Differential Gene Expression between Sensory Neocortical Areas: Potential Roles for Ten_m3 and Bcl6 in Patterning Visual and Somatosensory Pathways

Adult neocortical areas are characterized by marked differences in cytoarchitecture and connectivity that underlie their functional roles. The molecular determinants of these differences are largely unknown. We performed a microarray analysis to identify molecules that define the somatosensory and visual areas during the time when afferent and efferent projections are forming. We identified 122 molecules that are differentially expressed between the regions and confirmed by quantitative polymerase chain reaction 95% of the 20 genes tested. Two genes were chosen for further investigation: Bcl6 and Ten_m3. Bcl6 was highly expressed in the superficial cortical plate corresponding to developing layer IV of somatosensory cortex at postnatal day (P) 0. This had diminished by P3, but strong expression was found in layer V pyramidal cells of somatosensory cortex at postnatal day (P) 0. Ten_m3 was expressed in a graded pattern within layer V of caudal cortex that corresponds well with visual cortex. Retrograde tracing and immunostaining showed that Ten_m3 is highly expressed along axonal tracts of projection neurons of the developing visual pathway. Overexpression demonstrated that Ten_m3 promotes homophilic adhesion and neurite outgrowth in vivo. This suggests an important role for Ten_m3 in the development of the visual pathway.

Keywords: arealization, cortex, development, microarray, somatosensory, visual

Introduction

The adult cortex comprises discrete areas associated with distinct functions. Each area is characterized by unique patterns of cytoarchitecture, connectivity within and between cortical and subcortical regions, and functional roles. Recent work has provided strong evidence (reviewed in Sur and Rubenstein 2005) that factors both intrinsic and extrinsic to the cortex regulate the patterning and connectivity of cortical areas.

During early cortical development, secreted molecules such as bone morphogenetic proteins, fibroblast growth factors (FGFs), and Wnt proteins are released from signaling centers at the margins of the developing cortical mantle (Shimogori et al. 2004). These morphogens are believed to act in a concentration-dependent manner and cause the graded activation or repression of transcription factors in the proliferative ventricular zone. The molecules responsible for the generation of the abrupt boundaries in cytoarchitecture and connectivity characteristic of the mature cortex are likely to include not only transcription factors but also cell surface and secreted molecules that directly guide the formation of connections. Indeed, mutations of the transcription factors Emx2 and Pax6, or alterations in FGF8 signaling, lead to changes in the expression of transcription factors (Tbr1, Id3, and COUP-TFI), axon guidance (ephrinA5 and EphA7), and adhesion molecules (cadherins 6 and 8) (Bishop et al. 2000; Mallamaci et al. 2000; Fukuchi-Shimogori and Grove 2001; Garel et al. 2003; Hamasaki et al. 2004; Shimogori and Grove 2005).

Interactions between membrane-bound molecules are thought to directly regulate numerous aspects of cortical organization and connectivity. Mutations in ephrinA5 lead to inappropriate innervation of somatosensory cortex by limbic thalamic nuclei (Bear et al. 1985; Uziel et al. 2002), and mutations in ephrinA5 and EphA4 lead to disruption of topography and areal specificity of thalamocortical projections (Dufour et al. 2003). The topographic specificity of corticothalamic projections is dependent on EphA7 (Torii and Levitt 2005). Mutations of ephrinA5 or EphA7 lead to a decrease in the size of somatosensory cortex (Miller et al. 2006), and several features of primary visual cortex are impaired in ephrinA2/A3/A5 triple mutants (Cang et al. 2005). A member of the L1 family of cell adhesion molecules is required for the development of normal cytoarchitecture in the visual cortex (Demyanenko et al. 2004).

Given the remarkable complexity of the cerebral cortex, it seems likely that a number of molecules, many as yet unknown, will play fundamental roles in establishing its exquisite patterns of connectivity. We reasoned that such molecules are likely to be differentially expressed between cortical areas at the time when corticopetal and corticofugal projections are forming. We thus performed a screen to identify molecules that are differentially expressed between 2 major sensory neocortical regions, primary somatosensory and visual areas, in newborn mice. We report the results of this analysis and the confirmation of a number of differentially expressed genes. Of particular interest, we report differential expression of 3 members of the Ten_m/Odz family of transmembrane proteins and show that at least one of these, Ten_m3, is expressed in an area and layer-specific pattern by projection neurons of the developing visual system. In addition, we show that Bcl6, a transcriptional repressor, is expressed by specific projection neurons in the somatosensory cortex.

Methods

All studies were performed on C57/Black6 mice and were approved by the animal ethics committees of Massachusetts Institute of Technology (MIT) and/or the University of Sydney.

Expression Analysis

Mice within 24 h of birth, designated postnatal day (P) 0, were anesthetized on ice, decapitated, and the brains removed. Curettes
Funatsu et al. (2004) or Sansom et al. (2005), but the difference in regions and/or ages (E11, E13, or E16 vs. P0) sampled prevents this from being used as direct confirmation of our data.

