G Protein-Coupled Receptor-Dependent Development of Human Frontal Cortex

Xianhua Piao, *et al.*

Science **303**, 2033 (2004);
DOI: 10.1126/science.1092780

G Protein–Coupled Receptor–Dependent Development of Human Frontal Cortex

Xianhua Piao,[1,2] R. Sean Hill,[1] Adria Bodell,[1] Bernard S. Chang,[1] Lina Basel-Vanagaite,[3] Rachel Straussberg,[4] William B. Dobyns,[5] Bassam Qasrawi,[6] Robin M. Winter,[7]* A. Micheil Innes,[8] Thomas Voit,[9] M. Elizabeth Ross,[10] Jacques L. Michaud,[11] Jean-Claude Déscarie,[12] A. James Barkovich,[13] Christopher A. Walsh[1]†

The mammalian cerebral cortex is characterized by complex patterns of anatomical and functional areas that differ markedly between species, but the molecular basis for this functional subdivision is largely unknown. Here, we show that mutations in *GPR56*, which encodes an orphan G protein–coupled receptor (GPCR) with a large extracellular domain, cause a human brain cortical malformation called bilateral frontoparietal polymicrogyria (BFPP). BFPP is characterized by disorganized cortical lamination that is most severe in frontal cortex. Our data suggest that GPCR signaling plays an essential role in regional development of human cerebral cortex.

Although the human cerebral cortex is subdivided into dozens of specific areas with divergent functions, the genetic mechanisms that create these areas are very poorly understood. Because cortical neurons are formed deep in the forebrain, in specialized proliferative regions near the lining of the lateral ventricles, a fundamental question has been whether cortical areas are presaged by a "protomap" among the progenitor cells in the ventricular region (*1*). Alternatively, cortical areas may only be specified later by patterns of neuronal interconnections (*2*). Direct studies of cell lineage have not been decisive: Some clones of neurons, especially pyramidal neurons, maintain map relationships between the ventricle and cortex (*3*–*6*) via radially directed migration (*7*), whereas other clones, especially those generating interneurons, show little or no maintenance of map order (*4*–*6*, *8*, *9*). Ventricular zone progenitor cells have at least some positional information, imparted by gradients of transcription factors (*10*, *11*) or secreted factors (*12*), but how gradients are converted into distinct cytoarchitectonic areas is much less clear. Other genes with sharper expression borders have also been identified (*13*–*15*), but the roles of these genes in patterning the cortex are not well defined.

Another approach to studying the mechanisms of cortical specification can come from the genetic analysis of inherited conditions in which specific regions of the cortex are preferentially disrupted. Bilateral frontoparietal polymicrogyria (BFPP), a recessively inherited genetic disorder of human cerebral cortical development, shows severely abnormal architecture in the frontal lobes, with milder involvement of parietal and posterior parts of the cortex (*16*–*18*). Polymicrogyria (PMG), characterized by abnormally numerous and small gyri, is associated with disruption of the normal six-layered cerebral cortical structure into only four layers, or the absence of discernible layers altogether (*19*). BFPP patients show mental retardation, gait difficulty, language impairment, and seizures, consistent with frontal lobe dysfunction (*17*, *18*). Cranial magnetic resonance imaging (MRI) reveals a thinner than normal cortex thrown into innumerable small, irregular gyri and sulci, especially in the frontal lobe. Although some abnormalities affect parietal cortex as well, there is a striking change in the severity of the PMG in the depth of the central sulcus, which separates frontal (motor) cortex from parietal (sensory) cortex (Fig. 1A) and which represents one of the most striking regional changes in the microscopic structure of the human cortex. The exact prevalence of BFPP is difficult to estimate because five different names are in use for the same genetic and radiographic syndrome (*18*). Other PMG syndromes selectively affect other cortical regions, such as perisylvian regions, or occipital lobes (fig. S1) (*20*–*22*). These syndromes may lead to the identification of several genes that normally pattern the human cerebral cortex. Genetic loci associated with PMG have been mapped (*17*,

Table 1. Mutations in *GPR56* associated with BFPP. For each pedigree, consanguinity is indicated, although in one pedigree the exact consanguineous relationship was not defined. For details of the pedigrees and their original citations, see (*18*). NC, nonconsanguineous.

