Wiring the Brain by Clustered Protocadherin Neural Codes

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Abstract There are more than a thousand trillion specific synaptic connections in the human brain and over a million new specific connections are formed every second during the early years of life. The assembly of these staggeringly complex neuronal circuits requires specific cell-surface molecular tags to endow each neuron with a unique identity code to discriminate self from non-self. The clustered protocadherin (Pcdh) genes, which encode a tremendous diversity of cell-surface assemblies, are candidates for neuronal identity tags. We describe the adaptive evolution, genomic structure, and regulation of expression of the clustered Pcdhs. We specifically focus on the emerging 3-D architectural and biophysical mechanisms that generate an enormous number of diverse cell-surface Pcdhs as neural codes in the brain.

Keywords Clustered protocadherins · Genome architecture · Neuronal identity · Adhesion specificity · Self-avoidance · Cell recognition

Introduction

The human brain contains a staggering 86 billion neurons, each with numerous branches of dendrites covering receptive fields and of axons innervating diverse regions with minimal overlap. The correct patterning of dendritic and axonal arbors is central for establishing and maintaining enormously complex networks with specific neuronal connectivity in the brain. These vast networks of synaptic connections between axons and dendrites form specific neuronal circuits to fulfill complicated cognitive functions and to determine personality traits and behavior. Aberrant assemblies of neuronal circuits underlie neuropsychiatric diseases. Neuronal circuit assemblies require each neuron to have an identity code for self-recognition and non-self discrimination. How these fascinating and diverse neuronal networks are generated is of the utmost importance. In addition, how the limited size of the human genome encodes the enormous number of neuronal cell-surface identity codes is intriguing.

Over the past few decades, great progress has been made to uncover large families of adhesion proteins that are candidates for cell-surface identity codes for neuronal circuit assembly, such as neurexins [1], olfactory receptors [2], cadherins and families of other adhesion molecules [3–6]. For example, in Drosophila melanogaster, 38,016 isoforms of Dscam1 (Down syndrome cell adhesion molecule 1)—generated by alternative splicing—endow each neuron with a unique identity code to discriminate self from non-self [7–10]. In vertebrates, this is achieved through the stochastic and combinatorial expression of ~60 clustered protocadherin (Pcdh) genes [11–13].

Cadherins are a superfamily of Ca2+-dependent cell-adhesion proteins that are required for specific cell-cell recognition in metazoans. Members of the cadherin
superfamily include classical cadherins (type I and type II), clustered Pcdhs (α, β, γ), and non-clustered Pcdhs [6]. Compared with classical cadherins with five ectodomains (ECs), Pcdhs have six or more ECs with characteristic genome organization, in which multiple ECs are encoded by single unusually large exons [14, 15], and have diverse functions such as neuronal migration and axonal development [15, 16]. Clustered Pcdh genes are arranged in closely-linked clusters in one chromosomal region, while non-clustered Pcdh genes are scattered on different chromosomes [17]. As the largest subfamily of the cadherin superfamily, clustered Pcdh genes are prominently expressed in the brain, and each encodes a cadherin-like protein with six characteristic EC repeats. Their variable and constant genomic architectures are remarkably similar to those of the immunoglobulin (Ig), T cell receptor (Tcr), and UDP glucuronosyltransferase (Ugt) gene clusters, which generate tremendous diversity for the humoral immunity, cellular immunity, and chemical defense systems, respectively [11, 18].

In this review, we describe 3-D architectural and biophysical mechanisms for Pcdh neural codes in the brain. We first describe the 1-D genomic organization of the three Pcdh gene clusters and the 3-D architectural mechanisms that generate their combinatorial repertoires for single neurons. We then discuss cis- and trans-interactions between the extracellular domains of cell-surface Pcdh proteins to ensure neurons for self-recognition as well as self and non-self discrimination. These interactions transduce extracellular contact-dependent signals into the cytoplasm to induce actin dynamics and cytoskeletal remodeling through the common intracellular constant domains. It is this cytoskeletal remodeling that leads to the many functions of Pcdh such as neuronal migration, neurite morphogenesis, dendritic self-avoidance, axonal projection, spine elaboration, synaptogenesis, and neuronal connectivity. We refer interested readers to other excellent reviews discussing various aspects of the clustered Pcdh genes [5, 6, 19–25].

If It Looks Like a Code and Organizes Like a Code, It is a Code

Genetic studies have a long history of describing the phenomena of heredity. While individual genes determine certain phenotypes, the genome with the entire gene assembly holds the characteristics of a species and every creature has a genome that is passed on to the next generation. The genome encodes the brain, but the environment shapes and sharpens the brain: so-called neural epigenetics. The complexity of the brain determines the mind and consciousness. Both the brain and genome code and store information that is vital for the life of creatures. While the genome and genetic codes have been decoded [26, 27], the nature of the neural codes that wire the brain is still under intense investigation.

Setting the Stage for Neural Identity Codes

In the early 1940s, the Chemoaffinity Hypothesis posited that neurons express on their plasma membranes individual identification tags that specify synaptic connections [28]. Intensive efforts have since been devoted to uncovering the proposed neural codes but the exact nature of the neuronal chemoaffinity tags remains elusive [29, 30]. Among the four cell-adhesion families of cadherins, selectins, integrins, and Ig-containing proteins, cadherins are the only family that functions in direct Ca\(^{2+}\)-dependent plasma membrane-to-membrane homotypic interactions, and are thus strong candidates for the chemoaffinity tags of neural codes in the brain [3, 5, 6, 31, 32]. However, only about a dozen classical cadherin genes and a few Pcdh genes were cloned in the nineties [33, 34]. Using the yeast two-hybrid system, 2 full-length and 6 partial cadherin-related receptor genes were cloned from mouse brain tissues and found to be expressed at synaptic junctions in neuronal subpopulations [35]. However, where exactly these proteins are located remains to be determined.

