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The Role of Clustered Protocadherins in Neurodevelopment and Neuropsychiatric Diseases

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Abstract

During development, individual neurons extend highly branched arbors that innervate the surrounding territory, enabling the formation of appropriate synaptic connections. The clustered protocadherins (*cPCDH*), a family of diverse cell-surface homophilic proteins, provide each neuron with a cell specific identity required for distinguishing between self vs. non-self. While only 52 unique *cPcdh* isoforms are encoded in the human genome, a combination of stochastic promoter choice and the formation of a protein lattice through engagement of adjacent cPCDH protein cis/trans-tetramers confer the high degree of cellular specificity required for self-recognition. Studies of mice bearing deletions of individual *cPcdh* gene clusters have identified deficits in circuit formation and behavior. In humans, single nucleotide variants scattered across the cPCDH locus have been identified, which associate with multiple neurodevelopmental disorders, including autism and schizophrenia. To advance our understanding of cPCDH stochastic choice and maintenance, function across cell types, and contribution to neuropsychiatric disease pathogenesis, hiPSC-based models have been developed. Ultimately, integration of human genetic data, biochemical assays, and functional studies is needed to uncover the mechanism underlying neurite repulsion, which has been implicated in neurodevelopmental disorders.

Keywords

protocadherins; homophilic interactions; self-avoidance; neurodevelopmental disorders; stochastic expression; tiling; neural circuits

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DECLARATION OF INTEREST

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INTRODUCTION

Functional circuits established among the approximately 80 billion human neurons through trillions of synapses must be established during neuronal development. Failure to establish proper neural networks has been linked to neurodevelopmental and neuropsychiatric disorders[1,2]. During development, individual neurons extend highly branched neurites that innervate the surrounding territory with minimal overlap. To enable the formation appropriate synaptic connections, neurons have evolved self-avoidance mechanisms to distinguish self from non-self. These mechanisms ensure that neurites from the same neuron avoid one another, allowing extensive arborization without crossing or clumping, while neurites from different neurons can occupy the same field and engage in synaptic interactions[3,4]. Some neuronal subtypes, such as serotonergic neurons, tile throughout receptive fields in the brain ensuring that their neurites do not overlap sister neurites or neighboring neurites from other serotonergic neurons[1,5]. In *Drosophila*, self-avoidance is mediated by the Dscam1 gene, which can generate over 19,008 unique protein isoforms through an extraordinary example of stochastic alternative pre-mRNA splicing, providing the diversity needed for cellular identity[6–9]. In mammals, a different family of diverse cell-surface receptors, the clustered protocadherins (*cPcdh*), provide each neuron with the unique cell specific identity required for self-avoidance in the brain[10]. The protocadherins generate diversity by a remarkable mechanism that combines stochastic promoter choice, nearly random heterodimerization and a novel lattice formation at apposing membranes on the cell surface[11].

Consistent with their role in self vs. non-self identification, many studies examining deletion of *cPcdh* genes in mice have identified deficits in neuronal wiring across several neuronal subtypes in multiple brain regions and altered behaviors[1,5,10,12–15]. Moreover, large-scale DNA sequencing studies have identified single nucleotide variants (SNVs), scattered across the clustered protocadherin (*cPCDH*) locus, associated with multiple neurodevelopmental disorders, including ASD and SZ[16–18]. Here we summarize recent findings related to the regulation and function of clustered protocadherins, examine the contribution of protocadherins to the genetic risk for neurodevelopmental disorders, and highlight new models for functional genetic studies.

ORGANIZATION AND REGULATION OF PROTOCADHERIN GENE EXPRESSION

The clustered protocadherin (*cPCDH*) locus is organized into three closely linked gene clusters, *PCDHα*, *β*, and *γ* on human chromosome five, together spanning approximately 1 megabase of genomic DNA[19]. The *PCDHα* and *PCDHγ* clusters are more evolutionarily related and contain multiple variable exons that are stochastically chosen[20–22], along with two (α) or three (γ) variable exons that are expressed deterministically (*PCDH* c-types)[19,23,24]. Each variable exon is then spliced to a 3’ splice site located immediately upstream from three constant exons (Fig. 1A). Each variable exon encodes the extracellular cadherin and transmembrane domains, while the three constant exons encode a common intracellular domain for all of the protein isoforms in the *PCDH*α or *PCDH*γ clusters. By

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contrast, the *PCDHβ* genes, which are evolutionarily more divergent, consist only of stochastic variable exons, with no constant region encoding an intracellular domain[19].