Pedigree	*GPR56* mutation*	Resultant GPR56 abnormality	Pedigree comments		
			Consanguinity	Ethnicity	Number affected
1	IVS9+3G→C	Splicing mutation	First cousin	Palestinian§	3
2	IVS9+3G→C	Splicing mutation	First cousin	Palestinian§	2
3	E5–1G→C	Splicing mutation	NC	Pakistani	2
4	739_746 delCAGGACC	Frameshift†	NC	Indian	2
5	112C→T	R38W	First cousin	Qatari	2
6	739_746 delCAGGACC	Frameshift†	First cousin	Pakistani	1
7	739_746 delCAGGACC	Frameshift†	First cousin	Afghani	1
8	1036T→A	C346S	First cousin	Palestinian‖	2
9	1036T→A	C346S	First cousin	Palestinian‖	1
10	1693C→T	R565W	Consanguineous‡	Bedouin	3
11	272G→C	C91S	First cousin	Arabian	1
12	263A→G	Y88C	NC	French Canadian	2

*Base pair counted from starting codon ATG. IVS, intron; E, exon; +, intronic position 3′ of splice junction in donor; –, exonic position 5′ of splice junction in donor. For example, IVS9+3 means three bases of 3′ of the splice donor junction of intron 9. †Deletion of 7 bp that alters the translational reading frame, resulting in truncated protein with premature protein termination. ‡This pedigree consists of two nuclear families that are distantly related to one another. One family is first-cousin marriage and has two affected individuals; the other set of parents are consanguineous, although their exact relationship is not known. §These families are from the same village as one another. ‖These families are from the same village as one another but distinct from that of pedigrees 1 and 2.

[1]Howard Hughes Medical Institute, Beth Israel Deaconess Medical Center, and Department of Neurology, Harvard Medical School, Boston, MA 02115, USA. [2]Division of Newborn Medicine, Department of Medicine, Children's Hospital, Harvard Medical School, Boston, MA 02115, USA. [3]Department of Medical Genetics, Rabin Medical Center, Petah Tiqva 49100, Israel. [4]Neurogenetics Clinic, Department of Child Neurology, Schneider Children's Medical Center, Petah Tiqva 49202, Israel. [5]Department of Human Genetics, University of Chicago, Chicago, IL 60637, USA. [6]Social Welfare Institutes, Ministry of Social Affairs and Labor, Kuwait City 13006, Kuwait. [7]Clinical Genetics Unit, Institute of Child Health, London WC1N 1EH, UK. [8]Department of Medical Genetics, University of Calgary, Alberta Children's Hospital, S.W. Calgary, Alberta T2T 5C7, Canada. [9]Department of Pediatrics, University of Essen, 45122 Essen, Germany. [10]Department of Neurology and Neuroscience, Cornell University, New York, NY 10021, USA. [11]Division of Medical Genetics, [12]Department of Medical Imaging, Hôpital Ste-Justine, Montreal, Quebec H3T 1C5, Canada. [13]Pediatric Neuroradiology, Department of Radiology, University of California, San Francisco, CA 94143 USA.

*Deceased.

†To whom correspondence should be addressed. E-mail: cwalsh@bidmc.harvard.edu

23), but no genes that cause these conditions are known.

We fine-mapped the BFPP locus on chromosome 16q12.2-21 (*17*) to an interval of about 2.7 cM (Fig. 1B), bounded by D16S3110 and D16S3038 (*24*). This region contains 27 characterized genes (Fig. 1B), of which 17 were sequenced in the genomic DNA of BFPP patients. Eight independent mutations in *GPR56* were identified in 22 radiographically and clinically confirmed BFPP patients from 12 pedigrees, whereas no other genes showed any mutations.

