Expression profiling of mouse subplate reveals a dynamic gene network and disease association with autism and schizophrenia

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Edited* by Pasko Rakic, Yale University, New Haven, CT, and approved January 8, 2013 (received for review October 29, 2012)

The subplate zone is a highly dynamic transient sector of the developing cerebral cortex that contains some of the earliest generated neurons and the first functional synapses of the cerebral cortex. Subplate cells have important functions in early establishment and maturation of thalamocortical connections, as well as in the development of inhibitory cortical circuits in sensory areas. So far no role has been identified for cells in the subplate in the mature brain and disease association of the subplate-specific genes has not been analyzed systematically. Here we present gene expression evidence for distinct roles of the mouse subplate across development as well as unique molecular markers to extend the repertoire of subplate labels. Performing systematic comparisons between different ages (embryonic days 15 and 18, postnatal day 8, and adult), we reveal the dynamic and constant features of the markers labeling subplate cells during embryonic and early postnatal development and in the adult. This can be visualized using the online database of subplate gene expression at https://molnar.dpag.ox.ac.uk/subplate/. We also identify embryonic similarities in gene expression between the ventricular zones, intermediate zone, and subplate, and distinct postnatal similarities between subplate, layer 5, and layers 2/3. The genes expressed in a subplate-specific manner at some point during development show a statistically significant enrichment for association with autism spectrum disorders and schizophrenia. Our report emphasizes the importance of the study of transient features of the developing brain to better understand neurodevelopmental disorders.

RNAseq | microarray | Nxph4 | Tpd52l1 | interstitial white matter cells

The murine subplate (SP) layer contains a diverse group of cells, some of which are the earliest born neurons of the cerebral cortex (1–4). It is a distinct layer throughout the latter half of neurogenesis, cell migration, and the period of axon pathfinding. SP is essential for the guidance of thalamocortical axons through the pallial–subpallial boundary (5) and it forms the first compartment in which topographical connections from thalamus to the cortex are established (6, 7). The early postnatal SP neurons in rodents and cats are involved in generating oscillations in cortex (8, 9) and are necessary for the maturation of the inhibitory circuitry in cortical layer (L)4 (10, 11). The remaining adult SP/L6b cells in nonrodent species have been suggested to support corticocortical connectivity (1, 12–15).

The molecular mechanisms that underlie these various functions are poorly understood. Four recent studies profiled SP gene expression at different developmental stages, including early (16) and midembryonic (17), young postnatal, (18) and adult (19). Interestingly, the temporal pattern of most of the SP-specific gene expression in vivo appears to be tightly regulated, probably reflecting the changing functions of SP during this period. Our current analysis includes the heretofore unstudied period between embryonic day 15 (E15) and postnatal day 8 (P8). This period is particularly important as many of the known roles of SP in the mouse emerge in this time window.

In this study, we set out to characterize and compare gene expression in murine SP at E15, E18, P8, and adult (P56), thereby covering the entire developmental period. We provide data for E18 and reexamine the existing microarray and RNAseq data spanning development from E15 to adulthood to identify further molecular markers that label the SP layer between E15 and P8. We consider disease associations and biological pathway annotations of genes expressed in a SP-specific or -enriched manner.

Results

Identification of Subplate-Enriched Genes in Mouse. We used microarrays to compare gene expression levels between SP and cortical plate (CP) at E15, and E18, and between SP and layer 6a (L6a) at P8. The E15 and P8 data have been partially published before (17, 18), but were reanalyzed for this publication using different statistical methods. We used data from previously published high-throughput sequencing (19) to compare gene expression between layers 6a and 6b.

At E15, 159 genes were identified as 1.5-fold enriched in SP compared with CP, and 945 such genes at E18. At P8, 746 genes were identified as 1.5-fold enriched in SP compared with L6a, and 228 genes in the three adult samples (see Dataset S1 for a full list of identified probe sets and associated genes).

Considerable overlap was found between the different ages, with three genes reported as SP enriched in all four comparisons (Fig. S1). In total, 1,642 genes were identified as potentially SP enriched at least at one age. Of these, 416 genes were confirmed as SP enriched using publicly available or published in situ hybridization images (Dataset S2 and Fig. S1*B*). Sixty-eight of these were further classified as showing SP-specific expression at some ages (Fig. 1 and Fig. S1 *C* and *D*). Few of these genes are expressed SP specifically throughout cortical development and into adulthood (Fig. 2). The exceptions are *Nxph4*, *Nr4a2*/*Nurr1*, *Inpp4b*, *Ctgf*, *Htr1d*, *Tpd52l1*, and *Adra2a*. Of these, we have independently verified the SP-specific protein localization for NR4A2/NURR1 (18), CTGF, and TPD52L1 (Fig. S2).