The stochastic expression of *Pcdhα* genes is regulated through long-range DNA looping between individual promoters upstream of each *Pcdhα* variable exon, and a transcriptional enhancer designated HS5–1, located downstream of the *Pcdhα* gene cluster[25–27]. Each *Pcdhα* variable exon contains two CTCF binding sites, one in the promoter and the other within the protein coding sequence of the exon. These two sites have similar spacing and are in opposite orientation relative to the two CTCF binding sites located in the HS5–1 enhancer[25,27]. DNA methylation of the promoters appears to play an essential role in stochastic gene activation. The *Pcdhα* variable gene promoters are highly methylated prior to stochastic activation in ground state and thus do not bind to CTCF[23,28]. Remarkably, an "antisense" promoter was identified within the protein-coding region of each variable exon, and the stochastic activation of one of these promoters in the *Pcdhα* gene cluster results in transcription of an antisense polyadenylated lncRNA that extends through the upstream "sense" strand promoter resulting in the demethylation of the sense *Pcdhα* promoter by a TET demethylase[29]. This, in turn, permits binding of CTCF to the sense promoter and DNA looping to the HS5–1 enhancer. Consistent with a chromatin scanning mechanism, the demethylated promoter that is most proximal to the HS5–1 enhancer was shown to be the only promoter activated following demethylation, resulting the specific expression of individual *cPCDH* isoforms (Fig. 1B). In addition to DNA methylation, which was shown to be regulated by DMNT3b[28], H3 Lysine 9 trimethylation (H3K9me3) and Smchd1 binding are also critical for the regulation of *Pcdhα* expression[30,31]. In contrast, the regulated (as opposed to stochastic) expression of *Pcdhα*c2 and the stochastic expression of the *Pcdhβ* and *Pcdhγ* gene clusters are regulated by an independent, unidentified mechanism, as these genes do not generate an antisense lncRNA and the variable exons do not contain CTCF binding sites.

HOMOPHILIC MATCHING OF PROTOCADHERIN PROTEINS FORMS A PROTEIN LATTICE

While the protocadherin gene cluster encodes only 52 unique isoforms in humans, detailed structural and biochemical studies have demonstrated that complex, heterophilic *cis*-PCDH dimerization and homophilic *trans*-PCDH interactions confer the high degree of cellular specificity required proper self-avoidance[11,32–36]. The cPCDH proteins consist of six extracellular cadherin domains (EC1–6), which are required for specific cPCDH interactions. The crystal structure of a cPCDH cis-dimer and cell aggregation assays established that the EC5 and EC6 domains are required heterophilic cPCDH cis-dimerization, which leads to transport of cPCDH isoforms to the plasma membrane[33]. While PCDHβ and PCDHγ isoforms engage in dimers with all cPCDH isoforms, PCDH*a* isoforms (aside from PCDH*a*C2) must dimerize with either PCDHβ or PCDHγ to be transported to the plasma membrane[33].

Systematic *in vitro* cell aggregation assays in K562 cells, which lack the ability to repel, and thus aggregate, established that all cPCDH proteins (except PCDHαC*1* and PCDHγC4)

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engage in highly specific homophilic trans-interactions. An additional key finding was that the presence of a single mismatched isoform between two cells prevents aggregation in K562 cells expressing multiple *Pcdh* isoforms[32]. In contrast to classical cadherin family proteins, the cPCDH dimers on the cell surface engage their matching dimers on opposing membranes through anti-parallel protein-protein interactions (Fig. 1C). These interactions lead to the formation of an extended protein lattice, which was recently visualized by cryo-EM tomagraphy[36].

Taken together, these results support a model in which perfect cPCDH isoform matching leads to the formation extended lattice structure on the surface of sister neurites, which triggers the intracellular signaling mechanisms required for contact mediated neurite repulsion. While the intracellular domain of PCDHα and PCDHγ isoforms can bind to receptor tyrosine kinases including RET[37] and PYK2[38], the signaling mechanisms required for cPCDH-mediated self-avoidance remain unknown.

