

Gene Expression Analysis of the Embryonic Subplate

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The subplate layer of the cerebral cortex is comprised of a heterogeneous population of cells and contains some of the earliest-generated neurons. In the embryonic brain, subplate cells contribute to the guidance and areal targeting of thalamocortical axons. At later developmental stages, they are predominantly involved in the maturation and plasticity of the cortical circuitry and the establishment of functional modules. We aimed to further characterize the embryonic murine subplate population by establishing a gene expression profile at embryonic day (E) 15.5 using laser capture microdissection and microarrays. The microarray identified over 300 transcripts with higher expression in the subplate compared with the cortical plate at this stage. Using quantitative reverse transcription–polymerase chain reaction, in situ hybridization (ISH), and immunohistochemistry (IHC), we have confirmed specific expression in the E15.5 subplate for 13 selected genes, which have not been previously associated with this compartment (*Abca8a*, *Cdh10*, *Cdh18*, *Csmd3*, *Gabra5*, *Kcnt2*, *Ogfr1*, *Pls3*, *Rcan2*, *Sv2b*, *Slc8a2*, *Unc5c*, and *Zdhhc2*). In the *reeler* mutant, the expression of the majority of these genes (9 of 13) was shifted in accordance with the altered position of subplate. These genes belong to several functional groups and likely contribute to synapse formation and axonal growth and guidance in subplate cells.

Keywords: cerebral cortex, embryonic development, gene expression, laser capture microdissection, subplate

Introduction

The subplate layer of the cerebral cortex is a transient compartment that contains a heterogeneous population of the earliest-generated neurons (Kostović and Rakic 1990). From studies in various species over the last 60 years, it has become evident that the subplate is an important player in cortical development (Allendoerfer and Shatz 1994; Kanold and Luhmann 2010). At different developmental stages, subplate cells fulfill different developmental roles. Embryonic subplate cells are involved in the guidance of corticofugal and thalamocortical axons and the establishment of topographic connections (McConnell et al. 1989; Ghosh et al. 1990). The early postnatal subplate neurons in rodents and cats play a role in generating oscillatory activity (Dupont et al. 2006; Yang et al. 2009) and in the maturation and plasticity of the excitatory and inhibitory thalamocortical circuitry (Kanold et al. 2003; Kanold and Shatz 2006). Remaining adult subplate cells are suggested to be important in supporting corticocortical connectivity (Kostović

and Rakic 1990; Friedlander and Torres-Reveron 2009; Suárez-Solá et al. 2009; García-Marín et al. 2010; Kostovic et al. 2011).

Consistent with their important role in cortical development, subplate abnormalities have been implicated in the pathogenesis of various developmental disorders. These include visual and cognitive deficits following preterm hypoxia-ischemia (Volpe 1996; McQuillen and Ferriero 2005), schizophrenia (Anderson et al. 1996; Kirkpatrick et al. 2003; Eastwood and Harrison 2005), and autism spectrum disorders (Simms et al. 2009; Avino and Hutsler 2010).

Very little is known about the molecular mechanisms underlying subplate functions (Kwan et al. 2008). Recent studies have started to profile the gene expression of the murine subplate at different developmental stages (embryonic day [E] 12; Osheroff and Hatten 2009, postnatal day [P] 8; Hoerder-Suabedissen et al. 2009, and adult; Belgard et al. 2011). These studies identified a number of genes, which are expressed specifically in preplate neurons destined for the subplate (e.g., hippocalcin, glutamate receptor *Eaac1*) (Osheroff and Hatten 2009) or the postnatal and adult subplate cells (e.g., monooxygenase *MoxD1*, complexin 3) (Hoerder-Suabedissen et al. 2009). Interestingly, the temporal expression of most of these genes appears to be tightly regulated, which further emphasizes the dynamic nature and the changing roles of the developing subplate.

In this study, we set out to further characterize the embryonic murine subplate by establishing a comprehensive gene expression profile of this cell population at E15.5. This stage is different from the ones that previous studies have investigated, which were either earlier at E12 (Osheroff and Hatten 2009) or later at P8 (Hoerder-Suabedissen et al. 2009). The embryonic subplate is a highly dynamic and heterogeneous compartment, which not only contains postmigratory subplate neurons but also radially migrating excitatory neurons, tangentially migrating interneurons, developing blood vessels, and a rich extracellular matrix (Sheppard et al. 1991; Métin et al. 2006). The subplate neurons themselves are undergoing many developmental processes including cell maturation, synapse formation, and axonal and dendritic growth. In mouse, E15.5 is an important and very interesting phase in cortical and thalamocortical development as it is the time of the accumulation of monoaminergic and thalamic axons within subplate and the establishment of the first area-specific thalamocortical connections (Molnár and Blakemore 1995). In addition, embryonic subplate cells could be involved in the production of the extracellular matrix, the development of blood vessels, and the guidance of tangentially migrating interneurons (Denaxa et al. 2001; Shinozaki et al. 2002).

By providing a comprehensive gene expression profile at this stage, we aim to identify molecular mechanisms and pathways underlying known and novel characteristics and roles of the embryonic subplate. In addition, a gene expression analysis can provide us with valuable molecular markers to identify and specifically manipulate embryonic subplate cell populations. Most of the currently available subplate-specific genes have a late onset of expression (Hoerder-Suabedissen et al. 2009). Therefore, there is a need for novel markers to further elucidate subplate functions in normal embryonic development and to monitor subplate cells in early pathological conditions.

Materials and Methods

Tissue Preparation

All animal experiments were approved by a local ethical review committee and conducted in accordance with personal and project licenses. Embryos were obtained from time-mated pregnant females. The midday after a plug was detected was designated as embryonic day (E) 0.5. For microarray and quantitative reverse transcription polymerase chain reaction (qRT-PCR), whole heads of E15.5 C57/Bl6 mice were flash frozen directly in isopentane (VWR) on dry ice. Once hardened, the heads were embedded in O.C.T. compound (Tissue-Tek) and stored at -80°C . For in situ hybridization (ISH), heads from E13.5, E14.5, E15.5, and E17.5 and brains from postnatal day (P) 8 and adult C67/Bl6 and E15.5 reeler mice (Orleans mutant allele, *Re1n* [*rl-Orl*]) were embedded in O.C.T. and flash frozen. Brains of E15.5 and P8 Gad67-green fluorescent protein (GFP) knock-in mice (Tamamaki et al. 2003) were fixed in 4% paraformaldehyde (PFA) (TAAB) in phosphate-buffered saline (PBS) for 4 h (E15.5) or 24 h (P8) and cryoprotected in 30% sucrose before freezing. For immunohistochemistry (IHC), E13.5, E14.5, E15.5, and E17.5 brains from C57/Bl6 and E15.5 brains from reeler mice were immersion fixed in 4% PFA for at least 24 h. Fixed P8 and adult brains and E17.5 brains for γ -aminobutyric acid (GABA) IHC were obtained by deeply anesthetizing with pentobarbitone (Euthatal 150 mg/kg intraperitoneally; Merial Animal Health Ltd) and perfusing through the heart with 4% PFA or with 4% PFA and 0.25% glutaraldehyde (TAAB) for IHC against GABA. Further details on animal numbers and tissue preparation are listed in Table 1.

Laser Capture Microdissection

Subplate and lower cortical plate (in the future called cortical plate for simplicity) from anterior and posterior cortex were laser capture

microdissected as previously described (Wang et al. 2009). Briefly, E15.5 heads were sagittally sectioned to 20 μm on a cryostat (Jung CM3000; Leica, Germany) and mounted on membrane-coated 1-mm polyethylene naphthalate slides (Zeiss). Sections were fixed in 95% ethanol, rehydrated, stained with 1% cresyl violet (Sigma), and dehydrated. On stained sections, the border between the light-stained cell-sparse subplate and dark-stained cell-dense cortical plate was readily distinguishable. For the microdissection, subplate was defined as a cell band of 40 μm just below this border and the lower cortical plate as a 40- μm thick cell band just above this border (Fig. 1*a,b*). Laser capture microdissection was performed using a PALM Microbeam IP 230V Z (Zeiss), and microdissected tissue strips were collected in RNAlater (Ambion). For microarray, samples from 3 to 4 brains (a total of ~ 90 strips) from the same litter were pooled, and 4 biological replicates from 4 different litters were obtained in this way. For qRT-PCR, 2 additional sample sets were collected from individual animals (~ 40 strips each) from 2 different additional litters. Total RNA was extracted using the RNeasy micro kit (Qiagen) following the manufacturer's instruction. On-column DNase digestion step was performed. RNA integrity and quantity were analyzed on RNA 6000 Pico Chips (Agilent Technologies). All samples were undegraded (RNA integrity number > 7.6), and quantities were at least 20 ng per sample.

Preparation of cDNA and Microarray

Ten nanograms of RNA from each sample were reverse transcribed, and the cDNA was amplified using the Ovation Pico system (NuGEN) according to manufacturer's instruction. Two no template controls were processed together with the samples, and no significant amplification due to contamination was found. Amplified double-stranded cDNA was transformed into single-stranded sense cDNA using the Ovation Exon Module (NuGEN). Sense cDNA was fragmented and labeled with biotin using the FL-Ovation cDNA Biotin Module V2 (NuGEN). Successful fragmentation was confirmed for all samples on the BioAnalyzer showing a peak sequence length of around 200 nt. Fragmented and labeled single-stranded sense cDNA was hybridized to Affymetrix Mouse Gene 1.0 ST arrays at 45°C overnight. Arrays were washed and then stained using the GeneChip Hybridization, Wash, and Stain Kit (Affymetrix) according to manufacturer's instruction. The microarrays were scanned on an Affymetrix GeneChip Scanner 3000.

Microarray Data Analysis

All data processing was performed using Agilent GeneSpring GX (Agilent Technologies). Arrays were quantile normalized and intensity values summarized using the Probe Logarithmic Intensity Error (PLIER).

Table 1

Genotype, age, tissue preparation method, and numbers of mice used in this study

Application	Genotype	Age	Preparation	Number
Microarray	Wild type	E15.5	Fresh frozen	18 (from 4 litters)
	Wild type	E15.5	Fresh frozen	2 (from 2 litters)
	Wild type	E13.5	Fresh frozen	2
	Wild type	E14.5	Fresh frozen	3
	Wild type	E15.5	Fresh frozen	3
	Wild type	E17.5	Fresh frozen	2
	Wild type	P8	Fresh frozen	3
	Wild type	Adult	Fresh frozen	2
	Reeler	E15.5	Fresh frozen	3
Immunohistochemistry	Gad67-GFP	E15.5	Immersion fixed (PFA) and frozen	3
	Wild type	E13.5	Immersion fixed (PFA)	2
	Wild type	E14.5	Immersion fixed (PFA)	3
	Wild type	E15.5	Immersion fixed (PFA)	3
	Wild type	E17.5	Immersion fixed (PFA)	2
	Wild type	P8	Perfused (PFA)	3
	Wild type	Adult	Perfused (PFA)	3
	Reeler	E15.5	Immersion fixed (PFA)	2
		P8	Perfused (PFA)	2
	Gad67-GFP	P8	Immersion fixed (PFA)	3
	Wild type	E17.5	Perfused (PFA + glut)	2
	Wild type	P8	Perfused (PFA + glut)	3

Note: PFA, paraformaldehyde 4% in PBS; glut, 0.25% glutaraldehyde in PBS.

