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Pcdhαc2 is required for axonal tiling and assembly of serotonergic circuitries in mice

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Abstract

Serotonergic neurons project their axons pervasively throughout the brain and innervate various target fields in a space-filling manner, leading to tiled arrangements of their axon terminals to allow optimal allocation of serotonin among target neurons. Here we show that conditional deletion of the mouse protocadherin α (*Pcdh*α) gene cluster in serotonergic neurons disrupts local axonal tiling and global assembly of serotonergic circuitries and results in depression-like behaviors. Genetic dissection and expression profiling revealed that this role is specifically mediated by Pcdhαc2, which is the only Pcdhα isoform expressed in serotonergic neurons. We conclude that, in contrast to neurite self-avoidance, which requires single-cell identity mediated by Pcdh diversity, a single cell-type identity mediated by the common C-type Pcdh isoform is required for axonal tiling and assembly of serotonergic circuitries.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S12

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The human brain contains about 300,000 serotonergic neurons in the raphe nuclei located in the brainstem, a very small number compared to the billions of neurons they modulate. To accomplish this, serotonergic neurons project their axonal tracts throughout the entire central nervous system (CNS) and arborize in various target areas in a space-filling pattern, with their axon terminals orderly distributed among target neurons (1). In most target regions, serotonergic "synapses" are nonjunctional, lacking postsynaptic partners for target specificity. Serotonin is released from these presynaptic varicosities by volume transmission, interacting with diverse serotonin receptors that are broadly expressed in target neurons to modulate neural activities and behaviors (2, 3). As functional concentrations of serotonin are maintained only within a short distance from these release sites, the spacing between serotonergic axon terminals must be tightly controlled to allow for optimal distribution of serotonin in target areas. The underlying molecular and cellular mechanisms of this process, however, remain elusive.

Studies of simpler neuronal arbors have revealed that axonal and dendritic spacing is primarily controlled by two distinct mechanisms: self-avoidance and tiling (4, 5). Self-avoidance promotes repulsion between sibling neurites of the same cell, whereas tiling promotes repulsion between homotypic neurites from different neurons of the same functional cell type to maximize target coverage. In *Drosophila*, the Down syndrome cell adhesion molecules, Dscam1 and Dscam2, have been shown to mediate self-avoidance and tiling, respectively, through homophilic interaction followed by contact-dependent repulsion. In addition to these cell-intrinsic forces that regulate neurite spacing, axons and dendrites are also exposed to numerous extrinsic cues, and the precise pattern of their spatial arrangements results from the counterbalance between these developmental forces (4, 5).

Clustered protocadherins (Pcdhs) are a family of cadherin-like neuronal cell-adhesion molecules that are specific to the vertebrate lineage (6). In mammals, more than 50 Pcdh protein isoforms are encoded in three tightly linked gene clusters designated *Pcdh*α, *Pcdh*β, and *Pcdh*γ (Fig. 1A). Single-cell reverse transcription–polymerase chain reaction (RT-PCR) studies in Purkinje neurons showed that the alternate Pcdh isoforms are stochastically expressed in individual neurons, whereas the C-type isoforms are constitutively expressed (7–9). However, whether this expression pattern occurs in other types of neurons is not known. Biochemical and structural studies have shown that individual Pcdh isoforms are strictly homophilic (10, 11) and can form homo- or heterodimers in cis that engage in trans-homophilic interactions to enable highly specific self-recognition between apposing cell surfaces (11, 12). Conditional deletion of the entire *Pcdh*γ gene cluster in mice results in aberrant self-crossing and clumping of dendritic branches of starburst amacrine cells, indicating that the Pcdhγ proteins are required for dendritic self-avoidance in the mammalian nervous system (13). Previous studies of *Pcdh*α-hypomorphic mice reported defects in olfactory, serotonergic, and retinogeniculate axonal projections and cognitive function (14–17). However, the underlying mechanisms involved were not investigated.

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Depression-related behaviors in *Pcdh*α$^{-/-}$ mice

We generated a *Pcdh*α gene cluster deletion allele by chromosome engineering (Fig. 1A and fig. S1A). *Pcdh*α$^{-/-}$ mice are viable and fertile without obvious abnormalities, and they displayed largely normal motor and sensory functions in a behavioral screen (fig. S2A). By contrast, multiple independent assays revealed changes in cognitive and affective functions (fig. S2B). We validated the observed depression-related behaviors using a different cohort (Fig. 1B). *Pcdh*α$^{-/-}$ mutant mice displayed increased immobility time in both the tail-suspension test (TST) and forced swim test (FST), indicating enhanced behavioral despair, which is consistent with a depressive state. In addition, *Pcdh*α$^{-/-}$ mutant mice exhibited increased freezing time in the contextual–fear conditioning (CFC) paradigm, as previously reported for *Pcdh*α-hypomorphic mice (14), indicating enhanced fear memory, a core process in the development of mood disorders, including depression (18).