FUNCTIONAL STUDIES IN MOUSE MODELS

The role for cPCDH proteins in self-avoidance was first demonstrated in mouse starburst amacrine cells (SACs), which develop complex radial dendritic arbors that overlap the dendrites of nearby SACs, but do not overlap their own sister dendrites (Fig. 1D, left). Conditional deletion of the *Pcdh*γ cluster in mice led to a collapse and extensive crossover of sister SAC dendrites[10,39]. Subsequent studies investigated the role of *cPcdh* diversity using mature olfactory sensory neurons (OSNs) that project axons to precise locations within the olfactory bulb and converge to form glomeruli. Single-cell RNA-seq studies revealed that OSNs stochastically express distinct sets of alternate isoforms from all three *cPcdh* clusters and all individual, double and tri-cluster *cPcdh* gene cluster deletion mice failed to form of normal protoglomeruli and displayed a self-avoidance phenotype (Fig. 1D, middle)[15,40]. Overexpression of a distinct set of three *cPcdh* isoforms was unable to rescue glomeruli formation, likely due to inappropriate self-recognition and repulsion caused by the lack of cPCDH isoform diversity[15]. However, overexpression of the same *cPcdh* isoforms lacking the intracellular cytoplasmic domain (ICD) formed typical glomeruli structures, indicating that the ICD is required for cPCDH mediated neurite repulsion.

In addition to self-avoidance, *cPcdh* expression is essential for the proper tiling of serotonergic neurons throughout their target regions in the brain. The spacing between serotonergic axon terminals must be tightly regulated to properly maintain the concentration and distribution of serotonin within target regions. Genetic deletion of the complete *Pcdha* cluster and only the *Pcdha* C-type isoforms showed deficits in normal serotonergic tiling across many target regions[1,5] and exhibited deficits in cognitive and affective behaviors (Fig. 1D, right)[1,5]. In addition, translating ribosome affinity purification followed by RNA-seq (TRAP-seq) demonstrated that *Pcdhac2* is the predominant *Pcdha* isoform expressed in serotonergic neurons, indicating that *Pcdhac2* is required cell autonomously for proper homophilic repulsion and axonal tiling of serotonergic neurons and that these changes in serotonergic circuitry could lead to behavioral deficits seen across multiple neurological disorders.

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ASSOCIATION OF PROTOCADHERINS WITH NEURODEVELOPMENTAL DISORDERS

Failure to establish proper neural networks has been linked to neurodevelopmental disorders[1,2], including ASD[17,41–43] and SZ[44–47]. These multifaceted disorders are highly heritable and have few effective treatments, most of which are only effective for a subset of patients and/or symptoms[48], reflecting the complex genetic etiology. Large-scale sequencing studies have identified hundreds of genes that contribute to the genetic risk of neurodevelopment disorders[16,18,49–51]. These genes show enrichment for chromatin modifiers, synaptic proteins and cell adhesion molecules, including the protocadherins.

DNA sequencing of family structured cohorts have identified single nucleotide variants (SNVs) across the *PCDH* gene cluster that associate with ASD. Specifically, 5 SNPs were identified in *PCDHα*, which showed significant association with ASD in 841 families from the Autism Genetic Resource Exchange[17]. More recent whole exome sequencing of the Simon's Simplex Cohort further identified a number of additional *de novo* SNVs across the cPCDH locus in probands[16,52]. Additionally, epigenetic dysregulation of *cPCDH* locus has also been found in Williams-Beuren syndrome, and the reciprocal 7q11.23 duplication syndrome, which are complex neurodevelopmental disorders with ASD behaviors. Many of the genes within the 7q11.23 critical region encode DNA modification enzymes or chromatin remodeling complexes, a gene set significantly associated with ASD[53]. Consistent with this relationship, MeCP2 was shown to regulate *cPCDH* promoters[54], suggesting a possible convergence between epigenetic modifiers associated with ASD and *cPCDH* gene regulation.

Early genetic linkage studies first identified an association between chromosome region 5q31, harboring the c*PCDH* locus, and SZ[55,56]. More recently, genome wide association studies (GWAS) of over 20,000 SZ cases identified a significant risk locus upstream of the *PCDHα* cluster[18]. Examination of DNA looping demonstrated that the SZ risk locus upstream of the *PCDHα* cluster contained two bundles of DNA interactions in hiPSC-derived neural cells, one bundle interacting with the 3' end of the *PCDHα* cluster and the other interacting with the 3' end of the *PCDHγ* cluster[57]. Moreover, dosage of the top SZ GWAS risk SNP within the *cPCDH* locus is significantly correlated with increased expression of multiple *PCDHα* genes in the CommonMind dlPFC RNAseq dataset[57,58]. Furthermore, CRISPR mediated deletion of the region containing the SZ GWAS risk SNP within the cPCDH locus led to significantly decreased expression of *PCDHα*8 and *PCDHα*10, suggesting a mechanism in which the SZ-associated SNP impacts DNA looping to *PCDHα* target genes leading to changes in gene expression.