It turned out that these genes are members of the Pcdhx cluster which happens to be located upstream of the two other large gene clusters of Pcdhβ and Pcdhγ [11]. In total, there are 15 Pcdhx, 16 Pcdhβ, and 22 Pcdhγ genes that are highly similar and organized in tandem arrays in a single locus of the human genome. These large numbers and the striking organization immediately suggest that the clustered Pcdh genes are the long-sought neuronal address codes for the brain [4, 36–38]. These numbers are orders of magnitude less than that of neurons in the brain; however, mathematical analyses suggest that they are enough to encode the synaptic address codes required for geometrically constrained local brain regions or nuclei [39].

Genomic Organization of Clustered Pcdh Genes

The mammalian clustered Pcdh proteins are encoded by three closely-linked gene clusters (Pcdhx, Pcdhβ, and Pcdhγ) which span nearly 1 million base pairs [11]. The genomic arrangements of the Pcdhx and Pcdhγ clusters are similar, both with tandem arrays of large variable exons followed by respective single sets of three small constant exons (Fig. 1A) [11, 14, 40]. Within the Pcdhx and Pcdhγ clusters, each variable exon carries its own promoter and can be spliced to the single set of downstream constant exons of its respective cluster. Through stochastic promoter activation and cis-alternative splicing, clustered Pcdhs can generate dozens of different isoforms [41, 42].
The variable exons of \( \text{Pcdha} \) and \( \text{Pcdh} \gamma \) can be further divided into alternate and C-type gene groups based on their genomic location and sequence similarity (Fig. 1A). The mouse \( \text{Pcdha} \) cluster contains 12 alternate genes (\( \alpha_1-\alpha_{12} \)) and two C-type genes (\( \alpha c1 \) and \( \alpha c2 \)). The mouse \( \text{Pcdh} \beta \) cluster contains 19 alternate genes (12 A-types: \( \gamma a_1-\gamma a_{12} \); 7 B-types: \( \gamma b_1, \gamma b_2, \gamma b_4-\gamma b_8 \)) and three C-type genes (\( \gamma c_3-\gamma c_5 \)). Different from \( \text{Pcdha} \) and \( \text{Pcdh} \gamma \), the mouse \( \text{Pcdh} \beta \) cluster, however, contains 22 genes (\( \beta 1-\beta_{22} \)) and no C-type gene (Fig. 1A). In total, there are five C-type variable exons that are more similar to each other than to members of the alternate gene group [11, 40]. However, \( \text{Pcdh} \beta \) contains only large variable exons and lacks constant exons (Fig. 1A). Therefore, each member of the \( \text{Pcdh} \beta \) cluster is a single-exon gene [11, 40]. Together, these three clusters encode 58 Pcdh isoforms (14\( \alpha \), 22\( \beta \), and 22\( \gamma \)) in mice and 53 Pcdh isoforms (15\( \alpha \), 16\( \beta \), and 22\( \gamma \)) in humans (Fig. 1A).

In the \( \text{Pcdha} \) cluster, the promoter of each alternate gene is flanked by two CTCF-binding sites (CBS or CTCF sites). In the \( \text{Pcdh} \beta \) cluster, the promoter of each gene is associated with one CBS element except \( \beta 1 \) which has no CBS element (Fig. 1A). In the \( \text{Pcdh} \gamma \) cluster, the promoter of each alternate gene is associated with one CBS element. Finally, among the five C-type \( \text{Pcdh} \) genes, only the first C-type gene of the \( \text{Pcdha} \) cluster (\( \alpha c1 \)) and the first C-type gene of the \( \text{Pcdh} \gamma \) cluster (\( \gamma c3 \)) are associated with a CBS element (Fig. 1A).

Each variable exon encodes a signal peptide, followed by an extracellular domain containing 6 ECs, a transmembrane region, and a juxtamembrane variable cytoplasmic domain (VCD). The three constant exons encode a common membrane-distal intracellular constant domain (CD) shared by all isoforms of the \( \text{Pcdha} \) and \( \gamma \) cluster. There is a WAVE interacting receptor sequence (WIRS) motif located near the C-terminal end of the \( \text{Pcdha} \) CD that recruits the WAVE-regulatory complex and links to actin cytoskeletal dynamics.