GPR56 consists of 14 exons covering 15 kb of genomic sequence and has a 3-kb open reading frame (GenBank accession number AF106858) (*25*, *26*). DNA sequence analysis of *GPR56* revealed five distinct missense mutations in six pedigrees, two potential splicing mutations in three pedigrees, and one deletion/frameshift mutation in three pedigrees (Table 1 and Fig. 2C). Two mutations are presented in detail for illustration (Fig. 2, A and B). In each pedigree analyzed, the *GPR56* mutation segregated with the disease so that affected patients (including those from reportedly nonconsanguineous parents) displayed homozygous *GPR56* mutations, whereas parents who were clinically normal displayed heterozygosity for a normal and a disease allele, consistent with recessive inheritance. None of the mutations were observed in 260 control chromosomes (70 Europeans and 60 Middle Eastern Arabian individuals). Although most BFPP patients are from consanguineous families of Middle Eastern descent, where recessive conditions are more common by a factor of 2 to 3, we also identified a nonconsanguineous French Canadian family with BFPP and mutation in *GPR56* (Table 1), which suggests that BFPP is not geographically limited to the Middle East.

All missense mutations affect regions of the protein predicted to represent the extracellular portion of GPR56 (Fig. 3). One mutation found in pedigree 10 changes a conserved amino acid (R565W) (*27*) in a predicted extracellular loop of the protein (Figs. 2A and 3). The other missense mutations affected the extracellular N terminus of the protein (Table 1 and Fig. 3); three mutations occurred in exon 3 (Fig. 2C; R38W in pedigree 5, Y88C in pedigree 12, and C91S in pedigree 11), and one was found in exon 8 (Fig. 2, B and C; C346S in pedigrees 8 and 9). The C346S mutation was shared by two Palestinian families from the same village (along with shared alleles at flanking microsatellite markers), representing a founder mutation. GPR56 is a member of the family B GPCRs (G protein–coupled receptors), which, although they contain the seven-transmembrane domains seen in all GPCRs, also have long N termini characterized by an extracellular "cysteine box" motif and hydrophilic, potentially mucin-rich domains (*25*, *28*, *29*). The ligand binding for family B GPCRs involves both the N terminus and the extracellular loops between the seven-transmembrane domains (*30*). Therefore, the three mutations at the tip of the N terminus (R38W, Y88C, and C91S) as well as the R565W mutation in the second extracellular loop may affect ligand binding by GPR56.

Interestingly, pedigrees 4, 6, and 7, who originate from widely separated regions geographically, shared the same mutation that deletes 7 base pairs (bp) in exon 5 (Table 1, 739_746 delCAGGACC). This alteration creates a translational frameshift leading to a truncated protein that prematurely terminates 73 codons downstream. Pedigree 4 (*18*) is a nonconsanguineous family in which both parents originate from the Gujarat region of India. Pedigree 6 (*18*) is a consanguineous Pakistani family, whereas pedigree 7 (*18*) is a consanguineous family of Pashtun Afghani descent. Single-nucleotide polymorphism (SNP) analysis supports the existence of a common founder mutation in these three pedigrees, because all patients shared alleles for six SNP markers within the *GPR56* locus. Gujarat and Afghanistan both border Pakistan, and many tribes in Afghanistan are originally from India. Therefore, the common ancestral mutation in these three pedigrees is plausible but suggests a founder mutation maintained through a substantial population (>200 million people) and history.

The splicing mutations, frameshift mutation, and all missense mutations except one shared an indistinguishable phenotype and therefore are all probably null alleles; in contrast, one missense mutation caused a more severe phenotype. The C346S mutation found in pedigrees 8 and 9 substitutes serine for the first cysteine in the cysteine box of the GPR56 protein. Unlike all other BFPP patients, the affected individuals from these two families also have microcephaly (characterized by a smaller than normal overall cerebral cortical size). It is intriguing that the C346S mutation in the cysteine box has a more severe effect than abolishing the function of the gene encoding GPR56. The explanation for this finding