Author contributions: A.H.-S., F.M.O., and Z.M. designed research; A.H.-S., F.M.O., W.Z.W., S.L., and Z.M. performed research; M.L.K. and C.W. contributed new reagents/analytic tools; A.H.-S., F.M.O., M.L.K., T.G.B., S.L., C.W., E.P., and A.D.E. analyzed data; and A.H.-S., F.M.O., M.L.K., T.G.B., and Z.M. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

Data deposition: Data has been deposited at https://molnar.dpag.ox.ac.uk/subplate/.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1218510110/-/DCSupplemental.

Gene	E15	E18	P8	Adult	Image based
Cdh18	N/A	N/A	N/A	1.3	early
Kcnt2	N/A	N/A	N/A	2.5	early
Cdh9	N/A	N/A	N/A	1.3	early
9130024F1	N/A	N/A	N/A	1.3	early
Cdh11	N/A	1.7	1.6	1.5	early
Gabra5	1.9	2.5	1.5	1.3	early
Zdhhc2	1.8	1.6	N/A	1.3	early
Abca8a	2.1	1.5	1.4	1.3	early
Cacna2d3	1.3	1.5	2.0	1.8	early
Cdh7	1.8	1.5	0.7	1.3	early
Slc8a1	1.2	1.5	1.2	1.4	early
Csmd3	N/A	1.5	1.1	3.2	early
Gng11	1.8	2.0	1.2	1.5	early
Pde1a	1.8	2.1	4.2	1.8	early
Sv2b	1.6	1.9	1.3	1.2	early
Unc5c	1.6	1.5	0.9	1.7	early
Sema6a	1.3	2.8	0.9	1.5	early
Gsg1l	N/A	N/A	N/A	1.4	continuous
Nr4a2	1.4	1.9	1.9	1.3	continuous
Acvr2a	N/A	1.6	1.3	1.6	continuous
Mmp16	1.2	4.1	1.7	1.3	continuous
Lmbrd1	N/A	2.5	1.9	1.4	continuous
Chst2	1.1	2.4	1.7	1.3	continuous
Kcnab1	1.9	2.6	3.0	1.5	continuous
Nxph3	N/A	1.7	1.3	1.6	continuous
Prss12	N/A	2.3	2.8	1.5	continuous
Igf1r	N/A	1.5	0.9	1.7	continuous
Usp46	2.0	1.5	1.4	>100	continuous
Nxph4	1.2	1.5	1.6	3.4	continuous
Col11a1	1.1	2.8	2.5	1.5	continuous
Kcnc4	N/A	1.7	0.9	1.5	continuous
Adamts1	1.1	1.8	1.2	1.2	continuous
Slc1a2	N/A	2.5	1.5	1.1	continuous
Crtac1	N/A	N/A	N/A	3.2	late embryonic
Trp53i11	N/A	1.9	2.0	1.6	late embryonic
Ctnnb1	N/A	1.8	1.2	1.2	late embryonic
Alcam	0.8	1.7	2.7	1.3	late embryonic
Cck	1.5	1.5	0.8	1.9	late embryonic
Tmem163	N/A	2.0	5.3	1.7	late embryonic
Ctgf	N/A	3.1	3.4	1.7	late embryonic
Ngef	N/A	1.9	1.2	1.2	late embryonic
Trh	1.5	1.6	2.0	1.6	late embryonic
Chrna4	N/A	1.6	1.2	1.3	late embryonic
Etv5	N/A	1.7	1.2	83.6	late embryonic
Inpp4b	1.2	1.7	3.3	3.0	late embryonic
Tpd52l1	1.4	2.1	4.4	2.7	postnatal
Htr1d	1.2	1.5	1.6	1.0	postnatal
Gdf10	N/A	1.7	2.9	1.7	postnatal
Wnt4	N/A	2.3	1.5	1.4	postnatal
Drd1a	0.9	1.5	1.5	1.7	postnatal
Sema5b	N/A	3.2	2.0	1.5	postnatal
Nmbr	N/A	1.7	2.3	1.1	postnatal
Cplx3	N/A	1.8	6.7	3.2	postnatal
Adra2a	N/A	1.7	2.6	1.7	postnatal
Cacng5	N/A	2.1	0.9	1.8	postnatal
Impact	N/A	1.7	1.8	1.2	adult
Gng12	N/A	2.3	1.8	1.3	adult
Sulf2	1.6	2.2	1.7	1.2	adult
Chst11	1.2	1.6	2.4	1.3	adult
Rai14	N/A	1.7	1.8	1.3	adult
Ly6g6e	N/A	2.0	1.4	2.4	adult
Tpbg	1.8	1.8	1.4	2.7	adult
C1qtnf1	N/A	1.5	1.1	1.7	adult
Cidea	N/A	1.7	1.3	1.3	adult
Svil	N/A	2.1	2.0	1.2	adult
Thrsp	N/A	1.7	2.4	6.4	adult
Tnmd	N/A	1.7	2.5	2.7	adult
Clic5	N/A	1.8	1.1	2.0	adult