**Fig. 1** Genomic organization and domain structure of clustered protocadherins. A Mouse clustered protocadherin genes have 58 isoforms arranged into three closely-linked clusters: \( \text{Pcdha} \alpha, \beta, \) and \( \gamma \). The \( \text{Pcdh} \alpha \) and \( \gamma \) gene clusters contain more than a dozen of unusually large, highly similar, and repetitive variable exons, each of which is associated with a promoter and can be spliced to a common set of three downstream small constant exons within the respective cluster. These variable exons can be separated into alternate and C-type groups, based on the encoded protein sequence similarity. The \( \text{Pcdh} \beta \) gene cluster lacks constant exons and only contains 22 variable exons which can be further associated with one CBS element except \( \beta 1 \) which has no CBS element (Fig. 1A). Note that each \( \text{Pcdha} \) alternate promoter is flanked by two CBS elements (CSE and eCBS). HS, DNaseI hypersensitive site. B The domain organization of the encoded protein structure of clustered Pcdhs. Each large variable exon encodes an extracellular domain with a signal peptide, followed by 6 ectodomain (EC) repeats, a transmembrane (TM) domain, and a juxtamembrane variable cytoplasmic domain (VCD). The three small constant exons encode a common membrane-distal intracellular constant domain (CD) shared by all isoforms of the \( \text{Pcdha} \) and \( \gamma \) cluster. There is a WAVE interacting receptor sequence (WIRS) motif located near the C-terminal end of the \( \text{Pcdha} \) CD that recruits the WAVE-regulatory complex and links to actin cytoskeletal dynamics.
enhancer, composed of HS7L (HS7 like), HS5-1L (HS5-1 like), and HS18-22, was also identified downstream of the Pcdhγ cluster for both the Pcdhβ and Pcdhγ clusters (Fig. 1A) [43–48].

Fifteen DNaseI hypersensitive sites (HS15–HS1) were initially identified in the Pcdhα cluster, among which HS7 and HS5-1 have strong enhancer activity in a transgenic reporter assay [49]. In mice, genetic deletion of HS5-1, which is located 30 kb downstream of the last Pcdhα constant exon, results in a significant decrease in the expression levels of Pcdhα1–α12 and Pcdhαcl in the brain, but does not affect the expression of Pcdhαc2 [48, 50]. By contrast, deletion of HS7, which is located between the constant exons 2 and 3, results in a significant decrease of expression levels of all Pcdhα genes, including Pcdhαc2 [50].

**Adaptive Evolution of Clustered Pcdh Genes**

Initial studies on Pcdh genes showed that the encoded extracellular domain contains a “primordial” cadherin motif, similar to cadherin motifs in the *Drosophila* Fat protein [34]. It was thought that Pcdhs may be evolutionarily more ancient than the classical cadherins [34]. In addition, the Pcdh genes have characteristic genomic organizations in which multiple ECs are encoded by large exons, a feature that is distinct from the genomic organizations of classical cadherins [14]. Complete sequencing of the *Drosophila* genome revealed, however, that it does not contain clustered Pcdh genes [51]. Thus, the “proto” affix in the “protocadherin” nomenclature is a misnomer and the clustered Pcdh genes are thought to have adaptively evolved later and may be related to functions of more advanced nervous systems.

Similar to the human genome, the chimpanzee, mouse, and rat genomes contain the three Pcdh gene clusters [40, 52, 53]. Clustered Pcdh genes also exist in the anole lizard, frog, coelacanth, fugu, and zebrafish [52, 54–59]. The genome of the frog *Xenopus tropicalis* contains the Pcdhα and γ clusters but lacks Pcdhβ; however, the Pcdhγ cluster has been duplicated into two clusters [59]. In addition, the fugu and zebrafish genomes lack the Pcdhβ cluster but contain two Pcdhα and γ clusters because of the whole-genome duplication in the ray-finned lineage [52, 54, 57].

The anole and coelacanth genomes contain the Pcdhβ cluster [55, 58]. This suggests that the Pcdhβ cluster in mammals, anole, and coelacanth probably results from the duplication of variable exons of the Pcdhγ cluster. The duplicated variable exons subsequently lost their ability to be spliced to the constant exon 1 of the Pcdhγ cluster. Another possibility is that the Pcdhβ cluster results from duplication of the entire Pcdhγ cluster. The duplicated cluster then lost its constant exons through mutation or degeneration. Further research is needed to distinguish these two scenarios. Nevertheless, the topological regulation of both Pcdh β and γ clusters by a single super-enhancer composed of tandem arrays of CTCF sites (Fig. 1A) suggests that they share a common ancestor [48], consistent with their evolutionary trees [52]. Finally, molecular and structural analyses revealed that Pcdhβ and Pcdhγ share characteristics that are distinct from Pcdhα [12, 60].

The cartilaginous shark genome contains a single locus composed of four closely-linked Pcdh clusters that are para-orthologous to the three mammalian Pcdh gene clusters, suggesting that the ancestral jawed vertebrates contained seven Pcdh gene clusters [61]. During the evolution of the genomes of cartilaginous fish and bony vertebrates, this ancestral Pcdh locus experienced differential losses in that the mammalian lineages lost four clusters and the shark lineage lost three clusters [61]. Interestingly, clustered Pcdh genes are vastly expanded in the invertebrate octopus genome and enriched in neural tissues, consistent with their roles in establishing and maintaining the large and complex octopus nervous system [62, 63].

