

Regulation of the Protocadherin *Celsr3* Gene and Its Role in Globus Pallidus Development and Connectivity

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The globus pallidus (GP) is a central component of basal ganglia whose malfunctions cause a variety of neuropsychiatric disorders as well as cognitive impairments in neurodegenerative diseases such as Parkinson's disease. Here we report that the protocadherin gene *Celsr3* is regulated by the insulator CCCTC-binding factor (CTCF) and the repressor neuron-restrictive silencer factor (NRSF, also known as REST) and is required for the development and connectivity of GP. Specifically, CTCF/cohesin and NRSF inhibit the expression of *Celsr3* through specific binding to its promoter. In addition, we found that the *Celsr3* promoter interacts with CTCF/cohesin-occupied neighboring promoters. In *Celsr3* knockout mice, we found that the ventral GP is occupied by aberrant calbindin-positive cholinergic neurons ectopic from the nucleus basalis of Meynert. Furthermore, the guidepost cells for thalamocortical axonal development are missing in the caudal GP. Finally, axonal connections of GP with striatum, subthalamic nucleus, substantia nigra, and raphe are compromised. These data reveal the essential role of *Celsr3* in GP development in the basal forebrain and shed light on the mechanisms of the axonal defects caused by the *Celsr3* deletion.

The genes *Celsr1*, *Celsr2*, and *Celsr3* are members of the mammalian nonclustered protocadherin (*Pcdh*) family that are homologous to the fly *flamingo* gene (1). The *flamingo* gene plays important roles in dendrite development and self-avoidance, axonal projection, and planar cell polarity (PCP) in *Drosophila* (2–7). The mammalian *Celsr* genes are the so-called core PCP genes that have conserved as well as newly diversified functions. In particular, the vertebrate *Celsr3* gene has been shown to regulate axonal projections, dendrite development, neuronal migration, cilogenesis, and retina circuit development (8–16). However, very little is known about *Celsr* gene regulation.

The globus pallidus (GP) is a central nucleus of the basal ganglia (BG) (17, 18). Malfunctions of GP lead to several neuropathological conditions, such as Parkinson's disease, as well as many neuropsychiatric diseases (19). The mouse GP, which corresponds to the GPe in primates, contains approximately 44% of parvalbumin-positive (PV⁺) neurons and 1% of calretinin-positive (CR⁺) interneurons, but calbindin-positive (CB⁺) cells are rare (20, 21). The GP is traditionally thought to be derived from the medial ganglionic eminence (MGE) (22); however, recent studies have revealed that, in addition to MGE, subpopulations of GP cells are also generated from the lateral ganglionic eminence (LGE) and preoptic area (POA) (21, 23). The molecular requirements for GP development are not known, but specific transcription factors appear to be essential. For example, *Nkx2-1* is required for the generation of most GP neurons except the Npas1⁺ type from LGE (23). In addition, *Lhx6/8* double-mutant mice do not have a well-defined GP (24).

The GP functions through extensive axonal connections with other brain nuclei. For example, GP receives GABAergic axons from the striatum, glutamatergic fibers from the subthalamic nucleus (STN), dopaminergic axons from the substantia nigra pars compacta (SNc), and serotonergic axons from the raphe (18, 20). In turn, GP sends GABAergic fibers to the output nuclei: the entopeduncular nucleus (EP, or GPi in primates) and the STN

(18–20). Finally, the cells close to or associated with the developing GP might assist in the development of the major thalamocortical and corticofugal pathways as guidepost cells in the ventral forebrain (25–29). However, little is known about the molecular mechanisms of the development of GP connectivity.

We report that the transcription factors CCCTC-binding factor (CTCF) and neuron-restrictive silencer factor (NRSF) regulate the expression of *Celsr3* by directly binding to its promoter region. In particular, the *Celsr3* promoter interacts with several other promoters, most of which are enriched with the histone mark H3K4me3 and CTCF/cohesin, through long-distance DNA looping. In addition, we report the critical role of *Celsr3* in GP development and connectivity. Specifically, we have found that the ventral region of the *Celsr3* mutant GP is occupied by cholinergic neurons ectopic from the nucleus basalis of Meynert (NB) and that the axonal connections of the GP with other nuclei are compromised. Taken together, these data suggest that *Celsr3*, regulated by CTCF/cohesin and NRSF, is essential for GP development and connectivity.

