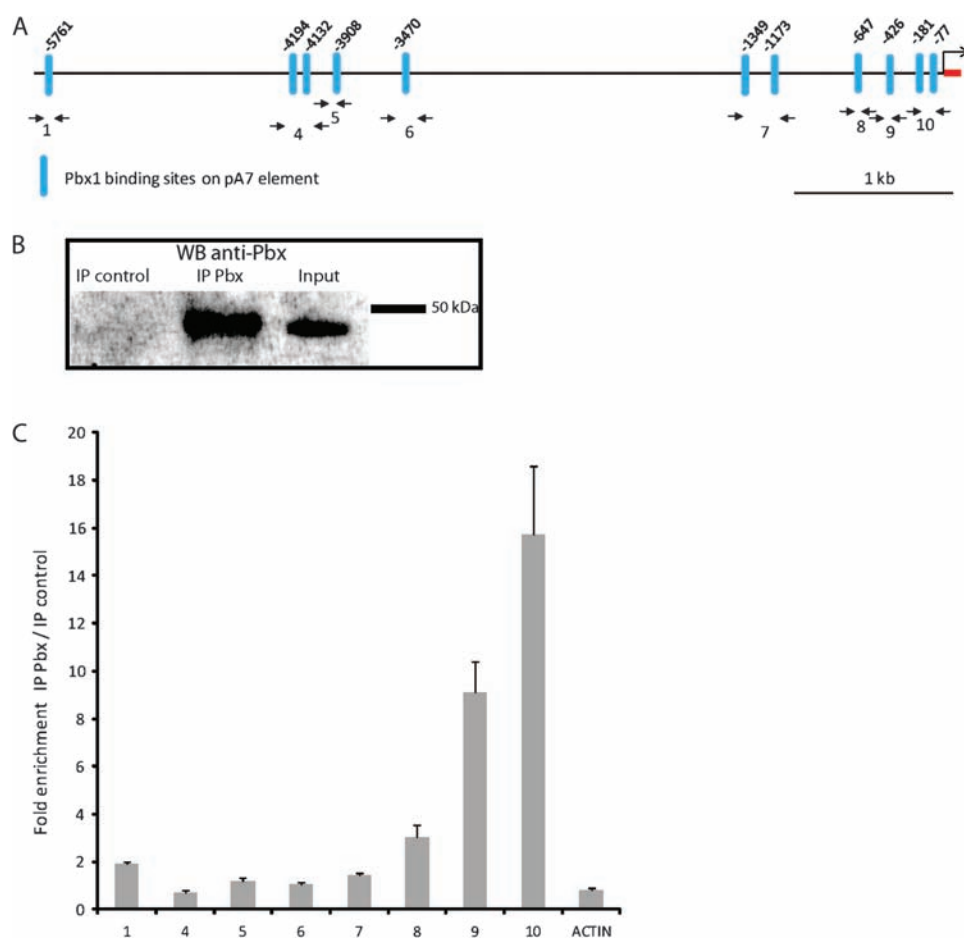
In order to test if the expression of the genes identified depends on thalamic input, we next examined their pattern in the cerebral cortex of mouse conditional mutants for *Celsr3* (*Celsr3* lox<sup>-/-</sup> and *Dlx5/6* cre<sup>+</sup>). In this model, the *Celsr3* gene is selectively disrupted within the ventral telencephalon, leaving the cortex genetically intact, but all thalamic sub-cortical projections fail to develop (Tissir et al. 2009; Zhou et al. 2010). The expression of all genes tested (*EphA7*, *Lmo1*, *Lhx2*, *Cpne4*, *EphA5*, *Fn1*, *Myt1l*, *Satb1*, *Helios*, and *FoxP1*) appeared to be unaffected by the lack of thalamic input, as a similar pattern of lateral-medial differential expression was observed in mutant and control littermates at the stage examined (E17) (Fig. 8). These data indicate that the patterned expression detected for these genes in the cortical plate are mainly dependent on mechanisms intrinsic to the cortex, in accordance with previous data on *EphA7* expression (Yun et al. 2003).