**3-D Genome Architecture of Clustered Pcdhs**

The three Pcdh gene clusters are organized as a large superTAD (super topologically associating domain) which can be divided into two subTADs of α and βγ (Fig. 2A) [45, 64]. The Pcdhα subTAD is formed by long-distance chromatin interactions between tandem arrays of forward CBS elements or CTCF sites of the variable region and the two reverse CBS elements within the promoter and super-enhancer regions (Fig. 2A). The Pcdhβγ subTAD is formed by long-distance chromatin interactions between tandem arrays of forward and reverse CBS elements within the promoter and super-enhancer regions. The topological chromatin loops between remote enhancers and target promoters. The topological chromatin loops between enhancers and promoters are formed by cohesin-mediated active loop extrusion [48]. Cohesin, a ring-shaped complex embracing double-stranded DNA, continuously extrudes

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chromatin fibers until blocked by CTCF-bound CBS elements. The cohesin loop extrusion brings the two remote DNA fragments with forward-reverse convergent CBS elements into close contact in the 3-D nuclear space [45, 65–67]. There is substantial evidence for a central role of CTCF/cohesin in clustered Pcdh gene regulation. First, knockdown of cohesin results in the loss of chromatin loops and downregulation of the clustered Pcdh genes [44, 68]. Second, knockdown of CTCF in cell lines also results in the loss of chromatin loops and a significant decrease of Pcdh expression levels [44, 68, 69]. Finally, conditional knockout of CTCF in neurons markedly downregulates a staggering 53 out of the total 58 clustered Pcdh genes in mice [70], providing strong evidence that CTCF is a master regulator for clustered Pcdhs [69].

Oriented CTCF Sites as Codes of Articulation Joints for Building 3-D Genome Architecture

Initial computational analyses identified a conserved sequence element (CSE), with a highly-conserved CGCT box, located at about the same distance upstream of the translational start codon of each member of the three Pcdh clusters (except for α2, β1, γc4, and γc5) [40]. These CSEs were later shown to bind CTCF proteins, and thus are CBS elements (Fig. 1A) [44, 68, 69].
In the Pcdh\textit{z} cluster, there is an additional CBS element located at \textasciitilde 700 bp downstream of the CSE within the coding region of each alternate variable exon (known as eCBS for exonic CBS) (Fig. 1A) [44, 68]. Thus, there are two CBS elements (CSE and eCBS) flanking each Pcdhz alternate promoter. However, there is only one CBS element (CSE) associated with the \textit{axl} promoter and no CBS element associated with the \textit{ax2} promoter (Fig. 1A). Interestingly, the HS5-1 enhancer is also flanked by two CBS elements, HS5-1a and HS5-1b, with an intervening distance similar to that between each promoter-flanking CBS pair of CSE and eCBS (Fig. 1A) [44, 68].

All of the CBS elements (CSE and eCBS) flanking the Pcdhz promoters are in the forward orientation. By contrast, the two CBS elements (HS5-1a and HS5-1b) flanking the HS5-1 enhancer are in the reverse orientation (Fig. 1A). Namely, the CBS elements in the Pcdhz promoter and enhancer regions are in the opposite orientation [44]. Forward-oriented CBS elements flanking a Pcdhz promoter and reverse-oriented CBS elements flanking the HS5-1 enhancer interact spatially to form a “double-clamp” transcription hub through CTCF/cohesin-mediated chromatin looping (Fig. 2B) [44, 71].

**CTCF Site Orientation Determines the Directionality of Chromatin Looping**

Inversion of the two enhancer CBS elements (HS5-1a and HS5-1b) in cells and mice by using the CRISPR/Cas9-mediated DNA fragment editing method provides strong evidence for the causality between CBS orientation and chromatin-looping directionality [45, 48]. Specifically, the reverse-oriented CBS elements flanking the HS5-1 enhancer normally form long-distance chromatin interactions with the forward-oriented CBS elements associated with the upstream Pcdhz promoters (Fig. 2A, B) [44]. After inversion by CRISPR DNA-fragment editing [72, 73], however, they no longer form long-distance chromatin interactions with the upstream Pcdhz promoters. Strikingly, the inverted CBS elements form long-distance chromatin interactions with the downstream CBS elements [45]. Thus, the relative orientation determines the directionality of long-distance chromatin looping [45]. In addition, spatial chromatin contacts are preferentially formed between forward-reverse CBS elements through CTCF/cohesin-mediated loop extrusion throughout the entire genome [45, 47, 48, 65, 67, 74]. Finally, these experiments also provide strong \textit{in vivo} evidence that enhancers do not function in an orientation-independent manner, at least those associated with CBS [45].

**Tandem CTCF Sites as Genome Topological Insulators**

In the Pcdh\textit{\gamma} clusters, only a single CTCF site is associated with each variable promoter (except \textit{\beta1}, \textit{\gamma4}, and \textit{\gamma5}) (Fig. 1A) [44, 68, 70]. Similar to the Pcdhz cluster, all of the promoter CTCF sites are in the forward orientation in the Pcdh\textit{\gamma} clusters. By contrast, the downstream super-enhancer contains several reverse-oriented CTCF sites organized in tandem (Fig. 1A) [45, 46, 48].

Genetic deletion of HS18-20 (part of the super-enhancer [46]) in mice results in a significant decrease of expression levels of the Pcdh\textit{\beta} genes [43]. In addition, deletion or inversion of HS5-1bL together with HS18-20 in mice totally abolishes the expression of all Pcdh\textit{\beta} genes, suggesting that these regulatory elements, bypassing the Pcdhz cluster, are enhancers for members of the Pcdh\textit{\beta} cluster [43, 47]. However, the expression levels of the Pcdh\textit{\gamma} genes are mostly unaffected in these deletions, leaving the regulation of the Pcdh\textit{\gamma} genes an unresolved question [43, 47].

The Pcdh\textit{\beta} genes are topologically regulated by the tandem CTCF sites of the downstream super-enhancer. Specifically, chromosome conformation capture experiments have revealed that the Pcdh\textit{\gamma} genes are in close spatial contact with the proximal CTCF sites of the super-enhancer (Fig. 2A, C). By contrast, the Pcdh\textit{\beta} genes are in close spatial contact with the distal CTCF sites of the super-enhancer (Fig. 2A, C) [48]. This topological regulation solves the long-standing mystery of Pcdh\textit{\gamma} gene regulation.