MATERIALS AND METHODS

Plasmid construction. The sequences for the mouse full-length *Celsr3* open reading frame (ORF) (9,930 bp) were cloned from brain total RNA preparations by reverse transcriptase PCR (RT-PCR) experiments. We first designed four pairs of primers (F1, AGTAG ATGGT GCCCA GAGAG CAGTG; R1, GTGCT GATGG CAAAT CCGTT CCGAG; F2, ACAGC GCCAT CAGCT ACCAA ATCAC; R2, CGTTG CAGTC ATCCA

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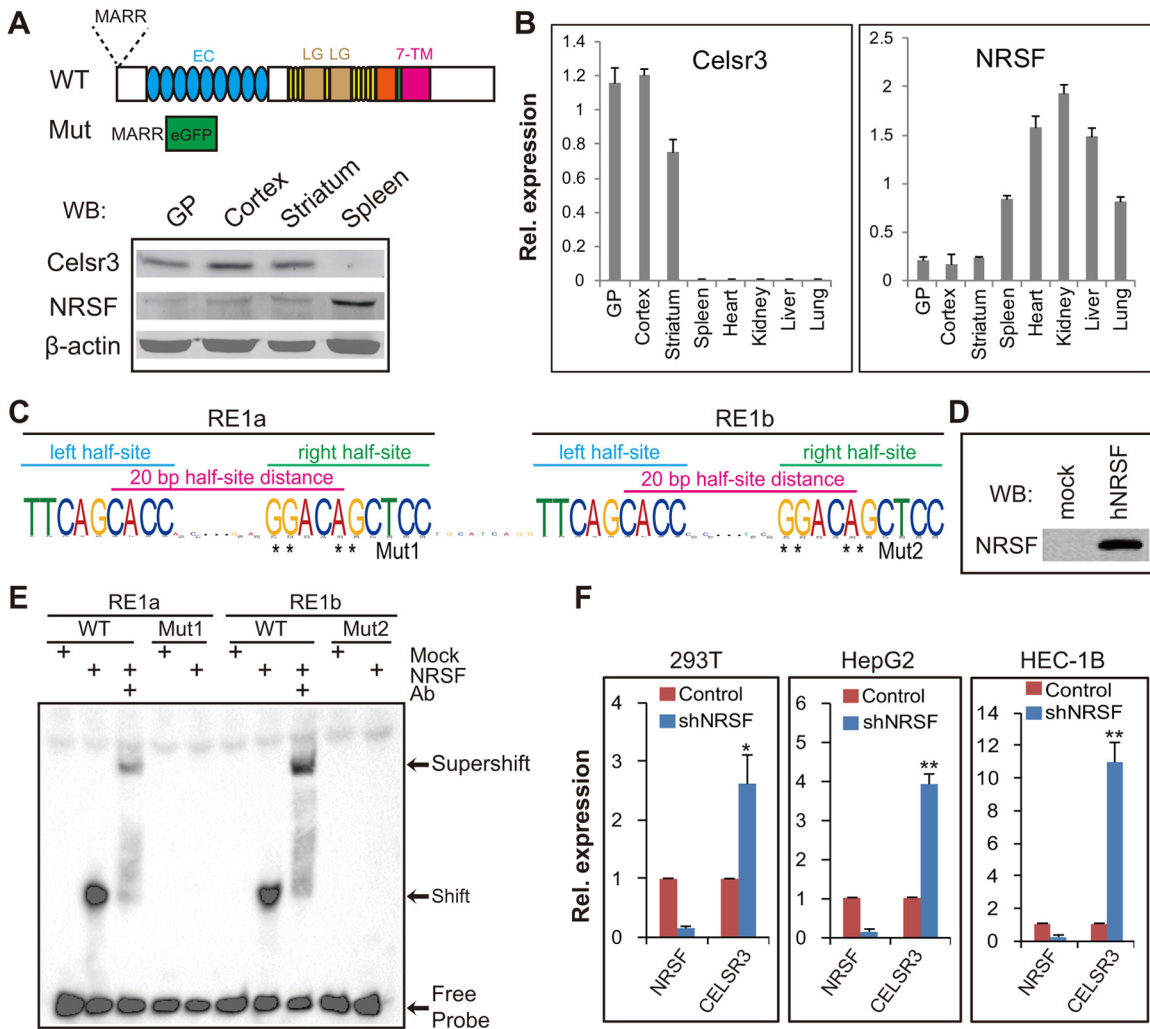