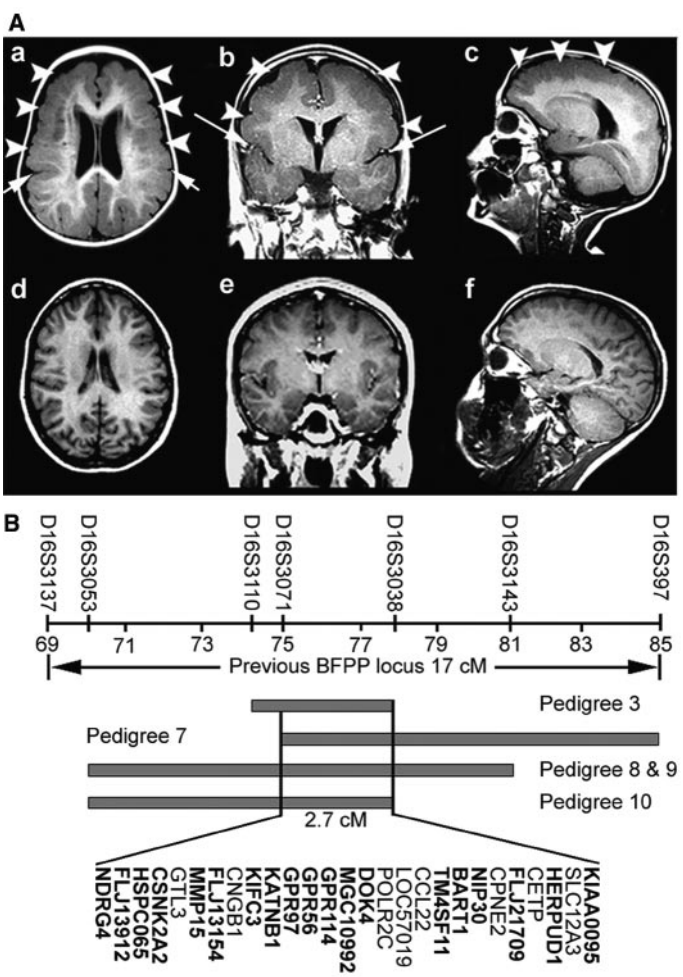

Fig. 1. Anatomical phenotype of BFPP and fine mapping of the BFPP locus. (**A**) T1-weighted axial (a and d), gadolinium-enhanced coronal (b and e), and sagittal (c and f) MRI images from individuals with BFPP (a to c) and normal controls (d to f). Arrowheads indicate the regions affected by PMG; short and long arrows indicate the central sulcus and sylvian fissure, respectively. These regions are characterized by an abnormally thin cortex with an irregular and scalloped gray-white junction, which creates a paradoxical increase in the apparent cortical thickness because many small, adjacent gyri are superimposed and signal-averaged in the MRI. The sharp regional differences in severity are best seen by comparing the severely abnormal frontal lobes to the relatively normal parietal lobes (a) or by comparing the abnormal frontal lobes to the almost normal temporal lobes (b). (**B**) The initial 17-cM BFPP locus was further narrowed to a 2.7-cM interval. All of the known genes in this region are listed. The genes in boldface are formal candidate genes for BFPP. All candidate genes were subjected to sequencing analysis in BFPP patients and family members except *GPR97* and *GPR114*, which were identified after we completed our mutational analysis.

likely involves the posttranslational processing of the GPR56 protein.

The extracellular cysteine box shared by many family B GPCRs contains four conserved cysteines arranged in a specific fashion (C-x_2-W-x_{6-16}-W-x_4-C-x_{10-22}-C-x-C) just before the first transmembrane domain (*28*, *29*, *31*) and serves as a cleavage site in some members of this group of GPCRs (*31*). Therefore, the cysteine box was also named the GPS (G protein–coupled receptor proteolytic site) domain. Mutations in this domain not only abolished proteolytic processing of α-latrotoxin, a member of family B GPCRs, but also prevented cells from expressing GPCRs at the cell surface (*31*). We speculate that the C346S mutation causes microcephaly by abolishing normal cleavage of GPR56 and affecting its proper trafficking, and possibly that of other proteins.