These proximal-to-proximal and distal-to-distal topological chromatin regulations were further confirmed by a series of genetic manipulations of the CTCF sites in the super-enhancer \textit{in vivo}. Specifically, when CTCF sites in the super-enhancer are deleted or inverted, the downstream reverse-oriented CTCF sites show increased chromatin interactions with members of the Pcdh\textit{\gamma} cluster and decreased chromatin interactions with members of the Pcdh\textit{\beta} cluster [47, 48]. Thus, tandem CTCF sites function as topological insulators to mitigate the chromatin contacts with and usage of the proximal Pcdh\textit{\gamma} promoters. In addition, these topological insulators, counter-intuitively, promote chromatin contacts with and usage of the distal Pcdh\textit{\beta} promoters. In conclusion, tandem arrays of oriented CBS elements determine the allocation of spatial resources of enhancers for promoters of both distal and proximal Pcdh genes.
Epigenetic Regulation of Chromatin Loops

Methylation of the CpG dinucleotide within the CGCT box of the CTCF sites of \textit{Pcdh} promoters precludes CTCF binding, suggesting epigenetic regulations of the clustered \textit{Pcdh} genes [44]. In each cell, these CTCF sites are differentially methylated, with one and only one alternate exon being activated through long-range chromatin contacts with the HS5-1 enhancer (Fig. 2B) [48, 75–77]. In the neuroblastoma cell line SK-N-SH, \textit{Pcdh} expression levels are inversely correlated with promoter methylation. Specifically, the CBS elements of expressed isoforms are unmethylated and bound by CTCF, but the CBS elements of silenced isoforms are methylated and devoid of CTCF. Consistently, demethylation of CBS elements activates \textit{Pcdh} gene expression [78]. Recently, recent structural analyses suggest that the addition of a methyl group at the 5th position of cytosine within the CpG interferes with the binding of CTCF zinc finger 3 to the CGCT box [79].

In neurons, the DNA methylation states of the \textit{Pcdh} promoters are also inversely correlated with the transcriptional states of the \textit{Pcdh} genes. For example, alternate \textit{Pcdh} genes, which are stochastically expressed by individual Purkinje cells, show mosaic and differential methylation patterns. In contrast, the C-type isoforms, which are constitutively expressed, are hypomethylated [75]. Thus, stochastic expression of \textit{Pcdh} isoforms is probably determined by the DNA methylation in individual neurons.

Recent studies revealed that the eCBS element of each alternate exon is associated with an antisense promoter which transcribes a long non-coding RNA (lncRNA) [78]. Stochastic transcription of this lncRNA extends through the sense promoter, leading to DNA demethylation of the corresponding CBS element. This CBS demethylation then facilitates CTCF binding and subsequent activation of the sense promoter [78]. Interestingly, the promoter activation mediated by antisense lncRNA transcription is only found in alternate but not C-type \textit{Pcdh} genes. This is consistent with the fact that the C-type \textit{Pcdh} variable exons do not contain an eCBS element.

Other Potential Regulatory Proteins

In addition to the architectural proteins CTCF and cohesin, other potential 3-D genome architectural proteins have been shown to regulate expression of the clustered \textit{Pcdh} genes. For example, a protein known as structural maintenance of chromosome hinge domain containing 1 (SMCHD1), which is critically involved in the pathogenesis of facioscapulohumeral muscular dystrophy, antagonizes CTCF in \textit{Pcdh} gene regulation [80]. The SMCHD1 occupancy at \textit{Pcdh} promoters and enhancers coincides with CTCF sites. Loss of \textit{Smchd1} results in increased CTCF binding to the \textit{Pcdh} alternate promoters and upregulation of \textit{Pcdh} \(\alpha\) and \(\beta\) gene expression [80]. However, the underlying mechanism by which SMCHD1 antagonizes CTCF DNA binding remains unknown.

SET domain bifurcated 1 (\textit{Setdb1}) is required for the maintenance of the superTAD structure in \textit{Pcdh} clusters [64]. Conditional knockout of \textit{Setdb1} in forebrain neurons results in the loss of H3K9me3, leading to demethylation of DNA and subsequent recruitment of CTCF to \textit{Pcdh} promoters [64]. The increased CTCF binding strengthens the chromatin interactions between \textit{Pcdh} promoters and enhancers, but weakens the chromatin interactions between the boundaries of the superTAD. Neurons without \textit{Setdb1} lose the stochastic constraint and express increased numbers of \textit{Pcdh} isoforms [64].

Neuron-restrictive silencer factor (\textit{NRSF}) regulates the neuron-restrictive expression of \textit{Pcdh} through binding to HS5-1 and \textit{Pcdh} variable exons [50, 81]. In addition, Wiz (widely-interspaced zinc finger-containing protein) defines cell identity by functioning as a DNA loop anchor in collaboration with CTCF and cohesin [82]. Wiz has been shown to regulate \textit{Pcdh}\(\beta\) gene expression in mice [83]. Consistently, Wiz proteins are enriched at all of the \textit{Pcdh}\(\beta\) promoters (except \textit{Pcdh}\(\beta1\), which is the only \textit{Pcdh}\(\beta\) gene with no CTCF site) and at the HS5-1\(b\) site of the \textit{Pcdh}\(\beta\) super-enhancer [83]. All in all, various transcription factors may regulate the stochastic expression of clustered \textit{Pcdh} by altering higher-order architectural chromatin loops between enhancers and promoters.