FIG 1 Inhibition of *CELSR3* expression by NRSF. (A) Schema of the wild-type (WT) and mutant (Mut) proteins and Western blot of *Celsr3* and NRSF in brain tissues of the P21 *Celsr3*^{+/-} mice, with spleen as a control. EC, cadherin ectodomain; LG, Laminin-A globular domain; 7-TM, seven transmembrane segments. (B) Quantitative real-time PCR of *Celsr3* and *NRSF* in P21 mouse tissues. (C) Two noncanonical NRSF motifs located in tandem in the *CELSR3* promoter region. (D) Western blot of the *in vitro*-translated NRSF proteins. (E) Gel shift and supershift of NRSF with wild-type and mutated *NRSF* motifs. (F) Relative expression of *CELSR3* genes upon the NRSF knockdown in three different nonneuronal cell lines. Data are means \pm SD ($n = 3$). Significance was determined with Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.

pared at a molar ratio of 1:1. The expression vectors were then mixed with 0.05% fast green as a tracer and injected through the uterine wall into the lateral ventricle. Five electrical pulses were applied at 32 V for a duration of 50 ms at 900-ms intervals. Six days later, the pregnant mice were sacrificed, and embryonic brains were fixed overnight. The brains were then mounted with 5% agarose and sectioned at 50 μ m with a vibratome.

Imaging and cell counting. Slides were imaged with a Zeiss Axiovert 200 microscope. The pictures in Fig. 3B, Fig. 9D, and Fig. 10D to F and H were photo merged from small pictures. Cell counting was performed on images at magnifications of $\times 200$. At least three sections of the rostral, middle, and caudal GP levels were counted. Data were expressed as means \pm standard deviations (SD). The significance of differences was assessed using the two-tailed Student *t* test.

Nucleotide sequence accession number. The sequence cloned here can be accessed through GenBank accession number KJ954288.

RESULTS

Inhibition of *CELSR3* by NRSF. To understand the mechanisms of developmental regulation of the *Celsr3* gene, we first investi-

gated the expression levels of the *Celsr3* proteins in several mouse brain regions by Western blotting with an anti-enhanced-GFP (anti-eGFP) antibody using an eGFP reporter mouse line (Fig. 1A) (11). The mouse line was constructed by inserting an eGFP reporter in frame with the first four amino acid residues of the *Celsr3* protein under the control of the endogenous transcription and translational signals. We found that *Celsr3* is expressed at high levels in the GP, cortex, and striatum, in contrast to no expression in the spleen and several nonneural tissues (Fig. 1A and B). This is consistent with previous reports that the expression of *Celsr3* is neuron specific (37). Interestingly, we found that NRSF, which is a transcription factor suppressing the expression of neuron-specific genes in nonneural tissues (38), is expressed at high levels in the mouse nonneural tissues but at low levels in the brain (Fig. 1A and B). We found two putative NRSF binding sites located in tandem in the promoter region of *CELSR3* (Fig. 1C), although most genes contain only one NRSF binding site (39). The two sites