Note: Ubiquitin specific phosphatase 6 Usp6, Kelch repeat and BTB domain 9 Kbtbd9, Lim only domain 3 Lmo3, Leucine zipper protein 2 Luzp2, Protein tyrosine phosphatase receptor O PTPrO, Neuropeptide Y NPY, Transforming growth factor beta receptor 1 TGFbR1, B-cell leukemia 6 Bcl6, Immunoglobulin superfamily 4/syncam Igsf4a, T-shirt 3 Tshz3, Lim only domain 4 Lmo4, RAR orphan receptor beta RORb/Nr1f2, Neuronal differentiation 1 NeuroD1, Dickkopf 3 Dkk3, EphrinA5 Epha5, Tank binding protein 1 Tbp1, BAR orphan receptor beta RobbN1T2, Fibrinogen leucine rich 3 F1r3, Immunoglobulin superfamily 4/syncam Igsf4a, Mu-crystallin Crym, Neuropilin 1 Nrp1, Teneurin 2 Ten_m2, Teneurin 3 Ten_m3, Mi-crystallin Crym, Transforming growth factor beta receptor 1 TGFbR1, Protein tyrosine phosphatase receptor O PTPrO, Neuropeptide Y NPY, Ketch repeat and BTB domain 9 Kbtbd9, Leucine zipper protein 2 Luzp2, Lim only domain 3 Lmo3, Usp6.

Table 1

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Note: +, genes confirmed here by PCR; *, genes confirmed here by in situ hybridization and/or immunohistochemistry; #, reported as differentially expressed in manner similar to that found here by Funatsu et al. (2004) or Sansom et al. (2005), but the difference in regions and/or ages (E11, E13, or E16 vs. P0) sampled prevents this from being used as direct confirmation of our data.
a representative cDNA template over a concentration range of 3 log orders (data not shown), using the equation \( E = 10^\frac{1}{\log_10} \) (Rasmussen 2001). Statistical analysis was performed using the relative expression software tool (REST 2005 BETA V1.9.12) (Pfaffl et al. 2002) and pairwise fixed reallocation randomization test (Pfaffl et al. 2004). Differences were considered significant at a level of \( P < 0.05 \).

Investigation of Spatial and Temporal Expression Patterns of Selected Candidates

Differential expression of selected genes was also confirmed using in situ hybridization and/or immunohistochemistry. For in situ hybridization, 200-bp long sense and anti-sense dioxygenin (DIG)-labeled riboprobes were synthesized and hybridized to 15-μm thick cryostat sections of fresh frozen brain tissue using standard techniques. Staining was developed using peroxidase-tagged anti-DIG Fab fragments. The complete data set showing expression values for all the genes is posted at the Web site http://www.physiol.usyd.edu.au/~cathy/. Genes were analyzed according to 3 criteria: the pairwise comparison of increase/decrease calls, fold change, and the relative difference score (see Methods). The pairwise comparison gave a list of 145 transcripts that fulfilled this criterion. The expression values for these transcripts in comparison to the entire population are plotted in Figure 1a, and the complete list is available at the Web site. Of these, 45 were upregulated in visual cortex in comparison to somatosensory cortex, and the remainder were upregulated in somatosensory in comparison to visual cortex. Genes were also analyzed for mean fold change. A threshold of 1.4 was chosen, and it was found that 1050 transcripts fulfilled this criterion. Their expression values are plotted in Figure 1b, and the genes are listed at the Web site given above. A SAM was also performed using a delta threshold of 1.2. The plot of relative versus expected values of the relative difference scores is shown in Figure 1c. The expression values for the 44 genes that fulfilled this criterion are indicated on the scatter plot in Figure 1d and are listed at the Web site.

The three lists of transcripts identified by each method were partially overlapping and corresponded to a total of 1091 transcripts. The lists obtained from each method reflect the biases of each selection procedure (see Discussion). In the interests of maximizing sensitivity while minimizing false discovery rates (FDRs), it was decided to combine these forms of analysis. For follow-up analysis, we required that genes fulfill at least 2 of the 3 criteria used here and, in addition, exceed a minimum threshold of 1.5 for their relative difference score. We found that 135 transcripts fulfilled these criteria, including 11 genes that were identified by all analysis criteria (SAM, pairwise comparison, and fold change). The complete list of genes upregulated in visual and somatosensory cortices, along with their expression values, is available at the Web site; the expression values for these genes are also plotted in Figure 1e. Of these 135 transcripts, 51 were upregulated in visual cortex and 84 were upregulated in somatosensory cortex. A number of the transcripts identified corresponded to genes that were represented multiple times on the microarray, leaving a total of 122 differentially expressed molecules, comprising 45 (32 genes and 13 expressed sequence tags) which were more highly expressed in samples from visual cortex and 77 (60 genes and 17 ESTs) which were more highly expressed in samples from somatosensory cortex. A heat map indicating the relative

Results

Gene Expression in Visual and Somatosensory Cortex

Microarray Analyses

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Quantification of Images

Two or 3 images through the peak of the labeling each from 3 different GFP and Ten_m3-GFP-transfected animals (a total of 7 sections from GFP controls and 8 sections from Ten_m3-GFP cases) were analyzed quantitatively. Images were converted to 8-bit tagged image files. Background was subtracted in Image J (the National Institutes of Health [NIH]) using a rolling ball radius of 500. Brightness and contrast levels were normalized in Photoshop (Adobe), and the resultant images were thresholded in Image J. The pixel coordinates for each image were written to text files that were further processed using the image analysis toolbox in Matlab where the size of labeled patches was measured in pixels. In order to be considered in the analysis, patches had to exceed a minimum size of 60 pixels. This threshold was chosen as it approximates the smallest regions that were clearly identifiable as cells in our images. One pixel is approximately equivalent to 0.6 μm². Mean patch size, maximum patch size, and the number of patches exceeding thresholds of 200, 350, 500, and 1000 pixels were calculated to identify patches of label corresponding to multiple cells. Statistical analysis was performed using the Wilcoxon rank-sum test.

