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Gene expression analysis of the embryonic subplate

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Abstract

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 stages, they are 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 15.5 using laser capture microdissection and microarrays. The microarray identified over 300 transcripts with higher expression in the subplate compared to the cortical plate at this stage. Using quantitative RT-PCR, *in situ* hybridization and immunohistochemistry, 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*, *Ogfrl1*, *Pls3*, *Rcan2*, *Sv2b*, *Slc8a2*, *Unc5c* and *Zdhhc2*). In the reeler mutant, the expression of the majority of these genes (9 out of 13) was shifted in accordance with the altered position of subplate. These genes belong to several functional groups and likely contribute to the maturation and electrophysiological properties of subplate cells and to axonal growth and guidance.

Keywords

Subplate; embryonic development; cerebral cortex; gene expression; laser capture microdissection

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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 sixty 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 fulfil 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 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 cortico-cortical connectivity (Kostović and Rakic 1990; Friedlander and Torres-Reveron 2009; Suárez-Solá et al. 2009).

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. 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. submitted)). 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 (e.g. monoxygenase *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 use 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-pregnant females and 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, 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, Reln [rl-Orl]) were embedded in O.C.T and flash-frozen. Brains of E15.5 and P8 Gad67-GFP knock-in mice (Tamamaki et al. 2003) were fixed in 4% paraformaldehye (TAAB) in PBS for 4h (E15.5) or 24h (P8) and cryoprotected in 30% sucrose before freezing. For immunohistochemistry, 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 24h. Fixed P8 and adult brains and E17.5 brains for GABA immunhistochemistry 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 immunohistochemistry 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 sagitally sectioned to 20μm on a cryostat (Jung CM3000, Leica) and mounted on membrane-coated 1mm PEN slides (Zeiss). Sections were fixed in 95% ethanol, rehydrated, stained with 1% cresyl violet (Sigma) and dehydrated. In the E15.5 mouse cortex, subplate was defined as a cell band of 40μm just below the lower border of the dark-stained cortical plate, and the lower cortical plate as a 40μm thick cell band just above this border (Fig 1a, b). Laser capture microdissection was

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performed using a PALM Microbeam IP 230V Z (Zeiss) and microdissected tissue strips were collected in RNAlater (Ambion). For microarray, samples from 3 or 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 two additional sample sets were collected from individual animals (~40 strips each) from two 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 was analyzed on RNA 6000 Pico Chips (Agilent Technologies). All samples were undegraded (RNA integrity number (RIN) > 7.6) and quantities were estimated to at least 20ng per sample.

Preparation of cDNA and microarray

Ten ng of RNA from each sample was reverse-transcribed and the cDNA was amplified using the Ovation^{TM} 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 200nt. 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 Affymetric GeneChip Scanner 3000.

Microarray data analysis

All data processing was performed using Affymetrix software or Agilent GeneSpring GX (Agilent Technologies). Arrays were quantile normalized and intensity values summarised using the Probe Logarithmic Error Intensity Estimate PLIER. 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 Welsh t-tests without correction for multiple testing. Genes with a fold-change ≥ 1.4 and a p-value < 0.05 were considered as differentially expressed.

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 (GO_BP) (Ashburner et al. 2000) and to pathways described in the Kyoto Encyclopedia of Genes and Genomes (KEGG) 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 EASE Score (a modified Fisher 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.

Quantitative RT-PCR

The remaining RNA from two replicates of the microarray as well as RNA from two 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. No template controls 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 centre and Primer3 software. The qRT-PCR reaction was performed using 100nM 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 10min followed by 40 or 50 two-step cycles of 95°C for 15sec and 60°C for 1min. 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 house-keeping 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 three 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 two 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 3. The resulting PCR products were ligated into the pST-Blue 1 plasmid (Novagen). The antisense and sense (a negative control) cRNA probes were transcribed using either SP6 or T7 RNA polymerase with digoxygenin (DIG)-labeled nucleotide mixture (Roche).

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

For co-localization, immersion-fixed E15.5 Gad67-GFP brains were cut into 16μm coronal cryosections and post-fixed with 4% PFA for 10min. Sections were treated with 2μg/ml proteinase K (Roche) in PBS for 10min at RT and deproteinized, acetylated and prehybridized as for permanent *in situ* hybridization. Hybridization with DIG-labeled probes and subsequent washes were performed at the lower temperature of 60°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 100mM Tris, pH 8.0, 400mM NaCl was applied for at least 6h. Sections were then incubated with donkey anti-rabbit Alexa488 1:250 (Invitrogen) in 1% donkey serum for 2h and counterstained with DAPI.

Immunohistochemistry

Permanent immunohistochemistry was performed on sections from 4% PFA-fixed brains cut to 40μm on a vibrating microtome (VT1000S, Leica). For immunohistochemistry against Sv2b, Kcnt2 and Plxna4 sections were boiled in sodium citrate (0.1M, pH 4.5) at 95°C for 8min to enhance labeling for synapse-enriched proteins (Gil et al. 2002). All sections were quenched in 1.5% hydrogen peroxide and blocked for 2h at RT with 5% donkey serum (Sigma) in TBS with 0.2% Triton-X100 (BDH). Sections were incubated with primary antibodyin 1% donkey serum and 0.2% Triton overnight at 4°C. The following primary antibodies and dilutions were used: Anti-Sv2b 1:1000 (AB15225, Millipore); anti-Kcnt2 1:1000 (ab77512, Abcam); anti-Plxna4 1:100 (ab39350, Abcam); anti-Rcan2 1:3000 (Porta et al. 2007); anti-NeuroD 1:200 (sc-1086, Santa Cruz Biotechnology). Biotinylated donkey anti-rabbit antibody 1:200 (ab6701, Abcam) in 1% donkey serum was applied for 2h at RT and reacted with ABC using the Vectastain Elite kit (Vector) and DAB according to the manufacturer's instructions.

For co-localization with Gad67-GFP, fluorescent immunohistochemistry against Rcan2 was performed on E15.5 16μm cryosections or P8 40μm microtome sections. Co-localization

between Rcan2 and reelin was performed on 40μm microtome sections of E15.5 brains fixed with 4% PFA and between Rcan2 and GABA on 40μm microtome sections from E17.5 and P8 brains fixed with 4% PFA and 0.25% glutaraldehyde, respectively. Cryosections were postfixed for 30min in 4% PFA and rinsed with PBS. All sections were blocked for 2h 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°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 2h at RT and sections were counterstained with DAPI(Invitrogen). The following secondary antibodies were used: donkey anti-rabbit Alexa568 1:500 (A10042, Invitrogen), donkey anti-rabbit Alexa488 1:500 (A21206, Invitrogen), goat anti-rabbit Alexa488 1:500 (A11008, Invitrogen), goat anti-guinea pig AlexaCy3 1:500 (106-165-003, Jackson ImmunoResearch), donkey anti-mouse Alexa568 1:500 (A10037, Invitrogen).

Results

Microarray analysis

We performed a microarray analysis in order to compare gene expression between subplate and lower cortical plate in two selected areas of the anterior and posterior cortex of embryonic E15.5 C57/Bl6 mice (Fig 1a,b) in four 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 while 327 genes had higher expression in the lower cortical plate (fold changes ≥ 1.4, Fig 1d and Supplementary Table 1).

A few genes with higher 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 two 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), and *Fezf2*, a transcription factor specifically expressed in layer V and VI projection neurons (Inoue et al. 2004; Chen et al. 2005).

