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Expression and Genetic Variability of *PCDH11Y*, a Gene Specific to *Homo sapiens* and Candidate for Susceptibility to Psychiatric Disorders

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Running title: *PCDH11X/Y in the human brain and psychiatric diseases*

Synaptogenesis, the formation of functional synapses, is a crucial step for the development of the central nervous system. Among the genes involved in this process, cell adhesion molecules, such as protocadherins and neuroligins, are essential factors for the identification of the appropriate partner cell and the formation of synapses. In this work, we studied the expression and the genetic variability of two closely related members of the protocadherin family *PCDH11X/Y*, located on the X and the Y chromosome, respectively. *PCDH11Y* is one of the rare genes specific to the hominoid lineage, absent in other primates. Expression analysis indicated that transcripts of the *PCDH11X/Y* genes are mainly detected in the cortex of the human brain. Mutation screening of thirty individuals with autism identified two *PCDH11Y* polymorphic amino acid changes, F885V and K980N. These variations are in complete association, appeared during human evolution approximately 40000 years ago and represent informative polymorphisms to study Y chromosome variability in populations. We studied the frequency of these variants in males with autism spectrum disorders (n = 110), attention deficit hyper-activity disorder (ADHD; n = 61), bipolar disorder (n = 61), obsessive-compulsive disorder (n = 51) or schizophrenia (n = 61) and observed no significant differences when compared to geographically-matched control populations. These findings do not support the role of *PCDH11Y*, or more generally of a frequent specific Y chromosome, in the susceptibility to these neuropsychiatric disorders.

KEYWORDS: Autism, schizophrenia, obsessive-compulsive disorder, bipolar disorder, synaptogenesis

INTRODUCTION

The role of neuronal cell adhesion molecules—including neuroligins (NLGN) and protocadherins (PCDH)—in psychiatric disorders was recently evidenced by the identification of mutations in *NLGN3* and *NLGN4* in autism and Asperger syndrome [Jamain et al., 2003]. In addition, a rare variant of the protocadherin *PCDH8* was observed in a family with two sibs with schizophrenia [Bray et al., 2002]. PCDH are neuronal cell-surface proteins that bind in a homophilic manner to each other and form symmetric intercellular junctions.

PCDH genes have a complex genomic organization with multiple variable exons and a set of constant exons, similar to the immunoglobulin (Ig) and T-cell receptor (TCR) genes. Furthermore, *PCDH* genes can be monoallelically expressed from a single neuron [Esumi et al., 2005]. Therefore, this huge diversity of PCDH expression can be used to specify individual neuronal cell identity and could play a crucial role in establishing the blueprint of the neuronal networks in humans [Hilschmann et al., 2001]. *PCDH11Y* is one of the rare genes specific of the hominoid lineage and absent from other primates [Blanco et al., 2000]. It is located on the Y chromosome and originates from a translocation of the *PCDH11X* gene after the divergence between chimpanzees and humans [Blanco et al., 2000; Blanco-Arias et al., 2004]. The X inactivation profile of *PCDH11X* as well as the function

and the neuronal specificity of the proteins PCDH11X and PCDH11Y remain unknown. However, the role of the PCDH during development and the recent origin of *PCDH11Y* make these genes strong candidates for susceptibility to psychiatric disorders [Giouzezi et al., 2004]. In particular, *PCDH11Y* could be a susceptibility gene for autism and attention deficit hyperactivity disorder (ADHD), in which males are more affected than females and for schizophrenia and obsessive compulsive disorder (OCD), in which the age of onset is younger in males.

In this paper, we report the expression pattern of both genes in the human brain and their sequence variability in patients with autism spectrum disorders, ADHD, bipolar disorder, OCD, and schizophrenia.

PATIENTS AND METHODS

French (n = 72) and Swedish (n = 38) male probands, fulfilling the DSM-IV and ICD-10 criteria for autistic disorder, Asperger syndrome or pervasive developmental disorder not otherwise specified were recruited by the Paris Autism Research International Sib-pair (PARIS) study. One hundred individuals (91%) met the Autism Diagnostic Interview-Revised (ADI-R) criteria for autism [Lord et al., 1994]. Subjects were included after a clinical and medical work-up comprising neuropsychological examination, standard karyotyping, fragile-X testing, and blood and urine analyses for metabolic screening; brain imaging and EEG were performed when possible. Patients with associated medical disorders were excluded from the study.