Cerebral Cortex
expression of the top 40 transcripts from each area as ranked by the relative difference score is shown in Figure 1f.

The identified genes included several that have previously been reported to be differentially expressed between somatosensory and visual cortex in early postnatal animals, such as ephrinA5, COUP-TfI (Nr2f1), RORb, Tbr1, Lmo3, and Lmo4 (Miyashita-Lin et al. 1999; Fukuchi-Shimogori and Grove 2001; Zhou et al. 2001; Bulchand et al. 2003; Garel et al. 2003). In addition, NeuroD1, Ten_m4, Bcl6, mu-crystallin (Crym), and TGFb1 have been reported to be differentially expressed between cortical regions at earlier developmental stages (Funatsu et al. 2004; Sansom et al. 2005). Although this cannot be taken as direct confirmation of our results due to the different ages sampled, the expression patterns previously reported are consistent with those found here. Together, the data indicate success of the assay and analysis method in identifying differentially expressed genes. In addition, a large number of genes not previously reported as being differentially expressed were also identified. These included a number of transcription factors including Bblbb2, Foxp1, Lbd2, and Luzp2. A number of genes associated with axon guidance such as neuropilin 1 (Nrp1), semaphorins 3C and 7A, and protein tyrosine phosphatase...
receptor O (PTPrO), which has recently been shown to modulate Eph receptor activity (Shintani et al. 2006), were identified as well. Table 1 provides a summary of the genes identified here as differentially expressed that have been confirmed either here (see below) or in other studies.

Molecules associated with specific morphogen signaling pathways, for example, 3 molecules associated with the transforming beta growth factor (TGFβ) pathway—TGFβ receptor 1 (TGFβr1), Mad homolog 1 (Smad1), and zinc finger homeobox b1 (Zfhxb1) transcription factor—were all upregulated in somatosensory cortex. Phosphorylation of TGFβr1 signals Smad proteins to translocate to the nucleus where they activate transcription (reviewed in Charron and Tessier-Lavigne 2005). Fibronectin leucine-rich transmembrane protein 3 (Fltr3) and Dickkopf 3 (Dkk3) were both upregulated in visual cortex. Fltr3 is associated with FGF signaling and can promote homophilic adhesion and neurite outgrowth (Tsuij et al. 2004; Haines et al. 2006; Karaulanov et al. 2006). Dkk3 is member of the Dickkopf family, which are secreted regulators of Wnt signaling (Brott and Sokol 2002). Although typically associated with the regulation of cell fate, a number of morphogens have recently been shown to also play roles in axon guidance (Charron and Tessier-Lavigne 2005). A number of adhesion molecules including pCAS130 (BCar1), immunoglobulin superfamily member 4a (Igolf4a or Syncam), cadherin 4, protocadherins 9 and 17, and plakophilin 4 were differentially expressed. Also of interest was the fact that 3 teneurin genes (Ten_m/Odz2, 3, and 4), which encode members of a family of transmembrane proteins, were all identified as being more highly expressed in visual cortex than somatosensory. Another recent study has also implicated these genes in arealization by showing that they may act downstream of Emx2 in defining caudal cortical regions (Li et al. 2006).

Real-Time PCR Analyses

Real-time PCR was used to confirm differential gene expression. Twenty genes and ESTs (10 each from somatosensory and visual cortical regions) that correspond to a total of 27 identified transcripts (20% of the total number of transcripts identified) and that spanned the range of values for fold change (from 1.4 to 6.2) and relative difference scores (from 1.7 to 21) were chosen. Genes chosen were predominantly not those previously reported as differentially expressed so as to provide novel data; a few of those previously reported were included as an independent control, however. Many of the genes chosen are associated with developmental processes and/or encode cell surface or extracellular proteins. All of the genes tested showed higher expression in the cortical region from which they were identified, consistent with the microarray analysis, and for 19 of the 20 genes, the differences were statistically significant (P < 0.05; pairwise fixed random reallocation test). The results are presented in Figure 2. The gene tested for which a significant difference in expression between the samples from somatosensory and visual cortex was not found was opioid cell adhesion molecule (Opm1/Obacm). The fact that 19 of the 20 genes (corresponding to 25 of the 27 transcripts) tested here were confirmed as significantly different, in addition to a number which have been independently confirmed by other studies (see above), indicates that the analysis used here reliably identified differentially expressed genes. In some cases, the fold change as determined by real-time PCR was notably higher than that suggested by the microarray. For example, it was found that Ten_m3 and Bcl6, which had mean fold changes of 1.7 and 2.7, respectively, according to the microarray analysis, both had fold changes of almost 8-fold according to the real-time PCR analysis. The observation that fold change as determined by the microarray analysis in many cases underestimated the fold change determined by quantitative PCR was also made in a recent survey of differences in gene expression between cortical neuron subtypes (Sugino et al. 2006). A plot showing the expression values as determined by the microarray analysis for the transcripts confirmed here and which highlights Ten_m3 and Bcl6 is shown in Figure 2c.
**Bcl6 Expression**

