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Protocadherin 11X/Y a Human-Specific Gene Pair: an Immunohistochemical Survey of Fetal and Adult Brains

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Protocadherins 11X and 11Y are cell adhesion molecules of the δ1-protocadherin family. Pcdh11X is present throughout the mammalian radiation; however, 6 million years ago (MYA), a reduplicative translocation of the Xq21.3 block onto what is now human Yp11 created the *Homo sapiens*-specific PCDH11Y. Therefore, modern human females express PCDH11X whereas males express both PCDH11X and PCDH11Y. PCDH11X/Y has been subject to accelerated evolution resulting in human-specific changes to both proteins, most notably 2 cysteine substitutions in the PCDH11X ectodomain that may alter binding characteristics. The PCDH11X/Y gene pair is postulated to be critical to aspects of human brain evolution related to the neural correlates of language. Therefore, we raised antibodies to investigate the temporal and spatial expression of PCDH11X/Y in cortical and sub-cortical areas of the human fetal brain between 12 and 34 postconceptional weeks. We then used the antibodies to determine if this expression was consistent in a series of adult brains. PCDH11X/Y immunoreactivity was detectable at all developmental stages. Strong expression was detected in the fetal neocortex, ganglionic eminences, cerebellum, and inferior olive. In the adult brain, the cerebral cortex, hippocampal formation, and cerebellum were strongly immunoreactive, with expression also detectable in the brainstem.

Keywords: human evolution, immunohistochemistry, language, neurodevelopment, Xq21.3/Yp11

Introduction

Many animals use complicated forms of communication, yet the infinitely generative nature of human language is unique to, and perhaps characteristic of, *Homo sapiens* (Hauser et al. 2002; Chance and Crow 2007). Therefore, one approach to the genetics of language is to search for genes that have been added to the human genome following the split from the chimpanzee lineage.

One candidate, the protocadherin 11X/Y (PCDH11X/Y) gene pair (Crow 2002; Priddle and Crow 2009; Priddle et al. 2010), arose 6 million years ago (MYA) by a reduplicative transposition from the X chromosome (Williams et al. 2006) on to what is now the human Y chromosome. Pcdh11X is present on the X chromosome throughout the mammalian radiation (Kalmady and Venkatasubramanian 2009); however, because of this translocation PCDH11X/Y is now X/Y homologous in humans and in no other extant mammal (Wilson et al. 2006). The case for a sex chromosomal locus for a gene related to language and its functional brain asymmetry is strengthened by observations of the neuropsychological deficits presented by individuals with sex chromosome aneuploidies. Klinefelter's (47,XXY) and triple X syndrome (47,XXX) individuals have delays in language acquisition (Visootsak and Graham 2006; Otter et al. 2010) and Turner's syndrome (45,X) patients have difficulties with spatial tasks (Kesler et al. 2004; Rae et al. 2004). These deficits correlate with the structural (Itti et al. 2006; Rezaie et al. 2008) and functional (Murphy et al. 1997; Itti et al. 2003) brain changes.

Members of the protocadherin family, to which the PCDH11X/Y gene pair belongs, are transmembrane cell adhesion molecules expressed predominantly in the brain (Frank and Kemler 2002) that make up the largest cadherin superfamily (Nollet et al. 2000; Hulpiau and van Roy 2009). PCDHs are classified into α, β, and γ sub-families on the basis of their clustered genetic organization (Wu and Maniatis 1999). An additional non-clustered group, termed δ-PCDHs, can be further subdivided, based on the number of cadherin repeats (ECs) and features of the cytoplasmic domain, into δ1- (the group containing PCDH11X/Y) and δ2-PCDHs (Redies et al. 2005; Vanhalst et al. 2005). Classical cadherins, as a class, are involved in the morphogenesis of diverse tissues through calcium-dependent homophilic cell adhesion mediated by a conserved motif in EC1 of the ectodomain (Gumbiner 2005). By contrast, this motif is absent in the PCDHs, thought to be less involved in the strength of cell–cell connections and more in specificity (Morishita and Yagi 2007). The δ1-family member NF protocadherin is required for the formation of the neural tube in *Xenopus* (Rashid et al. 2006), and the δ2-family member Pcdh19 is required for the correct neurulation of the forebrain in zebrafish (Emond et al. 2009) via an interaction with N-cadherin (Biswas et al. 2010). γ-Pcdhs are required for synaptic development in the mouse spinal cord and are thought to affect the maintenance or maturation of synapses (Weiner et al. 2005).

The PCDH11X/Y gene pair encodes 2 proteins each comprising an ectodomain of 7 ECs, a short transmembrane region, and a variable length cytoplasmic domain differing between isoforms. Following the translocation, PCDH11X/Y has undergone accelerated evolution in the human lineage (Williams et al. 2006). In the longest isoforms, there have been 5 human-specific changes to the PCDH11X ectodomain and 1 change in the cytoplasmic domain; PCDH11Y has accumulated 17 changes, 7 in the ectodomain, and 10 in the cytodomain (Williams et al. 2006). Three of the PCDH11X ectodomain changes are clustered within EC5: 3D homology modeling predicts that they are mapped closely to one another in space (Priddle et al. 2010). One change, Cys517, is located on the surface of the ectodomain, unpaired to any other cysteine residue and free to form a disulfide bond. Another cysteine (Cys680) is introduced between EC6 and EC7. Both these novel interaction sites may alter the binding characteristics of human PCDH11X through the formation of disulfide bonds, a mechanism previously described (Chen et al. 2007) for the *Xenopus* δ2-family member paraxial protocadherin, and γ-Pcdh-A3 tetramers (Schreiner and Weiner 2010). The cytoplasmic domain of PCDH11X/Y has been shown to interact with β-catenin and induces the *Wnt* signaling pathway in cultured prostate cancer cells (Yang et al. 2005). The cytoplasmic domain also contains a protein phosphatase 1α (PP1α)-binding motif, designated CM3, a defining characteristic of the δ1-PCDHs (Vanhalst et al. 2005).