### Discussion

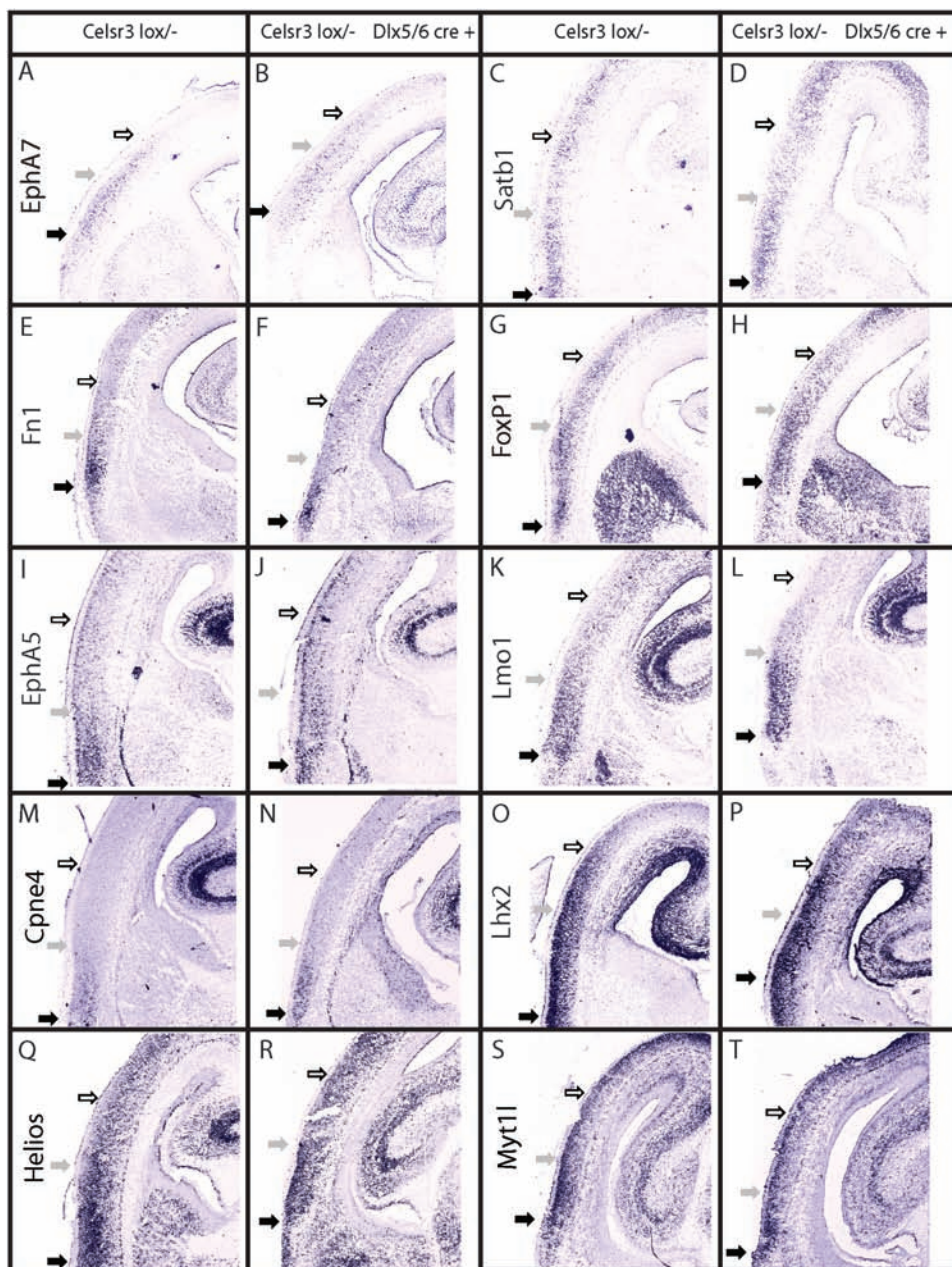
The generation of dozens of specific types of cortical neurons displaying selective hodological properties is at the core of the development of the cerebral cortex. The elucidation of the mechanisms by which the regional and temporal control of gene expression can lead to such a diversity of cortical neurons, remains a major challenge in developmental neurobiology.

