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Identification of candidate genes at the corticoseptal boundary during development

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Abstract

Cortical midline glia are critical to the formation of the corpus callosum during development. The glial wedge is a population of midline glia that is located at the corticoseptal boundary and expresses repulsive/growth-inhibitory molecules that guide callosal axons as they cross the midline. The glial wedge are the first cells within the cortex to express GFAP and thus may express molecules specific for glial maturation. The corticoseptal boundary is a genetically defined boundary between the cingulate cortex (dorsal telencephalon) and the septum (ventral telencephalon). The correct dorso-ventral position of this boundary is vital to the formation of both the glial wedge and the corpus callosum. Our aim was to identify genes expressed specifically within the glial wedge that might be involved in either glial differentiation, formation of the corticoseptal boundary or development of the corpus callosum. To identify such genes we have performed a differential display PCR screen comparing RNA isolated from the glial wedge with RNA isolated from control tissues such as the neocortex and septum, of embryonic day 17 mouse brains. Using 200 different combinations of primers, we identified and cloned 67 distinct gene fragments. In situ hybridization analysis confirmed the differential expression of many of the genes, and showed that clones G24F3, G39F8 and transcription factor LZIP have specific expression patterns in the telencephalon of embryonic and postnatal brains. An RNase Protection Assay (RPA) revealed that the expression of G39F8, G24F3 and LZIP increase markedly in the telencephalon at E16 and continue to be expressed until at least P0, during the period when the corpus callosum is forming.

Keywords: Midline glia; Glial wedge; Axon guidance; Differentiation; Patterning; Differential display PCR; tcf4; Zinc finger protein 288

The corpus callosum is the largest commissure in the brain, connecting neurons in the right and left cerebral hemispheres. Callosal axons first cross the midline at embryonic day 15.5 (E15.5) in mouse (Ozaki and Wahlsten, 1992; Rash and Richards, 2001) with fibers continually added from E16 to P0. Cortical midline glia are critical to the formation of the corpus callosum (Silver et al., 1982; 1993; Shu and Richards, 2001). Such glia form at the boundary between the septum and the cingulate cortex in a region known as the corticoseptal boundary. The corticoseptal boundary is both a morphological and genetic boundary between dorsally expressed genes such as Emx1 and 2 and ventrally expressed genes such as vax2 and Dlx. One glial population at the corticoseptal boundary is the glial wedge (GW) which is part of the radial glial scaffold of the cortex but expresses GFAP prior to any other region of cortex (Shu et al., 2003a). Furthermore, the differentiation of the GW from radial glia into glial fibrillary acidic protein positive astrocytes suggests that these cells may be among the first cells in the cortex to differentiate into mature glia and thus we wanted to learn more about the molecular profile of these specialized midline glia. The glial wedge also expresses the chemorepulsive molecule slit2, required for callosal axon guidance (Shu and Richards, 2001; Shu et al., 2003a,b). However, in a number of different mouse mutants that display agenesis of the corpus callosum and disruption of the glial wedge, Slit2 is still expressed and repels cortical axons in vitro (Shu et al., 2003c; Shen et al., 2002) indicating that additional molecules may be expressed by the glial wedge that could guide callosal axons at the midline.

To understand what makes these GW cells unique, whether they express additional guidance molecules, and how the corticoseptal boundary forms, we undertook a differential

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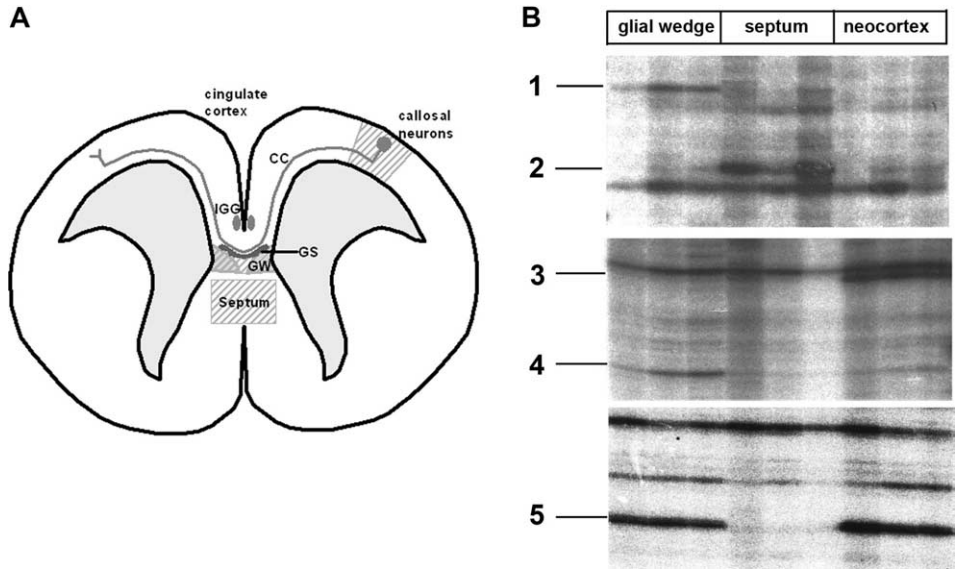

Fig. 1. Isolation of differential expressed bands. (A) Schematic of a coronal section of E17 brain, showing the regions (hatched boxes) where tissues were collected for DD-PCR. CC, corpus callosum; GW, glial wedge; GS, glial sling; IGG, indusium griseum glia. (B) A sequencing gel displaying differentially expressed bands. Total RNA for DD-PCR was isolated from the GW, septum and neocortex of E17 mouse brains. ^{32}P-labeled PCR products were separated on 6% denaturing polyacrylamide gels. To minimize false positives, each reaction was performed in triplicate from three independent preparations of total RNA. Examples of bands expressed only in the GW (1), septum (2) or neocortex (3) are shown as well as bands expressed in both the GW and the neocortex (4, 5). Only bands that were highly expressed in the GW (1, 4) were excised, reamplified and ligated into the pBluescript vector.

display PCR (DD–PCR) screen to identify genes specifically expressed within this region. The results of this screen identified genes previously known or predicted to be expressed in the glial wedge such as the glial-expressed gene vimentin and the transcription factor Nfib (Steele-Perkins et al., 2005), however we did not identify any genes specific to the boundary between the septum and the cingulate cortex nor any secreted molecules (potentially axon guidance ligands) expressed by the GW using this method.