Mechanisms for Generating Clustered \textit{Pcdh} Codes of Neuronal Identity

Combinatorial Expression of \textit{Pcdh} as Cell-Surface Identity Codes

Each cortical neuron stochastically expresses up to 2 alternate \textit{Pcdh}\(\alpha\) genes, 4 \textit{Pcdh}\(\beta\) genes, and 4 alternate \textit{Pcdh}\(\gamma\) genes as well as all of the 5 C-type \textit{Pcdh} genes (up to 15 in total) [48, 84]. These combinatorial expression patterns could generate the large number of address codes required for neuronal identity. For example, the 22 encoded \textit{Pcdh}\(\gamma\) proteins have been predicted to form up to 234,256 distinct tetrmers of cell-surface assemblies [85]. In conjunction with the encoded 15 \textit{Pcdh}\(\alpha\) and 22 \textit{Pcdh}\(\beta\) proteins, \textit{Pcdh} proteins could generate the enormous diversity of cell-surface assemblies required for coding single neurons in the brain. We summarize the mechanisms of \textit{Pcdh} promoter choice and expression regulation in this section.
Establishment and Maintenance of Clustered *Pcdh* Expression Patterns

A remarkable property of the clustered *Pcdh* genes is that their promoter choice is inherited and stably maintained by daughter cells as seen in the SK-N-SH cell line and differentiated neurons [44, 86]. This suggests that, once chosen, the expression patterns of clustered *Pcdh* genes are epigenetically inheritable. In addition, *Pcdh* promoter choice occurs early during the naive-to-primed conversion of ESCs (embryonic stem cells) [86]. The *Pcdh* promoters are modified with both active (H3K4me3) and repressive (H3K27me3) chromatin marks, so called bivalent promoters, in the primed ESCs before being activated. The chosen *Pcdh* genes are then stably inherited by differentiated neurons [86].

As the methylation states of promoters are inversely correlated with the expression levels of clustered *Pcdh* genes, a fundamental question is how single neurons achieve the stochastic activation of *Pcdh* promoters. On the one hand, stochastic activation of a *Pcdh* promoter could be achieved through demethylation of the chosen target promoter by antisense transcription of lncRNA [78]. On the other hand, this could be achieved through methylation of all of the non-chosen promoters [75]. Consistently, all of the *Pcdhα* alternate promoters are enriched with CTCF in naive ESCs, while only chosen promoters are enriched with CTCF in primed ESCs [86], suggesting hypomethylation-to-hypomethylated conversion of the non-chosen promoters during cellular differentiation. This indicates that the ground state of *Pcdh* promoters is unmethylated or hypomethylated and that the activation of specific promoters requires methylation of all of the other promoters (Fig. 3A, B).

Cell-Specific and Stochastic Expression of Clustered *Pcdh* Genes

Clustered *Pcdhs* are widely expressed in the developing and adult central nervous systems [11, 34, 35, 42, 53, 87–90]. The expression of members of the *Pcdhα* cluster is highly specific to the central nervous system. While members of the *Pcdhβ* and *γ* clusters are prominently expressed in the central nervous system, they are also expressed in several other tissues such as the kidney and lung [87, 89, 91]. Detailed expression patterns of each isoform were initially analyzed by *in situ* hybridization using isoform-specific probes, which showed that they are stochastically expressed in neuronal subpopulations in various brain nuclei or regions [35, 42, 53, 89, 90, 92].

Single Purkinje neurons express alternate members of clustered *Pcdh* genes in a stochastic and monoallelic manner (Fig. 3C) [92–94]. In addition, single cortical neurons also express alternate members of the three *Pcdh* clusters in a similar manner [48, 75, 84]. In the *Pcdhα* cluster, each tandem pair of the promoter CTCF sites (CSE and eCBS) functions as an insulator for all of its upstream *Pcdh* genes. A single chromatin loop between the HS5-1 enhancer and a variable promoter determines the expression of the chosen *Pcdhα* gene in each allele (Fig. 2A, B) [48].

In the *Pcdhβγ* clusters, the super-enhancer is composed of four CBS-containing elements. Up to two *Pcdhβ* genes (activated by enhancers with CTCF sites “de” and “fgh”) and two alternate *Pcdhγ* genes (activated by enhancers with CTCF sites “a” and “bc”) could be expressed from each allele through nested chromatin loops (Fig. 2A, C) [48].

In olfactory sensory neurons (OSNs), clustered *Pcdh* genes are stochastically expressed, except for the C-types (Fig. 3D) [76]. In addition, diploid chromatin conformation capture of single OSNs has shown that there are significant cell-to-cell heterogeneities of *Pcdh* chromatin architectures and that *Pcdh* enhancers communicate with distinct *Pcdh* promoters in different cells [95]. This may reflect the stochastic *Pcdh* promoter choice. Specifically, each OSN expresses a distinct set of up to 10 alternate *Pcdh* genes, among which 5 are stochastically and monoallelically expressed from each allele (Fig. 2). In summary, these findings suggest that the clustered *Pcdh* genes are stochastically expressed in single neurons of the cerebellum, cerebrum, and olfactory epithelium in a cell-specific manner.