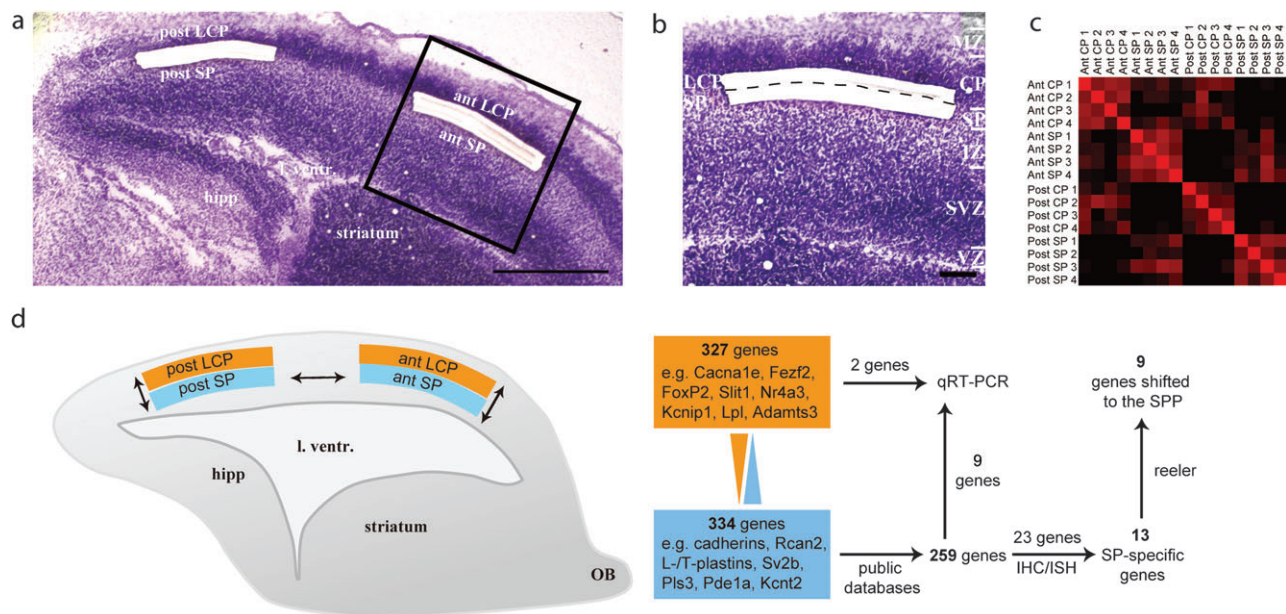


Figure 1. Overview of the experimental approach. Four cell populations were isolated by laser capture microdissection in 4 replicates, and gene expression was compared using microarrays. (a) Cresyl violet-stained cryosection after subplate (SP) and lower cortical plate (LCP) from anterior (ant) and posterior (post) cortex have been isolated. (b) High magnification of the anterior cortex showing the border between SP and LCP. (c) Heatmap of the correlation analysis across all 16 samples. Bright red squares denote a Pearson correlation coefficient of 1 and black squares a coefficient of 0. The heatmap indicates that the strongest correlations are between replicates of the same cell populations. (d) Transcript levels were compared between the ant SP and ant LCP and between the post SP and post LCP. 327 genes were more highly expressed in the SP and 334 were more highly expressed in the LCP. Based on public available datasets, 75 genes were excluded as false positives. Out of 23 selected genes, 13 were confirmed as specifically expressed in SP using IHC (IHC) or in situ hybridization (ISH). Scale bars: 500 μ m (a), and 100 μ m (b). hipp, hippocampus; l. ventr., lateral ventricle; MZ, marginal zone; OB, olfactory bulb.

Summarized signal intensities of each probe set were compared between anterior subplate and anterior cortical plate and between posterior subplate and posterior cortical plate using paired Student's *t*-tests. Correction for multiple testing was not used as current methods (e.g., Bonferroni, Benjamini-Hochberg) were too stringent for our data. Genes with a fold change ≥ 1.4 and a *P* value < 0.05 were considered as differentially expressed (Supplementary Tables 1 and 2). A complete list of all significantly different genes independent of the fold change (*P* value < 0.05) is accessible as supplementary material.

A correlation analysis across all 16 arrays was performed where the Pearson Correlation Coefficient was calculated for each pair of arrays and visualized as a correlation plot (Fig. 1c).

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics software was used to categorize the subplate gene expression data (Dennis et al. 2003). All genes were linked to Gene Ontology terms describing biological processes (Ashburner et al. 2000) and to pathways described in the Kyoto Encyclopedia of Genes and Genomes Pathway Database (Kanehisa and Goto 2000). Genes expressed in the subplate were analyzed for enriched terms by comparison with a background gene list containing all 28 869 genes on the Affymetrix Mouse Gene 1.0 ST array using Expression Analysis Systematic Explorer Score (a modified Fisher's Exact test). Terms with a *P* value < 0.05 were considered as significantly enriched, and similar terms were clustered into functional annotation groups using DAVID's fuzzy heuristic clustering algorithm. In addition, Fisher's Exact test implemented in the Ingenuity Pathway Analysis (IPA) software (<http://www.ingenuity.com>) was used to identify canonical signaling pathways associated with subplate-enriched genes. This analysis was performed on all genes with a significantly higher expression level in subplate (*P* value < 0.05 ; *n* = 1983), and pathways with *P* value < 0.05 were considered as significantly overrepresented.

Quantitative Reverse Transcription Polymerase Chain Reaction

The remaining RNA from 2 replicates of the microarray as well as RNA from 2 new sample sets were used for qRT-PCR. cDNA was generated using Superscript III reverse transcriptase and random hexamers (Invitrogen) according to manufacturer's instructions. Controls with no template were included for each reaction.

Primers for qRT-PCR were designed to amplify specific intron-spanning sequences of 120–150 bp (Table 2) using Roche's Universal ProbeLibrary Assay design center and Primer3 software. The qRT-PCR reaction was performed using 100 nM of forward and reverse primers and Power Sybr Green reagent (Applied Biosystems) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). PCR conditions were 95 °C for 10 min followed by 40 or 50 2-step cycles of 95 °C for 15 s and 60 °C for 1 min. Each primer set was verified to amplify a single PCR product by melt-curve analysis following qRT-PCR and by agarose gel electrophoresis.

Three commonly used housekeeping genes (beta-tubulin 2c [*Tbb2c*], peptidylprolyl isomerase A [*Ppia*], and beta-actin [*Actb*]) were initially selected based on the microarray data where they showed stable expression levels across all samples. They were further validated by qRT-PCR on additional samples of anterior and posterior subplate and cortical plate where all 3 genes showed very similar relative expression levels (relative standard deviation $< 10\%$). *Tbb2c* was subsequently chosen as the endogenous reference gene as it showed similar expression levels as the genes of interest.

Each reaction was performed in triplicates and ran with no template controls for each primer pair. *Tbb2c* was included on every qRT-PCR plate. LinRegPCR v11.4 software was used to determine baseline fluorescence and PCR efficiencies which were around 90% for all genes except for *Slit1* (82%) and *Nr4a3* (98%) and to calculate the starting concentrations (N_0) of each sample taking these 2 parameters into account (Ramakers et al. 2003; Ruijter et al. 2009). N_0 values were normalized to *Tbb2c*. Fold changes were determined by dividing normalized N_0 values of anterior subplate:anterior cortical plate and posterior subplate:posterior cortical plate. Fold changes were considered as significant if they were significantly different from 1 (one-sample one-tailed *t*-test, *P* < 0.05).

In Situ Hybridization

For riboprobe generation, total RNA of E15.5 whole cortex was extracted, and first strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) and random hexamers (Invitrogen) according to manufacturer's instruction. DNA fragments were amplified using the sets of gene-specific forward and reverse primers specified in

Table 2
Primer sequences for qRT-PCR

Gene	Forward primer	Reverse primer	Amplicon length (bp)
2610017109Rik	tgtgcccgggactgaag	cataccacttgatgaagctca	140
Abca8a	cccttatccaggccttct	tcctcttcaaacgctccag	140
Actb	ttctttgagctcctctgt	atggagggggaatacagccc	149
Cdh9	acaaccctctcgtgttc	tatatccagctcgcggttc	129
Csmd3	gggtctgagccacataatag	gggagcactaaatctcggtta	129
Epha3	tggtccttgagacgtttct	gttgcgagcagcaaggtc	142
Kcnip4	gctgattgaagcaggtttagaag	gaatctgaagctcttctgttg	128
Nr4a2	tcagagccacgtcgatt	gatctccatagagccggtca	143
Nr4a3	tcagagcgtcgacactgat	tggtcctttagctgtgtg	136
Ppia	gtcaaccaccgctgttct	agccaatccttctctccag	139
Sema6d	agaatcgcctggaacata	ccgagccttaagggaaggaag	128
Slit1	cccaggtgtgattggag	gcgagactctggtctgttg	146
Tbb2c	cttcgggagcagctctcagac	ggcagtcacagctctcagc	149
Unc5c	aggcagtcaggagacaat	ccaggcataggtactgagg	129

Table 3. The resulting PCR products were ligated into the pST-Blue 1 plasmid (Novagen). The antisense and sense (a negative control) complementary RNA (cRNA) probes were transcribed using either SP6 or T7 RNA polymerase with digoxigenin (DIG)-labeled nucleotide mixture (Roche).

For permanent ISH, fresh-frozen tissue was sectioned to 16 μ m on a cryostat (Jung CM3000; Leica). Sections were postfixed with 4% PFA for 20 min, deproteinized with 0.1 N HCl for 5 min, acetylated with acetic anhydride (0.25% in 0.1 M triethanolamine hydrochloride), and prehybridized at room temperature (RT) for at least 1 h in a solution containing 50% formamide, 10 mM Tris (pH 7.6), 200 μ g/mL *E. coli* transfer RNA, 1 $\times$ Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% sodium dodecyl sulfate, and 1 mM ethylenediaminetetraacetic acid. The sections were hybridized in the same buffer with the DIG-labeled probes overnight at 66–68 $^{\circ}$ C. After hybridization, sections were washed to a final stringency of 30 mM NaCl/3 mM sodium citrate at 66–68 $^{\circ}$ C and blocked with 1% blocking reagent (Roche) for at least 1 h. After overnight incubation with anti-DIG-alkaline phosphatase antibody 1:2000 (Roche), nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (Roche) in buffer containing 100 mM NaCl, 100 mM Tris-HCl, pH 9.5 and 50 mM MgCl₂, was applied for approximately 4–24 h. As a negative control, sections were labeled with sense cRNA probe and processed in parallel (see Supplementary Fig. 1).

For colocalization, immersion-fixed E15.5 Gad67-GFP brains were cut into 16 μ m coronal cryosections and postfixed with 4% PFA for 10 min. Sections were treated with 2 μ g/mL proteinase K (Roche) in PBS for 10 min at RT and deproteinized, acetylated, and prehybridized as for permanent ISH. Hybridization with DIG-labeled probes and subsequent washes were performed at the lower temperature of 60 $^{\circ}$ C in order to protect GFP protein structure. After blocking, sections were incubated with anti-DIG-alkaline phosphatase antibody 1:2000 (Roche) and rabbit anti-GFP antibody (1:500; Chemicon) overnight. For fluorescent color detection of probes, Fast Red (Roche) in 100 mM Tris, pH 8.0, 400 mM NaCl was applied for at least 6 h. Sections were then incubated with donkey antirabbit Alexa488 1:250 (Invitrogen) in 1% donkey serum for 2 h and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen).