As a first step to validate the microarray data, publicly available gene expression datasets Gensat (The Gene Expression Nervous System Atlas (GENSAT) Project), Allen Brain Atlas (Lein et al. 2007) and Genepaint (Visel 2004) were screened for all 334 genes with higher

expression levels in subplate. Based on the available E14.5 (Genepaint) and E15.5 (Allen Brain Atlas, Gensat) *in situ* hybridization images, we determined that 78 genes (23%) were expressed in subplate and/or the intermediate zone. These two zones were often impossible to distinguish on the available images. A further 75 genes (22%) showed clear expression patterns, mainly in the ventricular and subventricular zones, 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 was 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 1d). A gene ontology enrichment analysis was performed 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 2 for details).

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 1d). Surprisingly, we found that only 11 of these genes had higher expression levels in the anterior subplate (*Atp1b1*, *Cdh9*, *Cdh12*, *Cdh18*, *Fam84a*, *Gabra5*, *Gm10001*, *Kcnip4*, *Nt5dc2*, *Pls3* and *Ptn*) and 4 in the posterior subplate (*C3ar1*, *Nes*, *Pde1a* and *Slc4a4*). On public gene expression images for *Gabra5* and *Pls3*, a larger number of labeled cells are visible in the anterior subplate than in the posterior subplate, confirming the result of the microarray (not shown). Additionally, *Cdh9* has been described as a marker for the frontal cortex, at least in the adult mouse (Bedogni et al. 2010). Of the four 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, quantitative (q) RT-PCR was performed on nine randomly selected genes with higher expression in subplate and on two genes with higher expression in lower cortical plate (Fig 2). Quantitative RT-PCR was performed on two sample sets which had also been included in the microarray as well as on two additional sample sets specifically collected for this purpose from individual embryos. For eight of the nine subplate genes and the two 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 four biological replicates and in both anterior and posterior comparisons for all ten genes. However, the exact value of the fold-changes was relatively variable between replicates with

an average relative standard error of ~ 20%. Consequently, most but not all fold-changes were significantly different from 1 (p-value < 0.05; see Fig 2). The remaining subplate gene (*Kcnip4*) could not be sufficiently amplified with the designed primers to perform quantitative analysis.

Subplate-specific genes in the E15.5 mouse cortex

Based on expression pattern available in public databases and presumed subplate-relevant functions, a subset of 23 genes were chosen for further validation with *in situ* hybridization or immunohistochemistry (Table 4). An expression pattern was considered as subplate-specific if it was predominantly localized in a three to four cell-wide band just below the dense cortical plate. According to this criterion, we found 13 genes (*Abca8a*, *Cdh10*, *Cdh18*, *Csmd3*, *Gabra5*, *Kcnt2*, *Ogfrl1*, *Pls3*, *Rcan2*, *Sv2b*, *Slc8a2*, *Unc5c* and *Zdhhc2*) that are primarily expressed in the subplate (Fig 3). Seven further genes were primarily expressed in the intermediate zone and/or SVZ with additional weaker expression in the subplate in some cases (Supplementary Fig 2). For the remaining three 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 four type II cadherins as well as two 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 3b,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 3d). 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 CNS, *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 *in situ* hybridization, we found that at E15.5, *Unc5c* mRNA is specifically localized to the subplate (Fig 3e). 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). 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 3f). 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 Ca^{2+} (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 3g). 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 two 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 *in situ* hybridization.

The **zinc finger, DHHC domain containing 2** (Zdhhc2 aka Dhhc2) protein is a palmitoyl acyltransferase, an enzyme which mediates post-translational 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 3h). 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 3l) but occasionally, a larger number of cortical plate cells was labeled.

The **synaptic glycoprotein vesicle 2b** (Sv2b) is one of three 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 two dark bands at low magnification (Fig 3m). 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 ATP-binding cassette (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 *in situ* hybridization, we confirmed that *Abca8a* is very strongly and specifically expressed in the E15.5 subplate with no staining detectable in the cortical plate (Fig 3i).

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 foetal 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 3j) (fold-changes on the microarray: anterior: < 1.3; posterior: 1.6).

The **opioid growth factor-like receptor 1** (Ogfrl1) is a largely uncharacterized protein but is partially homologous (41.7% identity in a 314 out of 464 amino acid overlap) to the opioid growth factor receptor (Ogfr). Ogfr binds the endogeneous opioid peptide met-enkephalin, an inhibitory growth molecule influencing cell proliferation and tissue organization (Zagon et al. 1999; Zagon et al. 2002). *In situ* hybridization on E15.5 brains with a riboprobe against *Ogfrl1* resulted in a strong labeling of the subplate (Fig 3k) (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 which 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 3n) (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 3n). 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 4a). 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 4). *Abca8a*, *Cdh10*, *Cdh18*, *Csdm3* and *Gabra5* positive cells were all localized in a broad band just below the cortical pial surface (Fig 4b,c,d,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 4f,g). In contrast, we did not find any clear labeling for *Slc8a1*, *Zdhhc2*, Sv2b or *Ogfrl1* (Fig 4i,j,k and n) in the reeler cortex, neither in the superplate nor 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 4m,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 4m,o). As E15.5 is the onset of Rcan2 expression in the subplate, we also analyzed P8 reeler mice for Rcan2 labeling (Fig 4p). 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 intermediate zone, was unaffected in E15.5 reeler brains (Fig 4l).

Temporal expression profile of subplate-specific genes in the cortex

We analyzed the expression patterns of all 13 genes that showed a subplate-specific expression at E15.5 at additional developmental stages (E13.5, E14.5, E17.5, P8 and adult) in the cerebral cortex (Fig 5, Supplementary Figure 3 and Supplementary Table 3). Based on their temporal expression profile, genes were grouped into three 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 5a). 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 5b). 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 Figure 3a). *Slc8a1* is most strongly expressed in the subplate from E14.5 until adulthood which is always distinguishable as a dark band but additional weaker expression is found in other cortical layers (Supplementary Figure 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 Figure 3c). These moderately stained Rcan2 positive cells form a clear band in subplate at E17.5 which remains visible at P8 (Figure 4p) 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 Figure 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 5c). *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 5d).

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 Figure 3e). *Zdhhc2* 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 with additional weaker staining in all other layers (Supplementary Figure 3f). *Ogfrl1* 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, *Ogfrl1* is expressed in the deep cortical layers including subplate (Supplementary Figure 3d). *Csmd3* is expressed in the lateral cortex at E13.5 and in subplate and the later cortical plate at E14.5 and E15.5. From E17.5 onwards, *Csmd3* becomes weakly but ubiquitously expressed (Fig 5e). 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 Figure 3g,h).

Extracortical expression patterns of subplate-specific genes

A detailed analysis of the entire extracortical expression patterns of all genes would be beyond the scope of this study. We therefore limit the description to the most prominently labeled structures in the forebrain with special attention to the prethalamus (ventral thalamus) and thalamus (dorsal thalamus) and the claustroamygdalar complex. A few examples of extracortical expression patterns for each gene are shown in Fig 6 and more details can be found in Supplementary Table 3.

Overall, *Abca8a* showed the most restricted expression pattern as labeling was exclusively present in the subplate at E14.5 and E15.5 (Fig 6a). At later stages, *Abca8a* positive cells are also found in the claustrum, endopiriform nucleus and subiculum (Fig 6a'). Similarly, *Pls3* and *Ogfrl1* have a relatively restricted expression at early embryonic stages when they are only expressed in a few discrete structures including the hippocampal anlage (Fig 6b) and the olfactory bulb. *Pls3* is also expressed in the subpallial SVZ (Suppl Fig 3h) and Ogfrl1 faintly in the developing thalamus (Fig 6c). At later stages, *Pls3* and *Ogfrl1* positive cells are

additionally found in the claustroamygdalar complex and certain thalamic nuclei (Fig 6b',c').