Expression of Pcdhα proteins in limbic structures and neuromodulatory systems

To explore possible histological and/or circuitry changes that correlate with the observed behavioral alterations of *Pcdh*α$^{-/-}$ mice, we first generated a *Pcdh*α^{mCherry} reporter allele to examine the expression patterns of Pcdhα proteins (figs. S1B and S3A). Pcdhα proteins are broadly expressed in the CNS, including in multiple forebrain limbic structures that are essential for cognitive and emotional processing (fig. S3B). Limbic structures are pervasively innervated by multiple neuromodulatory systems, many implicated in the etiologies of depression (19). We found that Pcdhα proteins are enriched in both norepinerphrinergic (TH+) and serotonergic neurons (TPH2+) but are barely detectable in dopaminergic (TH+) or cholinergic (ChAT+) neurons (Fig. 1, C and D, and fig. S3C).

Clumping and redistribution of serotonergic axon terminals in target fields

No obvious histological changes in forebrain limbic structures were detected in *Pcdh*α-deficient mice, and adult neurogenesis in the hippocampus was normal (fig. S4). We therefore examined potential alterations in the neuromodulatory circuitries. Whereas the innervation patterns of norepinephrinergic axon terminals (NET+) in limbic structures remained unchanged in *Pcdh*α-deficient mice (fig. S5A), target innervation patterns of serotonergic axon terminals (SERT+) were profoundly altered (Fig. 1E and fig. S5, A to C), as previously reported in *Pcdh*α-hypomorphic mice (16). Compared to wild-type mice where serotonergic fibers are orderly arranged and evenly spaced in target fields, serotonergic fibers in *Pcdh*α$^{-/-}$ mutants became disorganized and lost their even spacing, displaying frequent tangling and clumping. In addition to fiber clumping, which occurs in a random fashion, as shown in the substantia innominata (SI) of ventral pallidum (Fig. 1E and movies S1 and S2), the unevenly spaced serotonergic fibers were redistributed in reproducible patterns in areas such as the hippocampus: In *Pcdh*α$^{-/-}$ mutants, fiber densities were increased in the stratum lacunosum moleculare (SLM) of field CA1 but were decreased in the dentate gyrus (DG) compared to fiber densities in wild-type mice (Fig. 1E), suggesting the involvement of target-derived cues in serotonergic innervation and rewiring. Similar scenarios of serotonergic-fiber clumping and redistribution were observed in other

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target fields throughout the CNS (Fig. 1E and fig. S5, A to C). Consistent with the lack of Pcdhα protein expression in dopaminergic and cholinergic neurons (fig. S3C), target-innervation patterns of these two neuromodulatory systems are normal in *Pcdhα*-deficient mice (fig. S5, B and C).

The number and organization of serotonergic neurons in raphe nuclei are not altered in *Pcdhα*-deficient mice (fig. S6A), and the formation and trajectory of major serotonergic axonal tracts are normal (fig. S6B). Differences in the target-innervation patterns (e.g., in the hippocampus) were only noticeable at postnatal day 5 (P5), were evident by P7, became progressively prominent during postnatal development, and then persisted throughout adulthood (fig. S6C). We conclude that the serotonergic-wiring defects occur during axon-terminal arborization and target innervation, the final step in serotonergic-circuit assembly (2, 3).

Cell autonomy of serotonergic-wiring and behavioral defects

To address the cell autonomy of the observed serotonergic-wiring and behavioral phenotypes, we used a *Pcdhα* conditional allele (20) and tissue-specific Cre drivers (fig. S7, A and B) to specifically abolish Pcdhα proteins in serotonergic neurons or in their target fields in the forebrain. Whereas serotonergic-innervation patterns remained unchanged with the deletion restricted to the forebrain (*Pcdhα$^{f/f}$*; *Camk2a::Cre*), the serotonergic-wiring defects were recapitulated in serotonergic neuron–specific knockouts (*Pcdhα$^{f/f}$*; *Slc6a4::Cre*) (Fig. 2, A and B, and fig. S7C). These observations were further validated with independent forebrain-specific (*Emx1::Cre*) and serotonergic neuron–specific (*Fev::Cre*) drivers (fig. S7, D and E). We conclude that the role of the *Pcdhα* gene cluster in serotonergic wiring is entirely cell autonomous to serotonergic neurons.