An antisense probe to the mouse *Gpr56* mRNA showed preferential expression in neuronal progenitor cells of the cerebral cortical ventricular and subventricular zones during periods of neurogenesis (Fig. 4, A to H), but no expression in the cortical plate or intermediate zone (*24*). We also observed expression in proliferative regions of the medial and lateral ganglionic eminence at E12 (embryonic day 12), E14, and E16, where up to one-third of cerebral cortical neurons, predominantly interneurons, are formed and subsequently migrate nonradially into the cerebral cortex (*8*, *32*) as well as several other brain areas. Cortical expression of *Gpr56* was strongest at E12 and E14 (when there are many progenitor cells in the cortical ventricular zone), weaker at E16, and greatly reduced at birth (when neurogenesis in the cortical ventricular zone is complete) (*33*); this pattern suggests that *Gpr56* is preferentially expressed in neuronal progenitors. In the adult brain, *Gpr56* expression was limited to scattered cells more common in white matter than in gray matter (Fig. 4, I to L). On the other hand, more intense *Gpr56* expression was observed in regions of adult brain that show postnatal neurogenesis, such as the dentate gyrus (Fig. 4, I and J) and the subventricular zone of the rostral migratory stream leading to the olfactory bulb (Fig. 4, K and L). Human *GPR56* expression has been shown in a wide range of different tissues (*25*), including heart, kidney, testis, pancreas, thyroid gland, and skeletal muscle. Our in situ hybridization also revealed strong signals in the spinal cord, somites, and liver, and weak signals in the intestine at E12 (*33*). However, no phenotype was observed outside of the central nervous system in BFPP patients.

The pattern of *Gpr56* expression and the anatomy of BFPP imply that *Gpr56* most likely regulates cortical patterning. The most severely affected cortical regions in BFPP are strikingly thin, and many forms of PMG show cortical alterations with reduction of the normal six cortical layers to four, suggesting possible roles in cell fate control that would be consistent with the expression of GPR56 in progenitor cells. However, further elucidation of GPR56's role will require mechanistic analysis in an animal model. Although there is no clear anterior-posterior gradient in expression of *Gpr56*, the severity of the phenotype in frontal cortex may reflect either regional differences in ligand expression or regional differences in the degree of dependence on GPR56-transduced signals.

Sequence analysis of GPR56 reveals substantial evolutionary differences between mammals and nonmammals. Of the more than 30 human GPCRs with long mucin-rich N termini that have been identified, GPR56 is most closely related to GPR97 and GPR114 (*29*). The three genes encoding these proteins are adjacent to one another on human chromosome 16 and are arranged in the same transcriptional direction (*26*). This gene order is conserved between mouse and human (*26*), which suggests that it may be the product of an ancestral gene duplication. However, outside of their homologies in the seven-transmembrane domains, GPR56, GPR97, and GPR114 share only 30% amino acid identity