Recent work has identified a number of genes involved in the patterning of cortical areas. Most of them, including *Pax6*, *Emx2*, *CoupTFI*, *Sp8* transcription factors, as well as morphogens of the FGF family, are mainly expressed in graded patterns among cortical progenitors at early stages of corticogenesis (Bishop et al. 2000; Mallamaci et al. 2000; Fukuchi-Shimogori and Grove 2003; Armentano et al. 2007; Sahara et al. 2007; Zembrzycki et al. 2007; Cholfin and Rubenstein 2008; Joshi et al. 2008; O'Leary and Sahara 2008), although recent work also points to areal-patterning genes expressed in cortical neurons, such as *bHLHb5* and *Tbr1* (Joshi et al. 2008; Bedogni et al. 2010).

In contrast, much less is known concerning the patterning of cortical areas that is still ongoing at late embryonic and perinatal stages. These important later steps control accurate



**Figure 7.** (A) Schematic structure of the pA7 element of the EphA7 gene promoter. Blue boxes depict putative Pbx1 binding sites, small arrows show the locations of the primers used in the ChIP experiments, large arrows and red box indicate the transcriptional start. (B) Western blot analysis of immunoprecipitation performed with a nonrelated antibody or Pbx1 antibody. (C) qPCR analysis performed on Pbx1 and control ChIP. On the y-axis is reported the fold enrichment ratio between the Pbx1 and the control ChIP for the different binding sites. Primers designed on the unrelated actin gene were used as a control.



**Figure 8.** (A–T) In situ hybridization analysis of genes differentially expressed between high and low GFP<sup>+</sup> cortical neurons in the parietal cortex in the absence of thalamic input. In situ hybridization on coronal sections of E17 embryonic brain from conditional mutants (*Celsr3lox<sup>-</sup>/Dlx5<sup>-</sup>/Cre<sup>+</sup>*) (B, D, F, H, J, L, N, P, R, T) and control littermates (*Celsr3lox<sup>-</sup>*) (A, C, E, G, I, K, M, O, Q, S). Genes analyzed are EphA7 (A, B), *Satb1* (C, D), *Fn1* (E, F), *FoxP1* (G, H), EphA5 (I, J), *Lmo1* (K, L), *Cpne4* (M, N), *Lhx2* (O, P), *Helios* (Q, R), and *Myt11* (S, T), at the level of parietal cortex. Lateral is left and dorsal up in all panels. Black, gray, and white arrows indicate boundaries of higher, intermediate, or lower levels of expression for each gene along the medial-lateral axis.

topographic mapping of thalamic input to the cortex, as well as specific and topographic cortical output to thalamus and subcerebral targets.

Here, we have focused on the transcriptional regulation of EphA7, an axon guidance receptor important for the development of cortical circuits, which is expressed in a graded pattern in several cortical areas at later stages of embryonic development, thus providing a potential link between early cortical patterning and later development of mature cortical areas. We identified a specific *cis* regulatory region of EphA7 that enables to recapitulate some key aspects of EphA7 cortical expression. Using a mouse re-

porter model, where GFP is expressed in the cortex, similarly to EphA7, we then purified and characterized the transcriptome of distinct populations of cortical neurons expressing different levels of EphA7, leading to the identification of a repertoire of genes potentially involved in cortical patterning. While some of the genes identified were already known to be expressed in cortical regional patterns and/or to be involved in cortical patterning (such as *Lhx2*, *ROR-β*, *Cadh8*, or EphA5) (Nakagawa et al. 1999; Bishop et al. 2002; Cooper et al. 2009), most of them have not been described before in the context of cortical patterning (Supplementary Tables 1–3).

Very little is known concerning the transcriptional mechanisms controlling patterns of expression in the developing cortex. Here, we identify pA7 as a regulatory region capable of driving expression in a medial-lateral gradient expression in the parietal cortex. It is remarkable that such a short element of less than 6 kb seems to be able to drive a specific and complex pattern of expression. In silico analysis of this element, combined with the transcriptome analyses, enabled to identify 26 transcription factors that are expressed differentially in cortical neurons in a similar way as EphA7 and that contain putative binding sites within the pA7 element of the EphA7 regulatory regions (Supplementary Table 3). This led us to demonstrate the binding of Pbx1 to the EphA7 promoter using *in vivo* ChIP, suggesting that it could act directly upstream of EphA7 in the developing cortex. Future work should enable to establish more directly the potential functional links between Pbx1, which is likely to act in combination with other transcription factors such as Meis2 (Toresson et al. 2000), and the regulation of EphA7 expression, to start dissecting the transcriptional programs underlying complex patterns of cortical expression.