Based on this analysis, 2 genes were selected for further investigation of their spatiotemporal expression patterns: Ten_m3 and Bcl6. Each of the genes met the microarray criteria for differential expression and had the highest quantitative PCR expression levels of all analyzed visual or somatosensory cortex genes. Bcl6 is a transcription repressor, mutations of which are associated with B-cell lymphomas (Ye et al. 1993). In situ hybridization for Bcl6 confirmed the differential expression of this gene along the rostrocaudal axis of the neocortex at P0, with strong expression in the superficial region of the cortical plate in a position that is consistent with the position of the developing somatosensory cortex (Fig. 3a). Expression was strongest in the superficial region of the cortical plate that corresponds predominantly to layer IV cells at this stage of development (Caviness 1982) though some fainter expression was also seen deep to this in the developing layer V. Importantly, no expression was seen in caudal neocortex corresponding to the position of visual cortex. Expression was, however, also seen in hippocampus, subiculum, and globus pallidus. The mediolateral distribution of label seen in a coronal section also corresponds well to layer IV of somatosensory cortex (Fig. 3b).

To determine whether the differential expression of Bcl6 expression is maintained at later developmental stages, expression in older animals was also investigated. At P3, expression in the somatosensory cortex had decreased to a level that was barely detectable using this technique (not shown) although expression was clearly visible in the hippocampus and subiculum of the same sections, suggesting the lack of expression in somatosensory cortex reflected a real decrease in expression levels in this region. Expression at P7 was strikingly different with expression in a subset of neurons in layer V of the rostral 2/3 of cortex (Fig. 3c). Strong expression was also present in the CA1 region of hippocampus and the subiculum. The robust expression in layer V was maintained in the adult. Interestingly, expression appeared to be associated with large pyramidal cells, suggesting the gene may be associated with large projection neurons in layer V. To investigate this possibility, retrograde tracing from the spinal cord, a major target of layer V pyramidal cells from the rostral 2/3 of cortex, was performed at P10 and analyzed at P14. The results of this analysis are presented in Figure 4. It was found that many of the retrogradely labeled corticospinal neurons (Fig. 4a) also expressed Bcl6 (Fig. 4a’,a”). Thus, Bcl6 is expressed by long-range projection neurons in layer V.

**Ten_m3 Expression**

We also investigated the expression pattern of Ten_m3. Ten_m3 belongs to a family of 4 homodimeric transmembrane proteins (Oohashi et al. 1999; Feng et al. 2002). In situ hybridization confirmed the results of the microarray analysis and revealed a remarkably restricted expression of Ten_m3 in the caudal region of cortex that correlates well with the position of developing visual cortex (Fig. 5a). Interestingly, expression was restricted not only in terms of region but also with respect to layer and was seen predominantly in the developing layer V (this is the region immediately deep to the densely packed undifferentiated superficial region of the cortical plate which contains mostly layer IV cells at this stage; Caviness 1982; Auladell et al. 2000). Immunostaining for Ten_m3 demonstrated that the protein showed a similar
distribution in terms of rostrocaudal extent as revealed by the in situ hybridization, but in addition to the staining in layer V, thin strands of label were seen to transverse layer VI and strong staining was seen in the intermediate zone (white matter) and in the internal capsule (Fig. 5). Together, these results suggest that Ten_m3 is expressed along the axons of cells projecting from the visual cortex. Because Ten_m3 was also observed in the dorsal lateral geniculate nucleus at this stage (not shown but see Fig. 6a), the immunostaining observed in the white matter may also reflect expression of Ten_m3 along geniculocortical axons.

We further investigated the expression pattern of Ten_m3 using in situ hybridization during the first postnatal week (Fig. 6). A similar pattern of expression to that seen at P0 was observed in sagittal sections at P3 with high expression in layer V of caudal cortex (Fig. 6a,b). At this stage of development, pale staining could also be barely discerned in layer IV of slightly more rostral cortex that likely corresponds to the developing somatosensory cortex (see inset Fig. 6a). This was much fainter than that seen in caudal cortex, however. Expression was also observed in the dLGN, subiculum, and a subregion of CA1; patches of staining were seen in the striatum. Strong staining was also seen in the medial entorhinal cortex (not shown). We wished to determine how the expression of Ten_m3 correlates with the visual cortex. For this, transneuronal tracing with WGA-HRP was performed to label geniculocortical terminals (Fig. 6c). The rostrocaudal distribution of label in the cortex corresponded remarkably well the region of strong expression in layer V of caudal cortex, suggesting that visual cortex does indeed express the gene. Interestingly, we also observed that there appears to be a gradient of Ten_m3 expression in visual cortex that is highest caudally and diminishes rostrally (Fig. 6a). We also examined Ten_m3 staining in coronal sections and again found that the distribution corresponded well with visual cortex, though some label was also observed more laterally suggesting that the gene may be expressed by more lateral regions including area 18 (Fig. 6a,e). The expression pattern described above was maintained until at least P7, but had begun to decline by P14 (not shown). Faint expression was observed in both layers V and VI of caudal cortex in the adult (not shown but see, e.g., the Allen Brain Atlas: http://www.brain-map.org/welcome.do).