Immunobistochemistry

Permanent IHC was performed on sections from 4% PFA-fixed brains cut to 40 μ m on a vibrating microtome (VT1000S; Leica). For IHC against Sv2b, Kcnt2 and Plxn4 sections were boiled in sodium citrate (0.1 M, pH 4.5) at 95 $^{\circ}$ C for 8 min to enhance labeling for synapse-enriched proteins (Gil et al. 2002). All sections were quenched in 1.5% hydrogen peroxide and blocked for 2 h at RT with 5% donkey serum (Sigma) in Tris Buffered Saline (TBS) with 0.2% Triton-X100 (BDH). Sections were incubated with primary antibody in 1% donkey serum and 0.2% Triton overnight at 4 $^{\circ}$ C. The following primary antibodies and dilutions were used: Anti-Sv2b 1:1000 (AB15225; Millipore), anti-Kcnt2 1:1000 (ab77512; Abcam), anti-Plxn4 1:100 (ab39350; Abcam), anti-

Table 3
Primer sequences for ISH riboprobes used to validate selected subplate-enriched genes

Gene	Forward primer	Reverse primer	Probe length (bp)
Pls3	tcgggaagaagaaccttc	tccgattctctagccatcg	601
Epha3	gtcatccagcttggtggaat	ccgttaagccaatcaccagt	608
Abca8a	ccagacctgggaattgagaa	gttgcatgccagggttaagt	621
Cdh9	cacagccagtggtctgaaa	gaacacaggggctcatcta	658
Csmd3	cagcaacaaaaactggctca	tcactccaagcagcaaacac	663
Unc5c	ttgggctggaagattactgg	gttcttgattgaggacca	680
Slc8a1	ggaccaacagctggagagag	aggagcacaaacagggaaga	643
Zdhhc2	ggacaaatggtctgctgat	ttccaatcggaaggtgttc	586
Gabra5	tgactgctcaactccactg	gagaggtggccctttatc	645
Ogfr1	gaaggaatgccaaacaaa	caaatcgaggagctttctc	649
Plxn4	ggacatcccagctacaaga	ctgtctcagctctctatcc	632
Cdh10	acgagcgactcaagagcat	ctatttgcaagagcaagacg	563
Cdh18	agccaaggtggaatgatg	ctctgtgccaggttttgg	615
Unc5d	aacgtatgccctcacagag	cgcagatcctctgtctgatg	566
Sema6d	acttcgagctctcagctct	agcatggtgatcctgttc	618
Nrp1	tgacagcgcaatagcaaaa	accagcgagatgttttctgg	648
1190002N15Rik	tcctttgctaaaagccttcg	ccccaaacttactgcaaaa	608
Nt5dc2	agtccttcagctttgtgga	cagctgagtgagccatgta	617
Cdh12	aagacctgatgtgggcag	caggatggtccatctgagt	572

Rcan2 1:3000 (Porta et al. 2007), anti-NeuroD 1:200 (sc-1086; Santa Cruz Biotechnology). Biotinylated donkey antirabbit antibody 1:200 (ab6701; Abcam) in 1% donkey serum was applied for 2 h at RT and reacted with ATP-binding cassette (ABC) using the Vectastain Elite kit (Vector) and 3,3'-Diaminobenzidine according to the manufacturer's instructions.

Colocalization of Gad67-GFP with Rcan2 by fluorescent IHC was performed on E15.5 16 μ m cryosections or P8 40 μ m microtome sections. Colocalization of Rcan2 and reelin was performed on 40 μ m microtome sections of E15.5 brains fixed with 4% PFA and between Rcan2 and GABA on 40 μ m microtome sections from E17.5 and P8 brains fixed with 4% PFA and 0.25% glutaraldehyde, respectively. Cryosections were fixed for 30 min in 4% PFA and rinsed with PBS. All sections were blocked for 2 h at RT with 5% serum (Sigma) in TBS with 0.2% Triton-X100. Sections were incubated with primary antibodies in 1% serum and 0.2% Triton overnight at 4 $^{\circ}$ C. The following primary antibodies were used: Rcan2 1:2000, Reelin 1:250 (E4; de Bergeyck et al. 1998), GABA 1:1000 (ab17413; Abcam). Fluorescent secondary antibodies were applied for 2 h at RT, and sections were counterstained with DAPI. The following secondary antibodies were used: donkey antirabbit Alexa568 1:500 (A10042; Invitrogen), donkey antirabbit Alexa488 1:500 (A21206; Invitrogen), goat antirabbit Alexa488 1:500 (A11008; Invitrogen), goat antiguinea pig AlexaCy3 1:500 (106-165-003; Jackson ImmunoResearch), donkey antimouse Alexa568 1:500 (A10037; Invitrogen).

Results

Microarray Analysis

We performed a microarray analysis in order to compare gene expression between subplate and cortical plate in 2 selected areas of the anterior and posterior cortex of embryonic E15.5 C57/Bl6 mice (Fig. 1a,b) in 4 replicates. A correlation analysis across all arrays shows the greatest correlation between replicates of the same cell population indicating that the microdissections have been performed consistently between the different litters (Fig. 1c). In addition, moderate correlation was observed between the anterior and posterior subplate and anterior and posterior cortical plate, respectively. This suggests that the differences between cells in subplate and cortical plate are more pronounced than the differences between anterior and posterior areas within the same layer.

A total of 334 genes were expressed at a higher level in subplate (Supplementary Table 1), while 327 genes had higher

expression in the cortical plate (Supplementary Table 2) using a fold-change cutoff ≥ 1.4 (Fig. 1*d*; a list of all differentially expressed genes independent of the fold change is available as supplementary material).

A few genes with higher messenger RNA (mRNA) levels in subplate have been previously described as associated with this compartment. These include *Nurr1* (*Nr4a2*), a known marker of the embryonic and postnatal subplate cells (Arimatsu et al. 2003; Hoerder-Suabedissen et al. 2009; Wang et al. 2009) and 2 genes encoding the extracellular matrix proteins fibronectin (*Fn1*) and laminin (*Lama4*) (Stewart and Pearlman 1987; Sheppard et al. 1991; Hunter et al. 1992). In agreement with previous reports (Andrews et al. 2007; López-Bendito et al. 2007), *Slit1* was more highly expressed in the cortical plate, while the Slit receptor *Robo2* was more highly expressed in subplate. The lower cortical plate samples showed enrichment in transcripts of *FoxP2*, a marker of layer VI neurons (Ferland et al. 2003), *Fzf2*, a transcription factor specifically expressed in layer V and VI projection neurons (Inoue et al. 2004; Chen et al. 2005), and *Rorb*, a marker of layer IV neurons (Schaeren-Wiemers et al. 1997).

As a first step to validate the microarray data, publicly available ISH datasets from Gensat (The Gene Expression Nervous System Atlas Project forthcoming), Allen Brain Atlas (Lein et al. 2007), and Genepaint (Visel 2004) were screened for all 334 genes that showed higher expression levels in subplate. Based on the available E14.5 (Genepaint) and E15.5 (Allen Brain Atlas; Gensat) ISH images, we determined that 78 genes (23%) were expressed in a band below the cortical plate. However, on the available images, it was often impossible to distinguish between expression in the subplate and the intermediate zone (IZ) and further analysis will be needed. A further 75 genes (22%) showed clear expression patterns, mainly in the ventricular zone (VZ) and subventricular zone (SVZ), but were not expressed in subplate. These genes were considered as false positives and excluded from the dataset in subsequent analyses. Thirty-seven genes (11%) appeared to be expressed in blood vessels. As previous studies have shown that the embryonic subplate can be enriched in plasma proteins and immunoglobulins (Sarantis and Saunders 1986; Møllgård et al. 1988; Upender et al. 1997), these genes were not excluded at this stage. Finally, the remaining 145 genes (38%) either showed no or very weak expression or expression data were unavailable. Based on these preliminary confirmation methods, a shorter list containing 259 potentially subplate-enriched genes was compiled from which confirmed false-positive genes were omitted (Fig. 1*d*).

A gene ontology enrichment analysis was performed using DAVID software on this curated list, and overrepresented terms associated with subplate-enriched genes were identified for biological processes and pathways. Among others, these terms include cell adhesion, focal adhesion, negative regulation of transcription, actin cytoskeleton organization, cell morphogenesis and axogenesis, axonal guidance, cell motion, and cation homeostasis (see Supplementary Table 3 for details). In addition, Ingenuity Pathway Analysis (IPA) was performed on all genes statistically enriched in subplate in order to identify canonical signaling networks specifically present in the developing subplate. We found over 40 pathways significantly associated to subplate (P value < 0.05) including several which could be very relevant to the function of this cell layer: G α 12/13 signaling, reelin signaling, axonal guidance signaling,

complement system, and α -adrenergic signaling. Many other highlighted pathways, however, appeared not to be directly linked to developing nervous system (see Supplementary Table 4 for a list of all pathways from IPA analysis).

The experimental design of this microarray study allows us to investigate whether genes are expressed in an anterior-posterior gradient. We compared the transcript levels of the 259 subplate-enriched genes in the anterior versus posterior subplate (Fig. 1*d*). Although many of these genes showed small differences in enrichment between anterior and posterior samples (Supplementary Table 1), only 11 of these genes had statistically significantly higher expression levels in the anterior subplate (*Atp1b1*, *Cdb9*, *Cdb12*, *Cdb18*, *Fam84a*, *Gabra5*, *Gm10001*, *Kcnp4*, *Nt5dc2/Stab1*, *Pls3*, and *Ptn*) and 4 in the posterior subplate (*C3ar1*, *Nes*, *Pde1a*, and *Slc4a4*) (fold change ≥ 1.4 ; P value < 0.05). On public gene expression images for *Gabra5*, *Cdb12*, *Ptn*, and *Pls3*, a larger number of labeled cells are visible in the anterior cortex than in the posterior cortex, confirming the result of the microarray (not shown). Additionally, *Cdb9* has been described as a marker for the frontal cortex, at least in the adult mouse (Bedogni et al. 2010). Of the 4 genes more strongly expressed in the posterior cortex on the microarray, publicly available gene expression data confirmed this gradient for *Nes* and *Pde1a* (not shown).

Validation with qRT-PCR

For further validation of the microarray dataset, qRT-PCR was performed on 9 randomly selected genes with higher expression in subplate and on 2 genes with higher expression in cortical plate (Table 4). qRT-PCR was performed on 2 sample sets, which had also been included in the microarray as well as on 2 additional sample sets specifically collected for this purpose from individual embryos. For 8 of the 9 subplate genes and the 2 cortical plate genes, qRT-PCR showed fold changes in the same direction as the microarray (i.e., >1 for subplate-enriched genes and <1 for cortical plate-enriched genes). Fold changes in the same directions were consistently found in all 4 biological replicates and in both anterior and posterior comparisons for all 10 genes. However, the exact value of the fold changes was relatively variable between replicates with an average relative standard error of $\sim 20\%$. Consequently, most but not all fold changes were significantly different from 1

Table 4

qRT-PCR for selected subplate- and cortical plate-enriched genes

	Microarray		qRT-PCR	
	Ant SP: Ant CP	Post SP: Post CP	Ant SP: Ant CP	Post SP: Post CP
<i>Nurr1/Nr4a2</i>	1.6	—	2.8 (0.9)	2.9 (0.5)*
<i>2610017I09Rik</i>	2.2	2.3	2.3 (0.3)*	3.3 (0.8)*
<i>Epha3</i>	1.5	1.4	1.7 (0.3)	2.3 (0.4)*
<i>Sema6d</i>	1.7	1.8	2.8 (0.2)*	3.9 (0.5)*
<i>Abca8a</i>	2.2	2.0	2.9 (0.1)*	5.1 (0.2)*
<i>Unc5c</i>	1.5	1.8	4.8 (1.2)*	6.0 (1.5)*
<i>Cdb9</i>	1.8	1.5	2.1 (0.3)*	4.2 (1.0)*
<i>Csmc3</i>	—	1.6	1.7 (0.2)*	3.5 (0.4)*
<i>Slit1</i>	0.7	0.6	0.4 (0.1)*	0.4 (0.1)*
<i>Nr4a3</i>	0.5	0.5	0.4 (0.1)*	0.5 (0.1)*

Note: qRT-PCR shows fold changes in the same direction as the microarray for 10 genes—8 with a higher expression in the subplate (SP) and 2 with a higher expression in the cortical plate (CP). Average fold changes were calculated as the ratio between anterior SP and CP and between posterior SP and CP, respectively. In brackets are standard error of the mean. Asterisks (*) indicates fold changes, which are significantly different from 1 (P value < 0.05).

(P value < 0.05; see Table 4). The remaining subplate gene (*Kcnp4*) could not be sufficiently amplified with the designed primers to perform quantitative analysis.