In contrast, the expression of *Gabra5*, *Slc8a1*, *Zdhhc2*, *Csmd3*, Sv2b and Kcnt2 is relatively widespread and involves many extracortical structures (Fig 6d-i,d'-i'). At early embryonic stages, all of these genes are expressed in the septum, the piriform cortex and some or all basal ganglia (Fig 6d-i). *Gabra5*, *Zdhhc2* and *Csmd3* show additional expression in the olfactory amygdala (Fig 6d,e,g). *Zdhhc2* is very strongly expressed in the prethalamus (Fig 6f). At late embryonic and postnatal stages, the expression of Sv2b, *Csmd3* and Kcnt2 becomes ubiquitous (Fig 6g',h',i'). *Slc8a1* is expressed in the majority of forebrain structures, particularly in some septal and thalamic nuclei (Fig 6e'), but not in the striatum. *Zdhhc2* is also moderately expressed in most forebrain structure but has a particularly high expression in the CA field of the hippocampus and the tRN in the prethalamus (Fig 6f'). Expression of *Gabra5* is particularly strong in the hippocampus (Fig 6d').

In the embryonic brain, Rcan2 is strongly expressed in the hippocampal anlage and the developing prethalamus and hypothalamus (Fig 6j). At postnatal stages, Rcan2 immunopositive cells are found scattered in most forebrain structures with particularly high numbers in the tRN (Fig 6j').

In the early embryonic brain, *Unc5c* is very strongly expressed in the claustrum, prethalamus, epithalamus and the ventromedial part of the thalamus (Fig 6k). At postnatal stages, the strongest expression is still found in the ventromedial thalamic nucleus and in sparse cells scattered throughout the hippocampus (Fig 6 k').

At embryonic stages, strong *Cdh10* expression is found in the subplate and the piriform cortex and weaker expression in the SVZ and lateral cortical plate (Fig 6l). In postnatal brains, strong labeling is additionally visible in some nuclei of the thalamus and the CA1/2 field of the hippocampus (Fig 6l'). *Cdh18* is expressed in the prethalamus and hypothalamus at embryonic stages. (Fig 6m) and strongly labeled *Cdh18* positive cells are found dispersed in the basal ganglia and the olfactory amygdala. *Cdh18* is also expressed in the claustrum throughout development (Fig 6m').

Co-localization with interneuron markers

The embryonic subplate is one of three 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 co-localize their expression with GFP in the Gad67-GFP mouse. In this transgenic line, almost all of cortical GABAergic cells are GFP-positive (Tamamaki et al. 2003). Co-localization analysis was performed for *Unc5c* and *Cdh10* with *in situ* hybridization and for Rcan2 with immunohistochemistry (Fig 7, Fig 8).

In the E15.5 cortex, *Cdh10* and *Unc5c* are specifically expressed in the subplate but do not co-localize with Gad67-GFP (Fig 8). Less than 1% of *Cdh10* positive cells were found to be expressing GFP (3 double-labeled cells out of 397 *Cdh10* positive cells and 212 Gad67-GFP

+ cells, n= 2 brains, Fig 7a-d). *Unc5c* never co-localized with Gad67-GFP (169 *Unc5c*+ cells and 239 Gad67-GFP+ cells, n=2 brains, Fig 7e-h).

Using fluorescent immunohistochemistry, Rcan2 immunoreactive cells were only detected in the marginal zone of the E15.5 Gad67-GFP cortex but not in the subplate (Fig 8A,B). Surprisingly, the Rcan2 positive cells in the marginal zone did not co-localize with Gad67-GFP (Fig 8A). Instead, they formed a distinct band of horizontally oriented cells localized above the stream of tangentially migrating Gad67-GFP neurons (Fig 8a,a'''). Based on their morphology and location, these Rcan2 positive cells are most likely Cajal-Retzius cells which is further supported by their co-expression of reelin (Fig 8B).

As we did not detect any Rcan2 positive cells in the subplate at E15.5 using fluorescent immunohistochemistry, co-localization of Rcan2 and GABA in the E17.5 cortex was analyzed (Fig 8C). 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 co-localize with GABA (32 double-labeled cells out 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 8D). As at E17.5, in the subplate, only a small proportion of Rcan2 positive cells co-localized with GABA (10.5%, 9 double-labeled cells out of 106 Rcan 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 out 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 co-localized with GFP (41 out of 305 cells, n=3 brains) while in layers V and VI over 80% of Rcan2 positive also expressed GFP (403 out of 499 cells, n=3 brains) (Fig 8D). The strong co-localization between Rcan2 and markers for interneurons in the cortical plate is consistent with previous findings in the adult cortex (Porta et al. 2007).

Discussion

Identification of 13 novel subplate-specific genes in the embryonic cortex

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 lower 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).

Interestingly, we found that only a very small proportion of the genes enriched in subplate are expressed in a anterior-posterior gradient (~6%; 15 out 259). In addition, the gene expression differences between subplate and lower cortical plate were much larger than the differences between anterior and posterior samples (Fig 1c). Similar observations have also been made in the P8 subplate gene analysis (Hoerder-Suabedissen et al. 2009) and in screens of other neuronal populations (Sugino et al. 2006). However, 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, the 15 genes with an anterior-posterior gradient identified are promising candidates for setting up the initial topography of thalamocortical axons and should be investigated in more detail.

Using our own optimized immunohistochemistry or *in situ* hybridization protocols, we were able to confirm the enrichment of 13 genes out of 23 selected in the E15.5 subplate zone (*Abca8a*, *Cdh10*, *Cdh18*, *Csmd3*, *Gabra5*, Kcnt2, *Ogfrl1*, *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. Morever, 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 were available for 12 out 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 out of 13) was shifted in accordance with the shifted position of subplate cells while the remaining genes no longer showed any clear expression pattern.

Our approach has several strengths, but also some limitations: Firstly, gene expression levels were not compared between subplate neurons and cortical plate neurons but between entire compartments likely to also contain mRNA from migrating neurons, glial cells, axons and dendrites and blood vessels. Secondly, genes identified with higher expression in subplate than in the lower cortical plate are not all necessarily restricted to subplate as they could be equally expressed in the subplate and in the intermediate zone or any other cortical structure. Thirdly, 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 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 co-localized with markers for GABAergic neurons. Due to technical challenges, this was only possible for three genes – Rcan2 against which a very specific antibody is available (Porta et al. 2007) and *Unc5c* and *Cdh10* 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 4).

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 Figure 3g,h; Figure 5e). 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 Csdm3 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 Figure 3a,b) could indicate that these two genes are involved in defining the electrophysiologcial 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)) (Figure 6m). Zdhhc2 may regulate synaptic plasticity by modifying PSD95 and synaptosomal-associated proteins 23 and 25 (Noritake et al. 2009; Greaves et al. 2010). Consistenly, 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 5a). 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 cadherin 18 are strongly expressed in the early embryonic subplate. As their expression is shifted to the superplate in reeler mice and *Cdh10* is not expressed in migrating interneurons (Fig 7a-d), we believe that the *Cdh10* and *Cdh18* positive cells are indeed postmitotic subplate neurons. Both cadherins show a clear antero-posterior expression gradient withing subplate and therefore might be involved in the targeting of areas-specific thalamocortical afferents. However, neither of these two cadherins are expressed in the developing thalamus (Fig 6b,c), 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 downregulated and becomes more evenly distributed (Fig 5d). 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 co-localize with Gad67-GFP (Fig 7e-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 Cordery 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 as it is very specifically expressed in subplate at all examined embryonic stages (E13.5 – E17.5). 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 5c).