**Figure 4.** Bcl6 is expressed in corticospinal neurons by P14. (a) Corticospinal neurons retrogradely labeled with CTB injected into the thoracic spinal cord at P10. (a’) The same section as in a photographed to reveal the in situ hybridization signal for Bcl6. (a”) An overlay of (a’) and (a”) shows that all of the retrogradely labeled corticospinal neurons express high levels of Bcl6. Arrows highlight the same cells in all 3 images. Scale bar: 100 μm.

**Figure 5.** Ten_m3 expression pattern confirms microarray analysis. (a) In situ hybridization for Ten_m3 (green) in a sagittal section at P0. In situ signal (green) is shown superimposed on a fluorescent nuclear counterstain (blue). Expression is highly localized to caudal cortex that corresponds well to the position of visual cortex. The signal is further localized to developing layer V. (b) Immunostaining for Ten_m3 from a nearby section to that shown in (a) confirms that the protein is expressed by cells in layer V of caudal cortex. Fine bands of label are also visible in layer VI, and label in the developing white matter suggests that the protein is expressed on axons growing to and/or from visual cortex. Scale bars: 500 μm. D: dorsal; R: rostral.
We wished to determine if Ten_m3 is expressed by projection neurons of layer V. To do this, we labeled cells retrogradely from structures that are major output targets of layer V of visual cortex, the ipsilateral superior colliculus and the contralateral visual cortex, and performed in situ hybridization for Ten_m3 (Fig. 7). Following injections of a retrograde tracer (CTB) into the superior colliculus, most of the labeled neurons expressed Ten_m3 clearly above background levels, thus suggesting that the gene is expressed by corticocortical projection neurons (Fig. 7a–a′). Retrograde tracing of another major output of layer V, the callosal projection, produced more equivocal results. The labeled callosal neurons were not Ten_m3 positive, suggesting the gene may be differentially expressed between these populations (Fig. 7b–b′). Given the nature of this analysis, however, it is not possible to definitively determine whether this is a consistent difference between the populations of neurons. Injections of CTB into visual cortex also resulted in labeling in dLGN (Fig. 7c). Although we cannot rule out the possibility that a proportion of this label represents corticogeniculate terminals, its appearance is strongly suggestive of somata and primary dendrites, which also express Ten_m3 (Fig. 7c′), indicating that geniculocortical neurons also express Ten_m3 (Fig. 7c′). These results therefore indicate that Ten_m3 is expressed in one or more subsets of projection neurons of the developing visual system, including the corticocortical projection and the geniculocortical projections.

Ten_m3 and Cell Adhesion

No functional role has been reported for Ten_m3, although other members of the Ten_m gene family encode transmembrane glycoproteins that are homophilic and homodimeric (Oohashi et al. 1999; Feng et al. 2002; Rubin et al. 2002), suggesting that Ten_m3 may also mediate cell adhesion. As a first step to determining a role for Ten_m3 in vivo, we examined the effects of localized overexpression of the gene using in utero electroporation. The full-length Ten_m3 construct was cloned into a mammalian expression vector downstream of an IRES-GFP site. The same vector with the IRES-GFP site only was used as a control. In preliminary experiments, GFP-only or Ten_m3-GFP was transfected into primary dissociated cortical cultures; in situ hybridization (not shown) and immunostaining revealed that Ten_m3 was being produced by the transfected cells. Confocal analysis revealed that Ten_m3 was being appropriately targeted to the membrane (Fig. 8a–a′).

In utero electroporation was used to transfected neurons in neocortex. Transfection with the control GFP-only construct on E14 resulted in the presence of a large cohort of GFP positive cells in layer IV (Fig. 8b,c). Transfection with Ten_m3-GFP typically resulted in GFP positive cells that appeared markedly different compared with GFP transfection alone (Fig. 8d,e). Rather than cells that were distributed in a seemingly random fashion within the transfected region as in controls, cells transfected with Ten_m3-GFP were typically grouped in clumps or clusters. At higher power, it can be seen that these clusters consist of groups of GFP positive somata (Fig. 8e) and their processes that are also intertwined. This effect was very consistent and markedly different from controls, where cells and their processes are clearly separate from each other. The effect on cellular clustering was quantified by measuring the size of GFP positive patches in thresholded images. Three parameters—mean patch size, maximum patch size, and the number of patches greater than threshold value which was larger than an average patch of label from control animals (the latter was treated both as an absolute number and as a proportion of the total number of patches)—were significantly greater (P < 0.01; Wilcoxon rank-sum test) from material from animals transfected with Ten_m3-GFP compared with GFP controls. These results are presented in Table 2. The clustering of cells made it difficult to ascertain whether there were also changes in the