Swedish male patients fulfilling diagnostic criteria according to the DSM-IV for ADHD (n= 61) were recruited at the Child Neuropsychiatric Clinic in Göteborg after a comprehensive psychiatric and medical assessment to assign diagnosis based on standard instruments such as the Conner's scale and to exclude associated somatic conditions.

French male patients with schizophrenia (n = 61) or bipolar disorder (n= 61) were recruited at the Albert Chenevier Hospital, Créteil, France. French male probands with OCD (n = 51) were recruited at the Robert Debré Hospital, Paris, France. All cases met DSM-IV criteria for one of these disorders [American Psychiatric Association, 1994]. Lifetime psychiatric evaluation was carried out during a direct interview by trained psychiatrists using the Diagnostic Interview for Genetic Studies (DIGS) [Nurnberger et al., 1994].

French (n= 86) and Swedish (n = 47) healthy male controls were recruited among blood donors at the Pitié-Salpêtrière Hospital in Paris, France and at the University of Göteborg in Göteborg, Sweden. The sample of French controls from European descent was included after being interviewed with the DIGS to confirm the absence of both personal and family history of psychiatric disorders in first- and second-degree relatives.

All patients and controls were of European descent. The local Research Ethics Boards reviewed and approved the study. Written informed consent was obtained from all controls, probands, and their parents. If the proband was under 18 years old, the proband's consent and written parental consent were obtained.

DNA and Statistical Analysis

DNA extracted from blood leukocytes or lymphoblastoid cell lines was used to amplify the *PCDH11X/Y* genes; sequence analysis was performed by direct sequencing of the PCR products, using a 373A automated DNA sequencer (Applied Biosystems, Foster City, CA). For expression studies, 1 µg of total RNA from different regions of the human brain was isolated by the acid-guanidium thiocyanate phenol chloroform method [Chomczynski and Sacchi, 1987] and reverse transcribed using the Gene Amp RNA PCR kit (Perkin-Elmer Corp., Norwalk, CT). Transcripts containing exons E4 and E5 were amplified with primers E4F (CCGGATGAAATATTTAGACTGGTT) and E5R1 (TATGGTCCGCATCCTTATCC). Transcripts containing exons E3, E4 and E5 were amplified with primers E3F (GTGGGTTTTAATTCAGATATT) and E5R2 (TGTTTCTTCCCTATCCAGTGG). Transcripts containing exons E4.1 and E5 were amplified with primers E4bF (CATGCATGTTTAGGGTTGGCT) and E5R2. For genotyping of the FK and VN *PCDH11Y* haplotypes, a fragment of exon 5 was amplified using primers E5F (CACAAGAGATCTGTTTGCAATCGA) and E5R3 (AGAAAGTTGTGTTAGAAACTTGG). After amplification, 15 µl of PCR products were digested with 1µl Tsp509 I (New England BioLabs, Ipswich, MA). The products were loaded on a 3% 1:1 agarose:nusieve gel. Differences in haplotype frequencies were evaluated using a homogeneity χ^2 test.

RESULTS

Expression of the *PCDH11X/Y* in the Human Brain

In order to study the pattern of expression of the *PCDH11X/Y* genes in the human brain, RT-PCRs of transcripts from both genes were performed on RNA isolated from different brain regions using primers with identical sequences for *PCDH11X* and *PCDH11Y* (Fig. 1). These RT-PCR results are nonquantitative and should be taken with care. Discrimination between the product amplified from *PCDH11X* and *PCDH11Y* mRNA was done by restriction analysis, taking advantage of a BsaAI site present only in the *PCDH11X* sequence. In males, *PCDH11Y* transcripts seem to be more abundant than *PCDH11X*. This difference could be due to the presence of an additional promoter upstream of exon 4 of *PCDH11Y*. The similar region appears to be non-functional on the X chromosome (Fig. 1). Expression of the *PCDH11X* and *PCDH11Y* genes (originating from two promoters in the case of *PCDH11Y*) is mainly restricted to the cortex (frontal, temporal, and occipital) and the hippocampus, is less abundant in the thalamus and is virtually absent from the cerebellum.