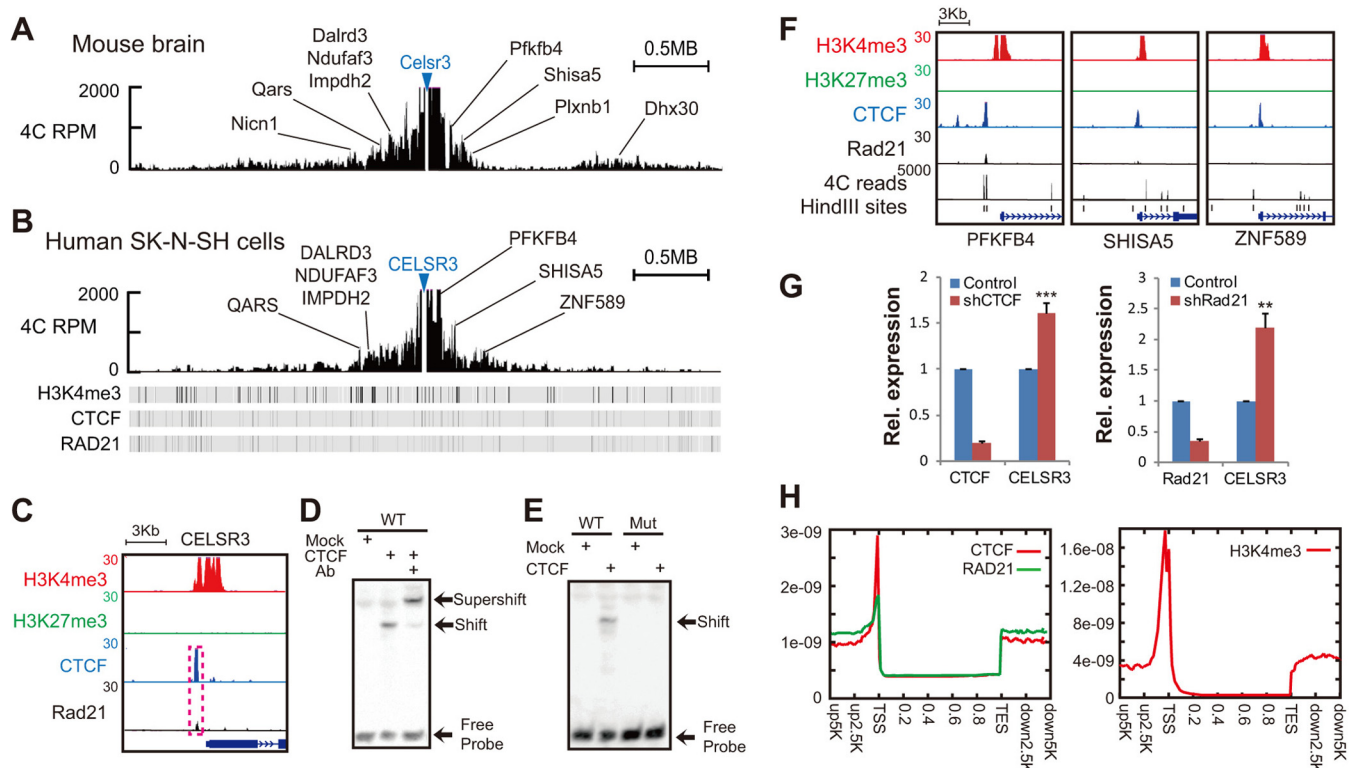


FIG 2 Spatial interactome of the *Celsr3* promoter region. (A and B) Chromosomal maps of 4C signals, in reads per million (RPM), anchored with the *Celsr3* promoter in E18.5 mouse brain tissues (A) and human SK-N-SH cells (B). (C) ChIP-seq signals of H3K4me3, H3K27me3, CTCF, and RAD21 in the *CELSR3* promoter. (D and E) Gel shift and supershift of CTCF with wild-type (WT) or mutated (Mut) probes of *CELSR3*. (F) Read density of H3K4me3, H3K27me3, CTCF, and RAD21, as well as 4C signals, in three interacting promoters. (G) Relative expression of *CELSR3* upon CTCF and RAD21 knockdown. (H) Genome-wide localization of CTCF, RAD21, and H3K4me3 immediately upstream of TSS. Data are means \pm SD ($n = 3$). Significance was determined with Student's *t* test. **, $P < 0.01$; ***, $P < 0.001$.

contain exactly the same binding motifs (Fig. 1C). Gel shift and supershift experiments with recombinant NRSF showed that it binds to both motifs (Fig. 1D and E). Knockdown of NRSF in several nonneuronal cell lines results in significant increases of *CELSR3* expression (Fig. 1F). Thus, NRSF binds directly to the *CELSR3* promoter region and inhibits its expression.