Figure 6. Ten_m3 expression is maintained during the first postnatal week. (a) In situ hybridization for Ten_m3 in a sagittal section at P3. A fluorescent nuclear stain for the same section is shown in (b). High expression is maintained in layer V of caudal cortex (approximate boundaries indicated by small arrows) where it appears to be in a high caudal to low rostral gradient. Expression is also high in dLGN (arrow). By this stage, faint expression can just be discerned in layer IV of slightly more rostral cortex consistent with the position of somatosensory cortex (arrowheads). This region is shown in more detail in the inset. High expression is also visible in subiculum (Sub.), a subregion of CA1, and patches within striatum (Str.). (c) Transneuronal labeling of the geniculocortical projection (small arrows) shows that the rostrocaudal extent of projections from visual thalamus matches well with the distribution of Ten_m3 expression within caudal cortex, suggesting that Ten_m3 is expressed in visual cortex. (d–e) In situ hybridization for Ten_m3 (d) and corresponding fluorescent nuclear stain (e) in a coronal section through visual cortex at P3. The mediolateral distribution of Ten_m3 staining correlates well with visual cortex although expression is also seen in more lateral regions suggesting that it may also be expressed in area 18 and other caudolateral cortex. Scale bars: 600 μm in (a–e) represents 250 μm in inset of (a).
morphology of individual neurons at this stage of development. A case where Ten_m3-GFP was transfected at E13 to target layer V cells is shown in Figure 8f. Again cells and their processes are clustered together. In this instance, where very large numbers of cells were transfected, the migration of the GFP positive neurons seems to have been delayed. This is evidenced by the fact that, unlike control cases where transfected cells are all aligned within a distinct lamina by P8 (Fig. 8b), in Ten_m3-GFP-transfected cases (Fig. 8d), some labeled cells are still present in the ventricular zone. In Figure 8f, numerous clusters of labeled cells are clearly visible throughout the depth of the cortex, suggesting that these cells are still migrating to their destination. We suggest that this delay is probably a consequence of the overexpression of an adhesive molecule rather than an indication that Ten_m3 normally plays a major role in neural migration. These results are consistent with the suggestion that Ten_m3 promotes adhesive interactions between cells that express it.

The long-term effects of overexpression of Ten_m3 were also examined in animals that were transfected at E15 and allowed to survive till adulthood. Although GFP was barely visible under the fluorescence microscope at this stage, immunostaining for GFP revealed that the protein was still present in significant quantities in material from control (Fig. 9a,b) and Ten_m3-GFP-transfected (Fig. 9c,d) animals. There was no evidence of inappropriate laminar positioning of cells at this stage, suggesting that if migration had been affected in these animals that it had not impacted the final position of the cells to any significant degree. The prominent clustering apparent at early stages was also not apparent. There was however a dramatic increase in the number of labeled neurites visible in the material from Ten_m3-GFP-transfected mice compared with controls. This is most apparent at high power (compare Fig. 9b,d). Due to the high density of labeled processes in Ten_m3-GFP-transfected material, it was not possible to reliably quantify changes in morphology at the single neuron level. Instead, quantification of the changes in neurite outgrowth was performed at the population level. This was based on a threshold analysis of labeled material in each image which showed that there was an almost 3-fold increase in the proportion of the image that contained labeled cells and processes in Ten_m3-GFP-transfected animals (38.9 ± 9.3%; mean ± standard deviation [SD] for n = 8 sections from 2 animals) compared with GFP alone (13.5 ± 5.6%; mean ± SD.

Figure 7. Ten_m3 is expressed in projection neurons of the developing visual system. (a–a’) Section through visual cortex at P5 photographed to show retrogradely labeled corticocollicular neurons (a) and Ten_m3 mRNA (a’). The images are merged in (a”) to show the relationship between the staining patterns. The same cells are indicated by arrows in all 3 images. The retrogradely labeled corticocollicular neurons are highly Ten_m3 positive. (b–b”) As for (a–a”), but following retrograde labeling of the callosal projection. Retrogradely labeled callosal neurons (b) are not highly Ten_m3 positive (b’, b”). (c–c”) Section through the dLGN showing that retrogradely labeled geniculocortical neurons (c) express Ten_m3 (c’, c”). In some cells, highlighted by arrows, Ten_m3 mRNA and the retrograde tracer can be seen in primary dendritic processes. Scale bars: 25 μm.
This difference ($P < 0.05$; $t$-test) is not due to differences in the numbers of labeled cells in the images, which were almost identical between the 2 sets of images: the thresholded proportion of each image per transfected cell was $3.3 \pm 1.4$ for control and $9.3 \pm 1.5$ for $\text{Ten}_m3$-GFP-transfected cases ($P < 0.05$; $t$-test). Qualitatively, similar effects on neurite outgrowth were observed regardless of area or layer transfected, suggesting that $\text{Ten}_m3$ can strongly promote neurite outgrowth in many types of neurons.
Table 2
Quantification of the effect of Ten_m3 on cellular clustering in cortical cells from P7-8 mice that were transfected with GFP-only or Ten_m3-GFP in utero

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GFP control</th>
<th>Ten_m3-GFP</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean patch size</td>
<td>182.6 ± 15.0</td>
<td>499.5 ± 56.4</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Maximum patch size</td>
<td>157.9 ± 303</td>
<td>16237 ± 3142</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Number patches &gt; threshold</td>
<td>7.7 ± 2.1</td>
<td>22.9 ± 3.2</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Percentage patches &gt; threshold</td>
<td>8.4 ± 1.8%</td>
<td>18.6 ± 1.4</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