The uneven spacing and clumping of *Pcdhα*-deficient serotonergic fibers may result from disrupted axonal self-avoidance or disrupted axonal tiling. To distinguish between the two possibilities, we injected Cre-dependent Brainbow-reporter adeno-associated viruses (AAVs) (21) into the dorsal raphe (DR) and median raphe (MR) nuclei of *Pcdhα$^{f/f}$*; *Slc6a4::Cre* mice, which differentially label individual serotonergic neurons and their axons with different colors after multiplex immunostaining (Fig. 2C and fig. S8). As evident with three-dimensional (3D) visualization and reconstruction, most mutant fiber clumps are comprised of differentially labeled axons originating from different serotonergic neurons (Fig. 2C), indicating that clumping occurs between homotypic serotonergic axons. We conclude that the uneven spacing and clumping of serotonergic fibers in target fields result from disrupted axonal tiling.

To examine whether the altered serotonergic wiring correlates with changes in cognitive-affective functions observed in *Pcdhα$^{-/-}$* mutants, we subjected the two types of conditional knockouts to behavioral testing. Whereas no significant differences in depression-related behaviors were observed in the forebrain-specific knockouts (*Pcdhα$^{f/f}$*; *Camk2a::Cre*), serotonergic neuron–specific knockouts (*Pcdhα$^{f/f}$*; *Slc6a4::Cre*) displayed increased immobility time in TST, FST, and CFC tests (Fig. 2D), as observed in *Pcdhα* constitutive knockouts (Fig. 1B). Consistent with these behavioral observations, fear context–induced

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expression of immediate-early genes c-*fos* and *Arc* (22) was unchanged in *Pcdh*α$^{f/f}$; *Camk2a::Cre* mutants, but expression of both was significantly elevated in *Pcdh*α$^{f/f}$; *Slc6a4::Cre* mice, further validating increased fear memory in the serotonergic neuron–specific knockouts (fig. S9). We conclude that the role of the *Pcdh*α gene cluster in both serotonergic wiring and cognitive-affective functions is strictly cell autonomous to serotonergic neurons and that the depression-related behaviors likely result from altered serotonergic circuitries.

Pcdh diversity is dispensable for serotonergic wiring

To investigate whether the other two *Pcdh* gene clusters may also be required for serotonergic wiring, we first generated the *Pcdh*β gene cluster deletion by chromosome engineering (Fig. 3A and fig. S1A). *Pcdh*β$^{-/-}$ mice are viable and fertile with no obvious abnormalities, and serotonergic-innervation patterns in the target fields are normal (Fig. 3A). To address the requirement of the *Pcdh*γ gene cluster, we used a *Pcdh*γ conditional allele (23) to circumvent neonatal lethality resulting from the *Pcdh*γ constitutive deletion (24) and found that serotonergic-wiring patterns in adult *Pcdh*γ$^{f/f}$; *Slc6a4::Cre* mice were indistinguishable from those of the wild-type mice (Fig. 3B). We conclude that both *Pcdh*β and *Pcdh*γ gene clusters are dispensable for serotonergic axonal arborization and that the observed serotonergic-wiring phenotypes result entirely from the loss of function of Pcdhα proteins.

The *Pcdh*α gene cluster encodes 12 alternate isoforms (Pcdhα1 to 12) and 2 C-type isoforms (Pcdhαc1 and Pcdhαc2), which are thought to be stochastically and constitutively expressed, respectively, on the basis of single-cell expression studies in Purkinje neurons (7–9). To address the roles of the two types of isoforms in serotonergic wiring, we first deleted genes for all 12 alternate isoforms using the clustered regularly interspaced short palindromic repeats (CRISPR) genome-editing technology, leaving only the two C-type genes intact (Fig. 3C and fig. S1C). *Pcdh*α$^{altko/altko}$ mice displayed normal patterns of serotonergic innervation (Fig. 3C), indicating that Pcdhα protein diversity is dispensable for serotonergic axonal tiling and circuit assembly, and, furthermore, none of the 12 alternate Pcdhα isoforms are required. We next used a double–C-type isoform knockout allele of the *Pcdh*α gene cluster (20) to determine whether the two C-type isoforms are required for serotonergic wiring. Similar to what was observed with the triple–C-type isoform knockouts of the *Pcdh*γ gene cluster (25), deletion of the C-type Pcdhα isoforms does not compromise the expression of alternate isoforms (fig. S1D). *Pcdh*α$^{dcko/dcko}$ mice displayed identical serotonergic-wiring defects as those observed in mice lacking the entire *Pcdh*α gene cluster (Fig. 3D). We conclude that one or both of the Pcdhα C-type isoforms are specifically required among all 58 Pcdh isoforms for axonal tiling and assembly of serotonergic circuitries.