PCDH11X/Y and Disease

Several SNPs in the ectodomain (Giouzeli et al. 2004) and cytoplasmic domain of PCDH11X (Giouzeli et al. 2004; Lopes et al. 2004) have been identified. Although SNPs causing coding changes in the cytoplasmic domain of PCDH11Y have been described (Giouzeli et al. 2004; Lopes et al. 2004; Durand et al. 2005), it is suggested that some of these may be X–Y paralogous sequence variants (Trombetta et al. 2010). No PCDH11X/Y sequence variation has been associated with schizophrenia, autism, bipolar disorder, or attention deficit disorder (Giouzeli et al. 2004; Durand et al. 2005). An intronic SNP in PCDH11X was reported in association with late onset Alzheimer's disease in women (Carrasquillo et al. 2009), but the association was not observed in subsequent studies (Beecham et al. 2010; Lescai et al. 2010; Wu et al. 2010; Miar et al. 2011).

PCDH11X/Y, Language, and Intellectual Function

Independent intragenic deletions in both Xq21.3 and Yp11 involving PCDH11X and PCDH11Y have been identified in a single case of a male child with a severe language delay (Speevak and Farrell 2011). The PCDH11X deletion was inherited from the (phenotypically normal) mother, but the PCDH11Y deletion was not present in the father and therefore appears to be a de novo occurrence. The authors postulate that the deletions interfere with the normal splicing, altering gene expression to disrupt the development of language. In another study (Whibley et al. 2010), 2 brothers with intellectual disability were identified with a 182-kb duplication within intron 2 of PCDH11X, although their mildly affected sister was found not to carry the duplication. One interpretation of these findings is that an interruption of PCDH11X is less well tolerated in males than in females, and this line of thinking has been suggested as a reason for the male propensity to autism and attention deficit hyperactivity disorder (Kopsida et al. 2009). Dibbens et al. (2008) invoked a related mechanism whereby PCDH11Y protects males from epilepsy and mental retardation limited to females associated with mutations of the X (only)-linked PCDH19. Both proposed mechanisms assume the presence of PCDH11Y in human males means that PCDH11X is no longer subject to "meiotic suppression of unsynapsed chromatin" (Turner 2007) and has an inactivation status that differs from that of Pcdh11X in all other animals. However, the inactivation status of PCDH11X/Y remains inconclusive: CpG islands in the promoters of both PCDH11X/Y alleles are unmethylated in male and female controls and all alleles present in Klinefelter's (47, XXY) syndrome are also unmethylated (Ross et al. 2006). In females, both alleles of PCDH11X are unmethylated and expression levels of PCDH11X are twice that of males (Lopes et al. 2006). These findings are consistent with the "escape from X-inactivation" that is held to be characteristic of genes on the X with a homolog on the Y, yet observations of the replication timing of both PCDH11X and PCDH11Y do not support this, suggesting complexity in the relevant epigenetic mechanisms (Wilson et al. 2007). The methylation status of PCDH11X/Y in psychiatric populations could be relevant to these conditions (Crow 2008; Isles and Wilkinson 2008).

Previous studies of the expression of PCDH11X/Y in humans have used the reverse transcription-polymerase chain reaction (RT-PCR) (Blanco et al. 2000; Blanco-Arias et al. 2004) and northern blotting (Yoshida and Sugano 1999), but have been limited to a few broad neuronal areas. Real-time PCR has demonstrated twice as much PCDH11X mRNA in adult female temporal lobes as in males (Lopes et al. 2006). A longitudinal study of the prefrontal cortex (Weickert et al. 2009) has shown that levels of PCDH11X/Y are highest in male neonates, decrease through childhood, and are lowest in adults of both sexes. Expression of PCDH11X/Y in fetal cortex, ganglionic eminence, hippocampal formation, and putamen and caudate, but limited to a few cases, was reported from a study with a major focus on PCDH19 (Dibbens et al. 2008).

A polyclonal antibody was raised against PCDH11X/Y for western blotting and immunoprecipitation of cultured prostate cells (Chen et al. 2002) but thus far, no immunohistochemical studies have addressed PCDH11X/Y expression in the human brain.

The aim of this study was to map the expression of PCDH11X/Y in a series of fetal and adult human brains, using antibodies raised in the absence of commercial products. While the study was under way, a commercial antibody against PCDH11X/Y became available and was subsequently included for comparison.

Materials and Methods

This study was conducted with the approval of the Oxfordshire Clinical Research Ethics Committee. Tissue blocks were taken from 12 fetal and 12 adult brains as detailed in Table 1. A full description of the areas used is provided in Supplementary Tables 1 (fetal) and 2 (adult).

Table 1
Specimens used

Case	Sex	Age
Fetal 1	Male	12 PCW
Fetal 2	Female	13 PCW
Fetal 3	Female	14 PCW
Fetal 4	Male	16 PCW
Fetal 5	Female	18 PCW
Fetal 6	Female	19 PCW
Fetal 7	Female	21 PCW
Fetal 8	Female	24 PCW
Fetal 9	Female	24 PCW
Fetal 10	Male	26 PCW
Fetal 11	Male	27 PCW
Fetal 12	?	34 PCW
Adult 1	Male	49 years
Adult 2	Male	54 years
Adult 3	Male	67 years
Adult 4	Male	66 years
Adult 5	Female	53 years
Adult 6	Female	82 years
Adult 7	Male	68 years
Adult 8	Female	72 years
Adult 9	Female	80 years
Adult 10	Female	67 years
Adult 11	Female	73 years
Adult 12	Male	43 years

PCW, postconceptional weeks.