Subplate-Specific Genes in the E15.5 Mouse Cortex

Based on the expression patterns available in public databases and presumed subplate-relevant functions, a subset of 23 genes were chosen for further validation by ISH or IHC (Table 5). An expression pattern was considered as subplate specific if it was predominantly localized in a 3–4 cell wide band just below the dense cortical plate. According to this criterion, we found 13 genes (*Abca8a*, *Cdh10*, *Cdh18*, *Csmd3*, *Gabra5*, *Kcnt2*, *Ogfr1*, *Pls3*, *Rcan2*, *Sv2b*, *Slc8a2*, *Unc5c*, and *Zdhc2*) that were primarily expressed in the subplate (Fig. 2). Seven further genes were primarily expressed in the IZ and/or SVZ with additional weaker expression in the subplate in some cases (Supplementary Fig. 2). For the remaining 3 genes, expression could not be detected with the designed riboprobes in the E15.5 cortex (data not shown). The 13 genes with a subplate-specific expression in the E15.5 cortex are presented here according to potential functional groups.

Cadherins

The microarray screen identified 4 type II cadherins as well as 2 protocadherin with higher expression in subplate (cadherins 9, 10, 12, and 18; protocadherin 10 and 18; Supplementary Table 1). Cadherin 10 (*Cdh10*) and cadherin 18 (*Cdh18*, aka *Cdh14*) showed the highest fold changes of all cadherins, both in anterior and posterior cortex (*Cdh10*: 2.4, 2.7; *Cdh18*: 2.7; 1.8). In situ hybridization on E15.5 brains confirmed that both *Cdh10* and *Cdh18* are specifically expressed in the subplate (Fig. 2*b,c*). The probes strongly labeled a substantial number of cells forming a large band in the subplate, while the cortical

plate appeared unlabeled. For both genes, the labeled cells band appeared to be thicker in the anterior than in the posterior subplate.

Proteins Involved in Axonal Growth and Guidance

Plastin 3 (*Pls3*, aka T-plastin) is an actin-bundling protein involved in neurite growth (Oprea et al. 2008). We found that in the E15.5 cortex, a riboprobe against *Pls3* primarily labeled cells in the subplate (Fig. 2*d*). More cells appeared to be labeled in the anterior than in the posterior subplate where only sparse cells were visible (fold changes based on the microarray: anterior: 1.4, posterior: <1.3). In the most anterior region of the cortex, a few *Pls3*-positive cells were also seen in the lower cortical plate.

The unc5 homologue *Unc5c* (aka *Unc5h3*) belongs to a family of netrin receptors, which mediate repulsion from a source of netrin. In the embryonic mouse central nervous system, *Unc5c* expression has been described in various structures including the spinal cord, mesencephalon, cerebellar plate, and prethalamus and thalamus but not in cortex (Przyborski et al. 1998; Braisted et al. 2000). Using ISH, we found that at E15.5, *Unc5c* mRNA is specifically localized to the subplate (Fig. 2*e*). Individual subplate cells with very strong labeling were seen across the entire rostrocaudal expanse of the cortex (fold changes on the microarray: anterior: 1.5; posterior: 1.8).

Proteins Involved in Neurotransmission

The GABA-A receptor alpha5 (*Gabra5*) is a subunit of the ionotropic GABA-A receptor. In the adult mouse and postnatal and adult rat brain, *Gabra5* shows the highest expression in the hippocampus with an additional weaker expression in other structures including the neocortex (Wisden et al. 1992; Liu et al. 1998; Sur et al. 1999; Ramos et al. 2004).

Table 5
Subplate-enriched genes selected for validation

Gene symbol	Name	Function (reference)	Expression
Specific expression in subplate			
<i>Abca8a</i>	ABC transporter A8a	Lipid transporter (Tsuruoka et al. 2002)	SP
<i>Cdh10</i>	Cadherin 10	Type II cadherin (Redies 2000)	SP
<i>Cdh18</i>	Cadherin 18	Type II cadherin (Shimoyama et al. 1999)	SP
<i>Csmd3</i>	CUB and Sushi multiple domains 3	Transmembrane protein of unknown function (Shimizu et al. 2003)	SP
<i>Gabra5</i>	GABA-A receptor $\alpha 5$	Ionotropic GABA receptor subunit (Fritschy and Mohler 1995)	SP
<i>Kcnt2</i>	Potassium channel subfamily T, member 2	Sodium-activated potassium channel (Bhattacharjee et al. 2003)	SP, MZ
<i>Ogfr1</i>	Opioid growth factor-like receptor 1	Homologous to the opioid growth factor receptor	SP
<i>Pls3</i>	L-plastin	Actin-bundling protein; involved in neurite growth (Oprea et al. 2008)	SP
<i>Rcan2</i>	Regulator of calcineurin 2	Regulator of calcineurin (Porta et al. 2007)	MZ, weak in SP
<i>Slc8a1</i>	Solute carrier 8a1	Sodium-calcium exchanger (Philipson et al. 2004)	SP
<i>Sv2b</i>	Synaptic vesicle 2b	Ca^{2+} -dependent regulation of neurotransmitter release (Janz et al. 1999)	SP
<i>Unc5c</i>	Unc homologue 5c	Repulsive netrin-1 receptor (Przyborski et al. 1998)	SP
<i>Zdhc2</i>	Zinc finger, DHHC domain-containing 2	Palmitoyl acyltransferase; involved in long-term potentiation (Noritake et al. 2009; Greaves et al. 2010)	SP
Main expression in IZ/SVZ			
1190002N15Rik	Riken clone	Unknown	SVZ and IZ
<i>Epha3</i>	Eph receptor a3	Ephrin receptor (Kudo et al. 2005)	SVZ, IZ, weak in SP
<i>Nrp1</i>	Neuropilin 1	Receptor for VEGF and Sema3a (Schwarz et al. 2004)	SVZ, IZ, weak in SP
<i>Nt5dc2</i>	5'-nucleotidase domain-containing 2	Dephosphorylation of nucleotides	SVZ and IZ
<i>Plxn4</i>	Plexin a4	Repulsive sema6a receptor (Suto et al. 2005)	SVZ, IZ, weak in SP
<i>Sema6d</i>	Semaphorin 6d	Guidance molecule (Taniguchi and Shimizu 2004)	SVZ, IZ
<i>Unc5d</i>	Unc homologue 5d	Repulsive netrin-1 receptor (Takemoto et al. 2011)	SVZ, IZ
No or weak expression in the neocortex			
<i>Cdh12</i>	Cadherin 12	Type II cadherin	Weak signal
<i>Cdh9</i>	Cadherin 9	Type II cadherin	Weak signal
<i>Neurod1</i>	Neurogenic differentiation 1	Transcription factor	Hippocampal anlage

Note: This table lists all 23 genes, which were selected for further validation by IHC or ISH. Thirteen genes were confirmed to be specifically localized in the E15.5 subplate (SP), sometimes with additional expression in the marginal zone (MZ); 7 genes were primarily expressed in the IZ and SVZ, sometimes with additional expression in SP; 3 genes were not expressed in the E15.5 neocortex. VEGF, vascular endothelial growth factor.

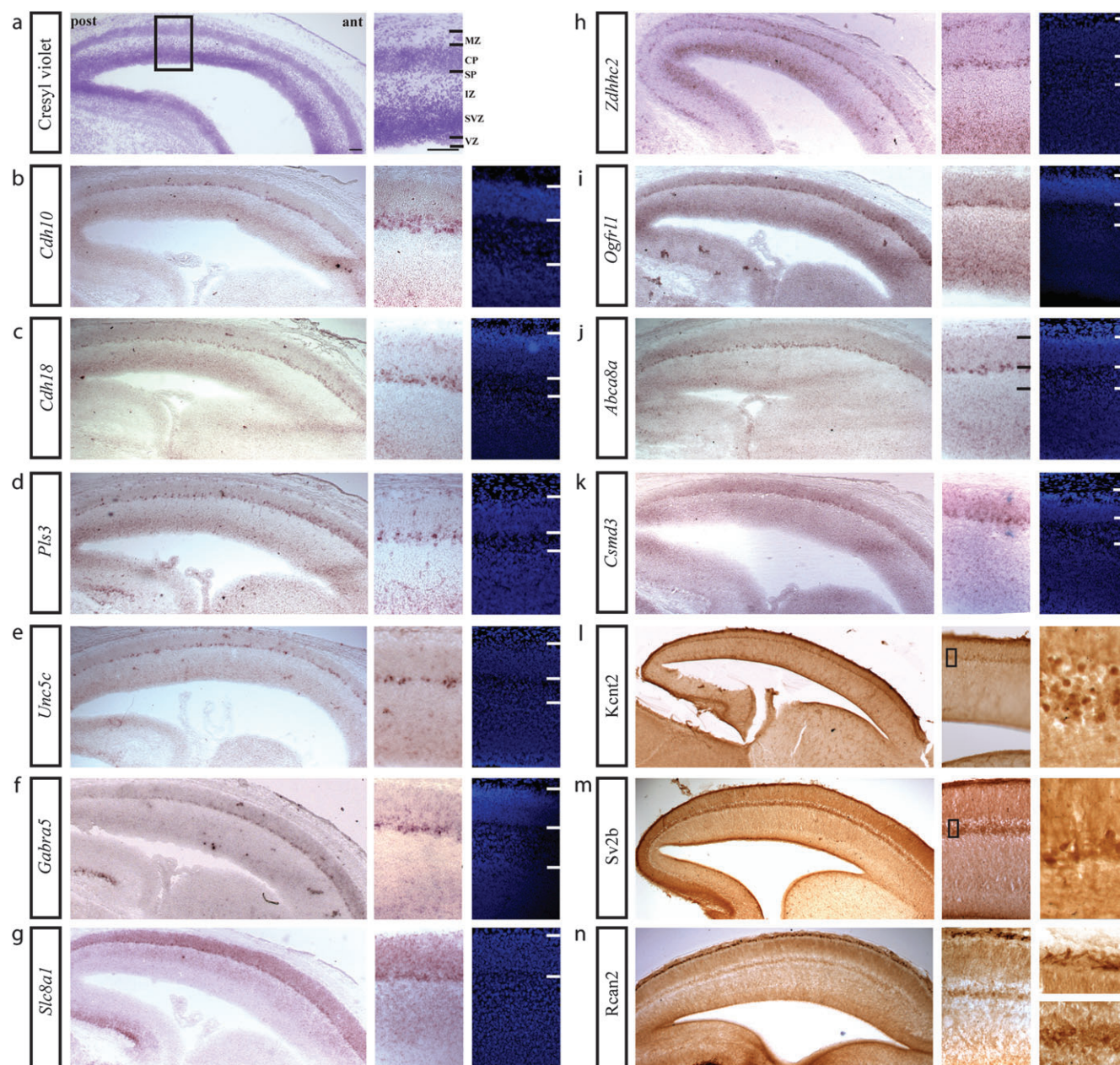


Figure 2. Genes with subplate-specific expression in the E15.5 cortex. In situ hybridization (*b–k*) or IHC (*l–n*) signals for 13 genes, which are expressed in the E15.5 subplate (SP) but not in the overlying cortical plate (CP). *Kcnt2* (*l*) and *Rcan2* (*n*) show additional expression in the marginal zone (MZ). A cresyl violet-stained section is shown for reference (*a*). The rightmost panels in (*b–k*) show DAPI counterstains of the same area as shown in the middle panels. Scale bars: 100 μ m.

Immunohistochemistry against *Gabra5* in adult rat was reported to prominently label neurons in layer VIb (Fritschy and Mohler 1995). We found that in the E15.5 cortex, *Gabra5* mRNA is localized in the subplate (Fig. 2*f*). Within subplate, *Gabra5* appeared to be expressed in an anterior high-posterior low gradient (fold changes on the microarray: anterior: 2.1; posterior: 1.9).