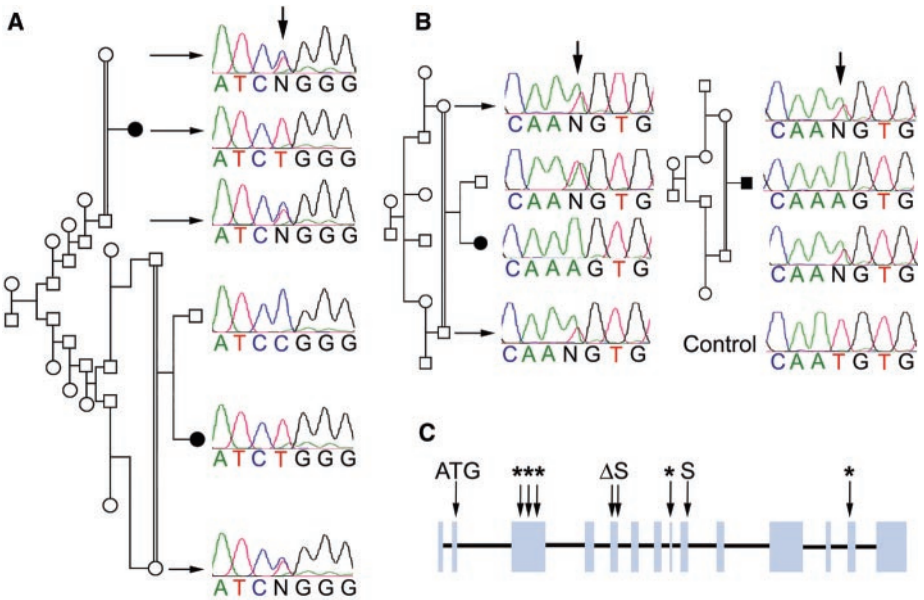

Fig. 2. Mutations in the *GPR56* gene. (**A**) A simplified drawing of pedigree 10 shows two nuclear families, each with distantly related consanguineous parents. A C→T mutation at base pair 1693 from the ATG results in an Arg → Trp change at amino acid 565. The mutation is homozygous in affected patients and heterozygous in unaffected parents. (**B**) Pedigrees 8 (simplified drawing) and 9 are of Palestinian descent and reside in the same village in Israel. Although there is no known relationship between these two families, mutational analysis revealed a founder mutation shared by the two families. A T→A mutation at base pair 1036 from ATG results in a Cys → Ser change at amino acid 346. (**C**) Positions of *GPR56* mutations in individuals with BFPP. *GPR56* consists of 14 exons; the starting codon ATG is located in the second exon. The five missense mutations are marked by asterisks. S denotes the splicing mutations; Δ denotes the 7-bp deletional mutation.

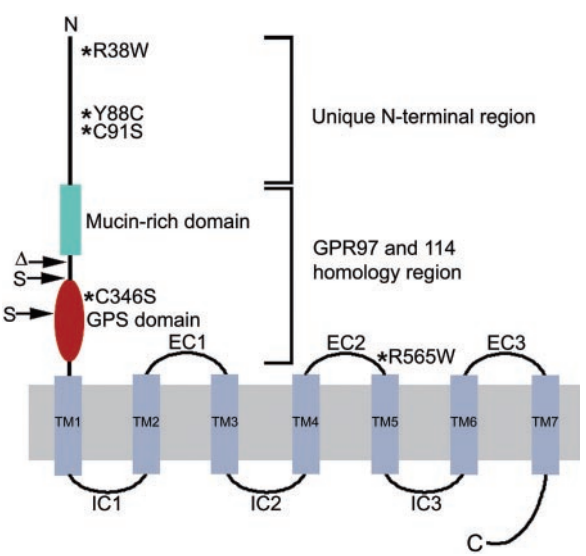

Fig. 3. Topology of the GPR56 protein. Asterisks indicate positions for the five missense mutations at the tip of the N terminus, for the C346S mutation in the cysteine box, and for the R565W mutation in the second extracellular loop of GPR56. Arrows point to the positions for the two splicing mutations and the 7-bp deletional mutation. IC, intracellular loop; EC, extracellular loop; Δ, the 7-bp deletional mutation; S, splicing mutation; TM, transmembrane domain.

over just a portion of their N-terminal regions (Fig. 3). The N-terminal 200 amino acids of GPR56 are distinct from these two genes and only 64% identical between mouse and human, versus ≥80% identical over the rest of the protein. Moreover, the unique N-terminal region has no readily identifiable protein domains (by cdart or RPS BLAST analysis) and no detectable amino acid homologies (by BLAST) outside of mammals. Thus, GPR56 is not only essential during human cerebral cortical development and patterning, but may also have been a key target in the evolution of the cerebral cortex, because the N-terminal domain that defines GPR56 is unique to animals that have a cerebral cortex. GPCRs represent the largest gene family in the human genome, comprising about 1% of all genes. They commonly function as gene families, such as olfactory receptors or pheromone receptors, raising the possibility that other orphan GPCRs may also play patterning roles in human cerebral cortex.

As has been pointed out by Rakic (*1*), the specification of cerebral cortical areas by genes that act in precursor cells creates a mechanism for the partially independent evolution of cortical areas, and hence of cognitive abilities. The frontal lobes, which are essential for motor function, social function, cognition, language, and problem solving, are particularly expanded in hominid primates. Evolutionary changes in GPR56 might alter the size or organization of frontal cortex preferentially, with little effect on other regions. Other genes, perhaps including those genes responsible for other syndromes of focal PMG in humans (fig. S1), may act independently of one another on other cortical areas mediating sensation, vision, or hearing.