Antibodies

Three antibodies against PCDH11X/Y were raised and used in this study. Procad1a is a mouse monoclonal antibody raised against a synthetic peptide [QEKNYTIREEMPE] corresponding to the N terminus (PCDH11Xa residues 24–36) of all PCDH11X/Y variants. Ex6 is another mouse monoclonal antibody raised against a synthetic peptide [EVPVSVHTRPTDST] corresponding to residues 1023–1037 of the C terminus of PCDH11Ya. X11 is a rabbit polyclonal antibody (made to order, BioCarta, San Diego, CA, United States of America) against a synthetic peptide [LHHSPPLTQATA] corresponding to a consensus sequence from a repeated motif (starting at residue 1158 of PCDH11Xc) within the cytoplasmic region of longer variants of PCDH11X/Y. We also used a commercial rabbit polyclonal antibody raised against a region common to all isoforms of PCDH11X and PCDH11Y (HPA000432, Sigma Aldrich).

Recombinant Proteins

A 357-bp sequence encoding EC1 (119 aa) of PCDH11Xa was directionally cloned into pET24a(+) (69749-3, Merck Chemicals Ltd.). A 333-bp sequence encoding the C terminus (111 aa) of PCDH11Ya was directionally cloned into pGEX-6P-1 (28-9546-48, GE Healthcare UK Ltd.). Large-scale bacterial cultures were grown, expression was induced, and proteins were extracted and purified.

Monoclonal Immunization

Mice were immunized with synthetic peptides, to produce monoclonal antibodies from spleen fusions. Sp2/0 myeloma cells were fused to splenocytes using polyethylene glycol (Harlow and Lane 1988). ClonaCell methylcellulose (03804, StemCell Technologies SARL) was used for cloning and re-cloning of cells following screening against formalin fixed paraffin-embedded human brain tissue. The ability of the antibodies to recognize PCDH11X/Y was assessed by screening with a solid-phase antibody capture enzyme linked immunosorbant assay against recombinant PCDH11Xa EC1 or PCDH11Ya cytodomain. Tissue culture supernatants were removed and diluted 1:5 in phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4) and 0.1% sodium azide.

Single-Label Immunohistochemistry

Formalin fixed paraffin-embedded tissues were sectioned at 10 μm. Sections were passed through a series of graded alcohols to remove the paraffin and then microwaved in antigen unmasking solution (H-3300, Vector Laboratories) at low power (without boiling) for 30 min to improve antigen detection (Evers and Uylings 1997). Once cooled, sections were treated with 3% H_2O_2 for 10 min, then placed into a humidified chamber on a rocking platform (to ensure an even coverage of solutions), and blocked for 90 min in 10% bovine serum and 0.1% Tween-20 diluted in Tris-buffered saline (0.05 M Tris, pH 7.6, 0.15 M sodium chloride).

Primary antibodies were diluted (Procad1a 1:150; Ex6 1:8; X11 1:150; commercial anti-PCDH11X/Y 1:250) in the blocking solution and applied to the tissues for 1 h at room temperature. The humidified chamber was then moved into a refrigerator and the incubation continued overnight (17 h). On the following day, all steps were performed at room temperature on the rocking platform. After a 1-h incubation with biotinylated goat secondary antibodies (anti-mouse 1:200, B 7151; anti-rabbit 1:400, B8895 Sigma Aldrich), the Vectorstain Elite ABC kit (PK-6100, Vector Laboratories) was used to locate the antibody complex. The peroxidase reaction was demonstrated with metal-enhanced 3,3′-diaminobenzidine (DAB, 34065, Perbio Science). The specificity of the immunohistochemical reactions was confirmed by the absence of specific immunoreactivity in control experiments in which the primary antibodies were omitted. Immunoreactivity was inhibited in a dose-dependent manner when Procad1a and Ex6 were incubated with their recombinant proteins and when X11 was incubated with its immunizing peptide prior to their use on tissue sections. A mouse monoclonal isotyping kit (MMT1, AbD Serotec) demonstrated that both Procad1a and Ex6 are immunoglobulins of the IgG1 κ subtype.

Double-Label Immunohistochemistry

Antigen retrieval was performed as for single labeling. Sections were treated with an Avidin/Biotin blocking kit (SP-2001, Vector Laboratories) and then blocked in 10% normal horse serum (NHS) in PBS for 1 h. Primary antibodies were diluted in 2% NHS/PBS at higher concentrations (Procad1a 1:15; Ex6 1:1; X11 1:15; commercial anti-PCDH11X/Y 1:25; doublecortin [DCX] 1:2000, Ab18723 Abcam; neuropeptide Y [NPY] 1:50, Ab10341 Abcam; calbindin D-28k 1:400, #300 Swant; calretinin 1:200, #6B3 Swant; parvalbumin 1:250, #235 Swant) than for single labeling owing to the reduced efficiency of immunofluorescent visualization (Hoffman et al. 2008) and applied to the tissues at 4°C for 17 h, with the exception of the calbindin incubation which lasted 3 days. Biotinylated goat secondary antibodies were diluted (anti-mouse 1:200; anti-rabbit 1:400; anti-guinea pig 1:500, Ab6907 Abcam) in 2% NHS/PBS and applied for 1 h before a 15-min incubation with Fluorescein Avidin distinct cell sorting (DCS) (20 μg/mL in PBS, A-2011, Vector Laboratories). The procedure (minus antigen retrieval) was then repeated for the second primary antibody and visualized with Texas Red Avidin DCS (15 min, 20 μg/mL in PBS, A-2016, Vector Laboratories). Finally, a 5-min incubation with 1% Sudan Black in 70% ethanol was used to reduce lipofuscin like autofluorescence (Schnell et al. 1999) and sections were mounted using Vecta Shield Hard Set (H-1400, Vector Laboratories).