Role of CTCF and cohesin in *CELSR3* gene regulation. To further investigate the mechanisms of *Celsr3* gene regulation, we performed 4C-seq experiments by using the *Celsr3* promoter region as an anchor in the mouse brain as well as in the human neural cell line SK-N-SH. We found that the *Celsr3* promoter is looped with the promoter regions of several neighboring genes as well as several nonpromoter regions in the mouse brain (Fig. 2A). Similarly, the human *CELSR3* promoter also interacts with promoters of several neighboring genes (Fig. 2B). ChIP-seq experiments revealed that all of these looped promoters are occupied by the active histone mark H3K4me3 (Fig. 2B). In particular, the *CELSR3* promoter is marked by H3K4me3 as well as the chromosomal architectural proteins CTCF and cohesin subunit RAD21 (Fig. 2C). Manual inspections identified a putative CTCF-binding motif in the *CELSR3* promoter region. Gel shift experiments showed that CTCF and the motif form a retarded band which is supershifted with a CTCF-specific antibody and is abolished by mutation of the core sequences (Fig. 2D and E). We noted that the vast majority of the looped promoters are enriched with CTCF and RAD21 (Fig. 2B and F). Finally, knockdown of CTCF or

RAD21 results in significant increases of *CELSR3* expression (Fig. 2G). Importantly, genome-wide analysis revealed that CTCF/cohesin complexes, as well as the H3K4me3 mark, are specifically enriched immediately upstream of transcription start sites (TSS) in SK-N-SH cells (Fig. 2H), suggesting an architectural role of CTCF/cohesin in mediating genome-wide promoter-promoter interactions. Taken together, we conclude that CTCF-mediated chromatin looping interactions between neighboring promoters are important in the regulation of *Celsr3* gene expression.

***Celsr3* expression patterns in GP.** To investigate whether *Celsr3* expression is regulated during neural differentiation, we used retinoic acid (RA) to induce differentiation of the human neuroblastoma cell line IMR-32. We found significant increases of human *CELSR3* expression upon RA-induced differentiation of IMR-32 cells (Fig. 3A), consistent with a previous report that *Celsr3* is expressed in differentiated brain nuclei (37). Interestingly, we found that *Celsr3* is especially enriched in the differentiated ventral forebrain nuclei of the GP in the developing mouse embryos (Fig. 3B). Costaining with the GP marker Nkx2-1 (21, 23) demonstrated that the vast majority of GP cells express *Celsr3* (Fig. 3C to E). Quantification revealed that $77.2\% \pm 2.8\%$ ($n = 3$ mice) of Nkx2-1⁺ (GP) cells express *Celsr3* at E15.5 (out of 506 Nkx2-1⁺ cells quantified, 391 cells are *Celsr3*⁺), $85.7\% \pm 4.6\%$ ($n = 8$ sections from 3 mice) at E18.5 (out of 675 Nkx2-1⁺ cells quantified, 578 cells are *Celsr3*⁺), and $97.0\% \pm 1.0\%$ ($n = 8$ sections from 3 mice) in the adult (out of 327 Nkx2-1⁺ cells quanti-