The solute carrier 8a1 (*Slc8a1*, aka NCX1) is a sodium/calcium exchanger, which typically exchanges 3 Na⁺ for 1 Ca²⁺ (Philipson et al. 2004). *Slc8a1* can be spliced into a large number of different isoforms, which show differential expression in different tissues and at different developmental stages in rat (Wakimoto et al. 2001). We used a riboprobe against all splice variants of *Slc8a1* and found strong staining in the E15.5

subplate (Fig. 2*g*). Subplate was clearly visible as a dark band, while the cortical plate showed weaker and more diffuse labeling (fold changes on the microarray: anterior: 1.4; posterior: <1.3). Due to the high homology between *Slc8a1* and the 2 other *Slc8* family members (*Slc8a2* and *Slc8a3*), we cannot exclude the possibility that the probe binds to a lesser degree to those mRNAs (probe homology with *Slc8a2*: 70%, *Slc8a3*: 75%), although high stringency conditions were used for the ISH.

The zinc finger, DHHC domain containing 2 (*Zdhhc2* aka *Dhhc2*) protein is a palmitoyl acyltransferase, an enzyme that mediates posttranslational protein modification by adding the fatty acid palmitate to specific cysteine residues. We found that in the E15.5 cortex, *Zdhhc2* is mainly expressed in the subplate

and the marginal zone with no labeling detectable in the cortical plate (Fig. 2*b*). Additional weaker staining was found in the VZ and SVZ (fold changes on the microarray: anterior: 2.0 posterior: 2.0).

The gene potassium channel subfamily T, member 2 (*Kcnt2* aka *Slick*) encodes a sodium-activated potassium channel, which is inhibited by ATP (Bhattacharjee et al. 2003). In the adult rat cortex, *Kcnt2* has been shown to be primarily localized to layers II/III and V (Bhattacharjee et al. 2005). We found that at E15.5, an anti-*Kcnt2* antibody labeled a large number of cells in the subplate and fewer cells in the marginal zone (fold changes on the microarray: anterior: 2.2; posterior: 1.9). In most brains, only a few scattered *Kcnt2*-immunoreactive cells were detected in the developing cortical plate (Fig. 2*d*), but occasionally, a larger number of cortical plate cells were labeled.

The synaptic glycoprotein vesicle 2b (*Sv2b*) is 1 of 3 members of the *Sv2* protein family and is involved in the calcium-dependant regulation of neurotransmitter release (Janz et al. 1999). *Sv2b* mRNA has been reported to be more strongly expressed in the upper layers of the developing cortical plate of the E17 rat and in the entire cortex of adult rat (Bajjalieh et al. 1994). We used an anti-*Sv2b* antibody on E15.5 sections and found specific labeling in the subplate and marginal zones, which were clearly visible as 2 dark bands at low magnification (Fig. 2*m*). This observation is consistent with Genepaint gene expression data at E14 and E15 where *Sv2b* mRNA is also primarily localized to the subplate (Genepaint IDs EH3733 and HB596) (fold changes on the microarray: anterior: 1.7; posterior: <1.3).

Proteins Highly Expressed in Subplate with Various Functions

ABC transporter A8a (*Abca8a*) belongs to the A family of ABC transporters, which constitute one of the largest protein superfamilies with 57 mammalian ABC genes identified so far (Li et al. 2007). Like other members of the A family, *Abca8a* is thought to transport mainly lipids (Tsuruoka et al. 2002). *Abca8a* showed consistent fold changes in both anterior and posterior cortex in the microarray screen (2.1 and 1.9, respectively). Using ISH, we confirmed that *Abca8a* is very strongly and specifically expressed in the E15.5 subplate with no staining detectable in the cortical plate (Fig. 2*j*).

CUB and Sushi multiple domains 3 (*Csmd3*) is a giant gene comprising 73 exons and is predicted to encode a transmembrane protein based on its amino acid sequence (Shimizu et al. 2003). *Csmd3* has been reported to be primarily expressed in fetal brain and testis (Shimizu et al. 2003). A riboprobe designed against *Csmd3* mainly labeled subplate cells in the E15.5 cortex, while staining of the overlying cortical plate was much weaker (Fig. 2*k*) (fold changes on the microarray: anterior: <1.3; posterior: 1.6).

The opioid growth factor-like receptor 1 (*Ogfr1*) is a largely uncharacterized protein but is partially homologous (41.7% identity in a 314 of 464 amino acid overlap) to the opioid growth factor receptor (*Ogfr*). *Ogfr* binds the endogenous opioid peptide met-enkephalin, an inhibitory growth molecule influencing cell proliferation and tissue organization (Zagon et al. 1999, 2002). In situ hybridization on E15.5 brains with a riboprobe against *Ogfr1* resulted in a strong labeling of the subplate (Fig. 2*i*) (fold changes on the microarray: anterior:

<1.3; posterior: 1.6). The lower cortical plate was also weakly labeled but subplate is clearly visible as a darker band.

Rcan2 acts a regulator of calcineurin, a phosphatase that is highly expressed in the brain and plays key roles in neurite outgrowth, neuronal death, and synaptic plasticity (Shibasaki et al. 2002). In both postnatal and adult cortex, *Rcan2* is primarily expressed in interneurons, although some pyramidal neurons, especially in layer VI, were also found to be *Rcan2* immunopositive (Porta et al. 2007). In the E15.5 cortex, we found that *Rcan2* protein was primarily located in horizontally orientated bipolar cells in the marginal zone (Fig. 2*n*) (fold changes on the microarray: anterior: 1.7; posterior: <1.3). The subplate also appeared to express *Rcan2* and was visible as a dark band but the labeling was weaker than in the marginal zone (compare right panels in Fig. 2*n*). The staining in subplate was detected in most but not all E15.5 brains. These brains generally appeared to be slightly more developed (i.e., with a thicker cortical plate), suggesting that E15.5 is the time of onset of *Rcan2* expression in the subplate.

Displacement of Subplate-Specific Genes in the Reeler Mutant

As an additional mean of verifying the subplate specificity of these 13 genes, we analyzed their expression patterns in the cortex of E15.5 reeler mutant mice. In these mice, the preplate fails to split and the cells normally contributing to the subplate are located underneath the pial surface (Caviness 1982) (Fig. 3*a*). Thalamic fibers first navigate to this mispositioned subplate (referred to as superplate) before turning back down to their targets in the cortical plate (Molnár et al. 1998). We found that in E15.5 reeler mice, the expression of the majority of subplate-specific genes was shifted to the superplate (Fig. 3). *Abca8a*, *Cdb10*, *Cdb18*, *Csmd3*, and *Gabra5*-positive cells were all localized in a broad band just below the cortical pial surface (Fig. 3*b–e,h*). Consistent with the sparser labeling of the subplate in wild-type animals, few but clearly labeled *Pls3*- and *Unc5c*-positive cells were visible in the superplate (Fig. 3*f,g*). In contrast, we did not find any clear labeling for *Slc8a1*, *Zdbbc2*, *Sv2b*, or *Ogfr1* (Fig. 3*i–k,n*) in the reeler cortex, either in the superplate or below the cortical cells. In wild-type cortices, *Kcnt2* and *Rcan2* are expressed both in the subplate and the marginal zone, while in the reeler cortex, *Kcnt2*- and *Rcan2*-immunoreactive cells were only visible in the superplate (Fig. 3*m,o*). The band of *Kcnt2*- and *Rcan2*-positive cells in the superplate appeared larger than the one in the marginal zone of wild-type animals, suggesting that the superplate contains both subplate and marginal zone cells positive for *Rcan2* and *Kcnt2* (Fig. 3*m,o*). As E15.5 is the onset of *Rcan2* expression in the subplate, we also analyzed P8 reeler mice for *Rcan2* labeling (Fig. 3*p*). In the P8 wild-type cortex, *Rcan2*-immunopositive cells are distributed throughout the whole cortex, but the subplate is clearly visible as a cell-dense band above the white matter. This band was absent in the reeler mutant brain and did not appear to be shifted to the superplate. In contrast to the shift or absence of expression of the 13 subplate-specific genes, the expression of neuropilin1 (*Nrp1*), which is strongly expressed in the IZ, was unaffected in E15.5 reeler brains (Fig. 3*l*).

Temporal Expression Profile of Subplate-Specific Genes

We analyzed the expression patterns of all 13 genes that showed a subplate-specific expression at E15.5 at additional

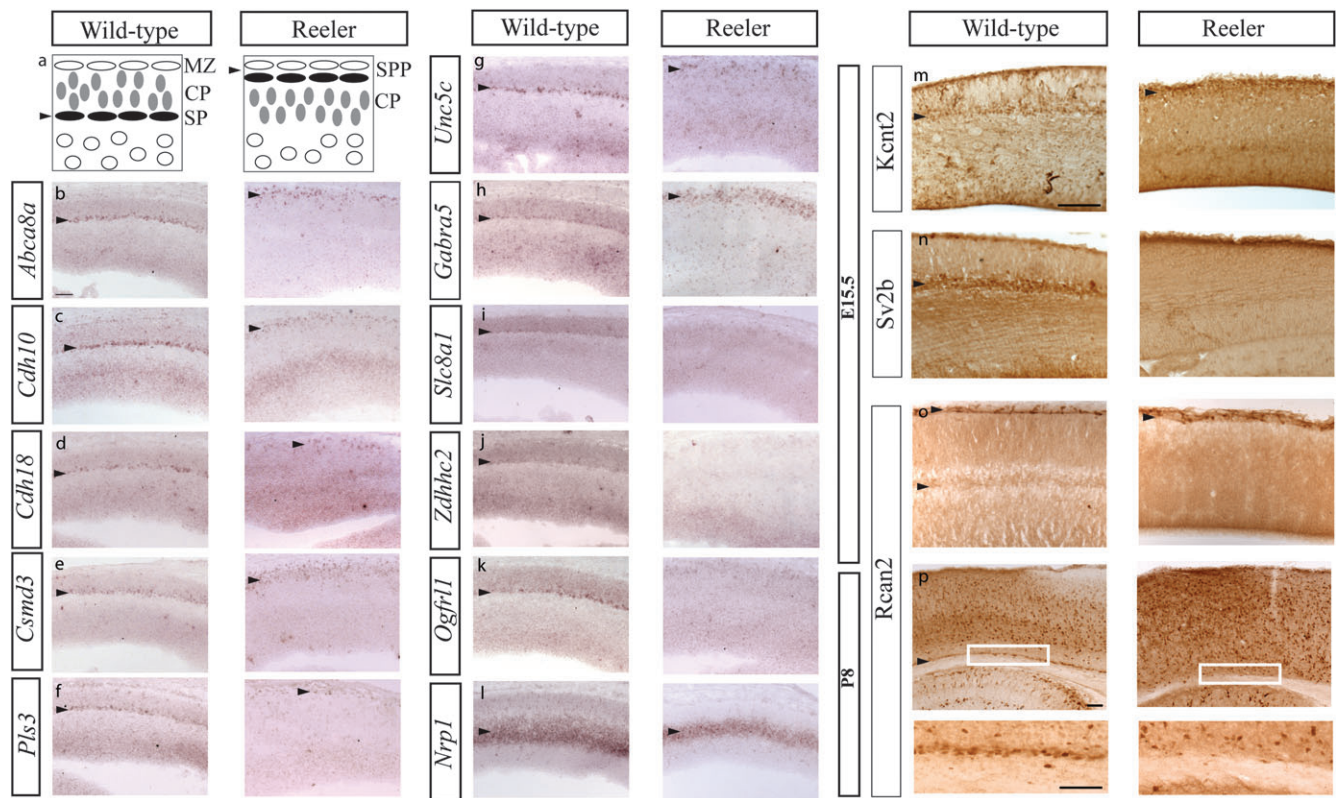


Figure 3. Shifted expression of subplate-specific genes in the reeler cortex. (a) Schema illustrating the inverted relative position of subplate (SP) and cortical plate (CP) cells in the reeler mutant. In the reeler, the preplate fails to split and remains at the top of the cortical wall as the superplate (SPP). (b–l) In situ hybridization signals in the reeler and wild-type E15.5 cortex. (m–p) Immunohistochemistry signals in the E15.5 (m–o) and P8 (p) reeler and wild-type cortex. Out of 13 genes with SP-specific expression in the wild-type cortex, 9 are expressed in the SPP in the reeler cortex (b–h, m, o). For the remaining 4 genes (i–k, n), no clear expression pattern is visible in the reeler brain. In contrast, the expression pattern of *Nrp1* (l), which is primarily localized in the IZ in the wild-type cortex, remains in the same position in the reeler cortex. Scale bars: 100 μ m.

developmental stages (E13.5, E14.5, E17.5, P8, and adult) in the cerebral cortex (Fig. 4, Supplementary Fig. 3 and Supplementary Table 5). Based on their temporal expression profile, genes were grouped into 3 broad categories.