Microscopy

We examined single-labeled sections using a conventional light microscope (Olympus BX50) and light box, and documented results with a digital camera, using the GNU Image Manipulation Program to adjust contrast and brightness on the digitized images. Double-labeled sections were examined using a fluorescent microscope (Nikon Eclipse E600) and Adobe Photoshop CS5 was used to produce merged images.

Results

All 4 PCDH11X/Y antibodies produced a pattern of immunoreactivity, predominantly in the cytoplasm of neurons, which was virtually identical in both groups of brains and will be considered together. Immunoreactivity produced by Procad1a and the commercial antibody directed against all predicted forms of PCDH11X/Y was co-localized within the same cells (Fig. 1*A*,*H*–*I*). Furthermore, immunoreactivity produced by Ex6 and X11 directed against the different cytoplasmic domains overlapped in the majority of cells (Fig. 1*B*,*C*).

Expression of PCDH11X/Y in the Fetal Human Brain

All PCDH11X/Y antibodies reacted with tissue from all ages, and immunoreactivity was detected in both sexes. The immunoreactivity was strongest in the cortical plate and the ventricular zone of the developing cerebral cortex (Figs 1*A*–*F* and 2*A*–*D*), the lateral and medial ganglionic eminences (Fig. 2*B*), the caudate (Fig. 2*A*), and the inferior olivary nucleus (Fig. 2*G*,*H*). Within the cerebellum, the Purkinje cells and the dentate nucleus were strongly immunoreactive (Fig. 2*F*). Moderate levels of immunoreactivity were detected in the hippocampal formation (Fig. 2*E*), the emboliform nucleus of the cerebellum (Fig. 2*F*), the gracile, cuneate, and spinal trigeminal nuclei (Fig. 2*I*), the abducens and facial nuclei (Fig. 2*F*), and the pontine nuclei. Weak immunoreactivity was detected in the subplate (Figs 1*A*–*F* and 2*A*–*C*), the thalamus (Fig. 2*B*), and the arcuate nucleus (Fig. 2*H*). PCDH11X/Y immunoreactivity was co-localized with DCX in the cortical plate, subplate and intermediate zone (Fig. 1*E*), and NPY within the subplate,