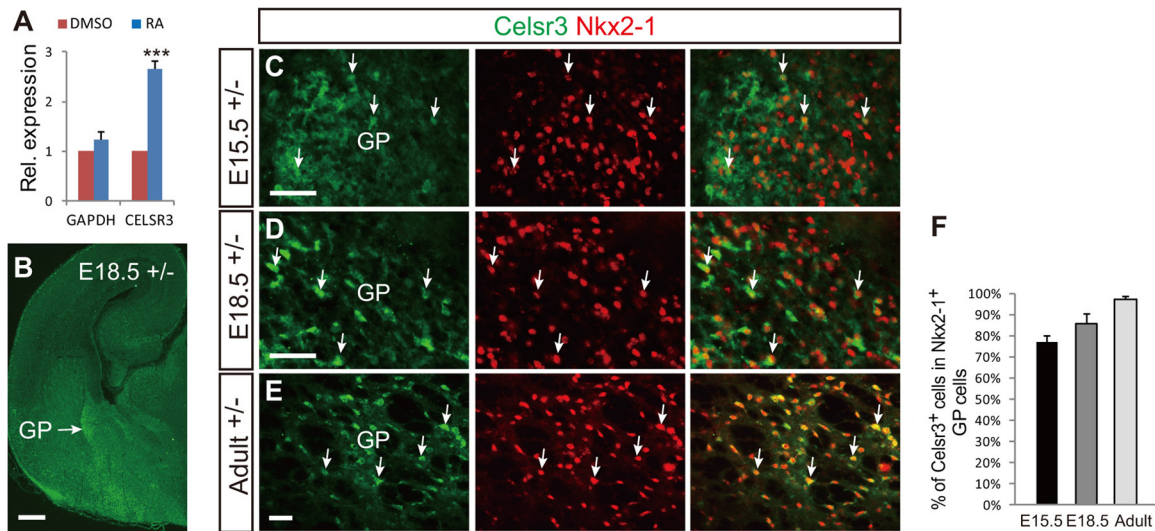


FIG 3 Prominent expression of *Celsr3* in GP. (A) Expression level of *Celsr3* in RA-treated IMR-32 cells. (B) *Celsr3* expression pattern in the mouse forebrain. (C to E) Expression of *Celsr3* (green) and *Nkx2-1* (red) in GP. Examples of coexpressed cells are indicated by arrows. (F) Quantification of the expression of *Celsr3* in *Nkx2-1*⁺ GP cells. Data are means \pm SD ($n = 3$ mice). Significance was determined with Student's *t* test. ***, $P < 0.001$. Scale bars, 300 μ m (B) and 50 μ m (C to E).

fied, 318 cells are *Celsr3*⁺) (Fig. 3F). This prominent expression of *Celsr3* in the GP suggests that *Celsr3* may play an important role in GP development and function.

Abnormal GP in *Celsr3* mutant mice. To investigate whether there are developmental defects of the GP in *Celsr3*-targeted mice, we performed DAPI and Nissl staining and found that an aberrant region where cells are sparse appears at E14.5 in the developing ventral GP of the *Celsr3* mutant mice (Fig. 4A). In addition, this defect is progressively more pronounced from E14.5 to E18.5 (Fig. 4A). Finally, we found many more GFAP⁺ astrocytes in the mutant GP than in the control, suggesting reactive gliosis resulting from potential increases of apoptosis (Fig. 4B). Indeed, activated caspase 3 immunostaining showed an increase of apoptosis in the mutant GP (Fig. 4C).

We analyzed the GP defect with its marker *Nkx2-1* by serial coronal sectioning from rostral to caudal levels and found that the distribution of *Nkx2-1*⁺ cells is disrupted by the same region with sparse cells (Fig. 4A and D). In particular, this region appears at the middle and caudal levels (Fig. 4D). Quantification revealed that the densities of *Nkx2-1*⁺ cells increase in the mutant dorsal GP but decrease in the mutant ventral GP (Fig. 4E). In addition, the difference in cell densities is progressively more pronounced from the middle to caudal levels (Fig. 4D and E).

Aberrant CB⁺ cells in the mutant GP. To investigate the etiology of the abnormal GP morphology, we performed *in situ* hybridization with a set of GP-specific transcription factor genes, such as *Er81*, *Lhx6*, and *Lmo3* (23). We found that mutant GP cells maintain the same levels of their expression, suggesting no fate determination defects in GP (Fig. 5A). In addition, we performed immunostaining with *Olig2* and *NG2*, which are oligodendrocyte markers in GP (23), and found no change of oligodendrocytes in the mutant GP (Fig. 5B and C). Third, we performed birth-dating experiments with *BrdU* injection at E10.5, E11.5, E12.5, and E13.5, which are the main stages of GP neurogenesis (21), and found no difference in birth timing of GP cells (Fig. 5D). Finally, we performed CB staining and found aberrant CB⁺ cells in the

mutant ventral GP (Fig. 5E). These CB⁺ cells in the mutant ventral GP have relatively big soma with leading processes pointing in different directions (Fig. 5E). Surprisingly, these CB⁺ cells do not express the GP marker *Nkx2-1* (Fig. 5F). This suggests that the CB⁺ cells in the mutant GP are of ectopic origin.

CB⁺ cells are cholinergic neurons. GP contains cholinergic neurons that represent an extension from the basal magnocellular complex (Ch4) (21). This Ch4 complex comprises cholinergic neurons scattered throughout the ventral pallidum, ansa lenticularis, substantia innominata, magnocellular preoptic nucleus (MCPO), and NB (40, 41). To determine the origin of the CB⁺ cells in the mutant ventral GP, we performed costaining with CB and ChAT, a marker of cholinergic neurons, and found that most of the CB⁺ cells in the mutant ventral GP are ChAT⁺ (Fig. 6A). Quantification showed that the vast majority of CB⁺ cells (88.9% \pm 7.6%; $n = 3$ mice) coexpress ChAT (out of 118 CB⁺ cells quantified, 103 cells are ChAT⁺) (Fig. 6B). Consistently, AChE histochemistry showed that the mutant ventral GP has more AChE signals than controls at the middle to caudal levels (Fig. 6C). Quantification revealed that cholinergic cells in the mutant GP are significantly increased, especially at the middle to caudal levels (Fig. 6D). Together, these data demonstrated that CB⁺ cells in the mutant ventral GP are cholinergic neurons.