Variation of *PCDH11X/Y* in Individuals With Psychiatric Disorders

Exons 3-5 of the *PCDH11X* and *PCDH11Y* genes were directly sequenced in a subset of 30 males with autism. No non-synonymous variation was identified in the *PCDH11X* gene. In contrast, two variations, F885V and K980N, were identified in exon 5 of the *PCDH11Y* gene. As recently reported, these variations were always found in association, thus defining only two haplotypes (FK and VN)

[Giouzeli *et al.*, 2004; Lopes *et al.*, 2004]. Both amino acids are conserved during evolution and are located in the intracellular part of the protein (Fig. 2a).

The FK haplotype is probably the ancestral one since the amino acids F885 and K980 are present in *PCDH11X* from human, chimpanzee and mouse, as well as in other protocadherins (Fig. 2a). We previously reported the Y chromosome haplotypes of 111 males with autism from France, Sweden and Norway [Jamain *et al.*, 2002]. Using this information, we could ascertain the origin of the novel VN haplotype of *PCDH11Y* in the P lineage of the human Y chromosome phylogeny [Y Chromosome Consortium, 2002]. Specifically, the VN haplotype is present in Y chromosomes carrying variation P-92R7-T (lineage P, variation 92R7, allele T), whereas the FK haplotype occurs in Y chromosomes carrying the variation P-92R7-C (not shown). The P lineage originated approximately 40,000 years ago and is the ancestor of P-M173 (lineage P, variation M173), the major Y chromosome haplotype in Europe [Wells *et al.*, 2001].

Frequencies of both the FK and VN haplotypes of *PCDH11Y* were determined in male patients with psychiatric diseases (Fig. 2b). None of the affected populations showed significant differences in their haplotype frequency (Fig 2c) compared to ethnically-matched controls. A significant difference was found when French and Swedish controls were compared ($p= 0.0046$). This genetic difference underlies the diverse migration of *Homo sapiens* during the settlement of Europe 40,000 years ago [Wells *et al.*, 2001].

DISCUSSION

PCDH11X/Y are potential functional candidates that might bear on susceptibility to psychiatric diseases for three main reasons. First, they are members of the PCDH family, which is known to play a major role in synapse recognition and neuronal path finding during brain development [Hilschmann *et al.*, 2001]. Second, *PCDH11Y* is one of the rare genes located on the Y chromosome with a strong regulated expression in the brain [Blanco *et al.*, 2000; Blanco-Arias *et al.*, 2004]. Third, *PCDH11Y* is specific to humans and may have acquired a new function in the wiring of the human brain. As suggested by Crow [Crow, 1999], this new connectivity may play a role in human specific cognitive functions, such as language, and consequently in conferring susceptibility to psychiatric disorders such as autism spectrum disorders and schizophrenia.

The RT-PCR analysis of *PCDH11X* and *PCDH11Y* transcripts revealed a strong expression in the cortex and hippocampus and less in the cerebellum. This pattern suggests that both proteins are involved in the recognition or path finding of neurons originating in the cortex and the hippocampus. During evolution, several Y chromosomal genes having a homologue on the X chromosome were rapidly converted into non-functional pseudogenes. Nevertheless, the presence of a specific *PCDH11Y* promoter with a regulated expression pattern, and the absence of a stop codon in the coding sequence strongly suggest that *PCDH11Y* is not a pseudogene.