1. Results and discussion

1.1. Identification of candidate genes expressed by the glial wedge

We used DD–PCR to isolate genes from E17 brains that were expressed at high levels in the GW compared with control tissues (septum and neocortex) which were chosen because they lie anatomically close to the GW but do not form part of the corticoseptal boundary (Fig. 1(A)). Genes selected for further analysis were highly expressed in the GW but not in the septum, and either not expressed or expressed at very low levels in the cortex of E17 brains. To perform the DD–PCR, ten base pair primers of arbitrary sequence were used to generate first strand cDNA to be used for the PCR reaction. ^{32}P-labeled DD–PCR products generated from the GW, neocortex and septum were separated on a 6% denaturing polyacrylamide gel. Though many differential display bands were found to be highly expressed in either the cortex or septum samples (Fig. 1(B)), we did not pursue these genes further because they were not the focus of our study. Differential bands that were expressed at a higher level in the GW were excised from the polyacrylamide gel (Fig. 1(B)), re-amplified and subcloned into the pBluescript SK vector. The sequences of the inserts were compared with the Genbank database using the BLAST search engine. Using 200 primer combinations, we identified 67 differential display fragments with sizes ranging from 120 to 800 bp. These cDNA clones could be grouped according to their expression patterns. The first group had a widespread pattern of expression in the brain but with higher levels in the midline region and ventricular zone (VZ)/subventricular zone (SVZ) of the telencephalon (data not shown). Preliminary analysis of these genes using BLAST suggested that they likely represent genes with more general cell transcription or 'housekeeping' roles. The second group had restricted expression patterns with high levels at the midline, VZ/SVZ, and/or other specific regions. This category of genes encoded different types of proteins including cytoskeletal/process outgrowth related proteins, extracellular matrix and adhesion molecules, transcription factors, cell surface receptors, intracellular signaling

Table 1
Overview of genes identified by DD–PCR

Number of DD–PCR primer combinations	200
Number of sequencing gels	44
Number of identified genes	67
Cytoskeletal and related molecules	4
Extracellular matrix and adhesion molecules	3
Transcription factors	12
Cell surface receptors	5
Intracellular signaling molecules	12
DNA-binding/chromosome structure genes	8
Miscellaneous genes	5
Genes without significant homologues	12
Total	67

Table 2
Differential expression of cDNA fragments and homologs identified

DD–PCR fragment	Identity	Genebank accession number
Extracellular matrix and adhesion molecules (3)		
G4F1	Tenascin C gene, hexamarchion (HXB)	XM_005348, X56160
G4F7	Mouse fat 1 cadherin; protocadherin	AJ250768, AF100960
G30F6	Mus musculus mRNA for L1 protein	NM_007697, X94310
Cytoskeleton and related molecules (4)		
G4F3, G18F1	Vimentin	NM_011701, X56397
G6F2, G18F3	Acid calponin 3	NM_019359, NM_028044
G9F8	Mouse ankyrin (epithelia specific Ank-3)	NM_009670
G30F5	Mouse actin-related protein 1 homolog A, ARP1 (centractin alpha)	NM_016860
Transcription factors (12)		
G1F3	Jumonji	NM_021878, D31967
G1F7	Nuclear factor I/B (NFI-B)	NM_008687, Y07687
G2F12	Similar to mouse transcription factor 4 (tcf-4)	NM_013685
G3F11	T-box brain gene 1 (Tbr1)	NM_009322, U16322
G7F13	Sall-like 1 protein	NM_021390, AJ271915
G9F1, G29F4	Mouse N-myc downstream regulated (Ndr2)	NM_013864
G11F3	CREB, cAMP responsive element binging protein	MMU46027
G15F3	LZIP protein gene, 5′-end, transcription factor, recognizing CRE and AP-1 element	L22167
G23F9A	Similar to mesoderm induction early response 1 gene (MI-ER1); immediately early gene, transcription factor	XM_233245, NM_020948
G30F11	NFI-X3 transcription factor	S81451, Y07688
G34F13	Mus musculus nuclear factor, erythroid drived 2; p45 NF-E2 related factor 2 (NRF 2)	NM010902, U20532
G40F10, G40F11	Mus musculus ring finger protein 23 (Rnf23), tripartite motif protein 39 (trim39). Transcription factor	NM_024468
Cell surface receptors (5)		
G1F1, G16F3, G25F7, G42F8	Eph receptor A4	NM_007936
G22F6	Mus musculus metabotropic glutamate receptor 8	U17252.1
G32F7	GABA-A receptor-associated protein-like protein 1	AF_180518
G25F9, G34F3, G39F10, G40F4	Mus musculus fibroblast growth factor (FGF) receptor-1	U22324.1, NM_010206
G32F2	Mus musculus notch B, mouse notch 2	X68279, D32210
Signaling pathway molecules (12)		
G1F10	NELL2 (protein kinase C-binding protein)	NM_016743, U59230
G16F8	Calcium binding protein, intestinal (Cai)	NM_009787
G25F8	Calcineurin-binding protein calsarcin-1	NM_016599
G16F9	Protein kinase piccolo	AF_128892
G17F4, G30F9	Rattus norvegicus protein kinase WNK1 (WNK1)	AF_227741
G17F5	Mus musculus Dlxin-1 (Dlxin1)	NM_019791
G32F5	Mus musculus Deltex 1 (DTX1)	AB015422
G8F29	Smad-interacting protein 1 (SIP 1)	AF_033116
G29F8A	Mus musculus similar to kinase D-interacting substance of 220 kDa; ankyrin repeat-rich membrane-spanning protein	NM_053795
G32F8	Homo sapiens protein tyrosine phosphatase, receptor type, F polypeptide (PTPRF), interacting protein (liprin), alpha 1 (PPFIAL)	XM_028889, NM_003626
G39F2	Mouse mRNA for Drctnnbla, down regulated by Ctnnbl	AB_030242, XM_033623
G34F7	Mouse WTAP protein, wilms' tumor 1-associating protein	AJ276707, XM_016021.2
DNA binding/chromosome structure (8)		
G9F7	Heterogeneous nuclear ribonucleo-protein A1 (hnrpa1)	NM_010447
G9F9	HMG1-related DNA-binding protein	S50213
G14F1	Apoptotic chromatin condensation inducer (Acinus)	NM_019567
G20F3	Mus musculus Swi/SNF related matrix associated, actin regulator of chromatin, subfamily a-like 1 (Snarcak1)	NM_018817
G20F4	Homo sapiens TATA box binding protein (TBP)-associated RNA polymerase II, A, 250KD (TAF2A)	XM_010137, NM004606
G22F4	Homo sapiens translocated promoter region (TPR)	XM_001737
G26F2	Mouse gene for 18S rRNA	X00686
G29F7	Snf2-related CBP activator protein (SRCAP)	XM034367
Genes similar to clones in the RIKEN full-length library (6)		
G1F4J	Mus musculus 13 day embryo head cDNA, RIKEN full-length enriched library, clone:3110037C01:homolog to hypothetical 22.1 Kda protein; Mus musculus X2CR1 mRNA	XM001863 AK014130

(*continued on next page*)

Table 2 (*continued*)