Genes which Are Strongly Expressed in Subplate Throughout Embryonic and Postnatal Development

Pls3 is specifically expressed in the subplate at E14.5, while at E15.5 and E17.5 additional labeling is seen in the anterior cortical plate. Postnatally, *Pls3* is strongly expressed in subplate with weaker expression in layer V (P8) or layers V and VI (adult) (Fig. 4a). Similarly, *Cdh18* is primarily expressed in subplate from E14.5 until adulthood with an additional weaker expression in layers V and VI at late embryonic and postnatal stages (Fig. 4b). Labeling with *Gabra5* is already visible in the lateral cortex at E13.5 and is localized specifically to the subplate at E14.5 and E15.5. From E17.5 onwards, *Gabra5* is additionally expressed in the cortical plate, in particular in layer V (Supplementary Fig. 3a). *Slc8a1* is most strongly expressed in the subplate from E14.5 until adulthood that is always distinguishable as a dark band, but additional weaker expression is found in other cortical layers (Supplementary Fig. 3b). *Rcan2*-immunopositive cells are localized exclusively in the marginal zone at E13.5 and E14.5. At E15.5, an additional, weaker labeling appears in subplate (Supplementary Fig. 3c). These moderately stained *Rcan2*-positive cells form a clear band in subplate at E17.5 that remains visible at P8 (Fig. 3p) and in adult. In P8 and adult, *Rcan2* staining is no longer visible in the marginal zone, instead, strongly labeled *Rcan2*-immunoreactive

cells are found scattered throughout the entire expanse of the cortex (Supplementary Fig. 3c).

Genes Which Are Downregulated in Subplate at Late Developmental Stages

Abca8a is specifically and strongly expressed in the embryonic subplate from E14.5 until E17.5. In the postnatal P8 cortex, however, *Abca8a* is absent or very weak in subplate, while strong expression is found in a subset of layer V and II/III cells. No clear expression pattern is visible in the adult (Fig. 4c). *Unc5c* is specifically expressed in the subplate at E14.5 and in subplate and the very lateral cortical plate at E15.5. At E17.5, the expression in subplate is weak, while strong labeling is visible in the lateral cortical plate. At P8 and in adult, expression of *Unc5c* is very weak with slightly stronger expression in layer V and upper layer VI (Fig. 4d).

Genes Which Have a Broad Expression Pattern at Late Developmental Stages

Cdh10 is very strongly expressed in subplate at E14.5 and E15.5. At E15.5, additional weaker expression is found in the anterior and lateral cortical plate and from E17.5 in the entire cortical plate. At P8 and adult, *Cdh10* is still strongly expressed in subplate as well as layer V and weaker in all other layers (Supplementary Fig. 3e). *Zdhc2* is most strongly expressed in subplate from E13.5 until E15.5. At E17.5, the subplate and upper cortical plate are most strongly labeled. In P8 and adult, the strongest staining is seen in subplate and layers IV and V

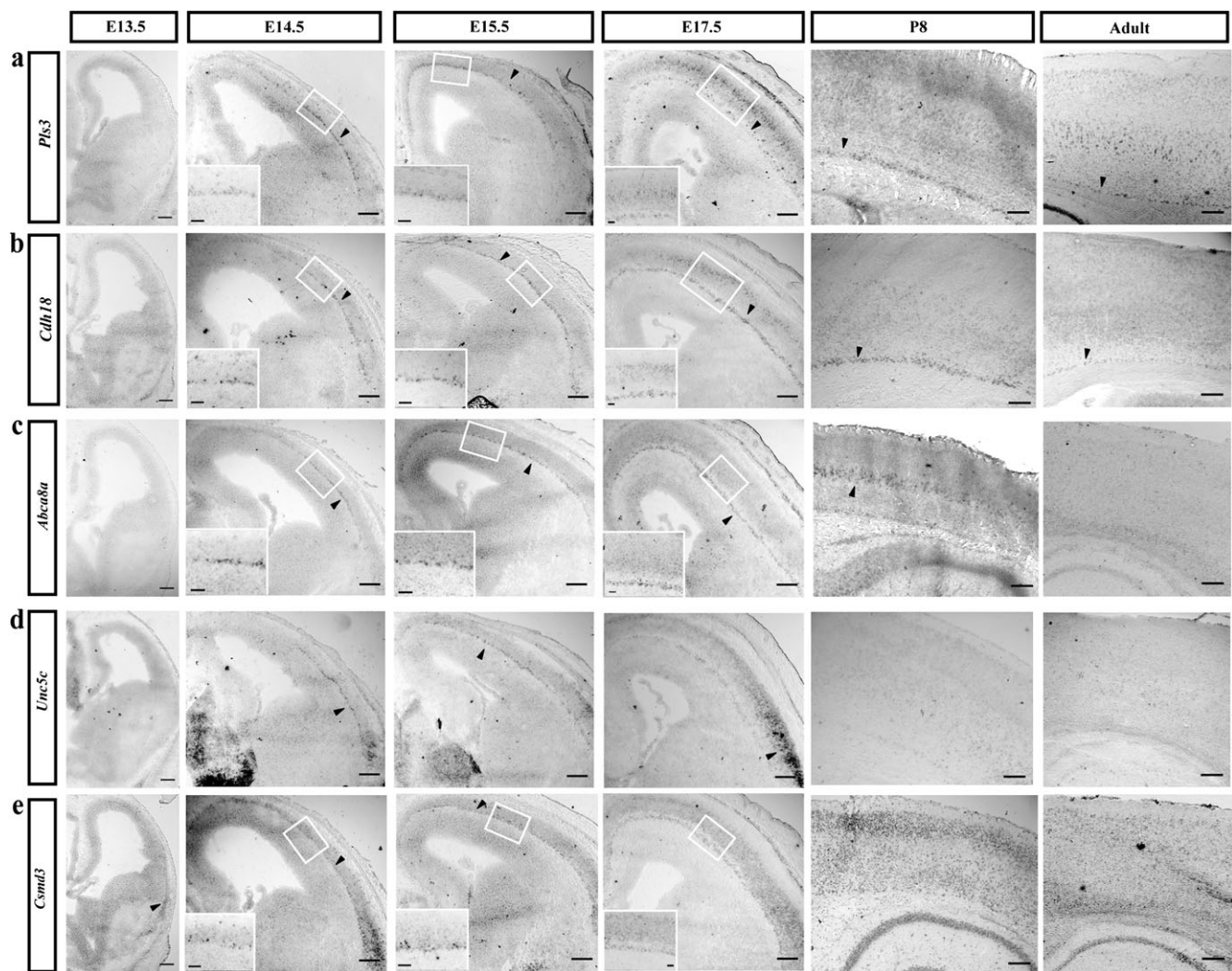


Figure 4. Developmental time course of expression of *Pls3*, *Cdh18*, *Abca8a*, *Unc5c*, and *Csm3*. In situ hybridization signals in the E13.5, E14.5, E15.5, E17.5, P8, and adult neocortex. At E13.5, only *Csm3* is expressed in the lateral cortex (arrowhead). At E14.5 and E15.5, all genes are specifically expressed in the subplate. At E17.5, *Abca8a* is still specifically expressed in the subplate (c), while *Pls3*(a) and *Cdh18* (b) are expressed in the subplate and upper cortical plate. *Unc5c* is primarily expressed in the lateral cortical plate (d) and *Csm3* is ubiquitously expressed (e). In postnatal and adult cortex, strong expression of *Pls3* (a) and *Cdh18* (b) is still visible in subplate. At P8, *Abca8a* (c) is specifically expressed in layer V, while in the adult, *Abca8a* expression is no longer visible. At both P8 and adult stages, *Unc5c* is no longer expressed (d), while *Csm3* is ubiquitously expressed (e). Scale bars: 200µm (panels); 50µm (insets).

with additional weaker staining in all other layers (Supplementary Fig. 3f). *Ogfr11* is expressed in the lateral cortex at E13.5 and in subplate and the very lateral cortical plate at E14.5 and E15.5. At later stages, *Ogfr11* is expressed in the deep cortical layers including subplate (Supplementary Fig. 3d). *Csm3* is expressed in the lateral cortex at E13.5 and in subplate and the lateral cortical plate at E14.5 and E15.5. From E17.5 onwards, *Csm3* becomes weakly but ubiquitously expressed (Fig. 4e). *Sv2b* and *Kcnt2* are specifically expressed in subplate from E13.5 until E15.5. *Kcnt2* is additionally expressed in the marginal zone. From E17 onwards, *Sv2b* and *Kcnt2* become ubiquitously expressed (Supplementary Fig. 3g,b).

We also analyzed the time course of extracortical expression patterns of all 13 genes in the forebrain with special attention to the prethalamus (ventral thalamus) and thalamus (dorsal thalamus) and the claustroramygdalar complex. A few examples of extracortical expression patterns for each gene are shown in Supplementary Figure 4 and more details can be found in Supplementary Table 5.

Colocalization with Interneuron Markers

The embryonic subplate is 1 of 3 zones where interneurons migrate tangentially from the ganglionic eminences into the cortex (Métin et al. 2006). In order to investigate whether some of the subplate-specific genes are expressed in these interneurons, we attempted to colocalize their expression with GFP in the Gad67-GFP mouse. In this transgenic line, almost all cortical GABAergic cells are GFP positive (Tamamaki et al. 2003). Colocalization analysis was performed for *Unc5c* and *Cdb10* with ISH and for *Rcan2* with IHC (Figs 5 and 6).

In the E15.5 cortex, *Cdb10* and *Unc5c* are specifically expressed in the subplate but do not colocalize with Gad67-GFP (Fig. 5). Less than 1% of *Cdb10*-positive cells were found to be expressing GFP (3 double-labeled cells of 397 *Cdb10*-positive cells and 212 Gad67-GFP+ cells, $n = 2$ brains, Fig. 5a-d). *Unc5c* never colocalized with Gad67-GFP (169 *Unc5c*+ cells and 239 Gad67-GFP+ cells, $n = 2$ brains, Fig. 5e-h).