Our co-localization studies suggest that Rcan2 is expressed in at least three different cell populations throughout development: 1) Cajal-Retzius cells; 2) Glutamatergic subplate cells; 3) Cortical interneurons in all layers including subplate. 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 three 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 varous 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 pahses of cortical development. The knowledge of these genes shall enable us to examine these proposed mechanisms in more detail.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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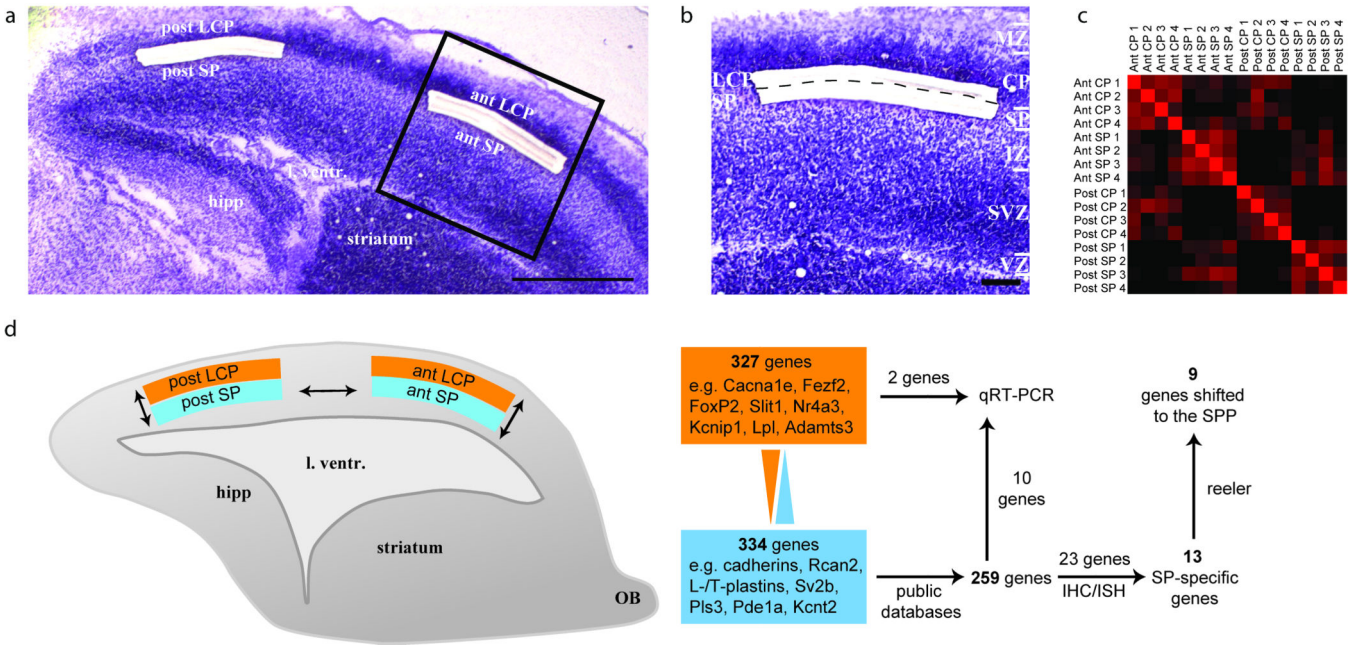

Figure 1. Overview of the experimental approach

Four cell populations were isolated by laser capture microdissection in four 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, 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. Combinedly, 327 genes were higher expressed in the SP and 334 were higher 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 immunohistochemistry (IHC) or *in situ* hybridization (ISH). Scalebars: a. 500µm. b. 100 µm. c. 200µm. hipp: hippocampus; l. ventr. = lateral ventricle; MZ: marginal zone; IZ: intermediate zone; SVZ: subventricular zone; VZ: ventricular zone; OB: olfactory bulb.

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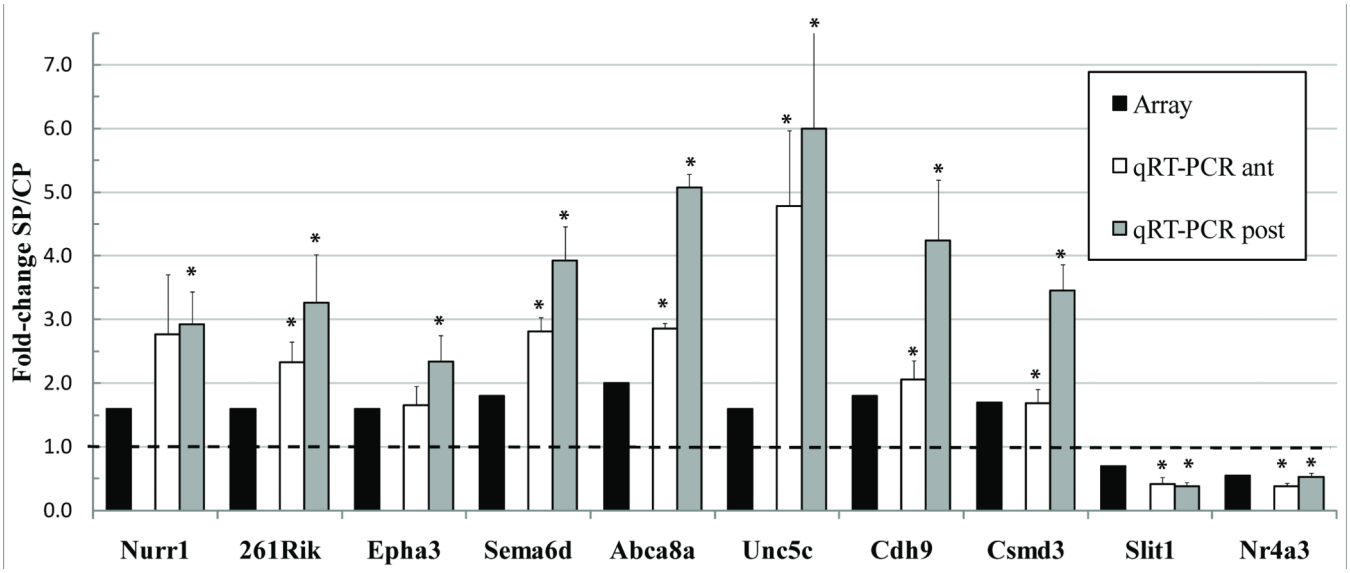

Figure 2. Quantitative RT-PCR for selected subplate- and cortical plate-enriched genes

Quantitative RT-PCR shows fold-changes in the same direction as the microarray for ten genes - eight with a higher expression in the subplate (SP) and two with a higher expression in the cortical plate (CP). Fold-changes were calculated as the ratio between anterior SP and CP and between posterior SP and CP, respectively. Error bars are SEM. Asterisks (*) indicate fold-changes which are significantly different from 1 (p-value < 0.05).