DD–PCR fragment	Identity	Genebank accession number
G16F1	Mus musculus RIKEN cDNA 2610507O21 gene (2610507O21Rik). Mus musculus 15 day embryo head cDNA, RIKEN full-length enriched library, clone:4021402N16; product:unnamed protein, putative similar to trans-membrane protein quicken [Xenopus laevis]	NM_028123 AK076213
G24F2A	Mus musculus 11 day embryo whole body cDNA, RIKEN full-length enriched library, clone:2700046A07; products: hypothetical protein	AK012381
G33F11D	Homo sapiens mRNA for KIAA0467 protein, partial cds	AB007936
G33F4A	Mus musculus adult male medulla oblongata cDNA, RIKEN full-length enriched library, clone:6330407J23, products: hypothetical glycine-rich region containing protein	AK018139
G34F6	Mus musculus 16 day embryo head cDNA, RIKEN full-length enriched library, clone:C130021H13 product: hypothetical Arginine-rich region/ Serine-rich region/Lysine-rich region containing protein	AK081496
Miscellaneous (3)		
G8F13	Mus musculus golgi reassembly stacking protein 2 (Grs2-pending)	NM_027352, XM_130256
G16F10A	Similar to mouse reticulon 1A (RTN-1A), a neuron-specific protein	BC030455, AB074899
G30F16	Mus musculus lyosomal membrane glycoprotein 1 (Lamp1)	NM_010684
G31F12	Mus musculus transgelin (TAGLN) mRNA	AF149291
G34F8	Mus musculus dolicho-phosphate (beta-D) mannosyltransferase (DPM1)	NM_010072

Novel, without significant homology 12 addition clones-see Table 3.

molecules, DNA-binding/chromosome structure-related proteins, and miscellaneous genes (Tables 1 and 2). Among these genes, many have been reported to function in developmental processes such as axonal pathfinding or neurite outgrowth (e.g. EphA4, L1-like protein, ankyrin, tenascin-C) (Demyanenko et al., 1999; Meiners et al., 1999; Kullander, et al., 2001; Takemoto et al., 2002), and neurogenesis/differentiation of neural progenitors (e.g. notch2, FGF receptor 1) (Morrison et al., 2000; Chambers et al., 2001). Some of them may be involved in the patterning of the dorsal telencephalon such as sip1 (smad-interacting protein 1) that may interact with smad, a BMP signaling pathway molecule (Verschueren et al., 1999), and tcf4 (transcription factor 4), a downstream target gene of the Wnt signaling cascade (Kolligs et al., 2002). In addition, we found 12 gene fragments that encode Expressed Sequence Tag (EST) genes or novel genes without significant homologs in Genbank (Table 3).

1.2. Expression analysis of genes identified by DD–PCR in mouse brain

The cell bodies of the GW are located in the medial wall of the telencephalic ventricle at the corticoseptal boundary. Thus, we expected that genes isolated from the GW would be expressed at higher levels at the midline and particularly within the VZ/SVZ. We analyzed the expression patterns of the DD–PCR fragments by in situ hybridization using ^{35}S-cRNA probes. These analyses confirmed that many of the genes were highly expressed in the GW as well as other regions of the telencephalon, although none were expressed in the septum (data not shown). Fig. 2 shows examples of (1) genes expressed at high levels in the VZ/SVZ (e.g. EphA4, LZIP and G39F8) as well as other regions (Fig. 2(A)); (2) genes whose expression was primarily in the dorsal telencephalon such as G24F3, tcf4, NFI-B and smad-interacting protein 1 (sip1) (Fig. 2(B)); and (3) genes whose expression was limited to the neuroepithelium and SVZ such as notch2 and Ndr2 (Fig. 2(C)).

Most of the genes isolated were known genes with functions in brain development. For instance, the nuclear factor I (NFI) gene family consists of NFI-A, -B, -C and -X (Gronostajski, 2000) We identified Nfib and Nfix in the DD–

Table 3
Summary of novel genes' expression patterns

Clones	Intensity of DD-PCR bands			Identity
	Glial wedge	Septum	Cortex	
G4F10	++	−	±	Predicted novel gene on mouse chromosome 11
G7F1	+	−	−	EST gene: U1-M-BZ1-bke-d-07-O-UI-3′
G8F14	+	−	±	XM_222179 Rattus norvegicus similar to CG17396 gene product [Drosophila melanogaster]. Predicted novel gene
G16F2	+	−	−	AL670462, Mouse DNA sequence from clone RP23-225F2 on chromosome X
G16F11	+	−	±	EST gene on mouse chromosome 10
G16F12	+	−	±	Genscan predicted protein
G22F3	+	−	−	EST gene on mouse chromosome 11
G24F3	++	−	±	Predicted gene: Transcription factor-4 (tcf4)
G30F10	++	−	±	Predicted gene: NULP1, a helix-loop-helix protein
G39F3	+	−	−	Predicted gene: Similar to C1-tetrahydrofolate synthase
G39F8, G39F12	+	−	−	Predicted gene: Zinc finger protein 288
G43F3	+	−	−	Mus Musculus chromosome 9 BAC clone MGS1-117K9 ES cell line

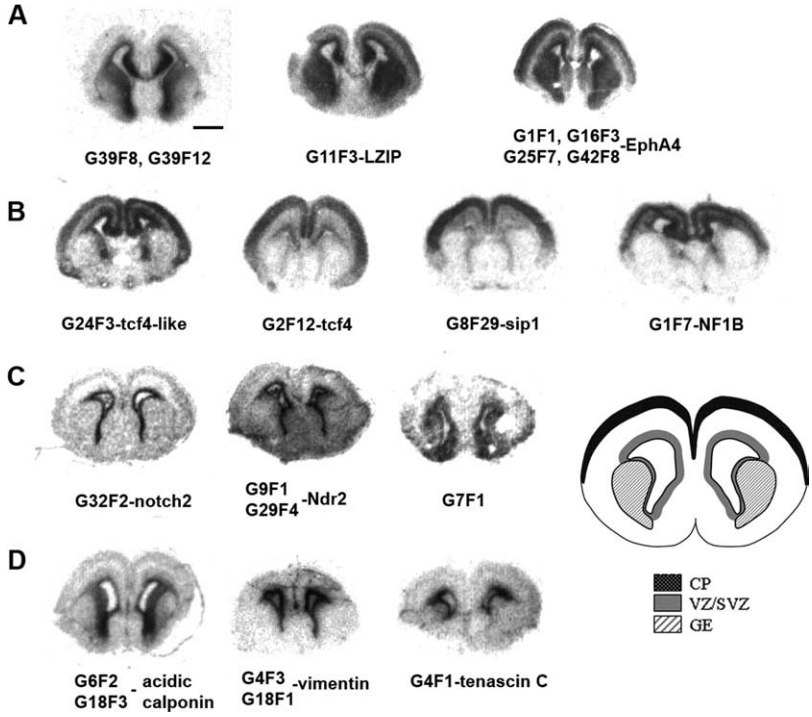

Fig. 2. In situ hybridization analysis of genes identified by DD-PCR in mouse brain. Bright-field images of film autoradiographs of the clones shown. Coronal sections of E17 mouse brain were hybridized with ^{35}S-labeled antisense probes. A schematic in the lower right corner describes the anatomical location of structures of interest labeled by the different probes. The key to the schematic is below; CP, cortical plate, VZ/SVZ, subventricular/ventricular zone and GE, ganglionic eminence. Genes identified by DD-PCR were grouped according to their expression patters. Panel A shows genes highly expressed in the VZ/SVZ. Genes restricted to the dorsal telencephalon are shown in Panel B, and panel C shows genes limited to VZ/SVZ. Scale bar in A = 1 mm for all sections in (A)–(D).