Normalized expression level coding

- >1 SD above average
- 0.5-1 SD above average
- average
- 0.5-1 SD below average
- >1 SD below average

Fig. 1. Table of subplate-specific genes. Genes were identified as SP-specific at least at one age. The column "image based" indicates when a gene is specific to SP (early, from E15 but not until adulthood; continuous, E15–adult; late embryonic, during perinatal period; postnatal, P4/P8–adult). Gray labeling indicates that the image-based classification was made with an incomplete time series of images. Values reflect the fold change between SP and CP/L6a, but only if there is a "best match" between the ST1.0 and 430A Affymetrix probe sets. Color-coding reflects the gene expression level at each age (N/A, fold change not available; SD, standard deviation).

Patterns of Coexpression of Subplate-Enriched Genes. Comparing SP gene expression levels against L6a or CP gene expression levels to identify genes with higher expression in SP inherently biases against certain coexpression patterns, namely uniform expression levels across SP and CP/L6a. No such constraint exists for other coexpression patterns. See Fig. 3 for selected examples of commonly occurring spatial coexpression.

Particularly common are coexpression in the intermediate zone (IZ) or the ventricular zone (VZ) at E15 (82 and 63 of 163 genes, respectively), and IZ/white matter (WM) or L5 at E18 (41 and 40 of 126 genes, respectively). Coexpression in L5 remains the most common pattern throughout postnatal development (75 of 131 and 97 of 220 genes for P4 and adult, respectively). Coexpression in CP (comprising layers 2–4) accounts for less than one-fifth of genes with SP-enriched expression (20 of 126 genes) at E18, and is virtually absent in L4 postnatally (1 and 2 genes at P4 and adult, respectively). These expression patterns and more can be browsed at https://molnar.dpag.ox.ac.uk/subplate/.

Using the adult sequencing data (19), it is possible to determine cortical layer similarity based on similarity in gene expression profiles. Overall, infragranular layers are more similar to other infragranular layers than to supragranular layers (Fig. S3). Additionally, a rough estimate of expected coexpression in other layers can be made. Overall, we observed 2.5 times more coexpression in L5 or L2/3 than expected. Conversely, we only observed a quarter of the expected genes to be coexpressed in subplate and L4.

Developmental Patterns of Subplate-Specific Gene Expression. Because genes that are expressed exclusively in SP at one age are rarely SP specific at all ages, we investigated the temporal patterns of gene expression among the 68 genes that are SP specific at least at one age. For this, gene expression was classified as not expressed, SP specific, SP enriched, uniform, or decreased in SP.

An especially common developmental pattern of gene expression is SP-specific expression at E15 but gene expression in additional layers, particularly L5, by E18 (13/43 genes; Figs. 2 and 3). Genes following this pattern include *Cacna2d3*, *Cdh11*, *Gabra5*, *Pde1a*, *Sema6a*, *Slc8a1*, and *Zdhhc2* (17). Less common is postnatal SP-specific expression (7/43). These genes are usually not expressed in the embryonic cortex. Examples of this are *Sema5b*, *Drd1a*, *Adra2a*, and *Cacng5*.

Biological Processes. These gene expression patterns are not stable over time, and neither are the currently known functions of the subplate. Thus, for each age, we tested for enrichments of gene ontology terms among SP-enriched genes. Genes were considered SP enriched at a particular age if the expression was confirmed by in situ hybridization. Across all ages, these genes were significantly enriched for 95 nonredundant functional terms (Benjamini–Hochberg false discovery rate, BH FDR <5%). Some functions were too broad to interpret, such as brain development, but four clear functional themes emerged: cell death/apoptosis and cell division pathways, cell–cell communication and signaling pathways, axon or dendrite growth and/or cell migration pathways, and myelination (Dataset S3 and Fig. S4). Functions only associated with particular time points include blood vessel morphogenesis and, surprisingly, mitotic sister chromatid segregation at E15, and changes in cell adhesion properties, membrane potential, and regulation of canonical Wnt receptor signaling at E18. Adult L6b is associated with regulation of transforming growth factor beta and G protein signaling.

Interestingly, cell adhesion varies across ages. At E15, SP appears to participate in homophilic cell adhesion and cell–matrix adhesion, followed by a period of decreased cell adhesion perinatally. A temporal pattern also emerges in relation to gliogenesis, with E15 SP-enriched genes overrepresented among glial differentiation genes, followed by enrichment in general gliogenesis at E18 and P8 and finally ensheathment of neurons/myelination in postnatal SP/L6b (P8 and adult).