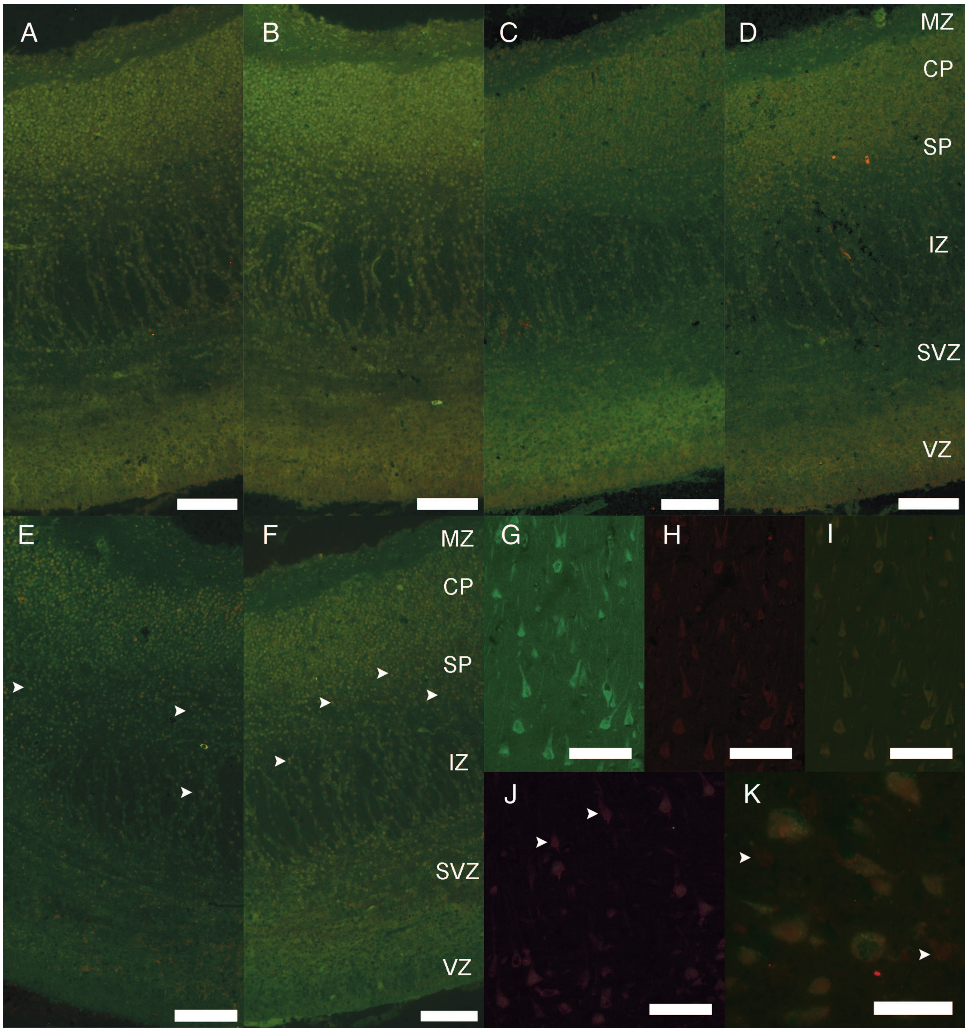

Figure 1. Double labeling of PCDH11X/Y. (*A–D*) Merged images showing co-localization of all PCDH11X/Y antibodies in the fetal cerebral cortex, 13 PCW female (*A*, Procad1a: green, anti-PCDH11X/Y: red; *B*, Procad1a: green, X11: red; *C*, Ex6: green, X11: red; *D*, Ex6: green, anti-PCDH11X/Y: red). (*E* and *F*) Merged images showing co-localization of PCDH11X/Y with DCX and NPY in the fetal cerebral cortex, 13 PCW female (*E*, Procad1a: green, DCX: red; *F*, Procad1a: green, NPY: red). Arrowheads in (*E* and *F*) highlight the co-expression of PCDH11X/Y in the SP and IZ with DCX and NPY, respectively. (*G–K*) Adult cerebral cortex, female (*G*, Procad1a: green; *H*, anti-PCDH11X/Y: red; *I*, merged image; *J*, calretinin: green, anti-PCDH11X/Y: red; *K*, calbindin: green, anti-PCDH11X/Y: red). Arrowheads in (*J* and *K*) highlight the expression of PCDH11X/Y in the absence of calretinin and calbindin, respectively. Scale bars: (*A–J*) 100 µm; (*K*): 25 µm. CP, cortical plate; IZ, intermediate zone; MZ, marginal zone; SP, subplate; SVZ, subventricular zone; VZ, ventricular zone.

intermediate zone, and subventricular zone (Fig. 1*F*). A summary of fetal results is shown in Table 2.

Expression of PCDH11X/Y in the Adult Human Brain

All PCDH11X/Y antibodies produced a pattern of immunoreactivity in male and female brains that was indistinguishable from each other, and reflected the immunoreactivity observed in the fetal brains. Strong PCDH11X/Y immunoreactivity was detected in neurons of layers II–VI in the frontal cortex (Fig. 3*A*), cingulate gyrus, occipital pole, and temporal cortex (Fig. 3*B*), with pyramidal cells prominently labeled (Figs 1*G–I* and 3*C*). As in the fetal brains, strong immunoreactivity was observed in the Purkinje cells (Fig. 3*F*) and dentate nucleus. Moderate immunoreactivity was seen in the hippocampal formation (Fig. 3*G*), amygdala, the inferior olivary nucleus (Fig. 3*I*), and the caudate and putamen (Fig. 3*D*). Weak immunoreactivity was detected in the thalamus (Fig. 3*H*), the dorsal raphe nucleus (Fig. 3*H*), the hypoglossal nucleus (Fig. 3*I*), the nucleus of the solitary tract (Fig. 3*I*), and the spinal cord. PCDH11X/Y immunoreactivity was co-localized with the calcium-binding proteins calretinin (Fig. 1*J*), calbindin (Fig. 1*K*), and parvalbumin. Results are summarized in Table 2.

Discussion

The first immunohistochemical investigation of the expression of PCDH11X/Y protein in the human brain has demonstrated PCDH11X/Y immunoreactivity in the cytoplasm of neurons of the developing and adult cerebral cortex in both sexes and on both sides of the brain.

In the fetal brains, immunoreactivity was prominent in the medial and lateral ganglionic eminences (Fig. 2*B*) and the developing neocortex (Figs 1*A–F* and 2*A–D*), resembling the pattern of PCDH11X/Y expression observed in situ with a common PCDH11X/Y riboprobe at 16–20 weeks gestation by