Note: Numbers represent numbers of pixels (mean ± standard error) that appear as a continuous patch when thresholded from images through the peak of the GFP or Ten_m3-GFP transfections. A pixel represents approximately 0.6 μm². Two or 3 sections each from 3 different animals were quantified in each case. Probabilities were calculated using the Wilcoxon rank-sum test. Thresholds for the data shown here were set at 500 pixels, 2–3 times larger than average patch size in control animals to identify clustered cells. Significantly different results for the GFP and Ten_m3-GFP-transfected cases groups were also obtained for thresholds set at 200, 350, and 1000 pixels. All measurements show patch size is significantly higher in material from Ten_m3-GFP-transfected cases compared with control, suggesting that Ten_m3-GFP-transfected cells are clustered much more frequently than GFP-transfected cells.

Discussion

The aim of this study was to identify genes that are differentially expressed between neocortical areas in neonatal mice. The day of birth was chosen because this is a time when many corticopetal and corticofugal projections—some of the key defining features of the nascent cortical areas—are forming. The 122 molecules identified here are thus candidates for playing a role in this process or other aspects of cortical organization. The success of our approach is indicated by the presence of most molecules previously reported to be differentially expressed between rostral and caudal cortex in neonatal mice in our list of candidate genes. The differential expression between cortical areas of many of the genes identified here is novel, however, and we anticipate that this will provide a useful framework for investigations of molecular determinants of cortical patterning and connectivity. It was not feasible to study the spatiotemporal expression of all the candidates. Our PCR data, however, strongly suggest that a large proportion of the genes identified here are indeed differentially expressed. Further, the results obtained for Bcl6 and Ten_m3 demonstrate that genes identified are differentially expressed between somatosensory and visual cortex. The expression pattern of Ten_m3 within visual cortex makes it a particularly strong candidate for future studies.

Microarray Analysis

The ability to perform rapid genome-wide screens is enormously powerful yet presents its own difficulties, particularly in terms of analysis. Standard statistical tests, such as the t-test, are not reliable when applied to 3–4 repeats of around 36 000 transcripts. The SAM was developed to circumvent some of these difficulties (Tusher et al. 2001) and has been used successfully by recent studies (Sansom et al. 2005; Tropea et al. 2006). This analysis is highly effective at identifying genes with low variability in their absolute expression levels across replicates as determined by the microarray analysis but tends to miss transcripts with variability in absolute expression even if they show consistent relative changes. In addition, the sample preparation and analysis procedures are not immune to error. Thus, the application of stringent criteria based on variability will tend to miss significant numbers of genes that are differentially expressed. Reducing the stringency of the criteria in an unbiased manner will however increase the FDR to high levels (Tusher et al. 2001). The pairwise comparison is sensitive to changes in relative expression levels between pairs of samples from different regions but tends to miss small changes. Fold change gives a measure of the relative expression levels but does not take variability between replicates into account. Consequently, it was decided to combine these approaches and require genes to fulfill at least 2 of the 3 criteria in combination with a minimum cutoff for the relative difference score. This proved highly successful at identifying differentially expressed transcripts, with 95% of genes tested confirmed as differentially expressed. Although similarly high success levels may have been achieved using the SAM analysis alone, many genes whose differential expression was confirmed (e.g., Ten_m3, Ten_m2, Bcl6, Lmo4) would have been missed unless the SAM criteria were dropped to levels corresponding to >50% FDR. In a study where Affymetrix criteria were used on their own, only around 50% of the identified genes were confirmed (Funatsu et al. 2004). We suggest that the combination of these forms of analysis provides a reliable and sensitive approach.

In addition to the microarray analysis, a major potential source of error here is the accuracy of the dissections. The regions chosen were based on preliminary tracing experiments that labeled appropriate thalamic nuclei. The confirmation of expression patterns by in situ hybridization suggests these were largely accurate. The possibility that cortical regions adjacent to somatosensory and visual cortex may have been included in some dissections cannot however be excluded. Although we estimate that 90% or more of the tissue included was indeed from the target regions, it is possible that some of the genes identified here may be differentially expressed between occipital and parietal regions rather than visual and somatosensory cortices per se. Because the development of the visual cortex is delayed with respect to the development of the somatosensory cortex by around 1 day in rodents (Bayer and Altman 1991), it is possible that some of the genes identified could reflect developmental rather areal differences. Our assay used tissue containing heterogeneous populations of cells. It is possible, therefore, that some differences between subtypes of cells from different areas may have been masked. The fact that we identified genes which are differentially expressed within specific laminae suggests that our assay was sufficiently sensitive to detect neurons differentially expressed within specific layers; it would be of interest to further refine this study to investigate lamina or neuron subtype specific differences (Arlotta et al. 2005; Christophe et al. 2005; Sugino et al. 2006) between cortical areas during development.