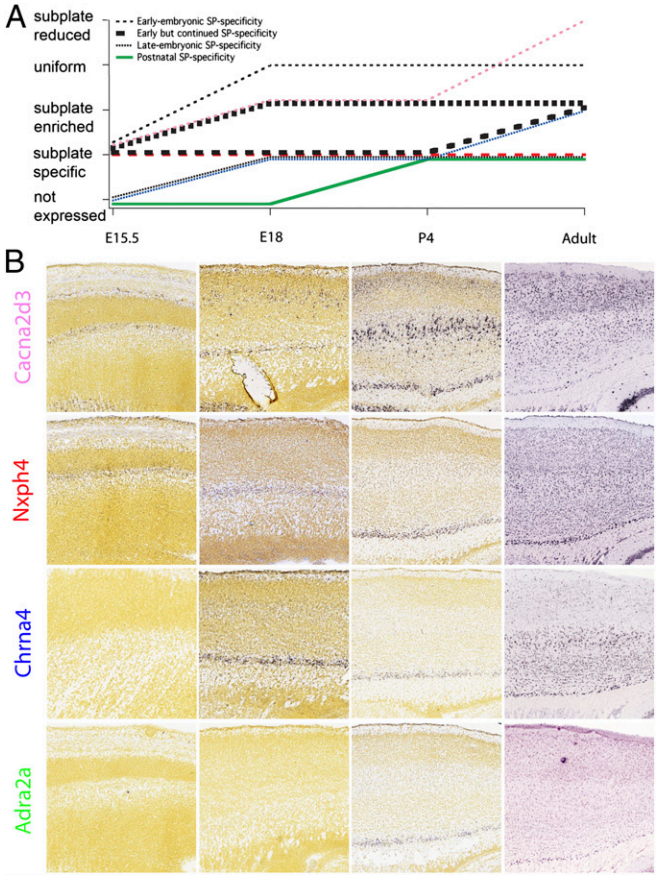

Fig. 2. Temporal patterns of expression of SP specific genes in other cortical layers (*A*) and selected examples (*B*). Genes were classified as "not expressed," "SP specific," "SP enriched," "uniform," or "SP reduced" for each age based on in situ hybridization [GenePaint (E14.5), Allen Developing Mouse Brain Atlas and Allen Mouse Brain Atlas (E15.5, E18.5, P4, and P56)]. Four common patterns emerged: early-embryonic SP-specific gene expression (13/43 genes, thin dashed lines, exemplified by *Cacna2d3*), early-onset but continued SP-specific gene expression (8/43 genes, thick dashed line, *Nxph4*), late-embryonic SP-specific gene expression (5/43 genes, dotted lines, *Chrna4*), and postnatal SP-specific gene expression (7/43 genes, solid line, *Adra2a*). For simplicity, only coexpression patterns containing at least two genes were included in the graph. Images were taken from the Allen Developing Mouse Brain Atlas and the Allen Mouse Brain Atlas (59).

We repeated the above analysis for all genes that are SP specific, but without differentiating by time point. These SP-specific genes are not significantly overrepresented in any Gene Ontology terms ($P > 0.05$).

Gene Coexpression Network and Protein Interaction Network. To gain further insight into possible functions of the subplate, we also reconstructed a coexpression network of genes significantly differentially expressed in SP versus L6, with no constraints on fold change or direction of expression.

To identify genes that are important throughout development, genes were retained if they were common to all three early time points. The input subset of 170 genes was derived by focusing on genes showing high variation in expression and differential expression in SP versus CP/L6a. The spacing between time points was integrated into the model, resulting in a partially directed network composed of a single connected component comprising 95 nodes and 253 edges (local FDR <5%; Fig. 4). Identifying genes with confirmed SP-enriched expression (see above) highlighted that the majority of these are not directly interconnected and are found mostly at the periphery of the network, which may indicate a previously unremarked role for genes that are more centrally located and important within the context of coexpression, but are not individually expressed at a high level. The network was annotated with Gene Ontology terms. This highlighted cell adhesion and cell proliferation as functions relevant to SP.

Additionally, interactions at the protein level were investigated by using the subset of 95 network genes as a starting point for the protein interaction algorithm DAPPLE (20). This identified one large, highly interconnected and one small (three protein) network. Within the large network, we identified five subnetworks comprising direct protein–protein interactions of SP-enriched proteins. They are functionally related to blood vessel generation and IGF-signaling regulation (VTN, IGF2, IGFBP7), cell division and neuronal differentiation (CCND1 and NEUROD1) and kinetocore assembly (MYT1, PLK1, CENPE, SPC25, SPC24, NDC80, and BIRC5; Fig. S5*B*), and neurite remodelling (NTM and TTK). The network comprising PTPN2, NR4A2, CAV1, EGFR, ALCAM, VEGFC, and FLT1 (Fig. S5*B*) may be involved in gliogenesis or glioma formation. These genes or proteins, however, have not all been confirmed for SP-enriched expression.