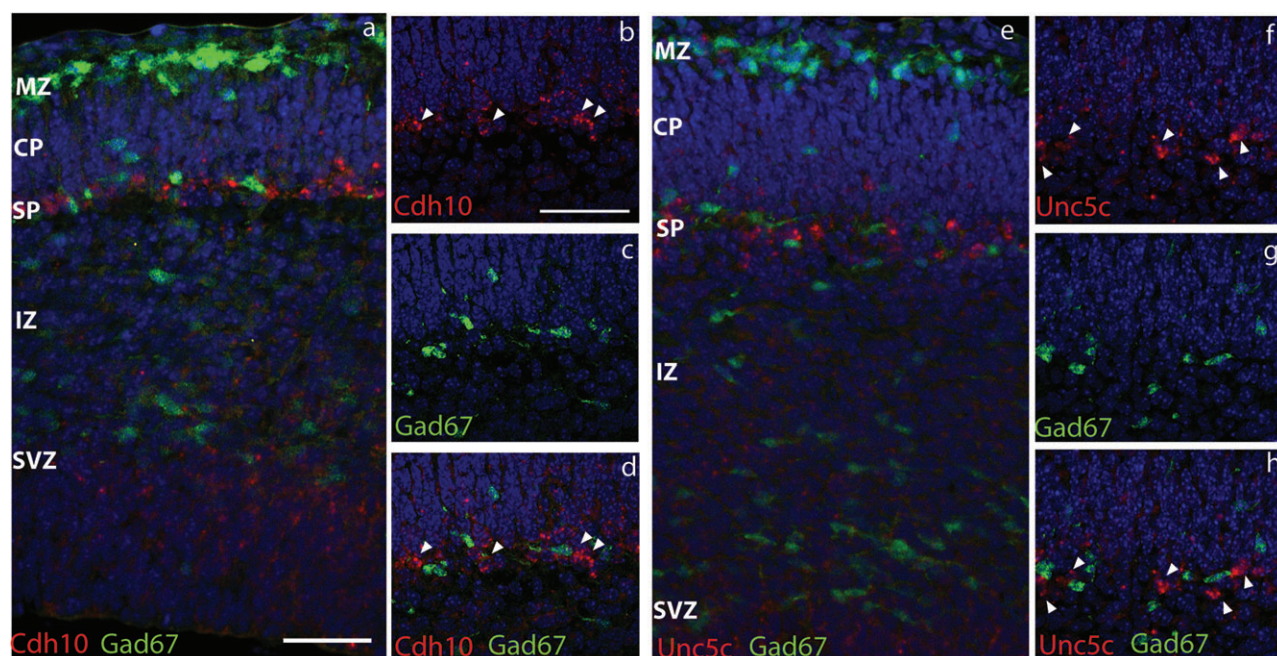


Figure 5. Expression of *Cdh10* and *Unc5c* in the Gad67-GFP cortex. In situ hybridization against *Cdh10* (a–d) and *Unc5c* (e–h) was performed on sections of Gad67-GFP E15.5 cortices. GFP-positive cells are localized in the marginal zone (MZ), in the subplate (SP), and at the border between the IZ and SVZ corresponding to the 3 routes of interneuron migration (a, e). *Cdh10*-positive cells (a–d; arrowheads) and *Unc5c*-positive cells (e–h; arrowheads) are localized in the SP. No colocalization was observed between *Cdh10* (red; arrowheads) and Gad67-GFP (green) (d) and between *Unc5c* (red; arrowheads) and Gad67-GFP (green) (h), respectively. Scale bars: 50µm.

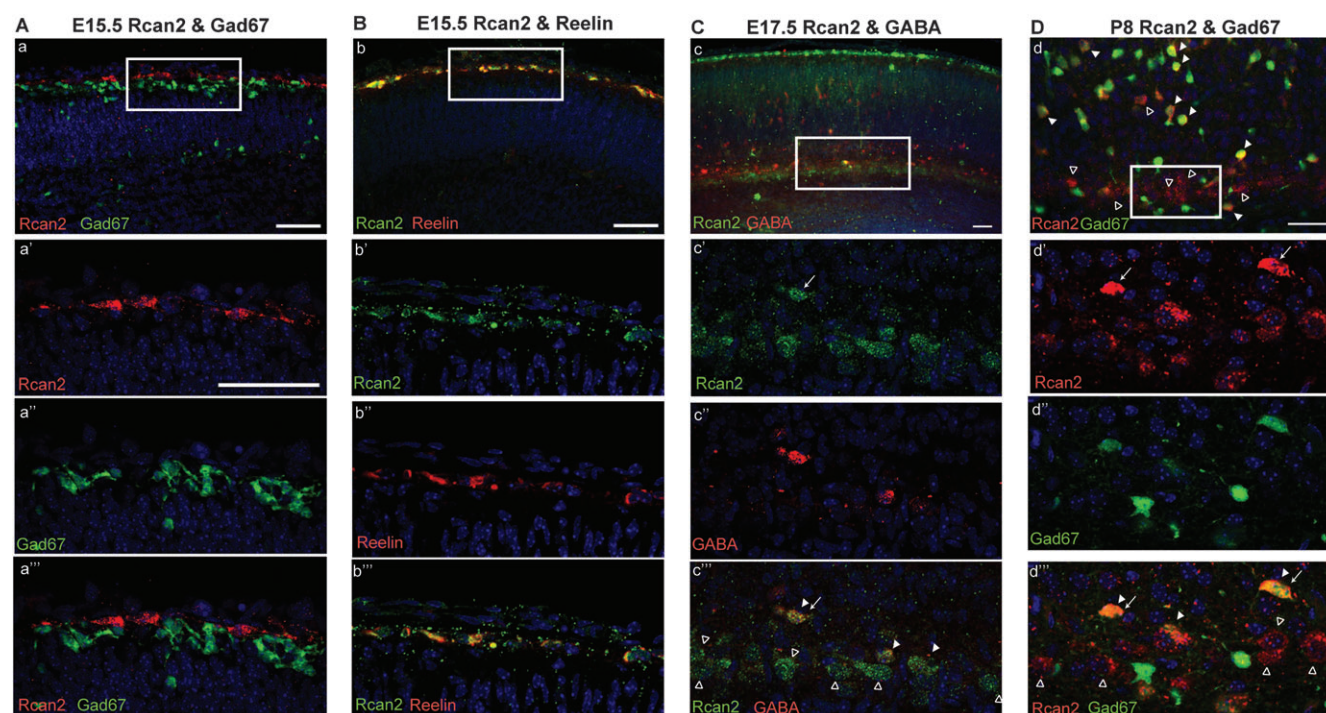


Figure 6. Colocalization between Rcan2, Gad67-GFP/GABA, and Reelin. (A) In the E15.5 cortex, IHC against Rcan2 primarily labels horizontally orientated cells at the outer edge of the marginal zone (red, a, a'). The Rcan2-positive cells are a distinct population from the Gad67-GFP-positive cells (green, a, a''), which are localized at the inner edge of the marginal zone (a'''). (B) The Rcan2-positive cells in the E15.5 marginal zone (green, b') coexpress reelin protein (red, b, b'', b'''). (C) At E17.5, Rcan2 is primarily expressed in the marginal zone and the subplate (green, c, c'). Sparse Rcan2-positive cells are also found within the cortical plate (arrow in c' and c'''). In the subplate, only a small proportion of Rcan2-positive cells coexpress GABA (red, c'''). (D) In the P8 cortex, strongly labeled Rcan2-positive cells are found dispersed in all layers (red, d). A band of moderately labeled Rcan2-positive cells is localized within the subplate (d, d'). Only few Rcan2-positive cells in the subplate coexpress Gad67-GFP (green, d, d'''). In contrast, most Rcan2-positive cells outside of subplate (arrows in d and d''') are positive for Gad67-GFP (d, d'''). Empty arrowheads point to single-labeled Rcan2-positive cells and filled arrowheads to double-labeled Rcan2-positive cells. Scale bars: 50µm.

Using fluorescent IHC, Rcan2-immunoreactive cells were only detected in the marginal zone of the E15.5 Gad67-GFP cortex but not in the subplate (Fig. 6A,B). Surprisingly, the Rcan2-positive cells in the marginal zone did not colocalize with Gad67-GFP (Fig. 6A). Instead, they formed a distinct band of horizontally oriented cells localized above the stream of tangentially migrating Gad67-GFP neurons (Fig. 6A,A'). Based on their morphology and location, these Rcan2-positive cells are most likely Cajal-Retzius cells, which is further supported by their coexpression of reelin (Fig. 6B).

As we did not detect any Rcan2-positive cells in the subplate at E15.5 using fluorescent IHC, colocalization of Rcan2 and GABA in the E17.5 cortex was analyzed (Fig. 6C). At this age, Rcan2-positive cells formed a distinct cell-dense band in the subplate. In the subplate, less than 10% of Rcan2-positive cells appeared to colocalize with GABA (32 double-labeled cells of 343 Rcan2+ cells, $n = 3$ brains).

At P8, a dense band of Rcan2-positive cells is still visible in the subplate, while many Rcan2-positive cells are scattered throughout the entire expanse of the cortex (Fig. 6D). As at E17.5, in the subplate, only a small proportion of Rcan2-positive cells colocalized with GABA (10.5%, 9 double-labeled cells of 106 Rcan2-positive cells, $n = 3$ brains). In contrast, in layers V and VI, the large majority of Rcan2 positive were GABAergic (88.3%, 106 double-labeled cells of 120 Rcan2-positive cells, $n = 3$ brains) (not shown). Similar results were also found in the P8 Gad67-GFP cortex: In the subplate, only 13.4% of Rcan2-positive cells colocalized with GFP (41 of 305 cells, $n = 3$ brains), while in layers V and VI over 80% of Rcan2 positive also expressed GFP (403 of 499 cells, $n = 3$ brains) (Fig. 6D). The strong colocalization between Rcan2 and markers for interneurons in the cortical plate is consistent with previous findings in the adult cortex (Porta et al. 2007).

Discussion

In this study, we established a gene expression profile of the embryonic E15.5 mouse subplate. Our approach was to microdissect strips of tissue containing subplate or cortical plate cells based on their position from anterior or posterior regions of developing cortex (Fig. 1A,B) and to compare mRNA levels using microarray-based expression analysis. We identified over 300 genes with higher expression in the subplate than in the overlying lower cortical plate (Fig. 1D; Supplementary Table 1).

Gene Ontology and Pathway Analyses

Gene ontology and pathway analyses of subplate-enriched genes revealed several functional gene groups and pathways associated with known characteristics and functions of the developing subplate. These include genes involved in focal adhesion, axogenesis, and axon guidance. In addition, several novel signaling pathways have been highlighted, which could be relevant to the function of the developing subplate (Supplementary Tables 3 and 4). It is important to note, however, that some genes in the subplate-enriched gene list are likely to be false positives, and that the available ontology and pathway databases are neither complete nor specific to the developing nervous system. Therefore, the here described findings can only be considered as a starting point for further analyses. The 2 pathways "signaling through the serotonergic receptor 5HT2" and " α -adrenergic signaling" (Supplementary

Fig. 5A) suggest that functional connections between the serotonergic and adrenergic system and subplate are present at E15.5. Indeed, monoaminergic afferents have been described to form a dense network within the subplate zone at E15 (Caviness and Korde 1981; Crandall and Caviness 1984), but whether they form synapses with subplate neurons has not been elucidated. Further, 2 signaling pathways involved in neuronal migration—"reelin signaling" (Caviness 1976) and " α 12/13 signaling" (Moers et al. 2008)—are associated with the developing subplate (Supplementary Fig. 5B,C). Further studies could reveal whether these signaling molecules are present in subplate cells themselves or in migrating neurons destined to the cortical plate. Finally, the enrichment of the functional categories "regulation of immune response" and "complement system" could be interesting to understand some further aspects of subplate development. Among other immune proteins, genes coding for subunits of the complement component C1q are enriched in subplate in the E15.5 microarray list (Supplementary Table 1). In the postnatal brain, C1q has been found to mediate synapse elimination that underlies activity-dependent synapse refinement (Stevens et al. 2007). It is an intriguing possibility that C1q plays a similar role in the developing subplate and could be involved both in the elimination of exuberant neurites and synapses as well as the subset of subplate neurons that dies through apoptosis.

Genes Expressed in an Anterior-Posterior Gradient

We found that only a very small proportion of the genes enriched in subplate are expressed in an anterior-posterior gradient (~6%; 15 of 259). Subplate gene expression gradients are of particular interest since they might be involved in regulating the topographic development of thalamocortical connections (López-Bendito and Molnár 2003; Shimogori and Grove 2005). Surprisingly, however, many of the molecular classes previously associated with cortical arealization and topography, including transcription factors, fibroblast growth factors, and semaphorin and ephrin receptors, did not appear to be expressed in a subplate-specific gradient. In addition, we found that for *Cdb18* and *Pls3*, the anterior-posterior expression gradients within subplate observed at E15.5 were no longer present at late embryonic and early postnatal stages (not shown). This suggests that at least some of the early gradients in subplate might reflect the anterior-posterior delay in maturation (Bayer and Altman 1991) rather than area-specific expression patterns. There are several possible reasons why our microarray analysis has not revealed more area-specific molecules. First, we have so far only analyzed genes with a subplate-enriched expression. However, many genes involved in arealization might not be specific to subplate but also present in other cortical layers (Shimogori and Grove 2005). Second, as the thalamocortical afferents have already arrived in the cortex at E15.5, some of the molecular gradients involved in early topographic guidance might already have been dissolved at this stage. Third, in order to obtain enough starting material for our microarray analysis, we microdissected and pooled relatively long tissue strips from different medio-lateral positions of the cortex. The samples thus likely contain transcripts from several putative cortical areas, and a more precise microdissection might be necessary to detect differentially expressed genes underlying arealization. Nevertheless, at least some of the genes with an anterior-posterior gradient

identified including the type II cadherins 9 and 12 or the growth factor pleiotrophin (Ptn) are promising candidates for setting up the initial topography of thalamocortical axons and should be investigated in more detail.