Singular expression of *Pcdh*α*c2* in serotonergic neurons

To investigate the question of why only the Pcdhα C-type isoforms are required for serotonergic wiring, we carried out translational profiling with translating ribosome affinity purification followed by RNA-sequencing technology (TRAP-Seq) to examine the

expression levels of all Pcdh isoforms in postnatal serotonergic neurons. We found that *Pcdhαc2* is the only *Pcdhα* isoform expressed in serotonergic neurons (Fig. 4A; fig. S10, A and B; and table S1). By comparison, RNA transcripts corresponding to the 12 alternate *Pcdhα* isoforms as well as *Pcdhαc1* were barely detectable. Among all 58 clustered *Pcdhs*, only *Pcdhαc2*, *Pcdhγc3*, and *Pcdhγc4* are expressed in serotonergic neurons at significant levels (Fig. 4A and table S1). This pattern of clustered *Pcdh* gene expression is distinct from that of adult motor neurons (26), where all *Pcdh* isoforms are expressed at comparable levels (fig. S10C and table S2). Consistent with the bulk-sequencing data, single-cell sequencing of multiple subtypes of serotonergic neurons (27) confirms that *Pcdhαc2* is predominately expressed among all 58 clustered *Pcdh* isoforms (Fig. 4B, fig. S11, and table S3). The specific requirement of Pcdhα C-type isoforms in serotonergic wiring, together with the singular expression of Pcdhαc2 in serotonergic neurons, indicates that axonal tiling of serotonergic neurons is mediated by a single C-type Pcdh isoform, Pcdhαc2.

Discussion

Despite the fundamental importance of the serotonergic system in neural development, behavior, and psychiatric disorders, the mechanism by which this vastly expansive neural network is assembled is largely unknown. Here we show that a single C-type Pcdh isoform of the *Pcdhα* gene cluster, Pcdhαc2, plays an essential role in this process. Results of genetic dissection and expression profiling strongly support the conclusion that Pcdhαc2-mediated homophilic interaction promotes tiling between serotonergic axon terminals, and this cell-intrinsic repulsive force counteracts target-derived cues to define local serotonergic-innervation patterns. Loss of function of Pcdhαc2 results in the loss of homophilic repulsion and disrupts axonal tiling, leading to uneven spacing and clumping between serotonergic fibers, as well as a loss of balance between the cell-intrinsic and -extrinsic forces regulating serotonergic axonal arborization (fig. S12). This, in turn, results in a global disruption of normal serotonergic-wiring patterns and consequently leads to defects in cognitive-affective function, as observed in *Pcdhα*-deficient mice.

Evidence presented here and in the accompanying paper (28) together reveal a notable evolutionary symmetry between the invertebrate Dscam proteins and the vertebrate clustered Pcdhs in mediating neurite spacing and neuronal wiring. The *Drosophila* Dscam1 and Dscam2 proteins mediate self-avoidance and tiling, respectively, both through homophilic interactions followed by contact-dependent repulsion (4, 5). However, Dscam1-mediated self-avoidance uses a diversity-dependent mechanism—extensive stochastic alternative RNA splicing—to provide single-cell identity for self-recognition and self-nonself discrimination. By contrast, Dscam2-mediated tiling is diversity independent; the *Dscam2* gene does not undergo substantial alternative RNA splicing, and thereby endows all cells of the same functional type with a common single cell–type identity for homotypic recognition and repulsion (29, 30). Similarly, whereas diverse clustered Pcdh isoforms generated by stochastic promoter choice are required to provide individual neurons with specific single-cell identities for self-recognition, a single Pcdh isoform, Pcdhαc2, mediates axonal tiling by endowing all serotonergic neurons with a common single cell-type identity. Therefore, both the fly Dscams and vertebrate clustered Pcdhs serve as “molecular identity codes” to

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distinguish single cells or single cell types from one another, providing cell-intrinsic forces for neurite spacing to maximize target coverage.

Whereas mature serotonergic neurons predominately express *Pcdhαc2* (and *Pcdhγc3* and *Pcdhγc4* at lower levels) but not the alternate Pcdh isoforms, mature olfactory sensory neurons (OSNs) predominantly express alternate isoforms but not C-type isoforms (28). These cell type–specific patterns of expression correlate with the distinct axonal-arborization patterns of the two types of neurons. As we show above, serotonergic axonal tiling requires the singular expression of *Pcdhαc2*, which endows all serotonergic neurons with a single cell-type identity. By contrast, mature OSNs, whose axons do not tile but instead converge and coexist to form a glomerulus, express distinct sets of multicluster alternate Pcdhs to provide individual OSNs with specific single-cell identities required for self-recognition and self-nonself discrimination. These observations suggest that the relative abundance of C-type and alternate Pcdh isoforms may play a role in balancing the cell-intrinsic forces required for neurite self-avoidance, tiling, and coexistence, and thereby regulate the arborization patterns appropriate for specific neuronal function.