A small group of 17 genes was expressed at higher levels in SP versus L6 across all three developmental time points; 13 of these genes are in the gene network (Fig. S5*A*) and 11 of them are first neighbors in the network. Their functions include synaptogenesis, axonal regeneration, neurite outgrowth, and AMPA receptor signaling. Five of the 17 genes also interact at the protein level in two clusters, one centered on THY1-LINGO1 and the other on NLGN1 (Fig. S5*C*).

Disease Association. The subplate layer and its neurons have been implicated in several neurodevelopmental pathologies (15), among them autism and schizophrenia. Increased numbers of SP remnants have been reported in postmortem histological specimens from schizophrenia patients (21–23) and an indistinct boundary between white and gray matter, possibly indicating supernumerary SP neurons, has been reported for brains of patients with autism spectrum disorders (24). On the other hand, decreases in cell number of peptidergic SP remnants have been reported in postmortem histological specimens of Alzheimer's disease patients (25). We therefore investigated whether the newly identified genes with SP-enriched expression are known disease susceptibility genes. We found that they are significantly overrepresented among autism-associated (26) ($P = 0.02$) and schizophrenia-associated genes (27) ($P = 0.04$), but not Alzheimer's disease-associated genes (28). By comparing the significance of these disease associations of genes enriched in SP to those expected by chance from 500 random sets of genes matched in number and general brain expression to the SP-enriched gene set (29), we confirmed the association with SP expression for both autism and schizophrenia ($P < 0.05$ for both). Additionally, the constituents of the gene network (see above) were also enriched for disease association genes ($P < 0.005$; see magenta symbols in Fig. 4 and Fig. S5)

Autism. The SP-enriched genes that are autism-associated are *Atp6a2*, *Cadps2*, *Cdh10*, *Cdh18*, *Cdh9*, *Gabra5*, *Nrxn1*, *Plp1*, *Prss12*, *Sema5a*, and *Tppp*. Of these, five are SP specific. As autism manifests in preschool age children and is thought of as a neurodevelopmental disorder, one would expect autism-associated genes to be expressed and play a functional role during development. Indeed, the large majority of the autism susceptibility genes are expressed in SP in the developing mouse brain.

These 11 autism-associated, SP-enriched genes are functionally and molecularly diverse, although the majority are transmembrane proteins. Their common link is likely to be their role in early SP formation and subsequent network maturation. TPPP/P25 is phosphorylated in a functionally significant manner by the cyclin-dependent kinase 5 (30), in the absence of which the cortical preplate fails to split into marginal zone and SP (31). SEMA5A is an axon guidance molecule and is possibly also involved in establishment of synapses (32). PRSS12 (neurotrypsin) is a serine protease, possibly involved in remodeling of the extracellular matrix and new synapse formation during learning and memory (33). An intronic single nucleotide polymorphism in *NRXN1* correlates with reduced frontal lobe white matter volume but also affects thalamic volume, suggesting an overall impaired