Cell Type-Specific Expression of Clustered *Pcdh* Genes

All of the C-type *Pcdh* genes appear to be constitutively and biallelically expressed in single neurons of the cerebellum and cerebrum in the mouse brain (Fig. 3B, C) [48, 75, 84, 92–94]. By contrast, none of the C-type *Pcdh* genes is expressed in mouse OSNs (Fig. 3D) [76]. Finally, only *Pcdhαc2* is predominantly expressed in serotonergic neurons (Fig. 3E) [96, 97]. Collectively, these studies suggest that C-type *Pcdh* genes are expressed in a cell-type-specific manner, in stark contrast to the stochastic expression of alternate *Pcdh* genes in the brain.

Molecular Logic of Neuronal Self-avoidance and Coexistence

Promiscuous Cis-interactions for Diverse Cell-Surface Assemblies

The *Pcdhα* proteins co-immunoprecipitate with *Pcdhγ* in cell lysates. In addition, cell-surface delivery of *Pcdhα* proteins (except for *Pcdhαc2*) requires the co-expression of *Pcdhγ* because *Pcdhα* alone cannot be sufficiently
expressed at the plasma membrane [12, 98], suggesting that Pcdhα and Pcdhγ may form heterodimers. Moreover, distinct members interact with each other in membrane fractions [85, 99]. Finally, each member of Pcdhβ or Pcdhγ (except for Pcdhγc4) can form homodimers or heterodimers; however, members of Pcdhα and Pcdhγc4 cannot form homodimers. They can only form heterodimers with Pcdhβ or other Pcdhγ isoforms [12, 100].

Structural studies support the formation of cis-homodimers or cis-heterodimers between isoforms of clustered Pcdhs. The cis-dimerization requires both EC5 and EC6 domains [13, 60, 101]. Specifically, the Pcdh cis-dimer
interfaces are asymmetric, with one molecule providing the EC5 and EC6 side of the interface, and the other providing only the EC6 side (Fig. 4A) [13, 60]. Isoforms of Pcdhβ and Pcdhγ (except for Pcdhγc4) form cis-homodimers or cis-heterodimers in that each isoform can participate as either the EC5–EC6 or EC6 side of the interface [13, 60]. However, isoforms of Pcdhx and Pcdhγc4 can only form cis-heterodimers and cannot form cis-homodimers because they cannot participate as the EC6 side of the interface. Namely, they participate only as the EC5–EC6 side of the heterodimer interface. They need isoforms of either Pcdhβ or Pcdhγ (also known as carrier isoforms, except for Pcdhγc4) to provide the EC6 side of the heterodimer interface [60].

In summary, clustered Pcdh isoforms appear as a cell-surface repertoire composed of homodimers and promiscuous heterodimers of members of all three Pcdh clusters on the plasma membrane of single neurons [12, 13, 60, 85, 100].

**Homophilic Trans-interactions for Self-recognition**

Great progress has been made in deciphering the trans-interactions of clustered Pcdh proteins for generating cell-recognition specificity. The trans-interactions of the Pcdh isoforms have been tested using an efficient cell-aggregation assay by transfecting two cell populations [12, 85, 101]. Different cell populations expressing the same combinations of Pcdh isoforms display strict homophilic interactions and can form cell aggregates, but those expressing different combinations of Pcdh isoforms cannot [12].

All of the clustered Pcdh β and γ isoforms, except for Pcdhγc4, can engage in robust and highly specific trans-homophilic interactions in cell aggregation assays. These isoforms are delivered to cell membrane, probably because they can form cis-homodimers [60]. Pcdhx (except for Pcdhx2) and Pcdhγc4, on the other hand, cannot form cis-homodimers and cannot be delivered to cell membrane by themselves. Therefore, they cannot induce cell aggregates [12]. Pcdhx2c2, however, is unique in that it can induce cell aggregates by itself because it can form cis-homodimers and be delivered to cell membrane [12].

The Pcdhx proteins can form cis-heterodimers with isoforms of Pcdh β and γ (except for Pcdhγc4). They can be delivered to cell membrane when they are co-expressed with Pcdh β or γ isoforms. Therefore, Pcdhx (except for Pcdhx2c1) does induce cell aggregates through homophilic trans-interactions when co-expressed with Pcdh β and γ isoforms (except for Pcdhγc4). Finally, homophilic interactions are abolished when there is a single mismatched isoform between the two transfected cell populations [12].

Structural analyses revealed that the trans-homophilic interactions are mediated by EC1–EC4 in an antiparallel manner. These trans-interactions form a zipper-like ribbon structure in apposed plasma membranes. Specifically, the EC1, EC2, EC3, and EC4 of one isoform at a cell surface interact with the EC4, EC3, EC2, and EC1 of the same isoform from the apposed cell surface, respectively [13, 101–105]. Among the six EC domains of clustered Pcdhs, EC2 and EC3 have been positively selected for diversity during evolution and are thus the most diversified ECs in amino-acid residues [52]. Consistently, they determine the stringent specificity of trans-homophilic interactions [12, 85, 104].