Aberrant CB⁺ ChAT⁺ cells are of ectopic origin. The expression of the LIM-homeobox transcription factor *Islet1* is specifically excluded from GP (42). However, there are aberrant *Islet1* expression patterns in the mutant ventral GP (Fig. 7A). Costaining of CB and *Islet1* revealed that the vast majority of CB⁺ cells (91.6% \pm 3.7%; $n = 3$ mice) in the mutant ventral GP also express *Islet1* (out of 69 CB⁺ cells quantified, 63 cells are *Islet1*⁺) (Fig. 7B and C). We costained ChAT and *Islet1* and found that they are coexpressed in the mutant ventral GP (Fig. 7D). Quantification revealed that 81.2% \pm 4.1% ($n = 3$ mice) of cholinergic neurons in the mutant ventral GP coexpress *Islet1* (out of 137 ChAT⁺ cells quantified, 111 cells are *Islet1*⁺) (Fig. 7E). The property of coexpression of ChAT and *Islet1* of these aberrant cells in the mutant is

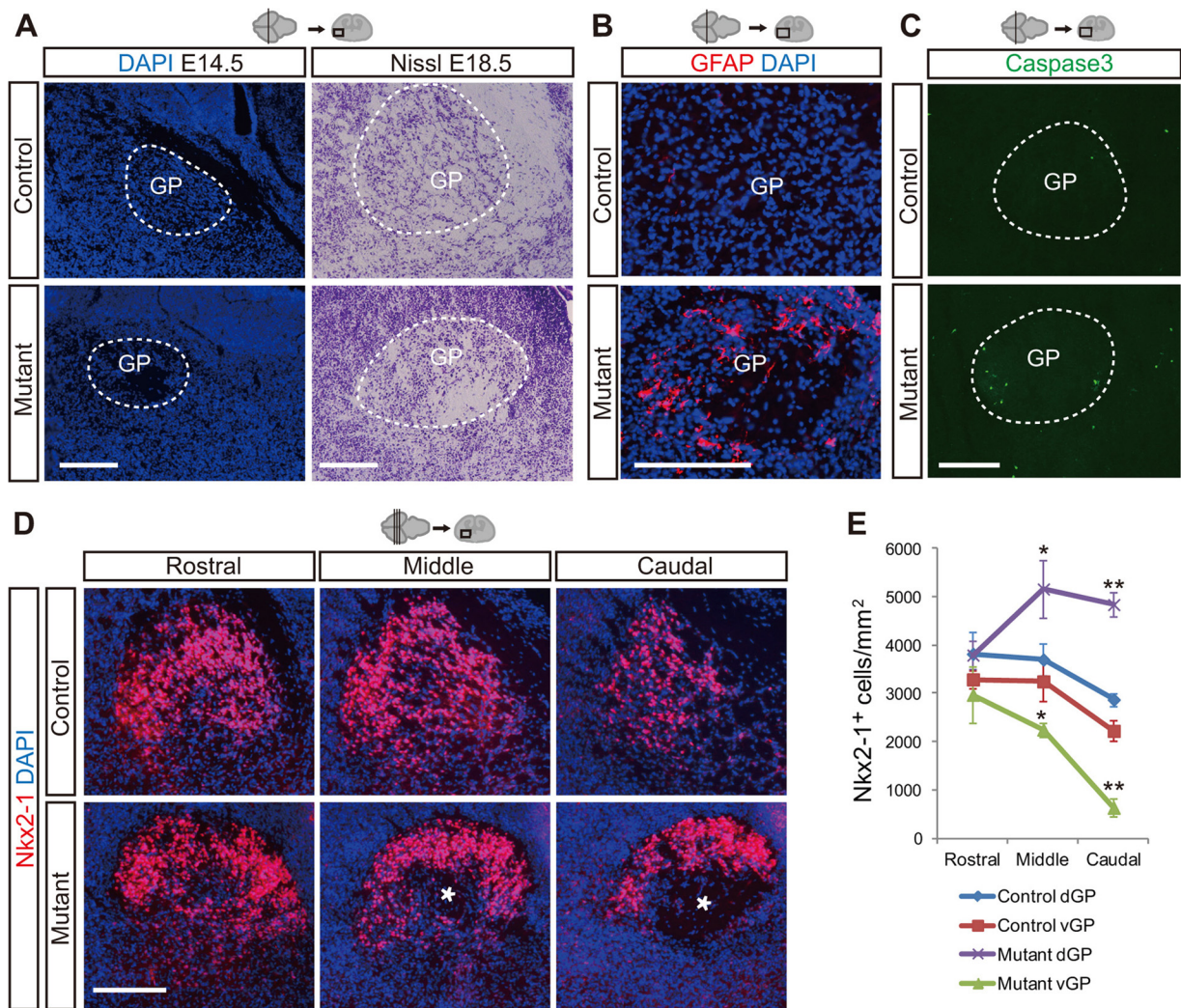


FIG 4 Abnormal GP morphology in *Celsr3* mutant mice. (A) DAPI and Nissl staining of the E14.5 and E18.5 GP, respectively. The GP region is indicated. (B) GFAP staining of the E18.5 GP. (C) Activated caspase 3 staining of the E18.5 GP. (D) Nkx2-1 and DAPI staining of the E18.5 GP at different rostrocaudal levels. The asterisk indicates the abnormal region with sparse cells in the mutant ventral GP. (E) Quantification of the Nkx2-1⁺ cell densities in the dorsal and ventral GP at different rostrocaudal levels. dGP, dorsal GP; vGP, ventral GP. Data are means \pm SD ($n = 3$ mice). Significance was determined with Student's *t* test. *, $P < 0.05$; **, $P < 0.01$. Scale bars, 200 μ m.

similar to that of ChAT⁺ Islet1⁺ NB cells in the control (Fig. 7D and E). Collectively, the data lead us to conclude that these aberrant CB⁺ ChAT⁺ Islet1⁺ cells are of ectopic origin.