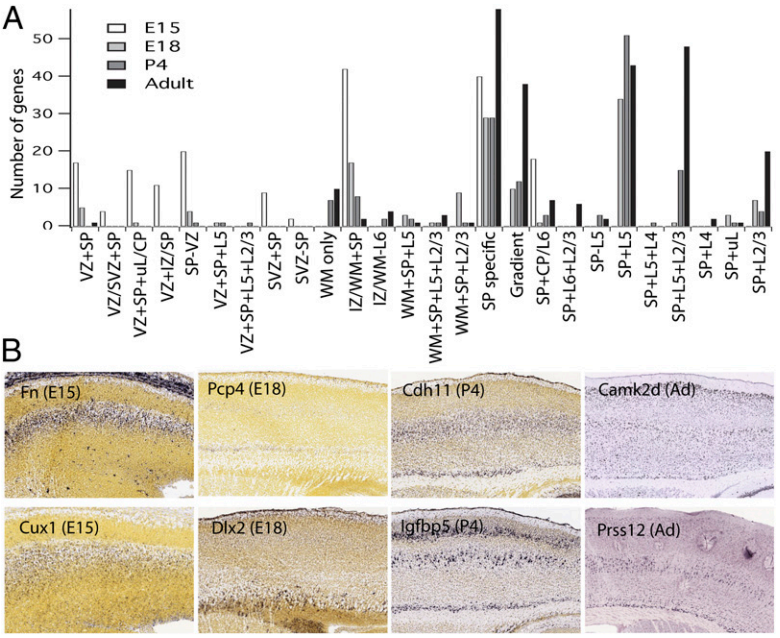

Fig. 3. Spatial coexpression patterns in other cortical layers and selected image examples. (*A*) Summarizes the spatial patterns of coexpression observed for genes that were expressed in a SP-enriched manner at some age, using the data available in Allen Developing Mouse Brain Atlas (E15.5, E18.5, P4, and adult) or Genepaint (E14.5). (*B*) Examples of genes representative of common coexpression patterns. At E15, coexpression in the ventricular zone (*Fn*) or intermediate zone (*Cux1*) is common. At E18, coexpression with layer 5 (*Pcp4*) or in the white matter/intermediate zone (*Dlx2*) is common. At P4 and in the adult, coexpression with layer 5 (*Cdh11* and *Prss1*) or layer 5 and layers 2/3 (*Igfbp5* and *Camk2d*) is common. Images were taken from Allen Developing Mouse Brain Atlas. CP, cortical plate; IZ, intermediate zone; SP, subplate; uL, upper layers; WM, white matter.

thalamocortical network (34). The protein products of a further three SP-expressed autism susceptibility genes (*Cdh9*, *-10*, and *-18*) are cell adhesion molecules. CDH9 expression is downregulated in *Tbr1* knockout mice, in which *Ctgf* expression is also abolished (35).

Schizophrenia. The SP-enriched genes were tested for significant association with schizophrenia-associated genes. For this, two data sets were used: a recent summary of copy number variant and genome-wide association studies (36) and a larger candidate gene list provided in the supplementary information of a recently published gene prioritization approach (27).

Of these, only the latter indicates a significant enrichment of SP-enriched genes ($P = 0.005$) among schizophrenia-associated genes. The SP-enriched genes that are schizophrenia associated are: *Apoe*, *Dbi*, *Ddr1*, *Drd1a*, *Fn1*, *Gad1*, *Insig2*, *Notch2*, *Nr4a2*, and *Slc1a2*. Three of these are SP specific, thereby also indicating significant ($P = 0.009$) enrichment of schizophrenia-associated genes among the SP-specific set of genes.

Several of the schizophrenia-associated genes are expressed in a SP-enriched pattern only during early development (e.g., *Apoe*, *Ddr1*, and *Fn1*). The 10 schizophrenia-associated, SP-enriched genes are functionally and molecularly diverse. *Ddr1*, *Drd1a*, *Notch2*, and *Slc1a2* encode transmembrane proteins (receptors and transporters). *Fn1* encodes a secreted glycoprotein, *Gad1* encodes the major enzyme for the biosynthesis of the inhibitory neurotransmitter GABA, and *Dbi* encodes a protein that modulates GABA-receptor activity. *Nr4a2* encodes a transcription factor with a known role in the specification of dopaminergic neurons.

Discussion

This study is unique in following gene expression in a cortical layer throughout development to adulthood and further increases the range of available marker molecules for the mouse subplate. Based on this comprehensive analysis, we propose additional roles for mouse SP and highlight the relevance of SP-specific gene expression to several neuropathologies.

Identification of Subplate-Enriched Genes. The experimental design for identifying genes with a higher expression level in SP has already been published and widely discussed (17–19, 37), although alternative approaches have also been taken (16, 38). Our study has systematically validated the SP-enriched gene lists.

Genes that are specifically expressed in SP at one time point are rarely expressed in this manner throughout development. Particularly common was early embryonic SP specificity and perinatal SP specificity, followed by broader expression later on. This could be a reflection of the relative maturity of SP compared with CP. Therefore, to identify genes contributing to SP-specific functions, analysis of SP-specific genes with no expression at other ages (such as *Htr1d* and *Tpd52l1*) or the few genes with a continuous SP-specific expression (*Nxph4*, *Nr4a2*, *Inpp4b*, *Ctgf*, and *Adra2a*) might be particularly informative.

Patterns of Coexpression of Subplate-Enriched Genes. Common and age-consistent coexpression patterns in other layers emerged that could indicate a functional relationship or shared role between SP and these other layers. The frequent coexpression observed between SP, L5, and L2/3 may be a consequence of the overall high numbers of genes being expressed specifically in L5 or L2/3 compared with other cortical layers (19), but it cannot explain the lack of coexpression of SP markers in L4, which shares functional similarities in terms of thalamocortical input with SP.