**The Chain-Termination Model for Non-self Discrimination**

The crystal structure of the full-length extracellular domain of Pcdhγb4 reveals a zipper-like lattice through cis-interactions mediated by EC5–EC6/EC6 and trans-interactions mediated by EC1–EC4 [13]. When tethered to liposomes, Pcdh extracellular domains spontaneously assemble into zipper-like linear arrays through trans-homophilic interactions between Pcdh dimers [13]. These linear assemblies extend through the contacted membranes as a chain to form a larger lattice (Fig. 4B). In this chain termination model, once a certain size threshold is reached, the assemblies trigger intrinsic Pcdh signaling pathways to regulate various cellular behaviors such as repulsion. By contrast, when mismatched isoforms are incorporated, the Pcdh chain extension terminates and the lattice size cannot reach the presumed signaling threshold (Fig. 4B) [13, 101]. This isoform-mismatch chain-termination model can explain the recognition initiation process of self and non-self discrimination mediated by the extracellular domains of clustered Pcdhs.

**Intracellular Signaling of Clustered Pcdhs Leads to Cytoskeletal Rearrangement and Morphological Remodeling**

The intracellular domains of the Pcdhx and Pcdhγ isoforms contain a respective common membrane-distal region encoded by constant exons that is shared by all isoforms from the same cluster [11, 14]. The Pcdhx and Pcdhγ isoforms are cleaved by metalloproteinase and subsequently by γ-secretase to generate a soluble extracellular fragment and an intracellular fragment that may function locally or translocate into the cell nucleus [106–109]. This proteolytic process requires endocytosis and is regulated during animal development and neuronal differentiation [110].
The Pcdhα and Pcdhγ proteins can bind and inhibit two cell-adhesion kinases, FAK (focal adhesion kinase) and Pyk2 (proline-rich tyrosine kinase 2), through the cytoplasmic domain (Fig. 4C) [111]. In the mouse hippocampus and cortex, Pcdhα and Pcdhγ regulate dendritic arborization and spine morphogenesis through inhibiting Pyk2 and FAK activity [112–114]. Knockout or knockdown of Pcdhα in hippocampal neurons results in the phosphorylation and activation of Pyk2 [113]. The activation of Pyk2 inhibits Rac1, leading to defects in dendritic and spine morphogenesis. Consistently, knockdown of Pyk2 or overexpression of Rac1 rescues the phenotype caused by Pcdh α or γ knockdown [113]. Pcdhγ knockout induces extensive neuronal apoptosis in the spinal cord [6], which could be related to aberrantly up-regulated Pyk2 activity. Consistent with this, over expression of Pyk2 also induces apoptosis [111]. Together, these data suggest that diverse extracellular signals acting on different Pcdhα and Pcdhγ isoforms converge into the same intracellular pathways through common downstream effectors of Pyk2 and FAK (Fig. 4C).

The common intracellular domain of Pcdhα isoforms, but not Pcdhγ isoforms, contains a conserved peptide WIRS (WAVE-interacting receptor sequence) motif that...
interacts with the WAVE (Wiskott-Aldrich syndrome family verprolin homologous protein) regulatory complex (WRC) to modulate cytoplasmic actin assembly (Fig. 1B) [115, 116]. Specifically, Pcdhα isoforms (except for Pcdhαc2) regulate cytoskeletal dynamics during cortical neuron migration and dendrite morphogenesis through the WAVE regulatory complex (Fig. 4C) [116]. Overexpression of Pcdhα isoforms (except for Pcdhαc2) rescues the migration defects caused by Pcdhα knockdown and the rescue is abolished by WIRS mutation. In addition, overexpression of WRC subunits also rescues the migration defects of Pcdhα knockdown [116]. Given that Pcdhα forms cis-heterodimers with Pcdh β or γ on the cell surface (Fig. 4A), the Pcdh β and γ isoforms may also modulate the WAVE complex through interacting with Pcdhα (Fig. 4C). Specifically, Pcdh β and γ proteins, together with Pcdhα, may regulate neuronal morphogenesis and dendrite self-avoidance through WAVE dynamics and cytoskeletal rearrangements (Fig. 4C). In summary, the establishment and maintenance of neuronal connectivity and self-avoidance likely require coordinated collaborations between members of all three Pcdh gene clusters.

Concluding Remarks and Future Perspectives

In the central nervous system, individual neurons stochastically express combinatorial sets of clustered Pcdhs. These Pcdh expression profiles constitute diverse cell-surface identity codes through cis-promiscuous pairing and discriminate self from non-self through strict trans-homophilic interactions. Their tremendous diversity is generated by intriguing 3-D genome architecture, stochastic promoter choice balanced by topological insulators, long-range spatial chromatin contacts between distal enhancers and target promoters, and alternative splicing.

Elucidating the regulatory mechanisms of clustered Pcdhs in different cell types throughout the nervous system will be of great importance in deciphering the molecular basis underlying neural-circuit coding. Several lines of investigation of the Pcdh clusters have provided deep insights into various aspects of gene expression mechanisms, from 1-D genomic organization to 2-D epigenetic regulation and 3-D chromatin architecture. However, many important questions remain unanswered. For example, when are Pcdh isoforms chosen to be expressed in neuronal progenitor cells during brain development? What is the mechanistic basis for the epigenetic memory of clustered Pcdh expression profiles? What are the mechanistic differences between the regulation of expression of alternate and C-type isoforms? How do serotonergic neurons selectively express only the Pcdhαc2 gene in a cell-type-specific manner? Finally, how do clustered Pcdhs collaborate with other families of cell-adhesion proteins to specify synaptic connectivity? Answering these questions about neural coding mechanisms in the brain requires interdisciplinary endeavors in the future.

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Conflict of interest

The authors declare that they have no conflict of interest.

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