Dysfunction of the serotonergic system has long been implicated in the disease mechanisms for multiple psychiatric disorders, including depression. However, almost all previous studies have focused on serotonin synthesis, transmission, and reuptake, rather than serotonergic wiring, as causal. By conditionally abolishing Pcdhα proteins in serotonergic neurons or in their target fields in forebrain limbic structures, we showed that both behavioral and wiring defects are cell autonomous to serotonergic neurons, which strongly suggests that the depressive-like behaviors result from serotonergic-wiring change. Notably, human genetic studies have implicated the *Pcdhα* gene cluster in schizophrenia (31) as well as in autism (32). Thus, our studies provide insights into circuitry mechanisms underlying depression, as well as other mental disorders associated with aberrant serotonergic signaling.

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Pattern formation in the brain

Neurons in the developing brain cooperate to build circuits. Mountoufaris *et al*. found that ~50 variable protocadherin genes support a combinatorial identity code that allows millions of olfactory neuron axons to sort into ~2000 glomeruli. Sharing olfactory receptors drives axons to one glomerulus, and protocadherin diversity allows the multiple axons to touch each other as they converge. On the other hand, Chen *et al*. found that a single C-type protocadherin underlies the tiled distribution of serotonergic neurons throughout the central nervous system. These neurons, which share protocadherin identity, enervate broad swaths evenly without touching neighboring neurons.

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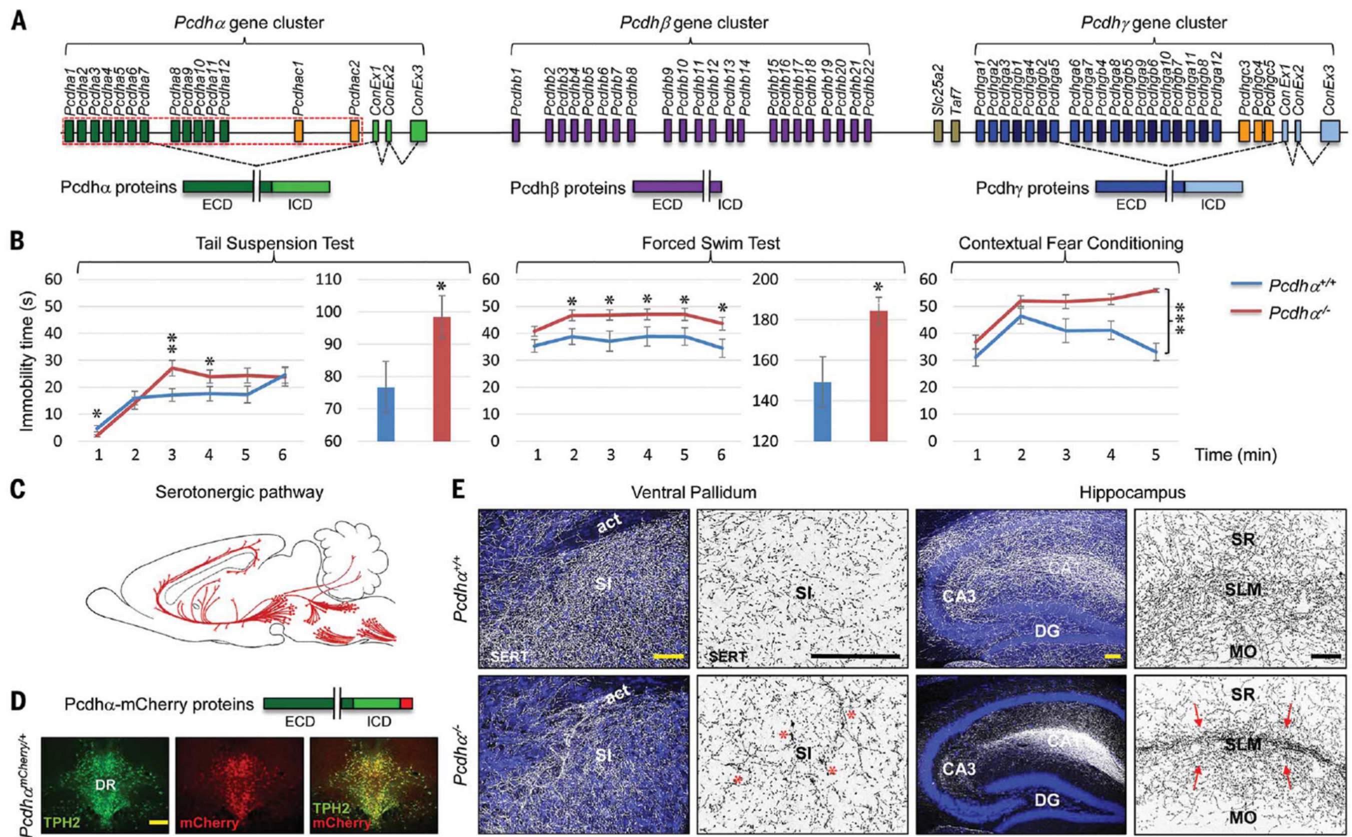