However, we observed more coexpression in L5 and L2/3 than expected even from the overall high number of genes expressed in these two layers. Thus, there may be additional similarities between these layers. SP and L5 are both output layers of the cortex and contain neurons with long-range projections to subcortical targets, which develop early during brain circuit formation (39). Thus, some genes enriched in both L5 and SP may be most relevant to developing pioneering axons or the metabolic demands of maintaining long axonal projections. Additionally, the axonal extension pattern and layer positioning of deep layer neurons from SP to L5 is affected by SOX5 (40). In the absence of SOX5, SP neurons are distributed throughout the cortex at P0 and ectopic SP neurons are colabeled with CTIP2. Thus, the similarities in expression patterns between SP and L5 could be a reflection of the "multipotential" of SP cells, in which a subcerebral projection fate is normally suppressed by SOX5 (40, 41). On the other hand, there is also frequent coexpression of genes in SP and L2/3 in the postnatal cortex without corresponding known functional similarities. We propose that expression of extracellular or transmembrane proteins in SP, L5, and L2/3 gives an alternating stripe patterning to the cortex, which may be relevant to give layer information to incoming connections or help delineate layer boundaries. This would also correlate well with the observed lack of coexpression in L4, and the relative overrepresentation of cell-adhesion molecules among SP-specific genes.

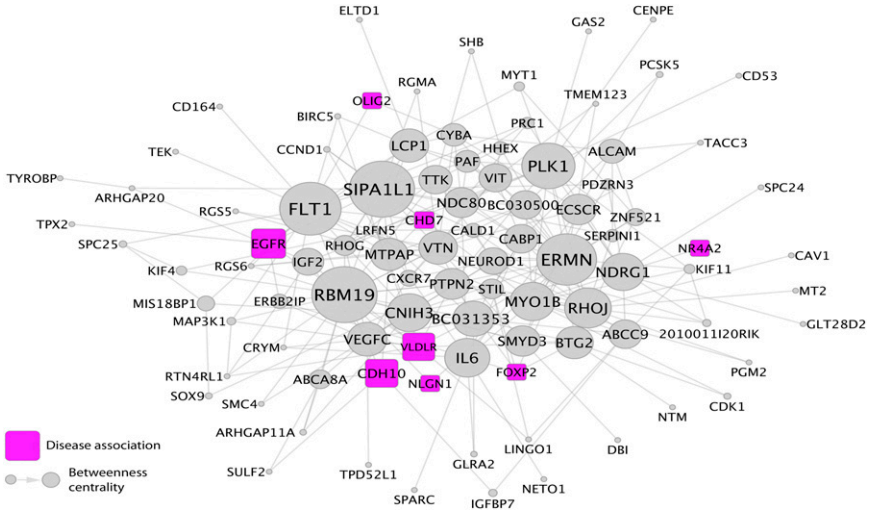

Fig. 4. Gene coexpression network. Coexpression network of genes differentially expressed in SP, with annotation for disease-implicated genes (FDR <5%).

Biological Processes. SP neurons have three commonly accepted roles in the developing cortex. Firstly, they pioneer subcortical projections (6, 42). Secondly, they meet with thalamic afferents in the internal capsule and provide guidance for them en route to the cortex (5, 43, 44). Lastly, they receive synaptic contacts from thalamic fibers (15, 45–49) and are essential for normal maturation of the thalamus–L4 circuitry (10, 11). As expected from these known functions and properties of SP, gene ontology-based analysis identified axon pathfinding/axonogenesis, cell–cell signaling and cell–cell adhesion as terms associated with SP-enriched genes in early development, and synapse regulation and/or plasticity-related processes and cell death in the perinatal period. However, the known roles of SP give no hypothesis for what to expect in the adult. Interestingly, gene ontology analysis identified myelination and glia-related processes during later development of SP. As the input list only contained confirmed, SP-enriched genes, it may be interesting to further investigate the role of SP in myelination, especially given that an increase in CTGF levels or *Ctgf*-expressing cells causes deterioration of oligodendrocyte maturation (50).

The methodology used here cannot identify genes that may be necessary to subplate specification, differentiation, or function but are equally expressed in layer 6a (such as Tbr1) (51), or expressed subplate specifically only before E15.5.

Coexpression Networks and Disease Association. Gene coexpression network analysis and protein-interaction networks were used as independent methods of identifying SP-related functions. The latter again highlighted that the SP compartment is particularly involved in cell adhesion, blood vessel formation, and also cell division, although the cell division term may be attributed to regulation of gliogenesis and terminal differentiation in oligodendrocyte precursors. Both the verified subplate-enriched genes and the inferred network of genes expressed at higher levels in the subplate are enriched for autism and schizophrenia disease association genes.

Here we provide a unique comprehensive association study of subplate-enriched gene expression and disease association with autism and schizophrenia.

Methods

Tissue and Animals Used. Tissue was collected in accordance with Home Office regulations and local ethical guidelines at the University of Oxford. Brain tissue was collected from C57/BL6 mice aged E15.5, E18.5 (plug date = E0.5), P8, and P56 (adult, birth = P0). Four biological replicates (each from a group of three to four littermates of unknown sex) were used for each time point up to P8. For adult, a total of eight males of two litters (four from each) were used. Tissue collection, layer dissection, and total RNA isolation have previously been described for the E15, P8, and adult samples (17–19, 52). The E18 samples were collected and processed identically to the P8 samples (18).