The two *PCDH11Y* non-synonymous variations, F885V and K980N, concern conserved amino acids and may modify the properties of the protein, such as the ability of *PCDH11Y* to perform signal

transduction. Interestingly, these two amino acid changes probably appeared on the Y chromosome at the same time or during a short time window, since we did not observe any FN or VK haplotypes in the individuals tested ($n = 477$). Comparison between the two control populations indicated that the VN haplotype is more frequent in France, whereas in Sweden, the FK haplotype is more common. Nevertheless, a comparison between probands and ethnically-matched controls did not show any significant difference in haplotype frequencies. These results suggest that any contribution of *PCDH11Y* to psychiatric diseases is likely to be weak, although the existence of rare variations of stronger effect cannot be excluded. Furthermore, using these Y-linked informative polymorphisms, we could document Y chromosome variability in autism, ADHD, OCD, bipolar disorder and schizophrenia. Given that most of the Y chromosome does not recombine, a unique sequence variation on this chromosome can be representative of a whole group of Y chromosomes (called haplogroups) [Y Chromosome Consortium, 2002]. This non-recombining property of the Y chromosome has been intensively used to understand the origin and the migration of human populations. Therefore, the absence of significant differences in haplotype frequencies between affected individuals and controls also supports the absence of a specific Y chromosome effect on the susceptibility to the neuropsychiatric disorders studied.

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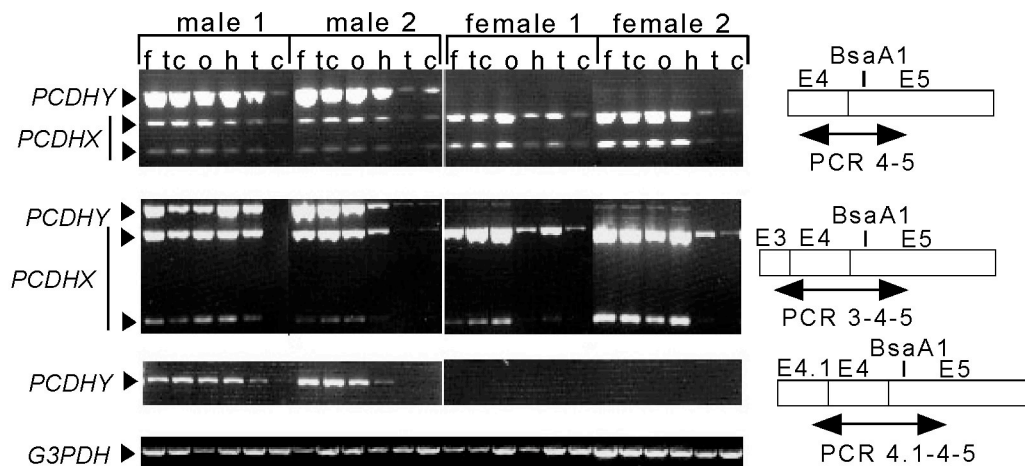


Fig. 1. Expression analysis of PCDH11X/Y in the human brain. Specific RT-PCRs were performed on total RNA from different brain regions using primers with identical sequences for both *PCDH11X* and *PCDH11Y*. Discrimination between *PCDH11X* and *PCDH11Y* mRNA was done by restriction analysis taking advantage of a BsaAI site present only in the *PCDH11X* sequence. Primers in exons 4 and 5 (**top panel**) amplified the E4E5 transcript, which contains the two exons (E4 and E5) retained in all isoforms of both *PCDH11X* and *PCDH11Y*. Primers in exons 3 and 5 (**middle panel**) amplified the isoforms retaining exon 3 and originating from the promoter upstream of exon 1. Primers in exons 4.1 and 5 (**bottom panel**) amplified the isoforms originating from the promoter upstream of exon 4.1 and specific to the *PCDH11Y* gene. Normal control human brains were obtained at autopsy under guidelines approved by the Ethics Committee. The ages of the two males and the two females studied were 74, 42, 55, and 36 year-old, with a post-mortem delay of 10, 21, 24, and 2 hr, respectively. f: frontal cortex, tc: temporal cortex, o: occipital cortex, h: hippocampus, t: thalamus; c: cerebellum.