PCR screen (Table 2), which are highly expressed in the cortex and at the cortical midline (Chaudhry et al., 1997; Gronostajski, 2000; Shu et al., 2003c; Steele-Perkins et al., 2005). Furthermore, mutations in either gene causes agenesis of corpus callosum and abnormalities in midline glial formation (Shu et al., 2003c; Steele-Perkins et al., 2005). Several genes such as EphA4, L1-like protein, and tenascin-C have been reported to be involved in axonal pathfinding or neurite outgrowth (Demyanenko et al., 1999; Meiners et al., 1999; Kullander et al., 2001; Takemoto et al., 2002; Goldshmit et al., 2004). In the EphA4 knockout mouse corticospinal axons display abnormal growth and pathfinding upon entering the gray matter of the spinal cord (Coonan et al., 2001). In situ hybridization analyses of EphA4 demonstrate that it is expressed in the GW and the indusium griseum at E17 (Fig. 2(A), and data not shown). Other genes identified in the screen, such as L1 and ankyrin, cause a reduction or complete agenesis of corpus callosum when mutated in mice (Scotland et al., 1998; Demyanenko et al., 1999). Interestingly, no secreted guidance factors were isolated from the screen. This included factors known to be expressed by the GW such as Slit2 nor any novel putative guidance factors and could suggest that DD–PCR is not the optimal method to use for isolating such genes.

Another group of genes we were interested in identifying were those that may be involved in patterning the dorso-ventral axis at the corticoseptal boundary. Our analysis of some of the isolated genes is continuing but several candidate transcription factors, including tcf4, sip1, and Nfib, were identified. These genes demonstrated restricted expression patterns in the dorsal telencephalon (Fig. 2(B)) and are known to interact with patterning molecules such as members of the BMP and Wnt gene families (Verschueren et al., 1999; Kolligs et al., 2002).

Finally, some genes identified may be involved in regulating the proliferation and differentiation of neuronal and glial progenitors. For instance, notch2 is a receptor gene that is highly expressed in the ventricular neuroepithelium (Fig. 2(C)) and may promote glial cell fate determination (Morrison et al., 2000; Chambers et al., 2001). Furthermore, glial markers such as Vimentin were present in the clones derived from the screen (Fig. 2(D)).

1.3. Expression of novel genes isolated from the DD–PCR screen

We expected that if a gene was critical for the development of the corpus callosum, its expression levels would be up-regulated at a developmental stage when the majority of the callosal axons cross the midline. We chose several gene candidates that were most specifically expressed in the GW to examine their expression throughout brain development using an RNase Protection Assay (RPA). RPA was used to determine the mRNA levels of five clones that were previously known genes (LZIP, EphA4, Calponin, Acinus and Jumonji) and five clones that did not have significant homologs in the Genbank database. Total RNA used in the RPA was isolated from mouse brains at E12, E14, E16, E17 and P0. We found that four of the five potentially novel genes, and one of the known genes, LZIP,

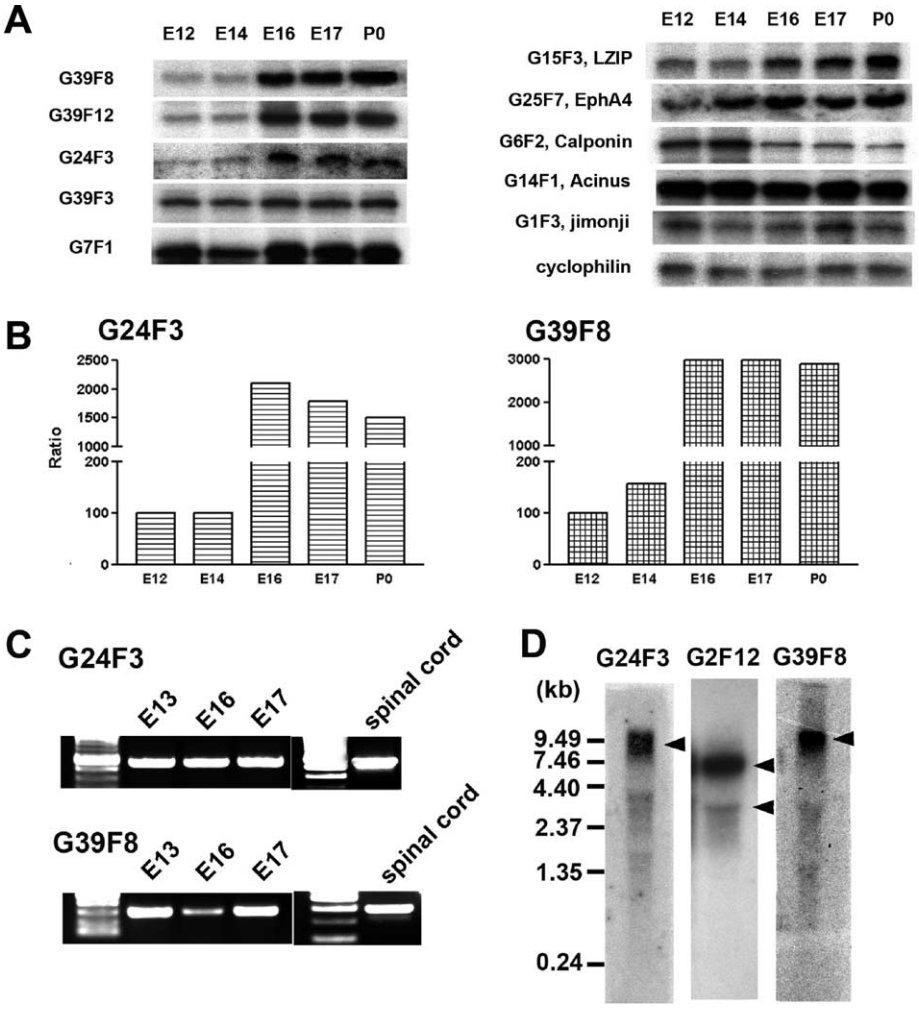

Fig. 3. Analysis of genes identified by DD-PCR. (A) RNase protection assay showing gene expression levels in whole mouse brain during development from E12 to P0 for each gene. G39F8, G39F12, G24F3 and LZIP expression levels increased significantly from E16 to P0 (left panel) whereas genes such as G39F3, G7F1, Acinus, jimonji and cyclophilin where expressed at similar levels throughout development. (B) Semiquantitative analysis of the protected bands shown in panel A for G24F3 and G39F8 clones indicates that their transcription levels increased by 25–30-fold from E16 through P0 compared with their expression levels at E12. (C) Confirmation of G24F3 and G39F8 expression in the brain from E13 to E17 by RT-PCR, showing both genes are expressed in brain and spinal cord. (Note that due to the non-quantitative nature of the RT-PCR method, although the band in the E16 lane appears lighter, we do not interpret this as reflecting a decrease in mRNA levels at this stage of development). The expected PCR products are 483 bp for G24F3 and 300 bp for G39F8. (D) Northern blot analysis indicated a transcript size of 9.5 kb for both G24F3 and G39F8, and 7 and 4 kb for G2F12.

were up-regulated at E16 (Fig. 3(A)). Semi-quantification of this data showed that the transcription levels of clones G24F3 and G39F8 increased by 25–30-fold from E12/E14 to E16 and then remained stable through P0 (Fig. 3(B)). RT-PCR confirmed the expression of both G24F3 and G39F8 in both the brain and spinal cord (Fig. 3(C)). G39F8 is expressed predominantly in brain and at a very low level in the heart, but not expressed in liver and limb (data not shown).