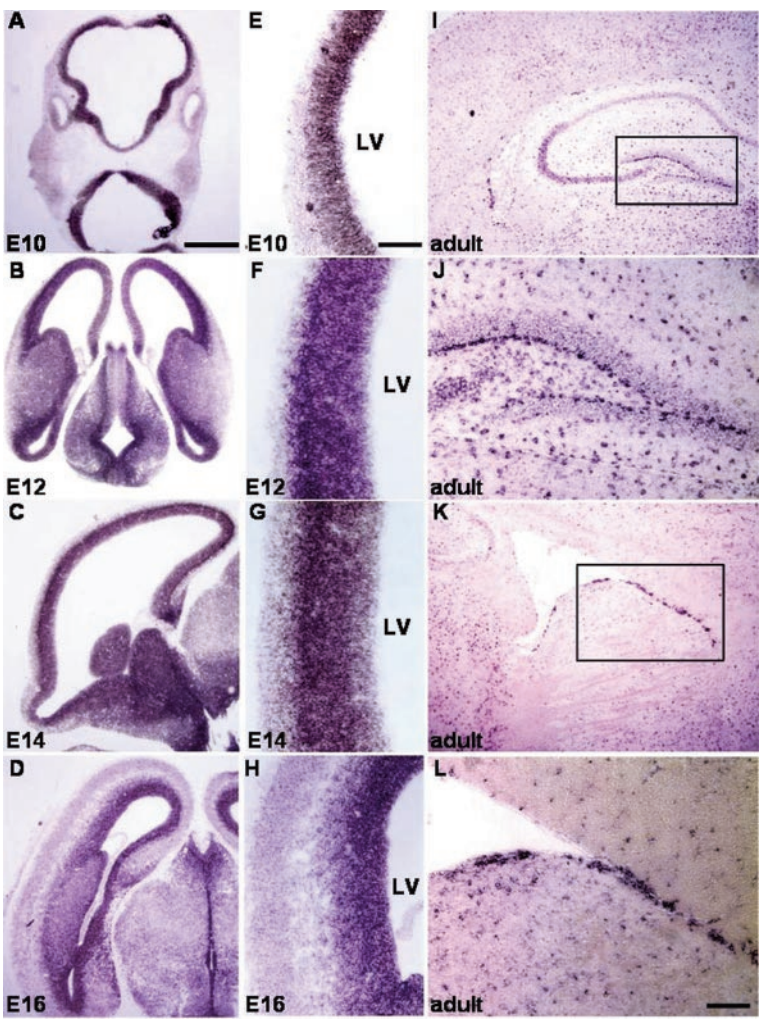

Fig. 4. Expression of *Gpr56* in mouse brain. (**A** to **H**) In situ hybridization experiments with a *Gpr56* probe were done on coronal and sagittal sections of mouse brain at E10, E12, E14, and E16. Strong expression was detected at the ventricular and subventricular zones of the cerebral cortex, but signal was minimal or absent in the cortical plate or intermediate zone, suggesting high expression in progenitor cells but low or no expression in postmitotic neurons. LV, lateral ventricle. (**I** to **L**) In adult brain, *Gpr56* hybridization was markedly reduced and limited to scattered cells, although it was still present in limited regions of the brain that show persistent postnatal neurogenesis, such as the dentate gyrus [(I) and (J)] and the subventricular zone of the rostral migratory stream leading to the olfactory bulb [(K) and (L)]. Boxed regions in (I) and (K) focus the areas for the fields shown in (J) and (L), respectively. Scale bars, 500 μm [(A) to (D), (I), and (K)]; 100 μm [(H), (J), and (L)]; 50 μm [(E) to (G)].