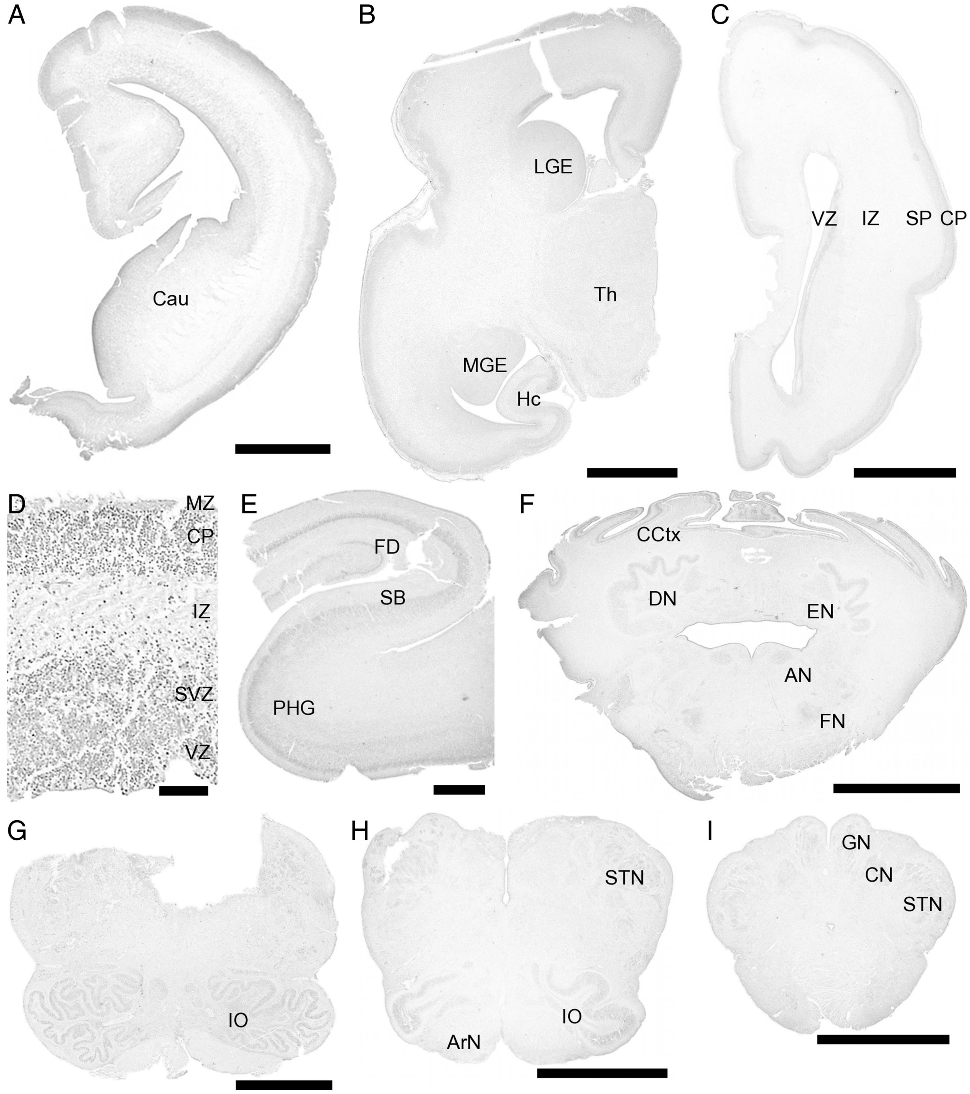

Figure 2. Expression of PCDH11X/Y in the fetal brain. Cerebral cortex (*A*, 14 PCW female; *B*, 16 PCW male; *C*, 19 PCW male; *D*, 12 PCW male), hippocampal formation (*E*, 18 PCW female), cerebellum and pons (*F*, 18 PCW female), and medulla oblongata (*G*, 27 PCW male; *H* and *I*, 18 PCW female). Scale bars: (*A*, *F–I*): 3000 μm; (*B*, *C*): 5000 μm; (*D*) 100 μm; (*E*) 1000 μm. AN, abducens nucleus; ArN, arcuate nucleus; Cau, caudate; CCtx, cerebellar cortex; CN, cuneate nucleus; CP, cortical plate; DN, dentate nucleus; EN, emboliform nucleus; FD, fascia dentata; FN, facial nucleus; GN, gracile nucleus; Hc, hippocampal formation; IO, inferior olivary nucleus; IZ, intermediate zone; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MZ, marginal zone; PHG, parahippocampal gyrus; SB, subiculum; SP, subplate; STN, spinal trigeminal nucleus; SVZ, subventricular zone; Th, thalamus; VZ, ventricular zone.

Dibbens et al. (2008). These authors also reported that PCDH11X/Y was expressed in the embryonic caudate nucleus exclusively in females; they detected no expression on northern blots of adult caudate from either sex. In our study, PCDH11X/Y immunoreactivity was detected in both sexes at all ages in which the caudate was present, including an 16-postconceptional weeks (PCW) male, and adults of both sexes. Furthermore, both PCDH11X and PCDH11Y transcripts have been detected in the adult caudate by RT-PCR and confirmed by restriction digests at sex-specific sites (Blanco et al. 2000; Blanco-Arias et al. 2004). This disparity may reflect mismatches between the presence of mRNA and the encoded protein that have been observed in brain (Tropea et al. 2001) and muscle (Andersen and Schiaffino 1997). Other areas of strong immunoreactivity in the fetal brains were the inferior olive (Fig. 2*G* and *H*) and the deep cerebellar nuclei (Fig. 2*F*). The strong immunoreactivity observed in the uppermost layers of the cortical plate and the ventricular zone at 12 PCW (e.g. Fig. 2*D*) may suggest PCDH11X/Y expression in recently migrated pyramidal neurons and their precursors, respectively. The co-localization of PCDH11X/Y and DCX (Fig. 1*E*), a microtubule-associated protein that is a marker of migrating neurons (Francis et al. 1999; Gleeson et al. 1999), in the subplate and cortical plate together with the strong immunoreactivity of pyramidal neurons in the adult cortex (Fig. 3*B*,*C*) supports this supposition. As the fetal cortex continues to develop, expression of PCDH11X/Y is also seen in the subplate, albeit less strongly than in the cortical plate and ventricular zone (e.g. Figs 1*A–F* and 2*B*,*C*). NPY is principally confined to neurons residing in the subplate (Delalle et al. 1997; Bayatti et al. 2008), and double labeling with NPY suggests that these resident subplate neurons also express PCDH11X/Y (Fig. 1*F*).

In the adult brains, expression was detected in all cortical areas in layers II–VI (Fig. 3*A*) with pyramidal neurons