1.4. Expression of G24F3 in the developing telencephalon, a new member of the tcf4 gene family

We have shown that the expression levels of G24F3 increase significantly from E16 through P0, a period during which the majority of callosal axons cross the midline, suggesting that up-regulation of these genes may be involved in callosal formation. To investigate their roles in callosal development, we performed in situ hybridization to obtain a detailed expression analysis in the developing and adult brain.

In situ hybridization analysis with the G24F3 probe demonstrated that G24F3 expression is restricted in the dorsal telencephalon from E12 through P10 (Fig. 4(A)). G24F3 is first expressed at high levels in the neuroepithelium as early as at E12 but is not detectable at E10 (data not shown). At E14, the highest level of G24F3 expression is in the VZ of the dorsal telencephalon and in the hippocampus, with lower levels of expression in ventral regions such as the VZ of ganglionic eminence (GE). As neurons generated in VZ/SVZ migrate radially to form the cortical plate, G24F3 mRNA is expressed in both the VZ/SVZ and the cortical plate at E15 through E17. At the cortical midline G24F3 is expressed in the GW (Fig. 6). In situ hybridization analysis on sagittal and horizontal sections of E17 brains indicated that G24F3 is expressed in a high caudal to low rostral gradient in the cortex (Fig. 4(A), E17

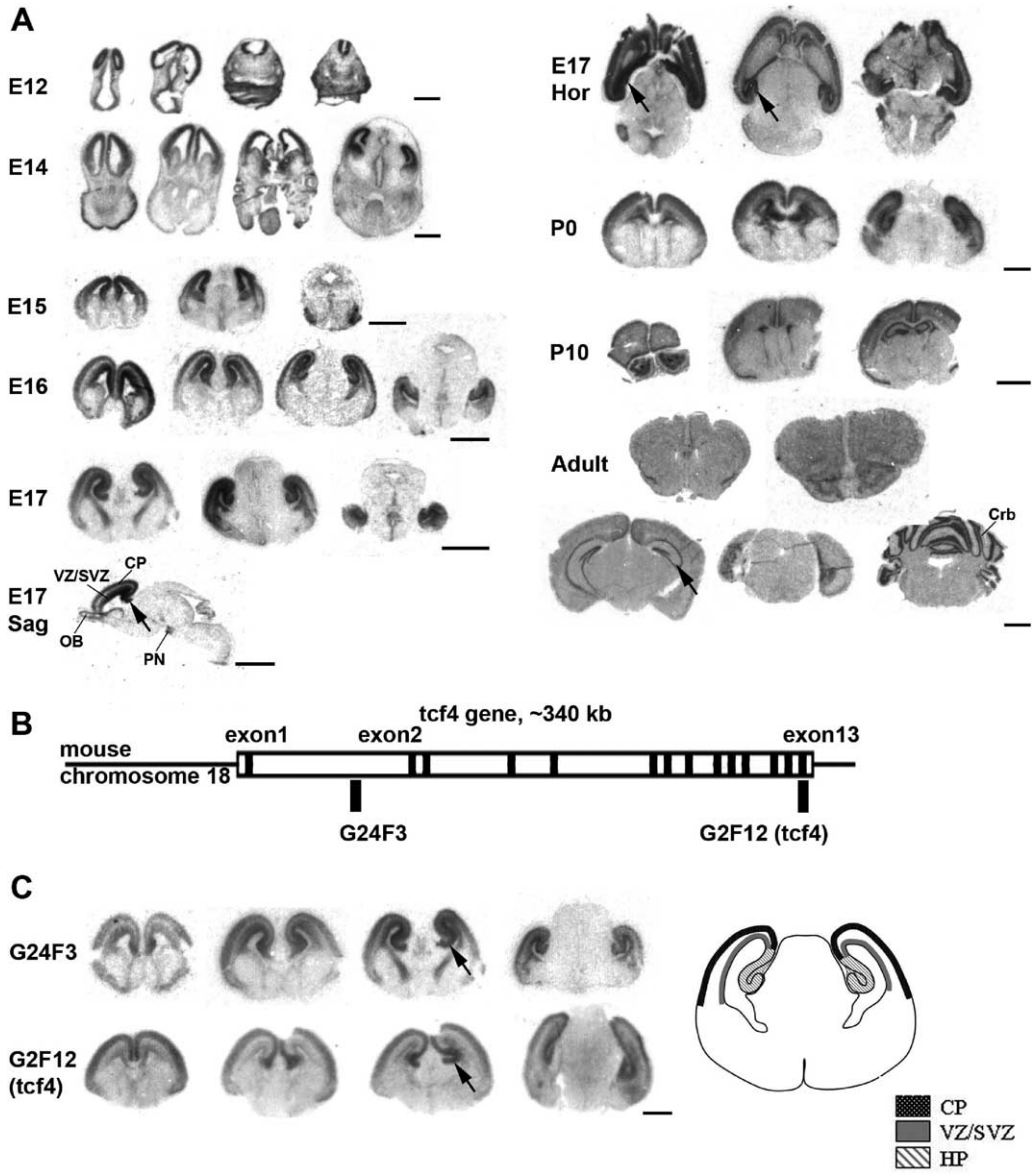

Fig. 4. G24F3 expression in mouse brain during development. (A) Bright-field images of film autoradiographs of the G24F3 clone. ^{35}S-labeled probes were hybridized to coronal sections (embryonic and/or adult brains); sagittal sections (E17 sag) and horizontal sections (E17 Hor). Expression of G24F3 is restricted to the dorsal telencephalon of developing brains. High expression levels between E14-P0 are shown in both the dorsal VZ, the cortical plate (CP) and the hippocampus (HP, and black arrow on sagittal and horizontal sections). G24F3 is also expressed in the pons (PN), olfactory bulb (OB) at E17 and in the cerebellum (Crb) in adult mouse. (B) Schematic demonstrating the overlap of G24F3 and G2F12 with the tcf4 gene in mouse chromosome 18. The G24F3 fragment does not match the cDNA sequences in the Genbank database, but the G2F12 fragment is identical to the tcf4 gene cDNA (Accession Number BC043050.1). (C) In situ hybridization analysis with G24F3 and G2F12 (tcf4) probes comparing the different expression patterns at E17. A schematic on the right shows the regions of interest on the sections and can be used for identification of these structures. G24F3 is highly expressed in both the VZ and the cortical plate, whereas G2F12 is highly expressed only in the cortical plate. Both clones are highly expressed in the hippocampus, but the expression of G2F12 is particularly high in the dentate gyrus/CA3 region compared to G24F3 which is uniformly expressed throughout the hippocampus (compare regions indicated by the arrows in (C). Scale bars in A = 500 μm (E12); 625 μm (E14), 750 μm (E15), 875 μm (E16), 975 μm (E17 coronal), 2.25 mm (E17 sagittal), 500 μm (P0 and E17 horizontal), 875 μm (P10),1.5 mm (Adult). Scale bar in C = 1 mm.

sagittal (E17 Sag) and horizontal (E17 Hor) sections). Sagittal sections also showed a low level of expression in the pons and the olfactory bulb at E17. G24F3 gene expression decreased in the cortex of postnatal brains from P0 and thereafter, but high levels of expression were maintained in the hippocampus and dentate gyrus. In the adult, G24F3 continued to be expressed only in the hippocampus and in granule cells of the cerebellum.