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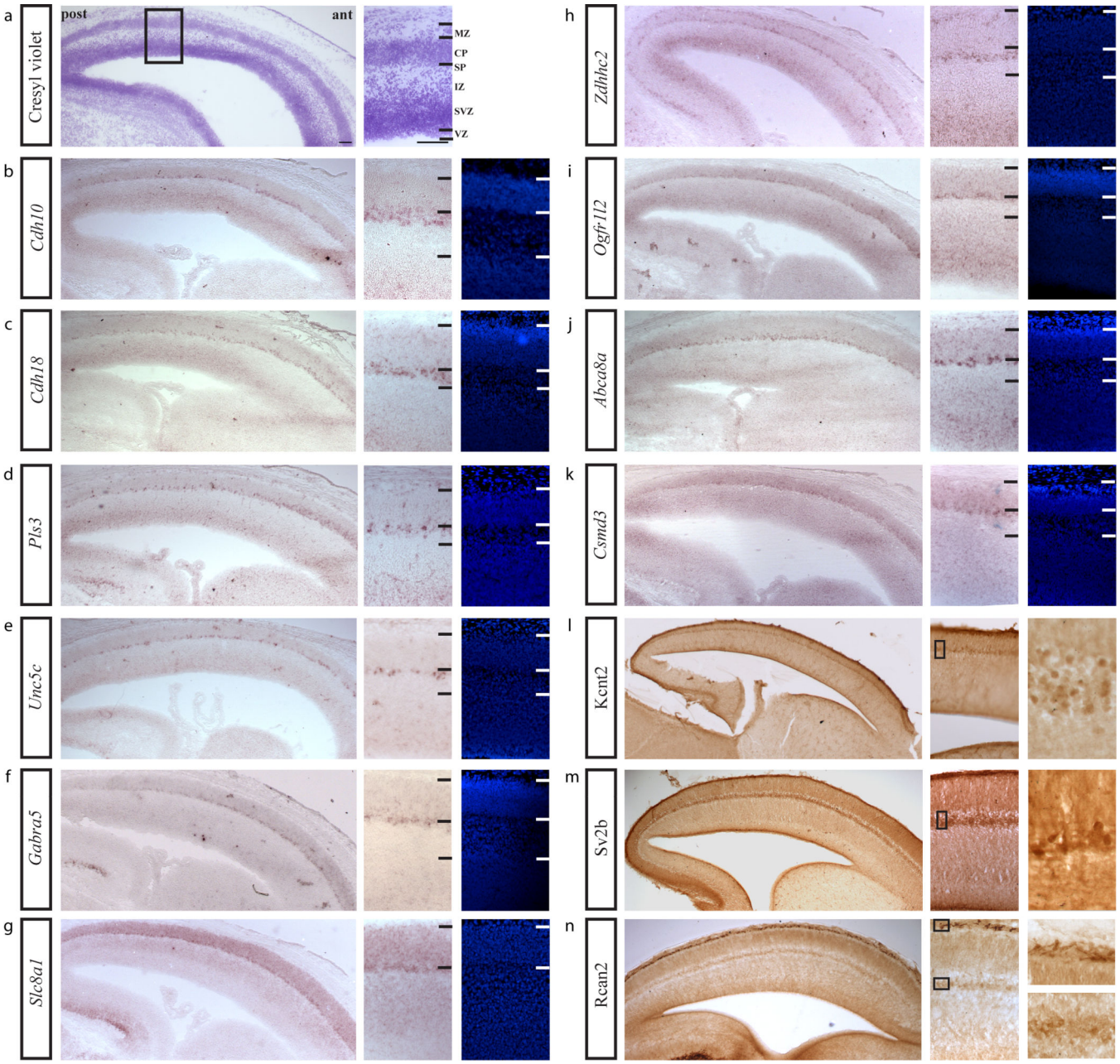

Figure 3. Genes with subplate-specific expression in the E15.5 cortex

In situ hybridization (b-k) or immunohistochemistry (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 right-most panels in b-k show DAPI counterstains of the same area as shown in the middle panels. Scalebars: 100μm. IZ: intermediate zone; SVZ: subventricular zone; VZ: ventricular zone.

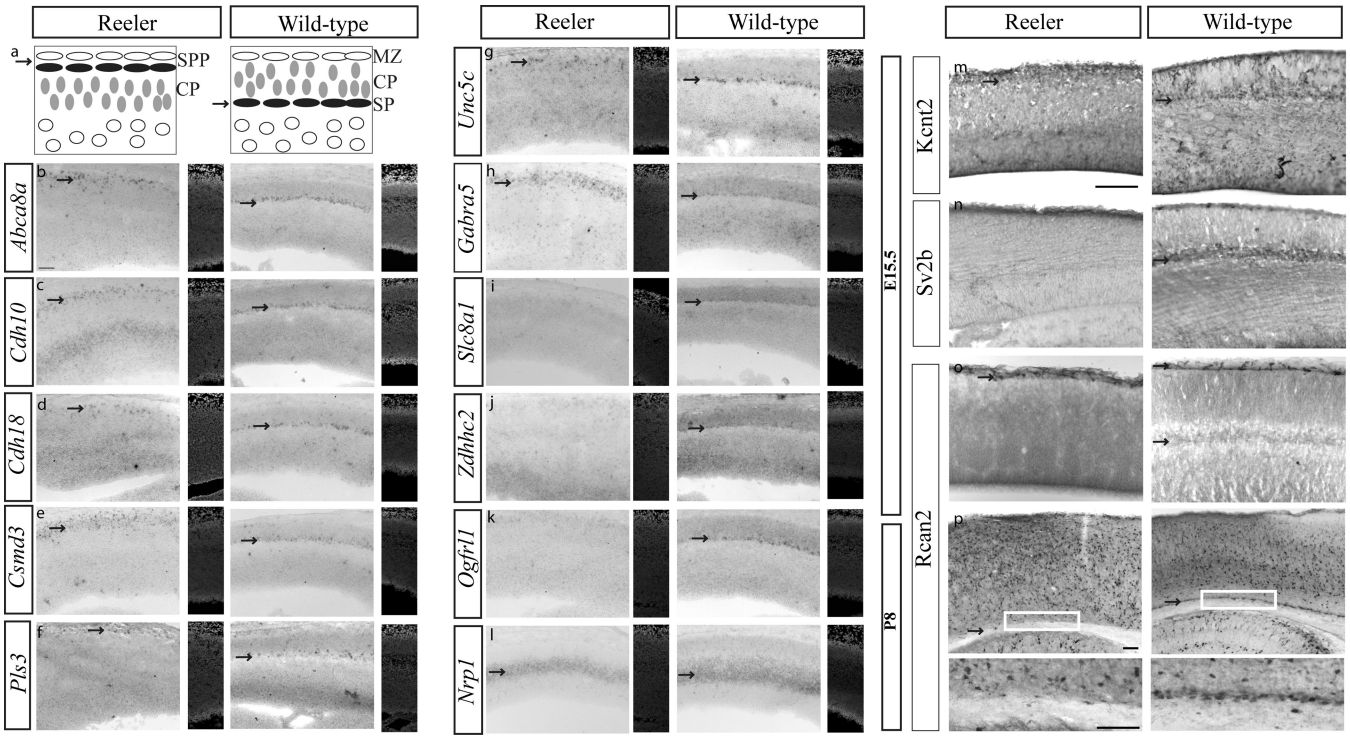

Figure 4. Shifted expression of subplate-specific genes in the reeler cortex

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. Small panels show DAPI counterstains of the same areas. 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, nine are expressed in the SPP in the reeler cortex (b-h, m, o). For the remaining four 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 intermediate zone in the wild-type cortex, remains in the same position in the reeler cortex. Scalebars: 100μm.