Table 2
Regional expression of PCDH11X/Y

Region	Fetal	Adult
Frontal cortex	CP and VZ	Layers II–VI
Anterior prefrontal		+++
Cingulate gyrus	+++	+++
Motor/premotor	+++	
Posterior orbital gyrus	+++	+++
Superior frontal gyrus	+++	
Middle frontal gyrus	+++	+++
Inferior frontal gyrus	+++	
Temporal cortex	CP and VZ	Layers II–VI
Insular	+++	
Superior temporal gyrus	+++	+++
Middle temporal gyrus	+++	+++
Inferior temporal gyrus	+++	
Parahippocampal gyrus	+++	+++
Temporal pole	+++	
Parietal cortex	CP and VZ	Layers II–VI
Postcentral gyrus	+++	T.U.
Superior lobule	+++	T.U.
Inferior lobule	+++	T.U.
Paracentral lobule	+++	T.U.
Occipital cortex	CP and VZ	Layers II–VI
Primary visual		+++
Ganglionic eminence		N/A
Medial	+++	
Lateral	+++	
Hippocampal formation		
CA1	++	++
CA2	++	++
CA3	++	++
CA4	++	++
Fascia dentata	++	++
Subiculum	++	++
Amygdala	T.U.	
Medial nucleus		+
Central nucleus		+
Basomedial nucleus		+
Basolateral nucleus		+
Basal ganglia		
Caudate	+++	++
Putamen	++	++
Thalamus		
Anterior nucleus		+
Lateral dorsal nucleus		+
Mediodorsal nucleus	+	+
Pulvinar nucleus	+	+
Cerebellar cortex		
Purkinje cells	+++	+++
Granule cells	+	+
Cerebellar nuclei		
Dentate	+++	++
Emboliform	++	++
Midbrain		
Dorsal raphe	+	+
Red nucleus	T.U.	+
Substantia nigra	+	+
Pons	+	+
Medulla oblongata		
Abducens nucleus	++	+
Arcuate nucleus	+	
Facial nucleus	++	+
Hypoglossal nucleus	++	+
Inferior olivary nucleus	+++	++
Spinal trigeminal nucleus	++	++
Solitary nucleus	T.U.	+
Spinal cord	T.U.	+

CP, cortical plate; N/A, Not applicable; T.U., Tissue unavailable; VZ, ventricular zone.

prominently labeled (Figs 1*G–I* and 2*B*,*C*). PCDH11X/Y expression was not confined to any subtype of interneurons as identified by the calcium-binding proteins, calretinin (Fig. 1*J*), calbindin (Fig. 1*K*), and parvalbumin.

We did not observe any asymmetric expression of PCDH11X/Y in the cerebral cortex nor was the expression in the superior temporal gyrus remarkable. The Purkinje cells (Fig. 3*F*) of the cerebellar cortex were intensely immunoreactive, as was the dentate nucleus. Immunoreactivity was also detected in the amygdala, hippocampal formation (Fig. 3*G*), caudate (Fig. 3*D*), and thalamus (Fig. 3*H*), coinciding with the prior RT-PCR data (Yoshida and Sugano 1999; Blanco et al. 2000; Blanco-Arias et al. 2004).

The pattern of expression in the developing human cortex was similar to that reported in the ferret (Krishna-K et al. 2009) and the rat (Kim et al. 2007), and our findings of expression in the adult hippocampal formation and amygdala are similar to observations made in the rat (Kim et al. 2010) and mouse (Hertel et al. 2008). Reports of Pcdh11 expression in the cortex of adult experimental animals are less consistent: Ranging from complete absence in rat (Kim et al. 2007), a subpopulation of neurons in layers IV–VI in the mouse somatosensory cortex (Krishna-K et al. 2011), to layers II–VI of the mouse motor cortex (Hertel and Redies 2011), and layers II–VI of the ferret visual cortex (Krishna-K et al. 2009). Interneuron expression was not specifically addressed by these studies; however, work on γ-Pcdhs demonstrates widespread interneuronal expression in many structures (Wang et al. 2002; Phillips et al. 2003; Lefebvre et al. 2008).

The prominent PCDH11X/Y expression we observed throughout the cerebral cortex may be a consequence of the putative gene dosage doubling at 6 MYA and/or the addition of the human-specific cysteines in the ectodomain. Further investigation of the human specificity of this gene pair may be directed at the predecessor cells (Bystron et al. 2006), the first neurons to populate the human cerebral cortex at Carnegie stage 12.

The antibodies used were broad in their range of specificity (Procad1a and anti-PCDH11X/Y: Common to all isoforms; Ex6: Most short forms; X11: Most long forms) and the location of individual PCDH11X/Y isoforms may yet reveal a more distinct expression pattern. Our repeated attempts to raise a PCDH11Y-specific antibody (i.e. Ex6) were unsuccessful. Despite careful screening and selection of clones that only reacted with male brains, antibodies were found to be also reactive with female brains. Ex6 immunoreactivity is ameliorated by incubation with the recombinant PCDH11Ya cytoplasmic protein suggesting specificity to a Y motif, but the high similarity between PCDH11Ya and PCDH11Xb within the cytodomain (and indeed the entire protein) makes it difficult to design antibodies and nucleotide probes that differentiate the PCDH11X and PCDH11Y. This is also a problem for longer isoforms, for example, a report of upregulation of PCDH11Xc in males using microarrays (Galfalvy et al. 2003) is dubious given that the probesets in question are 100% identical to PCDH11Yc (Lopes et al. 2006; Weickert et al. 2009) and, in another report, 3 PCDH11Y isoform-specific primer pairs produced product from female tissues (Ahn et al. 2010). To avoid such cross-reactivity, studies have isolated small fragments that exploit the few PCDH11X/Y sequence differences (Giouzeli et al. 2004) or used common riboprobes (Dibbens et al. 2008) or pan PCDH11X/Y antibodies (Chen et al. 2002) to examine PCDH11X/Y as a whole. Cyclophosphamide immunosuppression (Ou et al. 1991; Sleister and Rao 2001) has enabled the production of antibodies against highly similar neuronal antigens and should be considered when raising antibodies against PCDH11X/Y in the future.