Fig. 1. Characterization of the *Pcdh*α gene cluster–deletion mice

(**A**) The mouse *Pcdh*α, *Pcdh*β, and *Pcdh*γ gene clusters contain 14, 22, and 22 variable exons, respectively, each encoding the extracellular domain (ECD), transmembrane domain, and variable intracellular domain (ICD) of a Pcdh protein. In the *Pcdh*α and *Pcdh*γ gene clusters, these variable exons are spliced to three cluster-specific constant exons that encode the common ICD of the corresponding protein isoforms. All 14 *Pcdh*α variable exons are deleted from the *Pcdh*α gene cluster (boxed with red dashed line). (**B**) Depression-related behaviors in *Pcdh*α-deficient mice, including increased immobility time in TST, FST, and CFC tests. Average immobility times for minutes 3 to 6 in the TST and FST are shown in bar graphs. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. $N = 16$ to 25 per genotype group. (**C**) Schematic drawing of the serotonergic pathways in the brain. Serotonergic cell bodies and axonal projections are depicted in red. (**D**) Pcdhα proteins are enriched in serotonergic neurons (TPH2+), as shown with the $Pcdh\alpha^{mCherry/+}$ reporter mice. DR, dorsal raphe. (**E**) Serotonergic wiring defects in *Pcdh*α-deficient mice. Shown are serotonergic axon terminals (SERT+) in target fields, as indicated. Examples of serotonergic fiber clumps [crossovers on a single optical section ($Z = 0.5$ μm)] and patterns of redistribution are indicated with red asterisks and arrows, respectively. Scale bars, 100 μm. SI, substantia innominata; act, anterior commissure, temporal limb; CA, cornu ammonis; DG, dentate gyrus; SR, stratum radiatum; SLM, stratum lacunosum moleculare; MO, molecular layer.

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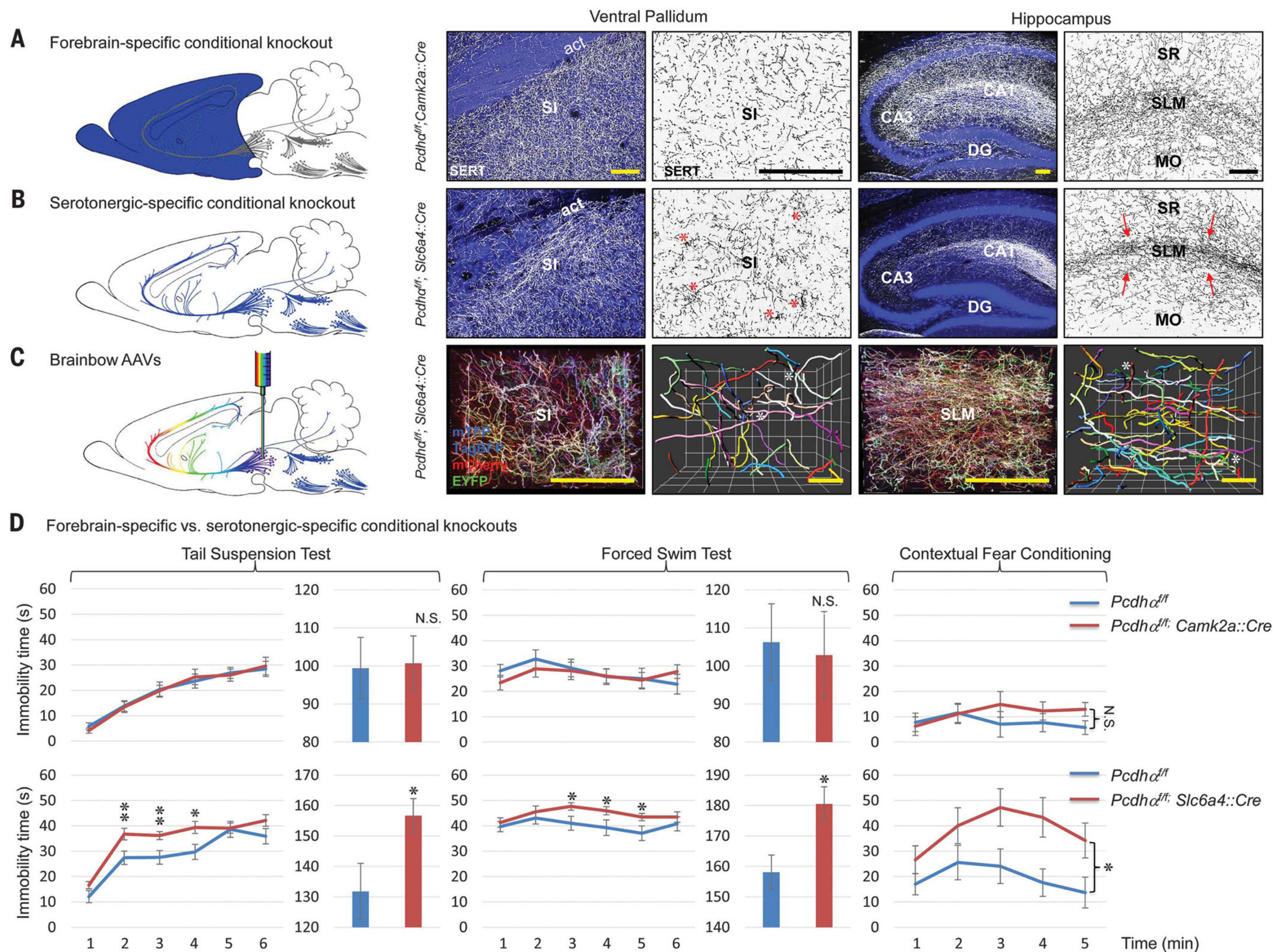