Genetic associations between *cPCDH* genes and other neurological disorders have also been reported. SNVs within the *PCDHγ* cluster were shown to segregate with dyslexia within a multi-incident family. Each of the *PCDHγ* SNVs fell within the extracellular cadherin domains critical for cPCDH interactions, suggesting a potential impact on cPCDH interactions[59]. Furthermore, *PCDHγC5* expression was shown to be increased in neurons following treatment with B-amyloid and amyloid precursor protein/presenilin-1, suggesting a role for *cPCDH* isoforms in the progression of Alzheimer's Disease[60]. Future work is

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needed to identify *cPCDH* variants in larger patient populations and to address the function of *cPCDH* isoforms across cell types, brain regions and in the context of disease.

Given the genomic complexity and extent of repetitive elements across the cPCDH locus, it is possible that there are both common and rare structural variants, which have not yet been uncovered. While large scale, long read sequencing studies have not yet been completed, some structural variants in cPCDH genes have already been identified. Initially, a 16.7kb deletion was identified as a common structural variant across multiple genetic backgrounds[61]. Moreover, there were a handful of structural variants (both insertions and deletions) identified through long read sequencing of genomes from 15 individuals[62]. The presence of these structural variants may make identifying diseases associated SNVs more difficult. Without large-scale, long read sequencing studies of individuals diagnosed with neurodevelopmental disorders, the association both *de novo* SNVs and structural variants across the cPCDH locus may be under represented.

USING HIPSC MODELS TO UNDERSTAND MECHANISMS OF PCDH FUNCTION

Studies of promoter choice in established cell lines, such as the human neuroblastoma cell line SK-N-SH have shown the promoter choice is maintained through cell division, suggesting that an epigenetic mechanism maintains enhancer/promoter choice, once it is made[29]. Although mouse models have provided many insights into cPCDH function, new models are beginning to emerge to understand *cPCDH* expression and relationship to disease. Investigation into the timing and maintenance of *cPCDH* stochastic choice in hESCs/hiPSCs and *in vitro* differentiated neurons demonstrated that the expression pattern of *cPCDH* isoforms in hiPSC-derived neurons could be predicted by the chromatin landscape of the parent hiPSC clone, suggesting stochastic choice of *cPCDH* isoforms occurs upstream of neural progenitors[63]. Moreover, evidence for restricted *cPCDH* isoform selection was found in fetal brains from the Allen BrainSpan collection, but not present in the adult brain samples, providing support for two modes of stochastic choice, one that restricts *cPCDH* choice during fetal development and a second that expands the repertoire of *cPCDH* isoforms expressed following an unknown developmental milestone after birth[63].

hiPSC models have also been implemented to investigate the contribution of cPCDH to neurological disorders. Recently, hiPSCs derived from major depressive disorder patients classified as either SSRI-remitters or SSRI-non-remitters were differentiated into serotonergic neurons to understand the molecular mechanisms underlying SRRI responsiveness. Serotonergic neurons generated from SSRI-non-remitters displayed deficits in neurite length, morphology, and decreased expression of multiple *PCDHα* genes[64]. shRNA mediated knockdown of *PCDHα6* and *PCDHα8* in control hiPSC-derived serotonergic neurons resulted in increased neurite outgrowth, suggesting *PCDHα* expression causally contributed to the increased length found in SSRI-non-remitters. In addition, hiPSC-derived cortical interneurons generated from individuals with schizophrenia, showed aberrant *cPCDH* gene expression and deficits in synaptic density and arborizaiton, which could be rescued by inhibition of protein kinase C, believed to participate in intracellular

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signaling pathways downstream of cPCDH proteins[65]. These studies demonstrate the utility of hiPSC-based models to understand cPCDH expression, function and relationship to neurological disorders; however, additional studies are needed to develop the intracellular signaling mechanisms leading to neuronal repulsion and the impact of individual genetic variants across the cPCDH gene clusters.