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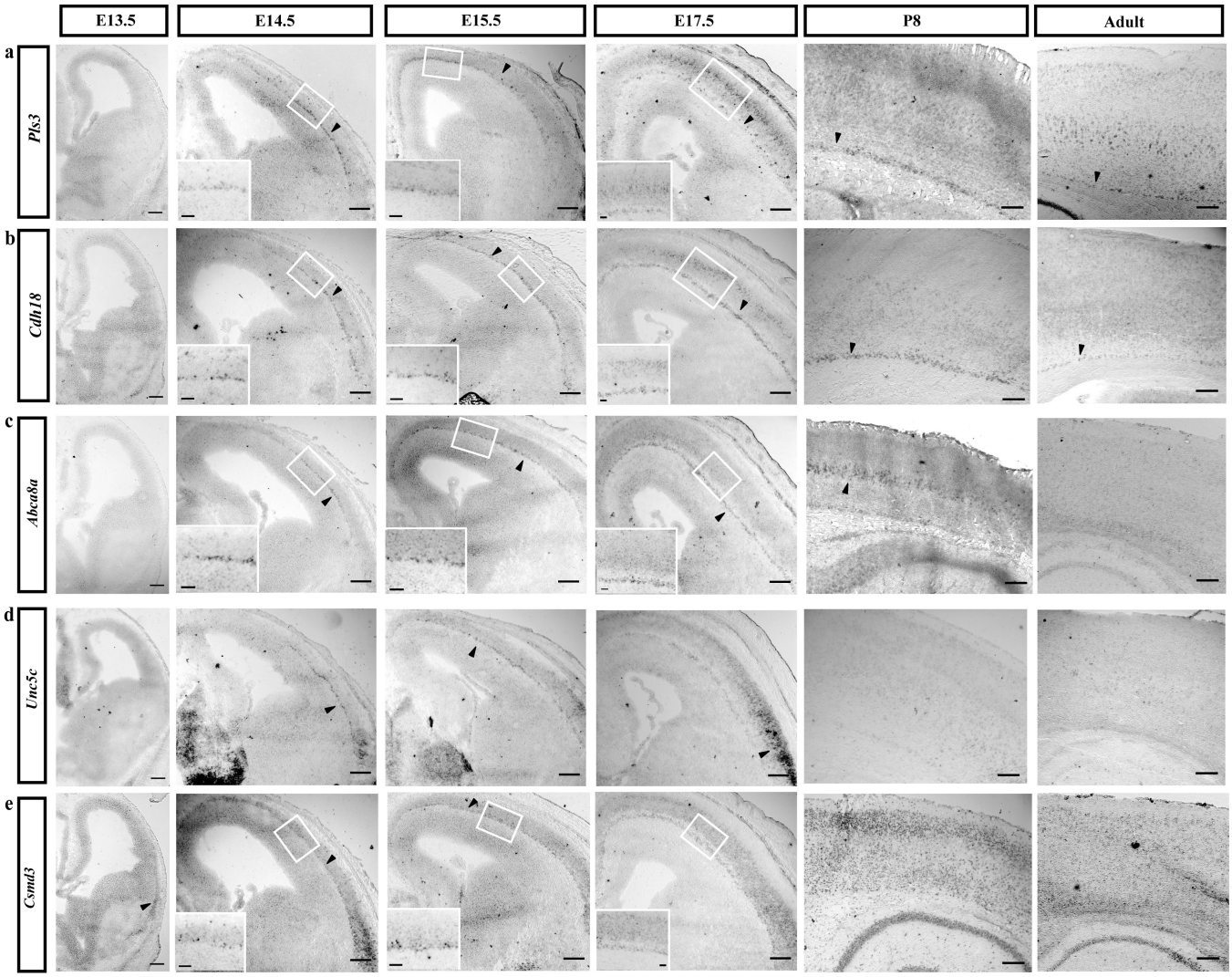

Figure 5. Developmental time-course of expression of *Pls3, Cdh18, Abca8a, Unc5c* and *Csmd3*

In situ hybridization signals in the E13.5, E14.5, E15.5, E17.5, P8 and adult neocortex. At E13.5 only *Csmd3* 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 *Csmd3* 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 *Csmd3* is ubiquitously expressed (e). Scalebars: 200μm (panels); 50μm (inserts).

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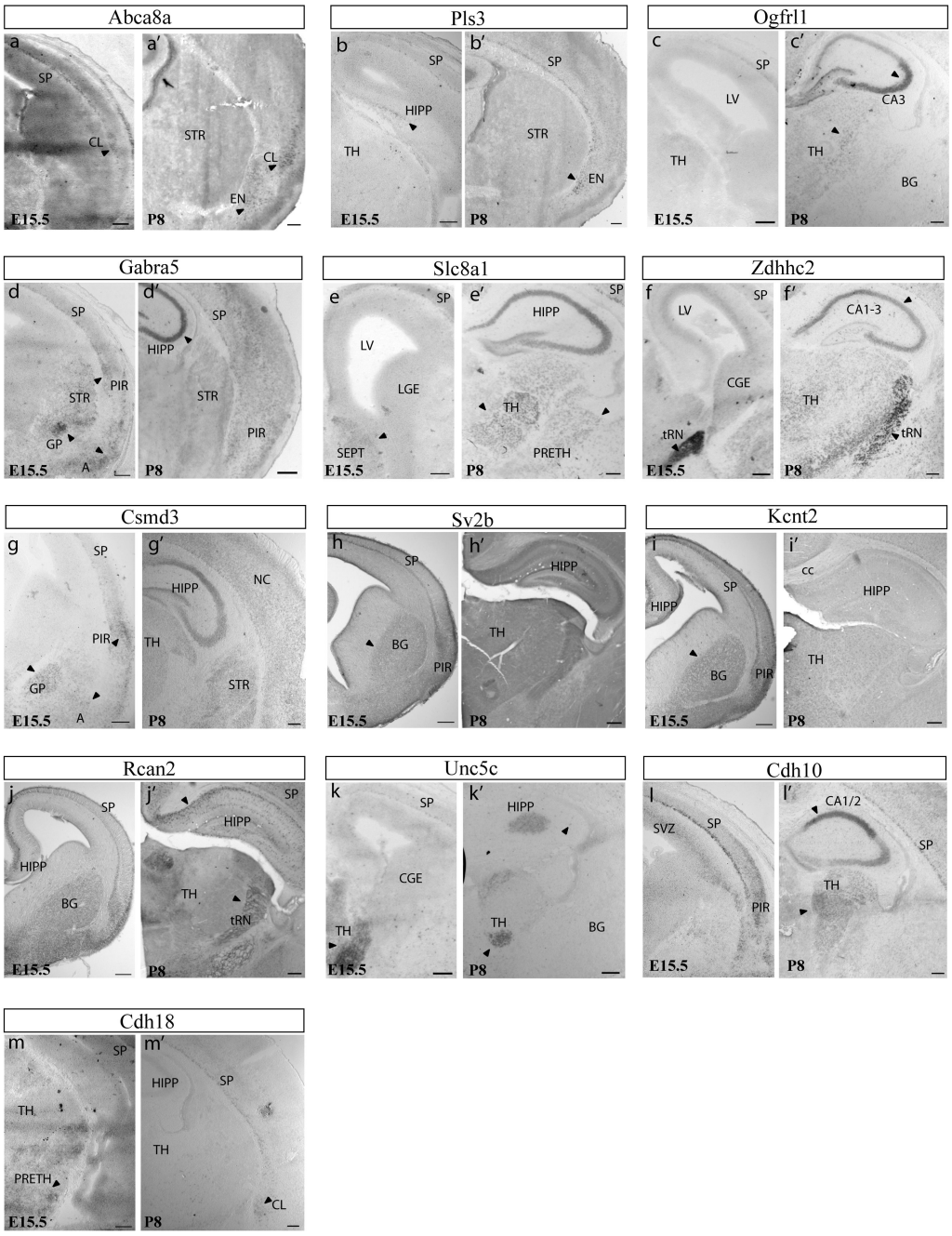

Figure 6. Extracortical expression patterns of subplate-enriched genes

In situ hybridization (a-g, k-m) or immunohistochemistry (h-j) signals at E15.5 and P8 of all 13 subplate-enriched genes. Images were selected to show extracortical areas of expression in the telencephalon and diencephalon (arrowheads). SP: subplate; CL: claustrum; EN: endopiriform nucleus; PRETH: prethalamus; TH: thalamus; PIR: piriform cortex; GP: globus pallidus; HIPP: hippocampus; BG: basal ganglia; A: amygdala; tRN: thalamic reticular nucleus; STR: striatum; LV: lateral ventricle; CGE: caudal ganglionic eminence; NC: neocortex; cc: corpus callosum. Scalebar: 200μm.