Fig. 2. Both serotonergic-wiring and behavioral defects result from *Pcdh*α loss of function in serotonergic neurons

(**A** and **B**) Serotonergic-wiring patterns in *Pcdh*α conditional knockouts. Whereas serotonergic-innervation patterns remain unchanged in forebrain-specific knockouts (*Pcdh*α$^{f/f}$; *Camk2a::Cre*), the serotonergic-wiring phenotype is recapitulated in serotonergic-specific knockouts (*Pcdh*α$^{f/f}$; *Slc6a4::Cre*). Scale bars, 100 μm. (**C**) 3D visualization (scale bar, 100 μm) and reconstruction (scale bar, 5 μm) of Brainbow AAV–labeled Pcdhα-deficient serotonergic axon terminals in target fields. Fiber clumps are indicated with white asterisks. mTFP, TagBFP, mCherry, and EYFP are reporter genes encoding green, blue, red and yellow fluorescent proteins, respectively. (**D**) Depression-related behaviors in *Pcdh*α conditional knockouts. Whereas no significant differences were observed in forebrain-specific knockouts, the behavioral phenotype was recapitulated in serotonergic-specific knockouts. *$P < 0.05$; **$P < 0.01$; N.S., not significant. $N = 17$ to 22 per genotype group.

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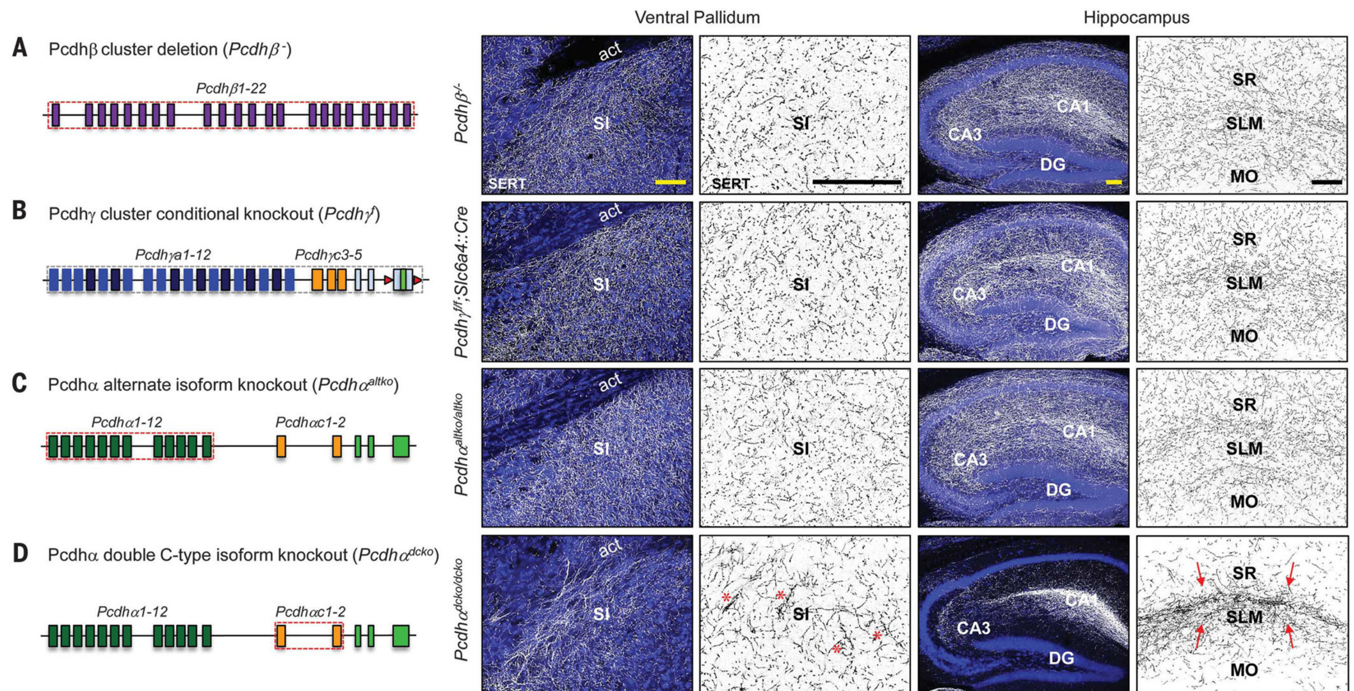