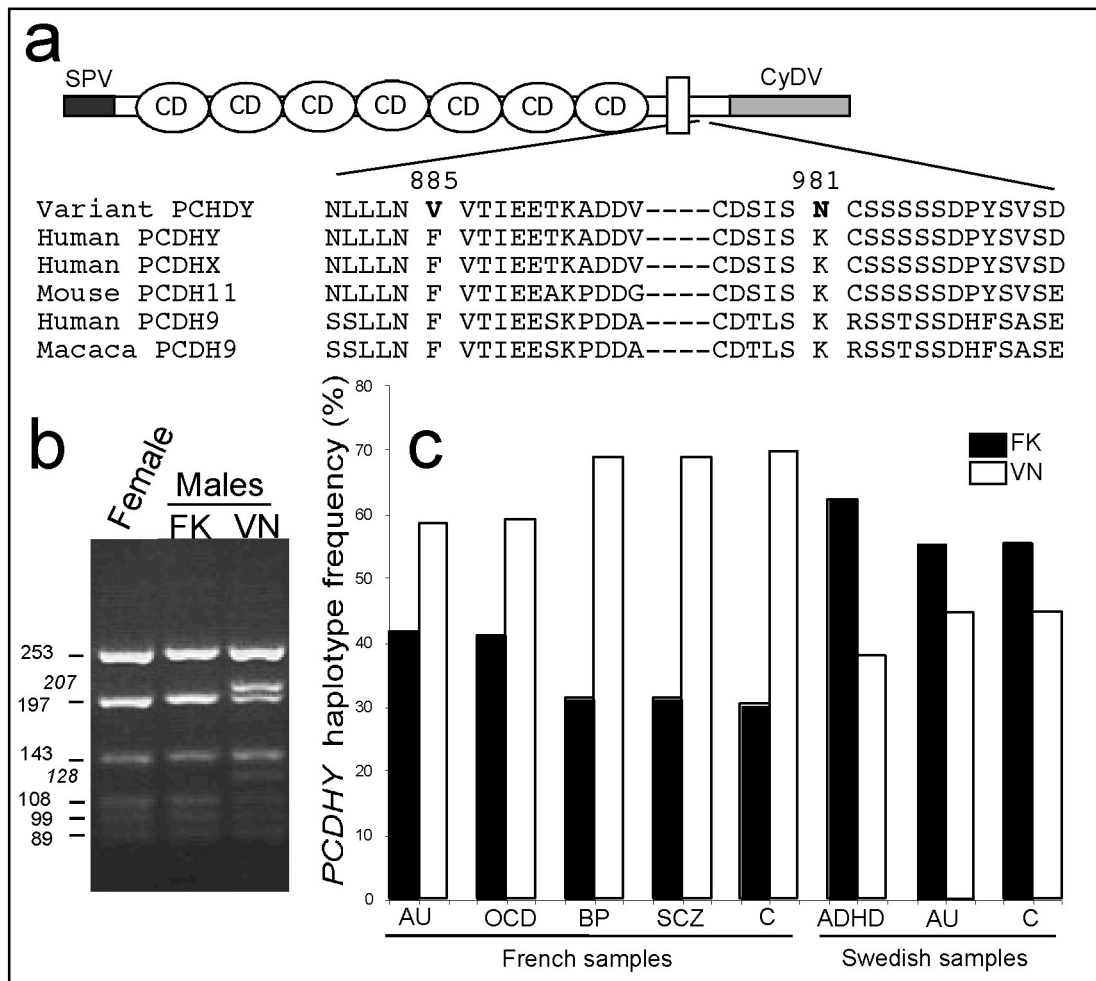


Fig. 2. Conservation, genotyping and frequencies of the two *PCDH11Y* haplotypes in control and affected populations. **a:** Protein structure and sequence alignment of protocadherins from different species. *PCDH9* is the closest homologue of *PCDH11* in the protocadherin phylogeny. SPV, signal peptide variations; Tm, transmembrane domain; CD, cadherin domain; CyDV, cytoplasmic domain variations. **b:** Genotyping of the two *PCDH11Y* haplotypes FK and VN by restriction analysis using the enzyme *Tsp509 I*. **c:** Frequencies of the *PCDH11Y* haplotypes FK and VN in the French and Swedish male samples. The French sample sizes are the following: autism spectrum disorder (AU, $n = 72$), obsessive-compulsive disorder (OCD, $n = 51$), bipolar disorder (BP, $n = 61$), schizophrenia (SCZ, $n = 61$) and controls (C, $n = 86$). The Swedish sample sizes are 61, 38 and 47 for ADHD, autism spectrum disorder (AU) and controls (C), respectively.