Gene Expression Profiling. To determine gene expression in SP and L6a/lower cortical plate (lCP) in primary sensory cortex S1 (or its anlage), the amplified and labeled cDNA libraries from the E15 to P8 time points were hybridized to Affymetrix microarrays (Affymetrix Mouse Gene 1.0 ST for E15 or Affymetrix 430 2.0) (17, 18). cDNA fragments corresponding to the adult mRNA were deep sequenced to a length of 51 or 76 nucleotides from each end, using Illumina's Genome Analyzer IIx. Sequence reads were mapped to the mouse genome (mm9), including splice sites, using TopHat, and known genes were quantified, using cufflinks, as previously described (19).

Differential Gene Expression. The data from all three microarrays were analyzed using the affy and limma Bioconductor packages (53–55). We normalized using the robust multiarray average (RMA) method and determined differential gene expression between SP and L6 using the linear model implemented in limma. Each age was analyzed separately. We obtained a candidate gene list having a nominal $P < 0.05$ and at least a 1.5-fold difference in expression levels.

For the adult deep sequencing, we compared expression between SP and L6 in S1, dorsal, and lateral cortex. Genes that were present on both microarrays (15,237 genes) and further had sufficient read density in the adult sequencing for statistical testing [9,671 genes, using a Benjamini–Hochberg FDR of 5%] were compared between SP and L6 using cuffdiff from the cufflinks package (56). Genes with at least 1.5-fold enrichment in SP were further analyzed.

Confirmation of Differential Gene Expression. All genes identified as SP enriched by the microarray or RNAseq experiments were assessed for mRNA distribution across the cerebral cortex using the publicly available image databases GenePaint (ages E14.5, P7, and P56) (57), the Allen Developing Mouse Brain Atlas (E15.5, E18.5, P4, and P14) (58, 59), and Allen Mouse Brain Atlas (Adult) (58) or our own in situ hybridization experiments (17, 18). Genes were classified as "SP enriched" if they were expressed at a higher level in SP than in L6/lCP at the level of S1. Genes were classified as "SP specific" if the in situ hybridization signal in the cerebral cortex was restricted to the SP zone at least at one age. Genes with uniform or no labeling, or for which no images could be found, were discarded from further analysis.

Pathway Analysis and Disease Association. Pathway analysis was performed using GO-Elite, with the list of SP-enriched or SP-specific genes used as the query (60). The comparison list consisted of the 15,235 genes present on both microarrays and in the adult deep sequencing (background list), but extended by a further 40 genes that had SP-enriched expression but were present on only one of the microarrays.

To identify enrichments of disease-associated genes, the SP-specific or SP-enriched genes were searched for significant overlap with autism (26), schizophrenia (27, 36), or Alzheimer's disease-associated (28) genes or chromosomal regions.

Gene–Gene and Protein Interaction Network Analysis. Developmental gene expression was investigated at E15.5, E18, and P8. Genes showing variation and differential expression between SP versus L6 at all ages were extracted (FDR <5%). A graphical Gaussian model (GGM) (33, 34) was applied to

reconstruct a gene coexpression network of these genes (FDR <5%). Genes up-regulated in SP at each age were found (FDR <5%) and a set of genes common to all three ages was extracted (*SI Methods*).

Enrichment for disease associations with autism and schizophrenia (26, 27) in the network was calculated. The network was annotated with Gene Ontology terms, and functional groupings and enrichment were assessed with the BinGO (61) and MOSAIC (62) packages on the Cytoscape platform. Interactions at the protein level were investigated by using the subset of the 95 connected network genes (identified by the GGM) as a starting point for a protein interaction algorithm (DAPPLE) (20).

ACKNOWLEDGMENTS. We thank Julia Steinberg for providing the algorithm for generating random expression sets and Helen Stolp for providing the brain tissue used for immunohistochemistry. This work was supported by the Medical Research Council (G00900901 and G0700377 to Z.M. and C.W.); a Wellcome Trust Studentship (to A.H.-S.); a Berrow Scholarship from Lincoln College, Oxford (to F.M.O.); a National Institute for Health Research Academic Clinical Fellowship (to M.L.K.); a Marshall–National Institutes of Health–Oxford Scholarship (to T.G.B.); the John Fell Fund (W.Z.W.); the Wellcome Trust Integrative Physiology Initiative in Ion Channels and Diseases of Electrically Excitable Cells (S.L.); and the European Union Seventh Framework Programme [241995, Project GENCODYS (C.W.)].

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