In summary, PCDH11X/Y is widely expressed throughout both the developing and adult human brain, with strong

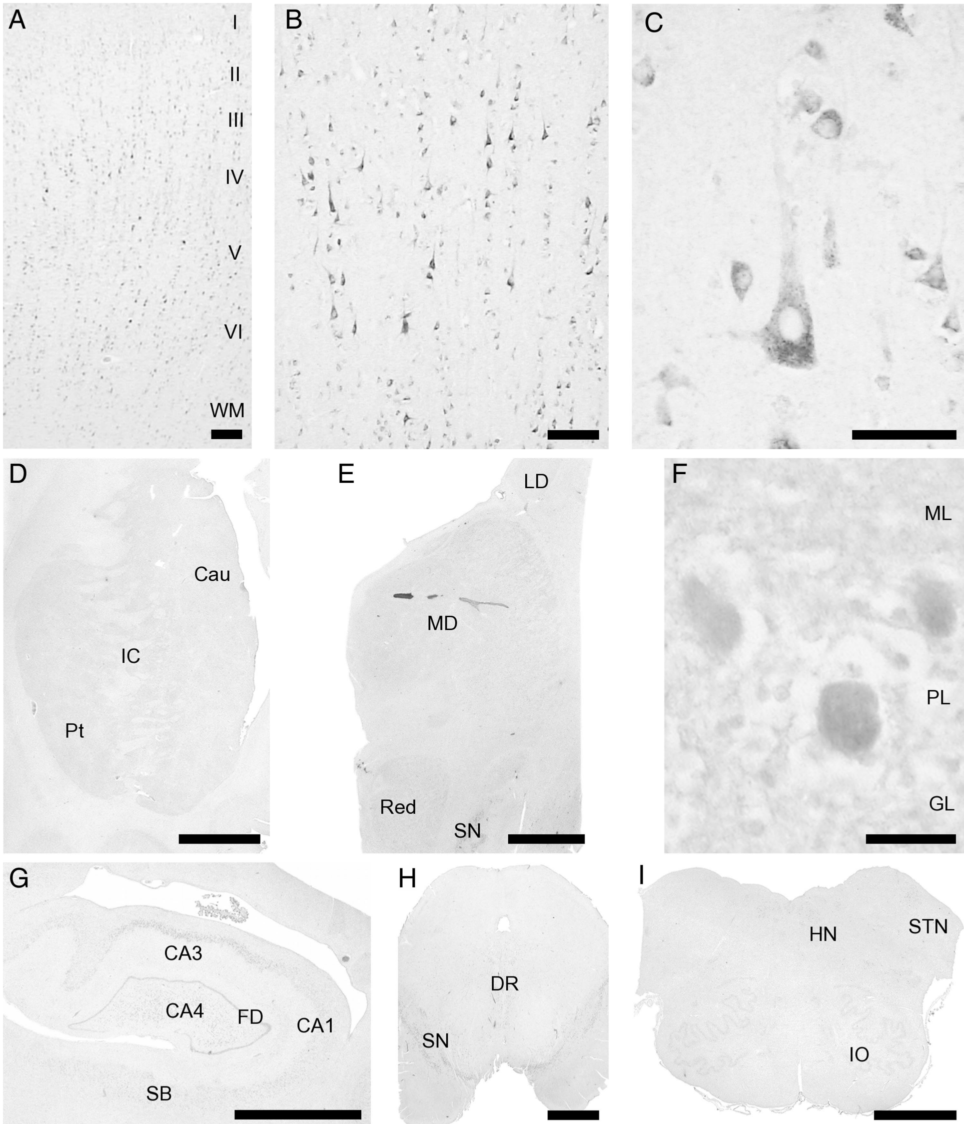

Figure 3. Expression of PCDH11X/Y in the adult brain. Frontal cortex (*A*, male), superior temporal gyrus (*B*, female) with prominent expression in pyramidal neurons (*C*, female), basal ganglia (*D*, male), thalamus (*E*, female), cerebellar cortex (*F*, male), hippocampal formation (*G*, female), midbrain (*H*, female), and medulla oblongata (*I*, male). Scale bars: (*A*) 200 μm; (*B*) 100 μm; (*C*) 50 μm; (*D*, *E G–I*) 5000 μm; (*F*) 25 μm. CA1-4, areas of Ammon's horn; Cau, caudate; DR, dorsal raphe; FD, fascia dentata; GL, granular layer; HN, hypoglossal nucleus; I–VI, cortical layers I–VI; IC, internal capsule; IO, inferior olivary nucleus; LD, lateral dorsal nucleus; MD, mediodorsal nucleus; ML, molecular layer; PL, Purkinje cell layer; Put, putamen; Red, red nucleus; SB, subiculum; SN, substantia nigra; WM, white matter.

expression observed in the cerebral cortex, ganglionic eminence, Purjkinje cells and dentate nucleus of the cerebellum, and the inferior olivary nucleus. It is interesting to note that many of the latter structures use γ-aminobutyric acid as their transmitter; however, our investigation did not find restricted PCDH11X/Y expression in a limited subset of interneurons and further work is required. The lack of an asymmetric distribution and the widespread expression in non cortical areas observed in the present study does not support PCDH11X/Y's role in human-specific faculties, but it is worth noting that the antibodies were broad in their specificity and individual isoforms may yet be found in a more restricted pattern in cortical areas related to language. In addition, Pcdh11X has been present in the mammalian radiation for some time and any functions attributed to the human-specific changes to PCDH11X and the entirely new PCDH11Y may build upon existing neuronal systems. These data confirm earlier reports using RT-PCR and in situ hybridization and identify PCDH11X/Y expression in areas that were previously untested. Methods for studying expression that are specific for the individual isoforms of PCDH11X and, more importantly, PCDH11Y together with the determination of the inactivation status and the functional significance of the PCDH11X ectodomain changes will be necessary to understand the role of the gene pair in disease and the evolution of the human brain.

Supplementary Material

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

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