Further analysis of the sequence of G24F3 suggested that it was either a novel gene or a new member of the transcription factor-4 (tcf4) gene family. Evidence for this is that first, a BLAST search using the G24F3 fragment sequence as a probe did not match any cDNA in the Genbank database. However, analysis of the genomic DNA database indicated that G24F3 is located on mouse chromosome 18 and overlaps with the gene encoding tcf4 (Fig. 4(B)). Tcf4 has been reported to be a downstream target of the Wnt signaling pathway (Kolligs et al., 2002) and Wnt has been shown to act in the dorso-ventral patterning (Altmann & Brivanlou, 2001). Second, G24F3 has a

different expression pattern from G2F12, a fragment identical to tcf4 cDNA (Accession Number BC043050.1). Third, although both genes overlap within the tcf4 gene on the chromosome 18, and are highly expressed in the dorsal telencephalon (Fig. 4(C)), the expression patterns in the dorsal cortex appeared different. G24F3 is expressed in both the VZ/SVZ and the cortical plate, whereas G2F12 is expressed at a high level in the cortical plate only (Fig. 4(A) and (C)). Furthermore, RT-PCR confirmed G24F3 expression in the embryonic brains (Fig. 3(C)) and Northern blot analysis confirmed that G24F3 has a larger transcript size (9.5 kb) than that of G2F12/tcf4 (7 and 4 kb) (Fig. 3(D)).

1.5. Expression of the novel gene, G39F8, during forebrain development

In situ hybridization with the G39F8 probe demonstrated that in the embryonic mouse brain, G39F8 is expressed in the telencephalon (Fig. 5(A)). G39F8 expression appeared in the neuroepithelium of telencephalon as early as E12. From E14 and thereafter, G39F8 is expressed in the VZ of the cortex and GE. From E16 to P10, the highest level of expression was in the indusium griseum and hippocampus, particularly the dentate gyrus. From P10 to adult, G39F8 expression decreases in the rostral forebrain in all areas except the hippocampus, dentate gyrus, and external granular layer of the cerebellum where the expression remains high.

Sequencing analysis showed that the G39F8 fragment overlapped with the zinc finger protein 288 (zfp288) gene on mouse chromosome 16 (Fig. 5(B)). However, it did not match any known cDNAs in the Genbank database. A possibility is that the G39F8 fragment may represent part of the 3prime;-untranslated region (UTR) of the zfp288 gene. To test this hypothesis, we screened a rat P1 brain cDNA library with the G39F8 fragment as probe and obtained a 4.7 kb clone (data not shown). A search of the Genbank and Genome databases

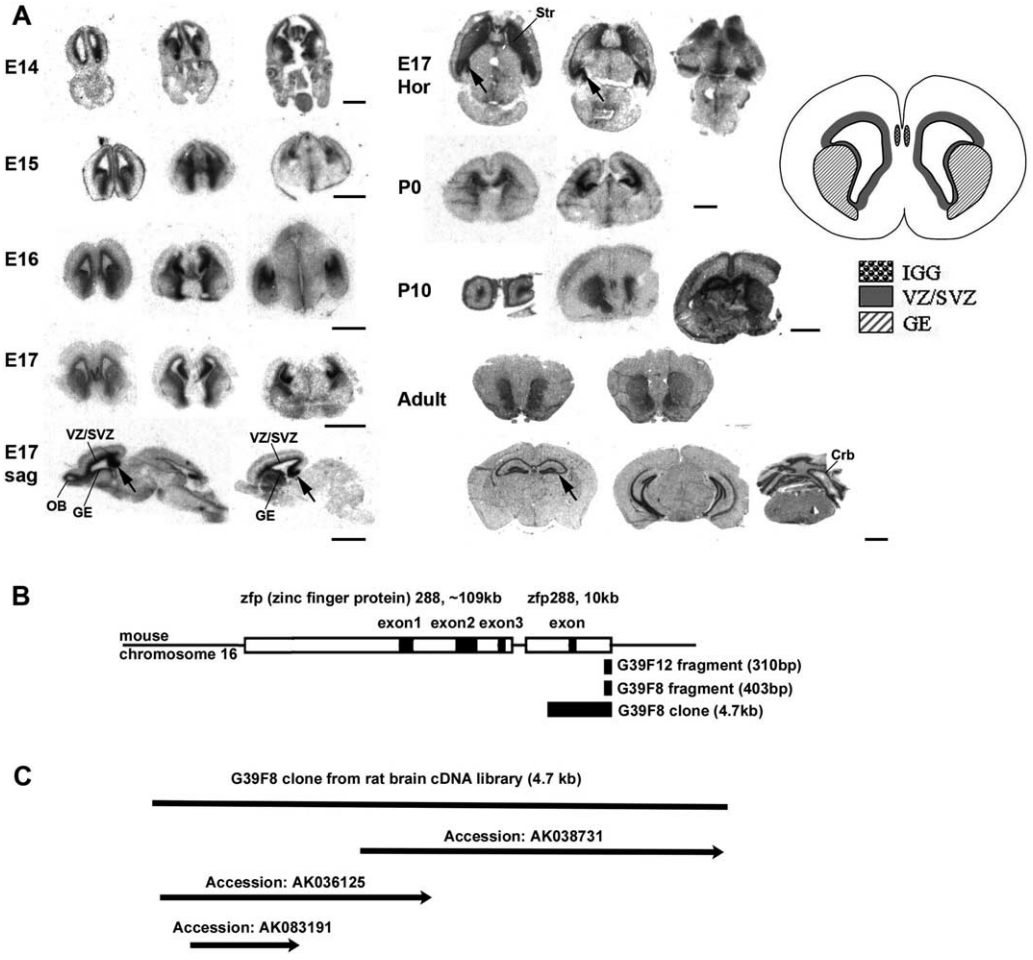

Fig. 5. The G39F8 clone is highly expressed in the dorsal telencephalon of developing brain. Bright-field images of film autoradiographs of the G39F8 clone. ^{35}S-labeled probes were hybridized to coronal sections (embryonic and/or adult brains), sagittal sections (E17 sag), and horizontal sections (E17 Hor). The schematic on the right demonstrates the location of structures of particular interest. G39F8 is expressed in the VZ/SVZ, ganglionic eminence (GE), olfactory bulb (OB), midline cell populations including the IG, the hippocampus (black arrow) and the striatum (Str; shown in the E17 horizontal section). In adult brains G39F8 is expressed in the hippocampus (arrow) and cerebellum (Crb). (B) Schematic demonstrating the overlap of the G39F8 fragment and the G39F8 library clone with the zfp288 gene on mouse chromosome 16. (C) The G39F8 fragment does not match any cDNA sequences in the Genbank database, but the G39F8 library clone (4.7 kb) is similar to clones in the Riken full-length enriched library (Accession number: AK036125, AK038731 and AK083191, respectively). Shown are overlaps in sequence homology between the G39F8 library clone and each clone from the Riken library (listed above). Scale bars in A = 625 μm (E14), 750 μm (E15), 875 μm (E16), 975 μm (E17 coronal), 2.25 mm (E17 sagittal), 500 μm (P0 and E17 horizontal), 875 μm (P10),1.5 mm (Adult).