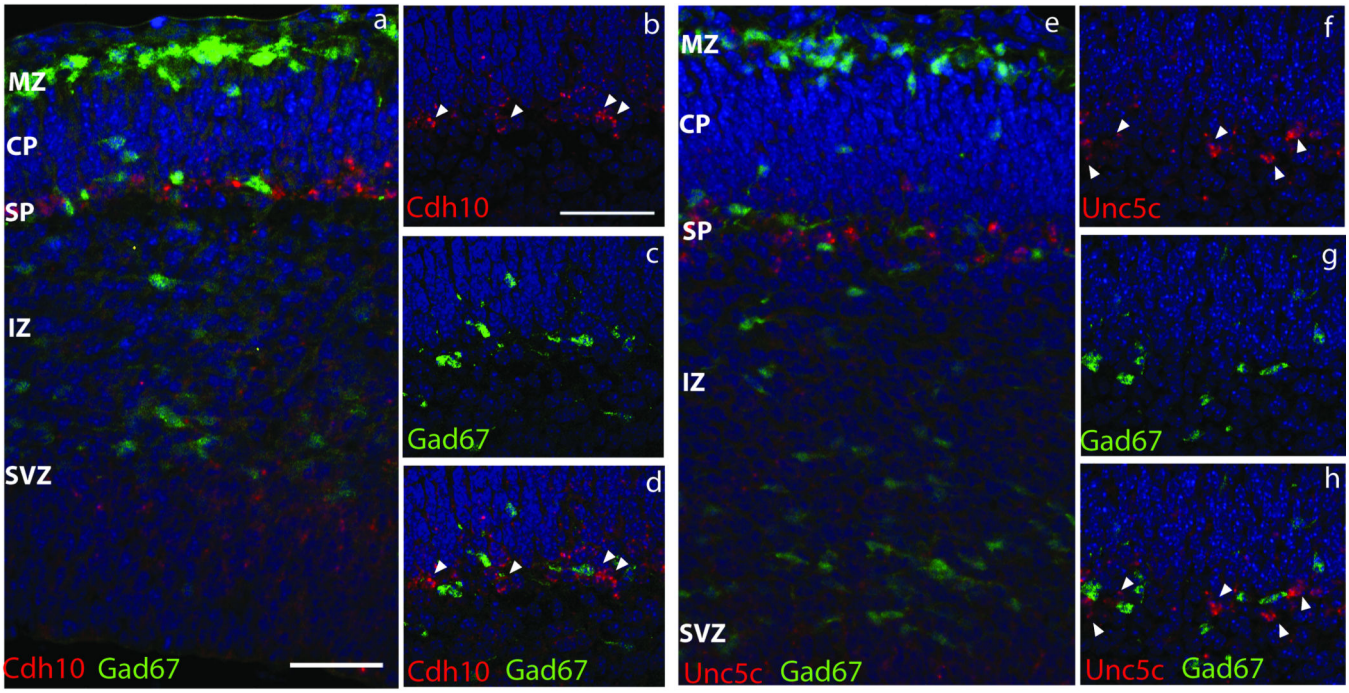

Figure 7. Expression of *Cdh10* and *Unc5c* in the Gad67-GFP cortex

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 intermediate zone (IZ) and subventricular zone (SVZ) corresponding to the three 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 co-localization was observed between *Cdh10* (red; arrowheads) and Gad67-GFP (green) (d) and between *Unc5c* (red; arrowheads) and Gad67-GFP (green) (h), respectively. Scalebars: 50μm.

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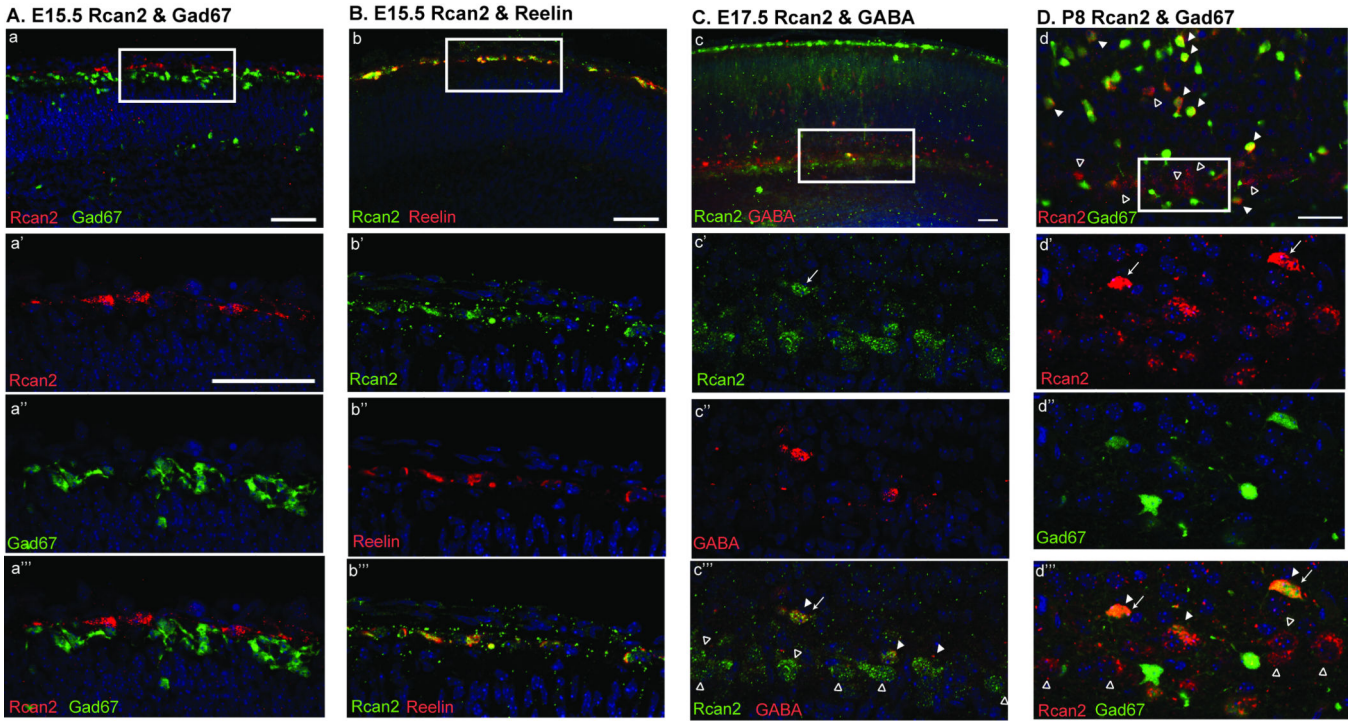

Figure 8. Co-localization between Rcan2, Gad67-GFP/GABA and Reelin

A. In the E15.5 cortex, immunohistochemistry 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') co-express 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 co-express 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 co-express Gad-67-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, filled arrowheads to double-labeled Rcan2 positive cells. Scalebars: 50μm.