Identification of 13 Novel Subplate-Specific Genes

Using our own optimized IHC or ISH protocols, we were able to confirm the enrichment of 13 genes of 23 selected in the E15.5 subplate zone (*Abca8a*, *Cdb10*, *Cdb18*, *Csmd3*, *Gabra5*, *Kcnt2*, *Ogfr11*, *Pls3*, *Rcan2*, *Slc8a1*, *Sv2b*, *Unc5c*, and *Zdhhc2*). Besides *Gabra5* which has been described as preferentially localized to subplate in the adult rat (Fritschy and Mohler 1995), none of these genes has been previously associated with the subplate compartment. Moreover, none of them have been identified by expression analyses at earlier (Osheroff and Hatten 2009) or later stages (Hoerder-Suabedissen et al. 2009). Publicly available gene expression datasets (Genesat, Allen Brain Atlas, and Genepaint) were available for 12 of 13 genes, but for half of them, expression patterns were either difficult to localize or failed to be detected. Further evidence that these genes are indeed expressed in subplate cells was gained from analysis of reeler mutant brains. In the reeler cortex, the expression of a majority of genes (9 of 13) was shifted in accordance with the shifted position of subplate cells, while the remaining genes no longer showed any clear expression pattern.

While we focused in this study on genes with higher expression in subplate than the lower cortical plate, it could be equally informative to identify gene pathways excluded from subplate but present in cortical plate neurons. Based on the microarray screen, over 300 genes are higher expressed in the cortical plate than in the subplate (Supplementary Table 2). Using public available ISH data, we confirmed the specific expression in the cortical plate of 9 of the 16 most highly enriched genes (fold change ≥ 2.0 ; Supplementary Fig. 6). Further analysis of genes enriched in the cortical plate could complement our current analysis.

Our approach has several strengths but also some limitations: First, gene expression levels were not compared between subplate neurons and cortical plate neurons but between entire compartments likely to also contain mRNA from tangentially and radially migrating neurons destined to other layers, glial cells, axons and dendrites, and blood vessels. Second, despite the precision of laser capture microdissection, it is difficult to accurately identify and distinguish the thin embryonic subplate zone from the underlying IZ on cresyl violet-stained sections. Third, genes identified with higher expression in subplate than in the cortical plate are not all necessarily restricted to subplate as they could be equally expressed in the subplate and in the IZ or any other cortical structure. Fourth, as a very large number of genes was compared on the microarray, the number of false-positive findings is inevitably high (i.e., a theoretical 5% for a *P* value of 0.05). Therefore, we limit our further discussion to the 13 genes, for which the subplate-specific expression has been confirmed with additional methods.

Potential Functions of Subplate-Specific Genes in Thalamocortical and Cortical Development

In order to gain more insight into possible functions of the 13 subplate-enriched genes, a developmental time course of

cortical and extracortical expression was established. As subplate is one of the compartments where interneurons migrate tangentially into the cortex, we also investigated whether some of these genes colocalized with markers for GABAergic neurons. Due to technical challenges, this was only possible for 3 genes—*Rcan2* against which a very specific antibody is available (Porta et al. 2007) and *Unc5c* and *Cdb10*, which can be strongly and specifically labeled with our riboprobes. Combining these results from our current study with findings from the literature, we can formulate some hypotheses on the possible roles these genes might play in the subplate (see also Table 5).

Genes Associated with Neuronal Maturation

Subplate neurons are a precocious population of cortical cells and show an early expression of markers of mature neurons such as microtubule-associated protein 2 (Map2) (Crandall et al. 1986; Chun et al. 1987). In our microarray study, we found that *Kcnt2*, *Sv2b*, and *Csmd3* are specifically expressed in subplate at early developmental stages but from E15.5 onwards their expression expands into the entire cortical plate (Supplementary Fig. 3*g,b*; Fig. 4*e*). In addition, all 3 genes show a widespread extracortical forebrain expression including the basal ganglia, septum, and piriform cortex (Supplementary Fig. 4*g-i*). *Sv2b* regulates Ca^{2+} -dependent vesicle release (Janz et al. 1999), while *Kcnt2* is a sodium-activated potassium channel possibly involved in adjusting the electric activity of a cell to its metabolic state (Bhattacharjee et al. 2003). The function of *Csmd3* is currently unknown. It seems probable that *Kcnt2*, *Sv2b*, and *Csmd3* are involved in fundamental neuronal functions and are first expressed in subplate neurons as these mature earlier than neurons of the cortical plate.

Electrophysiological Properties

There is abundant evidence for early electrical activity in subplate neurons and early functional thalamocortical interactions in subplate (Friauf et al. 1990; Hanganu et al. 2002; Higashi et al. 2002). In addition, subplate is involved in the regulation of the maturation and plasticity of early cortical circuits (Kanold and Luhmann 2010). We found several genes that could be associated to these interactions. The preferential expression of the ionotropic GABA receptor subunit *Gabra5* and the sodium-calcium exchanger *Slc8a1* in subplate (Supplementary Fig. 3*a,b*) could indicate that these 2 genes are involved in defining the electrophysiological properties of subplate neurons.

In the early embryonic cortex, *Zdhhc2* is specifically expressed in subplate and, outside of cortex, in the prethalamus (particularly in the thalamic reticular nucleus [tRN]) (Supplementary Fig. 4*f*). *Zdhhc2* may regulate synaptic plasticity by modifying PSD95 and synaptosomal-associated proteins 23 and 25 (Noritake et al. 2009; Greaves et al. 2010). Consistently, it has been shown that both subplate and tRN neurons can undergo long-term plasticity (Kaas 1999; Torres-Reveron and Friedlander 2007).

Axonal Growth and Maintenance

The subplate develops the earliest corticofugal projections and receives the first inputs into cortex (Crandall and Caviness 1984; McConnell et al. 1989; De Carlos and O'Leary 1992; Del Rio et al. 2000). Several genes in our screen could be linked to these developmental processes. The plastin *Pls3*, an actin-bundling

protein, starts to be expressed in the subplate around E14 and remains strongly expressed in subplate throughout late embryonic and postnatal period and into adulthood with an additional weaker expression in layer V and VI (Fig. 4*a*). *Pls3* has been shown to regulate axonal outgrowth and neurite length in motor neurons and neuronal cell lines (Oprea et al. 2008). It is plausible that *Pls3* plays a similar role in cortical projection neurons in subplate and layers V and VI. This proposition is further supported by the finding that *Pls3* expression in the embryonic subplate is relatively sparse indicating that only a subset of subplate cells expresses *Pls3* at this stage. It would be interesting to analyze whether the *Pls3*-positive neurons project to different targets than *Pls3*-negative neurons. *Pls3* is still expressed postnatally and in adulthood, when axons have been well established, which suggests that it is not only important in axonal growth but also maintenance.

Cadherin 10 and 18 are strongly expressed in the early embryonic subplate. As their expression is shifted to the superplate in reeler mice and *Cdb10* is not expressed in migrating interneurons (Fig. 5*a–d*), we believe that the *Cdb10*- and *Cdb18*-positive cells are indeed postmitotic subplate neurons. Both cadherins show a clear anteroposterior expression gradient within subplate and therefore might be involved in the targeting of areas-specific thalamocortical afferents. However, neither of these 2 cadherins are expressed in the developing dorsal thalamus (Supplementary Fig. 4*l,m*), which does not support the idea that these molecules would directly establish the topography between individual thalamic nuclei and cortical areas through homophilic interactions (Molnár and Blakemore 1995). Alternatively, these cadherins could play a role in the more fundamental organization of the cortex by maintaining interactions between cells in the subplate and later in other deep layers.

Unc5c is specifically expressed in subplate at E14.5 and in subplate and the lateral cortical plate at E15.5. At late embryonic and postnatal stages, *Unc5c* appears to be down-regulated and becomes more evenly distributed (Fig. 4*d*). *Unc5c* is a repulsive receptor for the guidance molecule netrin-1, which is strongly expressed in the mantle zone of the internal capsule (Braisted et al. 2000; Powell et al. 2008). Recently, netrin-1 has also been shown to be present in the marginal zone of the cortex where it plays a role in the guidance of migrating interneurons (Stanco et al. 2009). It is doubtful that *Unc5c* in the subplate is involved in directly regulating interneuron migration as it does not colocalize with Gad67-GFP (Fig. 5*e–h*). Instead, it seems most probable that *Unc5c* is involved in regulating the crossing of early corticofugal projections through the pallial-subpallial boundary and the subsequent growth of subplate projections through the internal capsule (Molnár and Cordero 1999).

Other Possible Functions

We found several genes with strong subplate expression for which a function is difficult to predict. *Abca8a* is a mouse-specific ABC transporter, which is postulated to transport lipids (Tsuruoka et al. 2002). *Abca8a* has an intriguing expression pattern in the forebrain as it is exclusively expressed in subplate at all examined embryonic stages (E13.5–E17.5) (Fig. 4*c*, Supplementary Fig. 4*a*). Postnatally, however, the expression of *Abca8a* is shifted to layer V and II/III in restricted areas of the cortex and then is downregulated in adulthood (Fig. 4*c*), while extracortically, *Abca8a*-positive cells are also found in

the claustrum, endopiriform nucleus, and subiculum (Supplementary Fig. 4*a'*).

Our colocalization studies suggest that within the cortex, *Rcan2* is expressed in at least 3 different cell populations throughout development: 1) Cajal–Retzius cells, 2) glutamatergic subplate cells, and 3) cortical interneurons in all layers including subplate (Fig. 6). *Rcan2* is a regulator of calcineurin, a phosphatase, which plays key roles in regulating neurite outgrowth and guidance, neuronal death, and synaptic plasticity. As *Rcan2* can be involved in the regulation of such a large number of mechanisms, it is possible that it plays very different roles in the 3 cell populations identified in this study.

In conclusion, our study revealed a gene expression profile of the embryonic mouse subplate and validated several potentially interesting genes at various embryonic and postnatal stages. E15.5 is a stage when thalamocortical projections start to accumulate in subplate, a zone that develops extensive intra- and extracortical projections and serves as a compartment of tangential migration of GABAergic interneurons. Combining our validated gene expression patterns in embryonic subplate with the knowledge of the developmental timeframe and cellular interactions, we postulated several hypotheses on the specific molecular mechanisms involved in specific phases of cortical development. The knowledge of these genes shall enable us to examine these proposed mechanisms in more detail.

Supplementary material

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

Funding

Medical Research Council (grants G0700377 and, G00900901 to Z.M.); the Berrow Foundation, Lincoln College, Oxford (to F.M.O.); the University of Oxford John Fell Fund (to Z.M. and W.Z.W.); the Spanish Ministerio de Educación (to F.G.-M.). The microarray facility was funded by the Wellcome Trust Integrative Physiology Initiative on Ion Channels (OXION).

Notes

We are very grateful to Prof. John Parnavelas, Dr Anna Hoerder-Suabedissen, and Miss Eleanor Grant for their comments on an earlier version of this manuscript and to Dr Andreas Heger for his help with some of the statistical analyses of the microarray data. *Conflict of Interest*: None declared.

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