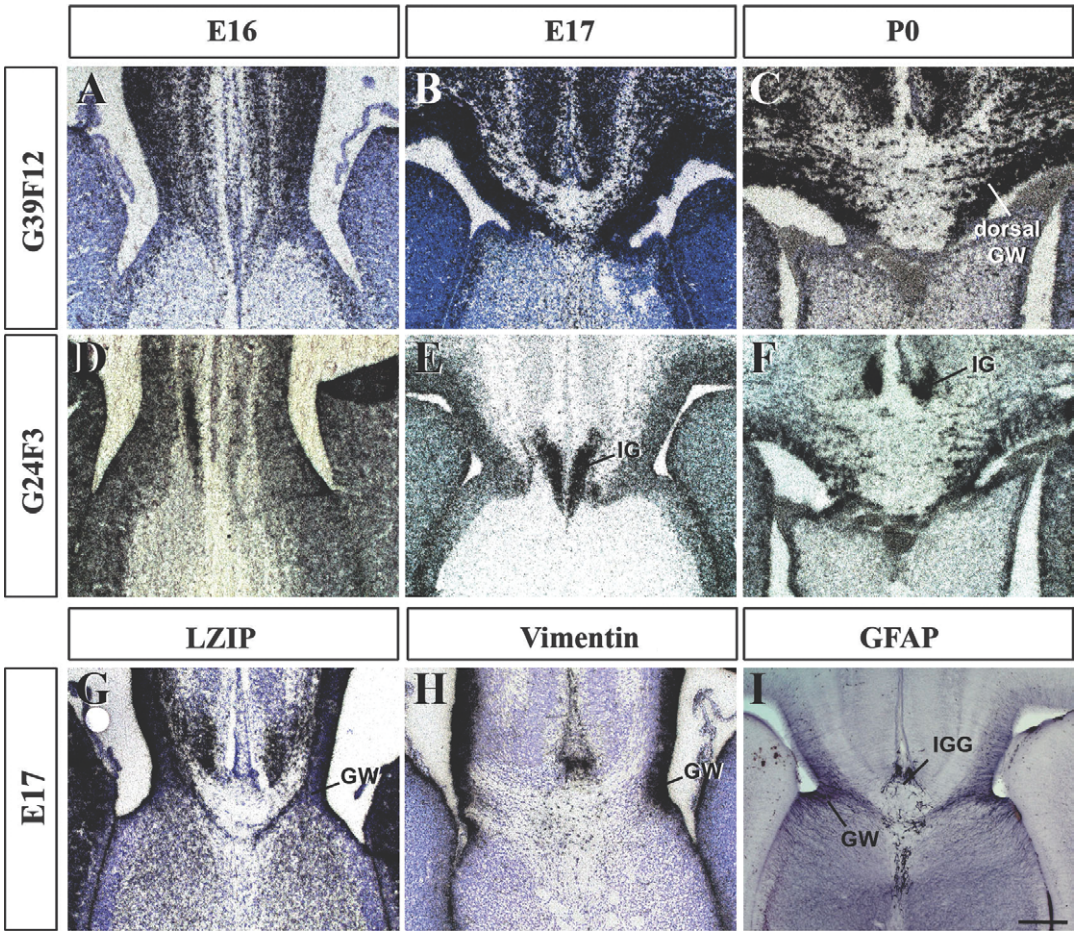

Fig. 6. Developmental expression of the G39F12, G24F3, LZIP and Vimentin clones obtained from the DD–PCR screen. Coronal sections were labeled by in situ hybridization with ^{35}S-labeled probes of either the G39F12 (A to C, from E16 to P0, respectively) or G24F3 clones (D to F, from E16 to P0 coronal sections, respectively). Shown are high power views of sections at the corticoseptal boundary to demonstrate expression within the region of the midline glial populations. Sections were dipped in radiographic emulsion and then developed to display the expression patterns. For orientation and comparison purposes, panel I shows a similar section stained with GFAP to label the midline glial populations (the indusium griseum glia (IGG) and the glial wedge (GW)). Both clones were highly expressed in the IGG and GW with clone G24F3 being most highly expressed in the indusium griseum (IG, E and F). Panels G and H are radioactive in situ hybridizations at E17 showing the expression of LZIP and Vimentin, respectively. Sections A to H were counterstained with cresyl violet. Scale bar in I = 200 μm in all panels.

indicated that this 4.7 kb clone is similar to clones in the RIKEN full-length enriched library (Accession numbers ak038731, ak083191, ak036125) (Fig. 5(C)), does not match completely any member of the zinc finger gene family, but overlaps with the zfp288 gene on mouse chromosome 16 (Fig. 5(B)). This evidence suggests that G39F8 is a new member or a new isoform of this zinc finger protein family.

Since our primary purpose of the screen was to identify genes expressed by the glial wedge we wanted to confirm the expression of G24F3, G39F12 and LZIP in the glial wedge (Fig. 6(A)–(G)). To do this we compared the expression of these genes with the known glial markers, glial fibrillary acidic protein (GFAP; Fig. 6(I))) and vimentin (Fig. 6(H)) expressed by the glial wedge. We examined the expression of G39F12 and G24F3 at E16, E17 and P0 and found that G39F12 was expressed only in the dorsal region of the VZ/glial wedge region whereas G24F3 and LZIP were expressed throughout the VZ/glial wedge region. LZIP is a transcription factor that has been shown to bind the chemokine receptor CCR1 and modulate its response to the chemokine leukotactin-1 (Ko, Jang, Kim, Kim, Sung and Kim, 2004). It is generally ubiquitously expressed and thus its role, if any, in glial development or callosal axon guidance is unclear. Finally, G24F3 was highly expressed within the indusium griseum throughout development (Fig. 6(D)–(F); IG). Both neurons and glia reside within this region and thus at present we do not know whether this expression is cell type specific or expressed by both populations.

In conclusion, genes isolated from the GW by DD-PCR may function in brain developmental processes such as axon guidance, dorsal patterning and neurogenesis/differentiation of neural progenitors. Our data suggested that clone G24F3 is either a novel gene or a new member of the transcription factor-4 (tcf4) gene family which may be involved in the dorsal patterning of the telencephalon, and that clone G39F8 is a new

member or an isoform of zinc finger protein 288 (zfp288) gene family. Further analysis is required to identify the functions of these novel isoforms in embryonic brain development.