References and Notes

1. P. Rakic, *Science* **241**, 170 (1988).
2. D. D. O'Leary, *Trends Neurosci.* **12**, 400 (1989).
3. M. B. Luskin, A. L. Pearlman, J. R. Sanes, *Neuron* **1**, 635 (1988).
4. C. Walsh, C. L. Cepko, *Science* **255**, 434 (1992).
5. C. Walsh, C. L. Cepko, *Science* **241**, 1342 (1988).
6. S. S. Tan *et al.*, *Neuron* **21**, 295 (1998).
7. P. Rakic, *J. Comp. Neurol.* **145**, 61 (1972).
8. S. A. Anderson, D. D. Eisenstat, L. Shi, J. L. Rubenstein, *Science* **278**, 474 (1997).
9. K. Letinic, R. Zoncu, P. Rakic, *Nature* **417**, 645 (2002).
10. K. M. Bishop, G. Goudreau, D. D. O'Leary, *Science* **288**, 344 (2000).
11. A. Mallamaci, L. Muzio, C. H. Chan, J. Parnavelas, E. Boncinelli, *Nature Neurosci.* **3**, 679 (2000).
12. T. Fukuchi-Shimogori, E. A. Grove, *Science* **294**, 1071 (2001).
13. M. J. Donoghue, P. Rakic, *Cereb. Cortex* **9**, 586 (1999).
14. Q. Liu, N. D. Dwyer, D. D. O'Leary, *J. Neurosci.* **20**, 7682 (2000).
15. R. F. Hevner *et al.*, *Neuron* **29**, 353 (2001).
16. L. Sztriha, M. Nork, *Pediatr. Neurol.* **22**, 240 (2000).
17. X. Piao *et al.*, *Am. J. Hum. Genet.* **70**, 1028 (2002).
18. B. S. Chang *et al.*, *Ann. Neurol.* **53**, 596 (2003).
19. I. Ferrer, I. Catala, *Anat. Embryol. (Berlin)* **184**, 517 (1991).
20. R. Kuzniecky, F. Andermann, R. Guerrini, *Lancet* **341**, 608 (1993).
21. M. M. Guerreiro *et al.*, *Ann. Neurol.* **48**, 39 (2000).
22. R. Guerrini *et al.*, *Ann. Neurol.* **41**, 65 (1997).
23. L. Villard *et al.*, *Am. J. Hum. Genet.* **70**, 1003 (2002).
24. See supporting data on *Science* Online.
25. M. Liu *et al.*, *Genomics* **55**, 296 (1999).
26. UCSC Genome Bioinformatics Site (http://genome.ucsc.edu).
27. Abbreviations for amino acid residues: C, Cys; R, Arg; S, Ser; W, Trp; Y, Tyr.
28. M. Stacey, H. H. Lin, S. Gordon, A. J. McKnight, *Trends Biochem. Sci.* **25**, 284 (2000).
29. R. Fredriksson, D. E. Gloriam, P. J. Hoglund, M. C. Lagerstrom, H. B. Schioth, *Biochem. Biophys. Res. Commun.* **301**, 725 (2003).
30. J. Bockaert, J. P. Pin, *EMBO J.* **18**, 1723 (1999).
31. V. Krasnoperov *et al.*, *J. Biol. Chem.* **277**, 46518 (2002).
32. H. Wichterle, J. M. Garcia-Verdugo, D. G. Herrera, A. Alvarez-Buylla, *Nature Neurosci.* **2**, 461 (1999).
33. X. Piao *et al.*, data not shown. *GPR56* had been previously identified as *Cyt28*, a gene expressed in both hematopoietic and neural stem cells (*34*). Review of our patient records, however, shows no evidence of clinical abnormalities of hematopoietic lineage.
34. A. V. Terskikh *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 7934 (2001).

35. We are deeply grateful to the patients and their families for their participation in this study, and to other clinical collaborators who provided information and patient samples not directly involved in this study. Supported by NIH program grant HD07466 (X.P.), National Institute of Neurological Disorders and Stroke grants K08 NS045762-01A1 (X.P.) and R37 NS35129 (C.A.W.), the March of Dimes, and the McKnight Foundation. C.A.W. is an Investigator of the Howard Hughes Medical Institute. We thank N. D. Dwyer, E. C. Olson, and the rest of the members of the Walsh lab for comments on the manuscript. This paper is dedicated to the memory of Dr. Robin Winter for his contributions to the teaching and practice of human genetics.

Supporting Online Material

www.sciencemag.org/cgi/content/full/303/5666/2033/DC1
Materials and Methods
Fig. S1
References

21 October 2003; accepted 21 January 2004