FUTURE CONSIDERATIONS AND CONCLUSIONS

The mechanisms by which neurite repulsion occurs is fundamental to understanding how neural circuits are established during vertebrate development, and this understanding has important implications for neurodevelopmental and neuropsychiatric disorders[1,2]. Significant progress has been made in understanding how genomic organization, DNA methylation and chromosome looping leads to the stochastic promoter choice required to generate random sets of *Pcdhα* isoforms in individual neurons, but we do not yet understand the mechanisms involved in stochastic promoter choice in the *Pcdhβ* and γ gene clusters[66]. Structural and biochemical studies along with homophilic binding studies have provided an answer to the question of how a relatively small number of protein isoforms can generate sufficient functional diversity for self-recognition[35]. However, a key unanswered question is how homophilic engagement of cPCDHs at the cell surface can lead to neurite repulsion. It seems clear that Adam protease and γ-secretase are involved[67,68] and the intracellular domains of Pcdhα and γ are required. Biochemical studies have identified protein kinases that associate with cPCDHs[38,69], but their role in self-avoidance is not understood. Studies addressing these outstanding questions are likely to continue to provide novel insights into the mechanisms underlying vertebrate neural circuit development and their contribution to neurodevelopmental disorders.

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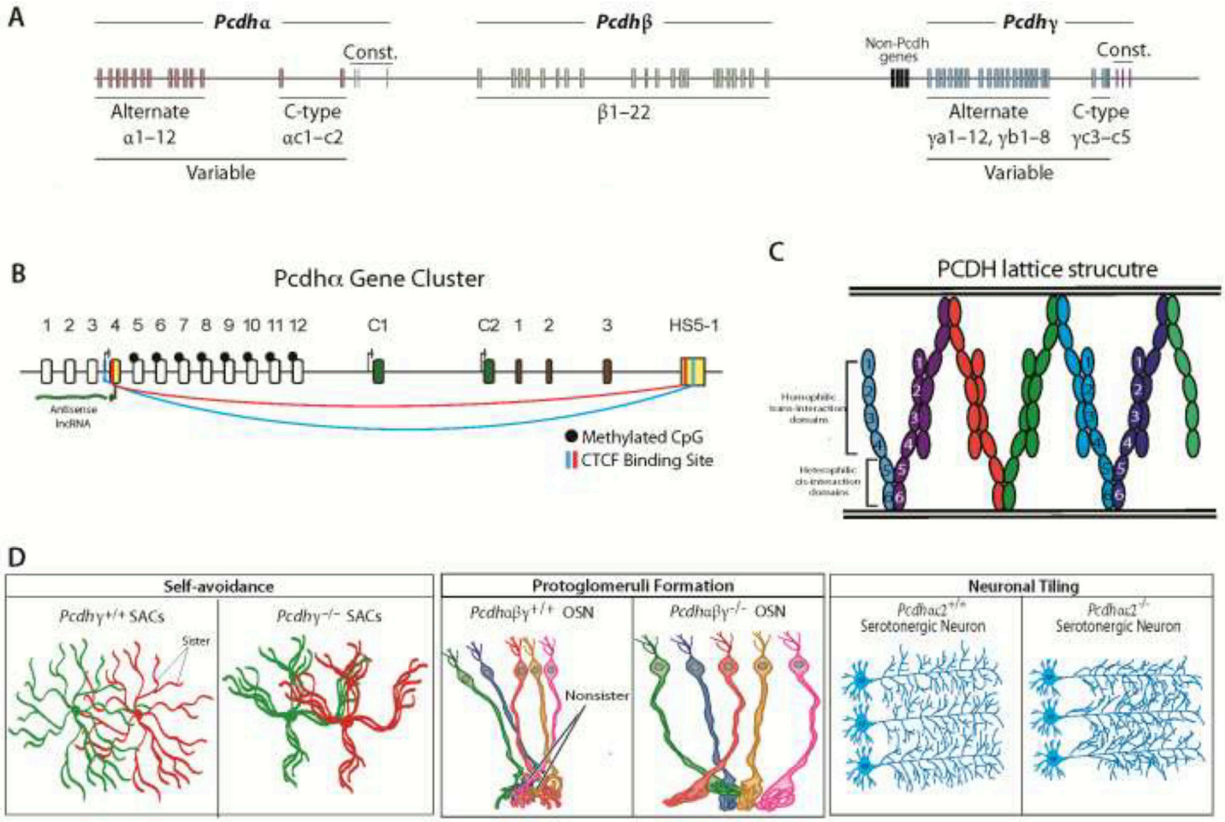

Figure 1:

Overview of the protocadherin gene cluster. **1A)** Schematic of the cPCDH gene cluster; 1B) Schematic highlighting the mechanism of stochastic choice used to express individual PCDHα isoforms; **1C)** Representation of a cPCDH protein lattice formed through homophilic interaction of cis-cPCDH dimers; **1D)** Deficits in self-avoidance and neuronal tiling found in mouse cPCDH deletion models demonstrating their role in self vs. non-self identification