Fig. 3. Serotonergic wiring is specifically mediated by Pcdhα C-type isoforms

(**A** and **B**) *Pcdhβ* and *Pcdhγ* gene clusters are not required for serotonergic wiring in the brain. No alterations in serotonergic-innervation patterns were observed in *Pcdhβ*-cluster knockouts (A) or serotonergic-specific *Pcdhγ* conditional knockouts (B). (**C** and **D**) Genetic dissection of *Pcdhα* gene cluster. Whereas mice lacking all 12 Pcdhα alternative isoforms display normal serotonergic-innervation patterns (C), the serotonergic-wiring phenotype is recapitulated in *Pcdhα* C-type–isoform knockouts (D). Scale bars, 100 μm.

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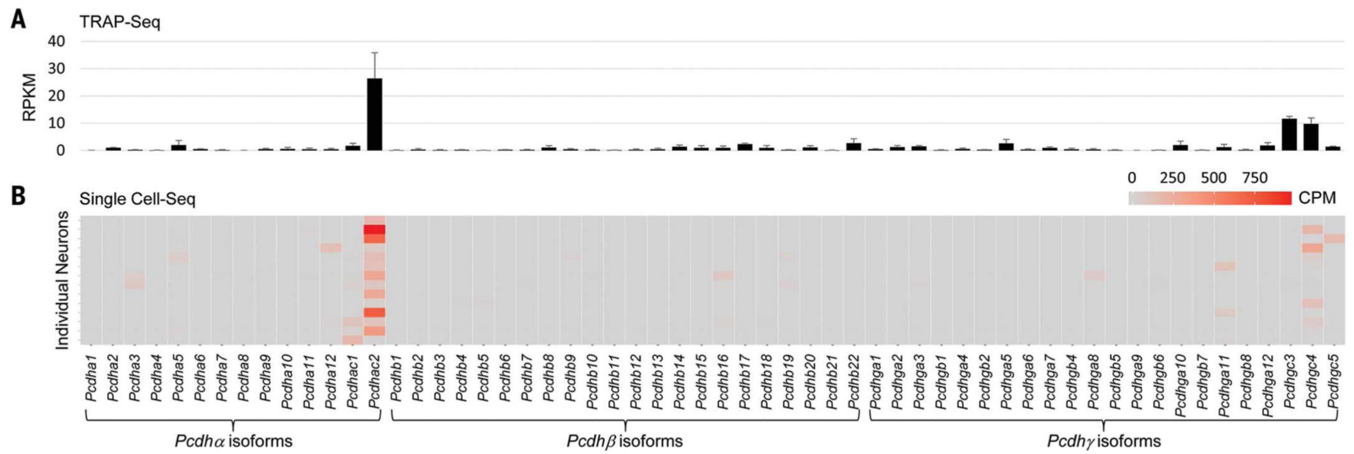

Fig. 4. *Pcdhαc2* is the predominantly expressed Pcdh isoform in serotonergic neurons

(**A**) Bulk expression levels of variable exons of the corresponding clustered *Pcdh* isoforms in serotonergic neurons, as shown with TRAP-Seq. RPKM, reads per kilobase of transcript per million mapped reads. (**B**) Single-cell expression profiles of clustered *Pcdh* isoforms in various subtypes of serotonergic neurons. Shown here are neurons with high levels of marker-gene expression (*Slc6a4* and *Tph2*, CPM > 5000). CPM, counts per million mapped reads.