2. Experimental procedures

2.1. Animals

C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were bred on site at The University of Maryland animal facility under the care and approval of the accredited University of Maryland, Baltimore Institutional Animal Care and Use Committee. The date of vaginal plug detection is designated as embryonic day (E) 0 and the date of birth as postnatal day (P) 0.

2.2. RNA Isolation

Living E17 C57BL/6J mouse embryonic brains were blocked and embedded in 3% low melting point agar (Sea plaque; FMC Bioproducts, Rockland, ME) and vibratome-sectioned at 350 μm. Glial wedge, cortex and septum were dissected from the regions shown in Fig. 1(A). Three independent samples for each tissue were prepared from E17 brains. Total RNA was isolated using an RNeasy mini kit (Qiagen), treated with RNase-free DNase I (Gibco BRL) and re-purified over an RNeasy column (Qiagen).

2.3. Differential display PCR

Differential display PCR (DD–PCR) was carried out following the procedure as described (Gesemann et al., 2001). Briefly, reverse transcription reactions were performed using the Superscript reverse-transcriptase II (Gibco BRL) and 10 base pair primers of arbitrary sequence (Operon Technologies). cDNA from this reaction was re-purified and used as templates for DD–PCR. ^{32}P-labelled PCR products were separated on 6% denaturing polyacrylamide gels. Gels were exposed to X-ray film overnight. Differences in the intensity of the bands were determined by visual inspection of films and they were aligned with the gels so that the differentially expressed bands could be excised. To minimize false positives, each reaction was performed in triplicate from three separate preparations of total RNA. Differentially expressed DNA fragments were re-amplified and cloned into pBluescript vectors. Clones containing the correct inserts were sequenced and compared with sequences in the NIH Genbank Database. DD–PCR fragments were designated as G###F## (G## indicated the polyacrylamide gel number and F## the excised fragment number).

2.4. In situ hybridization

^{35}S-UTP labeled DD–PCR fragments were used as probes for in situ hybridization. Probe labeling performed as previously described (Shu et al., 2003c). Briefly, 20 μm cryostat sections of embryonic mouse brains were mounted on cold RNase-free gelatin-coated microscope slides and prefixed in 4% paraformaldehyde, treated with proteinase K (Ambion, 20 mg/ml), re-fixed, and acetylated in 100 mM triethanolamine with acetic anhydride (TEA), then dehydrated through a graded series of ethanols.

The probes were diluted with hybridization buffer which contains 50% deionized formamide, 10% dextran sulfate, 1×Denhardts solution, 0.3 M NaCl, 10 mM Trirs pH7.5, 10 mM sodium phosphate pH6.8, 5 mM EDTA, 25 mM DTT and 50 mM β-mercaptoethanol (β-ME) to a concentration of 50,000 cpm/ul. Sections were hybridized with 80–90 μl of the denatured probe mixture in a humidified box and incubated overnight at 55 °C. Sections were washed at 55 °C for 30 min in 5×SSC and 20 mM β-ME, 45 minutes at 65 °C and 2–3 h at 37 °C in 2×SSC, 50% formamide, 20 mM β-ME, 15 min at 37 °C in 0.5 M NaCl in TE. Sections were treated with 20 μg/ml of RNase A for 15 min at 37 °C, and washed for 5 min each in 2X then 0.2X SSC at room temperature. Slides were dehydrated through a graded series of ethanols. Air-dried slides were then exposed to X-ray film (Kodak) for 5 days. Following this sections were dipped in autoradiographic emulsion (NTB2, Kodak), air-dried and stored in light-proof boxes at room temperature for 4 weeks before being developed in D-19 and fixer (Kodak). After developping, sections were counterstained with cresyl violet, and dehydrated through a series of alcohols before being coverslipped with DPX (Electron Microscopy Sciences) mounting medium.

2.5. Immunohistochemistry

Brains were embedded in 3% agarose (Noble Agar, Difco, Detroit, MI) and all sections were cut at 45 μm on a Vibratome (Leica, Deerfield, IL). Immunostaing was performed as previously described (Shu et al., 2003c). Briefly sections were blocked in 0.2% Triton X-100 (Sigma) and 2% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS for 2 h. The sections were then incubated overnight in rabbit anti-glial fibrillary acidic protein (GFAP, 1/30K; Dako, Carpinteria, CA). Sections were washed with PBS and incubated for 1 h with secondary antibody (biotinylated goat anti-rabbit, 1/500; Vector Laboratories). The signal was amplifiyed using AB solution (Vectastain elite kit, Vector Laboratories) for 1 h. Then staining was revealed using Nickel DAB (3,3-diaminobenzidine tetrahydrochloride; Sigma). Sections were mounted on 2% gelatin-coated glass slides, dehydrated through a series of alcohols and coverslipped with DPX (Electron Microscopy Sciences) mounting medium.

2.6. RNase protection assay

Plasmid DNAs containing DD–PCR fragments were linearized. Radio-labelled antisense riboprobes were transcribed using T7 or T3 RNA polymerases (Promega), and [α-^{32}P]UTP (ICN, 800 and 10 mCi/ml). The labeled probes had a specific activity of 7×10^7 cpm/μg or higher. As an internal control, an antisense riboprobe specific for cyclophilin was also prepared. Approximately, 1.5×10^5 cpm of labeled probe was hybridized to 10 μg of total RNA isolated from E12, E14, E16, E17 and P0 mouse brains. Non-hybridized RNA was removed using RNase A (Roche, 10 mg/ml) and RNase T1 (Gibco BRL, 1359 U/μl), and samples were then treated with proteinase K (Ambion, 20 mg/ml). The protected hybrids were separated on 6% denaturing polyacrylamide gels. The density of the cyclophilin signal was roughly equivalent in all of the samples, suggesting that cyclophilin expression is similar across these tissues. Semi-quantitative analysis was performed by using QuantiScan software on RNase protection assay (RPA) bands (Biosoft).

2.7. RT-PCR

All PCR reagents were purchased from Applied Biosystems (ABI, Foster City, USA).

Total RNA was isolated from the telencephalon of embryonic brains with Trizol reagent (Invitrogen), and reverse transcribed into cDNA with Superscript II (Invitrogen). Reverse transcription without Superscript II was used as a negative control. The PCR conditions were as follows: initial activation at 94 °C for 2 min; denaturation at 94 °C for 30 s, annealing at 53 °C for 1 min, and extension at 72 °C for 1 min 30 cycles; followed by extension at 72 °C for 10 min and then a hold cycle at 4 °C. Primers for PCR were designed based on the DD–PCR fragments. PCR primers for G24F3 were: forward CCCTACACAACAACAGTCTGC, reverse GGGTCAAACCTTTGACAGC, with an expected PCR product of 483 bp. Primers for G39F8 were: forward TCCTAGCTTTGTGATTGG, reverse 1: TGCTAACATCACGTCAGG, with an expected PCR product of 300 bp; reverse 2: GCTAAGGGAA-CAAAAAGG,with an expected PCR product of 254 bp.

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