Table 1

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

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

Application	Genotype	Age	Preparation	Number
Microarray	wild-type	E15.5	Fresh-frozen	18 (from 4 litters)
qRT-PCR	wild-type	E15.5	"	2 (from 2 litters)
In situ hybridization	wild-type	E13.5	Fresh-frozen	2
	"	E14.5	"	3
	"	E15.5	"	3
	"	E17.5	"	2
	"	P8	"	3
	"	Adult	"	2
	reeler	E15.5	"	3
	Gad67-GFP	E15.5	Immersion-fixed (PFA) and frozen	3
Immunohistochemistry	wild-type	E13.5	Immersion-fixed (PFA)	2
	"	E14.5	"	3
	"	E15.5	"	3
	"	E17.5	"	2
	"	P8	Perfused (PFA)	3
	"	Adult	"	3
	reeler	E15.5	Immersion-fixed (PFA)	2
		P8	Perfused (PFA)	2
	Gad67-GFP	P8	Immersion-fixed (PFA)	3
Immunohistochemistry against GABA	wild-type	E17.5	Perfused (PFA + glut)	2
	"	P8	"	3

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Table 2

Primer sequences for quantitative RT-PCR.

Gene	Forward primer	Reverse primer	Amplicon length (bp)
2610017I09Rik	tgtgcccgggactgtaag	cataccacttgatgaaggctca	140
Abca8a	cctttatcccaggccttctt	tcctcttcaaaaccgtccag	140
Actb	ttctttgcagctccttcgtt	atggaggggaatacagccc	149
Cacna2d1	aatggctatgtcttattgcatcc	tcattttatttcgaatctccacttt	127
Cdh9	acaaccctcctcggttcc	tatattccagctcggcgttc	129
Csmd3	ggttctgagccacctacaatatg	gggagcactaaatcctcggta	129
Epha3	tggctccttggacagtttct	gttgcgagcagcaaggtc	142
Kcnip4	gctgattgaagcaggtttagaag	gaatctgaagctctttcttggtg	128
Nr4a2	tcagagcccacgtcgatt	gatctccatagagccggtca	143
Nr4a3	tgcagagcctgaaccttgat	tggtcctttaagctgcttgtg	136
Ppia	gtcaaccccaccgtgttct	agccaaatcctttctctccag	139
Rlbp1l2	gccgcattgcttagtgactc	tgattctctctttgtgtgtccaa	135
Sema6d	agaaatcgccgtggaacata	ccgagccttaaggaaggaag	128
Slit1	ccccgaggtgtatttggag	gcgagactctggatcttgttg	146
Tbb2c	cttcgggcagatcttcagac	ggcagtcacagctctcagc	149
Unc5c	aggcagtgcagggacaat	ccagggcataggtactgagg	129

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Table 3

Primer sequences for in situ hybridization riboprobes used to validate selected subplate-enriched genes.

Gene	Forward primer	Reverse primer	Probe length (bp)
Pls3	tcgggaagaaagaaccttcc	tccgattcttctagccatcg	601
Epha3	gtcatccagcttgtgggaat	ccgttaagccaatcaccagt	608
Abca8a	ccagacctgggaattgagaa	gttgcatgccagggtaaagt	621
Cdh9	cacagccagtgttcctgaaa	gaacacaggggctcatcta	658
Csdm3	cagcaacaaaaactggctca	tcactccaagcagcaaacac	663
Unc5c	ttgggcctgaagattactgg	gttcttggattggaggacca	680
Slc8a1	ggaccaacagctggagagag	aggagcacaaacagggaaga	643
Zdhhc2	ggacaaatggtctgcctgat	ttccaatcggaaggttgttc	586
Gabra5	tgactgctcacttccacctg	gagaggtggccccttttatc	645
Ogfrl1	gaaggaaatgccaaaccaaa	caaatcgcaggagctttctc	649
Plxna4	ggacatccccagctacaaga	ctgtcctcagctcctcatcc	632
Cdh10	acgagcgactcaaagagcat	tcatttgcaagagcaagacg	563
Cdh18	agccaaggtggaaaatgatg	ctctctgtccaggcttttgg	615
Unc5d	aacgtatgccctcacaggag	cgcagatcctctgtctgatg	566
Sema6d	acttcgacgtcctgcagtct	aagcatggtgatccttgtcc	618
Nrp1	tgacagcgcaatagcaaaag	accagacggatgttttctgg	648
1190002N15Rik	tcctttgctaaaaggcttcg	ccccaaactttactgcaaaca	608
Nt5dc2	agtcccttcagctttgtgga	cagctgagtgaggccatgta	617
Cdh12	aagaccttgatgtgggcagt	caggatggtcccatctgagt	572

Table 4
Subplate-enriched genes selected for validation

This table lists all 23 genes which were selected for further validation by immunohistochemistry or in situ hybridization. 13 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 intermediate zone (IZ) and subventricular zone (SVZ), sometimes with additional expression in SP; 3 genes were not expressed in the E15.5 neocortex.

Gene symbol	Name	Function (Reference)	Expression
Specific expression in subplate			
Abca8a	ABC transporter A8a	Lipid transporter (Tsuruoka et al. 2002)	SP
Cdh10	Cadherin 10	Type II cadherin (Redies 2000)	SP
Cdh18	Cadherin 18	Type II cadherin (Shimoyama et al. 1999)	SP
Csmd3	CUB and Sushi multiple domains 3	Transmembrane protein of unknown function (Shimizu et al. 2003)	SP
Gabra5	GABA A receptor α5	Ionotropic GABA receptor subunit (Fritschy and Mohler 1995)	SP
Kcnt2	potassium channel subfamily T, member 2	Sodium-activated potassium channel (Bhattacharjee et al. 2003)	SP, MZ
Ogfrl1	opioid growth factor-like receptor 1	Homologous to the opioid growth factor receptor	SP
Pls3	L-plastin	Actin-bundling protein; involved in neurite growth (Oprea et al. 2008)	SP
Rcan2	Regulator of calcineurin 2	Regulator of calcineurin (Porta et al. 2007)	MZ, weak in SP
Slc8a1	solute-carrier 8a1	Sodium-calcium exchanger (Philipson et al. 2004)	SP
Sv2b	Synaptic vesicle 2b	Ca^{2+-}dependant regulation of neurotransmitter release (Janz et al. 1999)	SP
Unc5c	Unc homologue 5c	Repulsive netrin-1 receptor (Przyborski et al. 1998)	SP
Zdhhc2	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 intermediate zone/subventricular zone			
1190002N15Rik	Riken clone	Unknown	SVZ and IZ
Epha3	Eph receptor a3	Ephrin receptor (Kudo et al. 2005)	SVZ, IZ, weak in SP
Nrp1	Neuropilin 1	Receptor for VEGF and Sema3a (Schwarz et al. 2004)	SVZ, IZ, weak in SP
Nt5dc2	5′-nucleotidase domain containing 2	Dephosphorylation of nucleotides	SVZ and IZ
Plxna4	Plexin a4	Repulsive sema6a receptor (Suto et al. 2005)	SVZ, IZ, weak in SP
Sema6d	Semaphorin 6d	Guidance molecule (Taniguchi and Shimizu 2004)	SVZ, IZ
Unc5d	Unc homologue 5d	Repulsive netrin-1 receptor (Takemoto et al. 2011)	SVZ, IZ
No or weak expression in the neocortex			
Cdh12	Cadherin 12	Type II cadherin	weak signal
Cdh9	Cadherin 9	Type II cadherin	weak signal
Neurod1	Neurogenic differentiation 1	Transcription factor	hippocampal anlage