The validity of our analysis is supported not only by the fact that almost all of the genes whose differential expression was tested were confirmed but also by the presence of most genes previously found to be differentially expressed between rostral and caudal neonatal cortex in our screen. These include RORβ, Tbr1, ephrinA5, COUP-TFI1, Lmo3, and Lmo4 (Miyashita-Lin et al. 1999; Fukuchi-Shimogori and Grove 2001; Zhou et al. 2001; Bulchand et al. 2003; Garel et al. 2003). There are however a few genes that seem conspicuous by their absence, most notably cadherin 8 (Cad8) and EphA7. Although EphA7 is consistently reported as expressed in visual cortex, its expression is not limited to this region (Miller et al. 2006) and shows a graded expression pattern within visual cortex (Cang et al. 2005). Cad8 has been used by a number of studies as a marker for visual cortex (Fukuchi-Shimogori and Grove 2001; Hamasaki et al. 2004; Cang et al. 2005), so its absence in our analysis was of
some concern. However, data presented in a recent study (Miller et al. 2006) show a surprisingly uniform distribution of Cad8 along the rostrocaudal axis of the cortex; its absence in our analysis is consistent with this.

The genes selected for confirmation by real-time PCR spanned a range of relative difference scores to test the validity of our analysis. Many of the genes tested were chosen because they encode cell surface or extracellular proteins and/or are associated with developmental processes. Of particular interest was the differential expression of genes associated with signaling pathways of morphogens as well as adhesion and axon guidance molecules (see Results). A few of the other genes identified here (Lmo4, Crym, Dkk3, and S100a10) were also identified by Arlotta et al. (2005); differences in experimental design make it difficult to make useful comparisons between the results.

**Bcl6**

Our demonstration that Bcl6 is expressed in corticospinal neurons by P14 is consistent with the work of Arlotta et al. (2005). Our data also show, however, that expression of Bcl6 in developing cortex is highly dynamic. A dynamic pattern of Bcl6 expression has also been found in the olfactory epithelium (Otaki et al. 2005). These authors proposed that Bcl6 may play a role in the terminal differentiation of olfactory sensory neurons consistent with its role in the differentiation of germinal center B cells (Dent et al. 1997; Fukuda et al. 1997; Ye et al. 1997). A role for this gene in cortical development is yet to be determined. The high level of expression of Bcl6 in corticospinal neurons that is maintained into adulthood is particularly intriguing and warrants further investigation.

**Ten_m3**

Three members of the Ten_m family were identified as more highly expressed in visual cortex. The Ten_ms encode a highly conserved family of 4 type II transmembrane glycoproteins that are the vertebrate homologs (Oohashi et al. 1999) of the late-acting Drosophila pair-rule gene Ten_m/Odz (Baumgartner et al. 1994; Levine et al. 1994). Expression patterns in the developing mammalian embryo (Zhou et al. 2003) suggest important roles during early development. Ten_ms 1 and 2 are expressed in complimentary patterns in the developing avian visual system, and roles in adhesion and neurite outgrowth have been reported in vitro (Rubin et al. 1999, 2002). Other studies have shown that the intracellular domains can be cleaved and translocate to the nucleus where interactions with zic1 (Bagutti et al. 2003) and methyl binding domain 1 and CAP/ponsin (Nunes et al. 2005) have been reported. The carboxy terminal has also been found to produce a neuromodulatory peptide (Wang et al. 2005; Tucker and Chiquet-Ehrismann 2006). This information, combined with the fact that Ten_m3 showed the greatest fold change in expression between somatosensory and visual cortex in the real-time PCR analysis, led us to investigate the expression pattern and potential role of Ten_m3 in some detail. A recent study has reported that Ten_ms 2, 3, and 4 are downstream targets of Emx2 signaling (Li et al. 2006). Our independent discovery of their differential expression in visual cortex is largely in agreement with these observations and highlights the success of our screen in identifying previously unknown arealization candidates. Our characterization of Ten_m3 also provides considerable novel data on this intriguing but currently little known family of molecules.

Our in situ hybridization and immunohistochemistry data show that Ten_m3 is not only differentially expressed between somatosensory and visual cortex but also highly expressed by

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**Figure 9.** (a–d) GFP immunostaining in low (a, c) and high (b, d) power images of sections through cortex from adult mice that were transfected with GFP (a, b) or Ten_m3-GFP (c, d) in utero. Although similar numbers of cells are labeled in each image, there is a dramatic increase in neurite outgrowth in material from Ten_m3-transfected animals. This increase was highly consistent and was independent of region or layer transfected. Scale bars: (a) 50 µm, applies to (c); (b) 20 µm, applies to (d).
specific subsets of neurons within this region, most notably the
corticoocular projection neurons of layer V of visual cortex.
Our data also suggest that it is expressed by other projection
eurons of the developing visual system, such as the geniculo-
cortical projection. Most interestingly, immunohistochemistry
showed that the protein is expressed along the trajectories of
growing axons, suggesting a potential role in axon targeting.
Our data also show that Ten_m3 strongly promotes both homophilic adhesion and neurite outgrowth in vivo. To our
knowledge, this represents the first demonstration of a role for
any Ten_m in vivo in vertebrates. Together, these data suggest
important roles for Ten_m3, and potentially other members of the
Ten_m family, in mediating patterns of connectivity in the
developing mammalian visual system.

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

Notes
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Address correspondence to Catherine A. Leamey, Department of
Physiology, F13, University of Sydney, Sydney NSW 2006, Australia.
Email: cathy@physiol.usyd.edu.au.

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