Recent progress has been made to identify transcription factors involved in the control of cortical projections, which enable projection neurons of different cortical layers to adopt selective patterns of axonal output to the cortex or to subcortical targets (Molyneaux et al. 2007; Leone et al. 2008; Gaspard and Vanderhaeghen 2011). On the other hand, very little is known concerning the transcriptional control of patterning of topography of this cortical output, which for instance controls the somatotopic organization of the motor and somatosensory output to the midbrain/hindbrain and spinal cord. Some of the transcription factor genes identified here (such as Pbx1), which are differentially expressed among cortical projection neurons at late developmental stages, could participate to this important process of refinement of cortical connectivity. Conversely, some of these genes could be involved in setting up the coordinates enabling to generate the fine topography of thalamic input to the cortex, upstream of ephrin/Eph, and other thalamic axon guidance cues.

A number of the differentially expressed genes identified in this study correspond to signaling factors or guidance molecules (Supplementary Table 2) that could act more directly to guide cortico-efferents of thalamic afferents, in particular in the somatosensory/motor cortex. These include members of the ephrin/Eph family (EphA5, EphB2, ephrin-B3), which could cooperate with EphA7 and ephrin-A5 (Vanderhaeghen et al. 2000; Dufour et al. 2003, 2006; Cang et al. 2005; Torii and Levitt 2005), but also members of the Semaphorin pathway (Plxna3, Sema3a, Nrp1), some of which were already identified to control the area-specific output of visual cortex efferents (Low et al. 2008). Other interesting candidate guidance/adhesion genes include Fn1 (Fibronectin1), which was found to be expressed in striking differential patterns in the cortical plate, while it was previously proposed to interact with Eph signaling in non-neuronal cells (de Saint-Vis et al. 2003).

While cortical patterning results from the interplay of intrinsic and extrinsic mechanisms (Sur and Rubenstein 2005; O'Leary and Sahara 2008; Rakic et al. 2009), genetic data indicate that a remarkably high degree of cortical organization seems to be acquired intrinsically, in absence of input from the rest of the brain (Miyashita-Lin et al. 1999; Nakagawa et al. 1999; Molnar et al. 2002; Yun et al. 2003; Zhou et al. 2010). As the genes identified in this study were found to be

differentially expressed in the late embryonic cortex, a stage where thalamic axons gave started to invade the cortex, we thus tested the influence of thalamic projections on their expression. To this end, we used a newly described mouse model where the *Celsr3* gene is selectively disrupted from the VT (Zhou et al. 2008). While this model is characterized by a complete absence of thalamic input to the cortex, the cerebral cortex develops surprisingly normally up to postnatal stages (Zhou et al. 2008, 2010). We found that the expression of all candidate genes tested was unchanged in these mutants, like previously found for EphA7 in another mutant defective for thalamocortical projections (Yun et al. 2003). Our data confirm the recent findings obtained with the same mouse model (Zhou et al. 2010), strengthening the notion that most of the critical steps of cortical patterning can occur intrinsically, at least until birth. Conversely, it will be interesting to test the expression of the genes identified in this study, in mouse models defective for early cortical patterning, such as *Emx2* or *Sp8* mutants, in which the pattern of expression of EphA7 was found to be disrupted (Bishop et al. 2000; Zembrzycki et al. 2007).

Overall, this study points to the identification of a specific *cis* regulatory element that drives graded expression among cortical neurons and to an array of genes differentially expressed in a similar way, that could work upstream or together with EphA7 to control intrinsic cortical patterning. Future work should aim at defining better which genes control the pA7 element to drive graded patterns of expression of EphA7 in the cortex and most importantly how they interplay to generate the unique organization of cortical networks.

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### Supplementary Material

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

### Notes

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