Ectopic CB⁺ ChAT⁺ Islet1⁺ neurons are from NB. We found that the vast majority of cholinergic neurons in NB and MCPO express *Celsr3* (Fig. 8A and B). However, CB is expressed in cholinergic neurons from NB (43) but not MCPO, consistent with the fact that there is no difference between the MCPO regions of mutant and control mice (Fig. 8C). The strong expression of CB by the cholinergic neurons in the mutant ventral GP (Fig. 6A) suggests that these neurons may be ectopic from NB. Indeed, the region ventral to GP contains numerous cholinergic NB neurons in control mice (Fig. 8D). In mutant mice, however, the cholinergic neurons of NB appear to be located within the ventral GP, and there are no cholinergic neurons ventral to GP (Fig. 8D). As Islet1 is expressed in about 80% of cholinergic neurons in NB in the ventral telencephalon (41) (Fig. 7D and E), and taking this in conjunction with the fact that NB is absent in the corresponding

region of the mutant mice (Fig. 8D), we concluded that the ChAT⁺ CB⁺ Islet1⁺ neurons in the mutant ventral GP are ectopic from NB, as shown in the schema in Fig. 8E. To see whether *Celsr3* plays cell-autonomous or non-cell-autonomous roles in GP, we transiently expressed *Celsr3* in GP by *in utero* electroporation and found that the ventral GP still has similar defects (Fig. 8F), suggesting a non-cell-autonomous role of *Celsr3* that is consistent with its known cell adhesion activities (2).

Missing guidepost cells in the caudal GP. Expression of *Celsr3* in the basal forebrain is required for the thalamocortical axon (TCA) pathfinding (8, 10). However, the underlying mechanisms are unknown. It has been proposed that cells associated with developing internal capsule (IC cells) act as transient guideposts for TCA pathfinding (25–28, 44). We first confirmed the role of *Celsr3* in the development of thalamocortical connections by transiently knocking down *Celsr3* in the ventral forebrain through *in utero* electroporation using CR as a marker for TCA (Fig. 9A and B) (8, 10, 42). Second, we found that Nkx2-1⁺ cells in the mutant GP

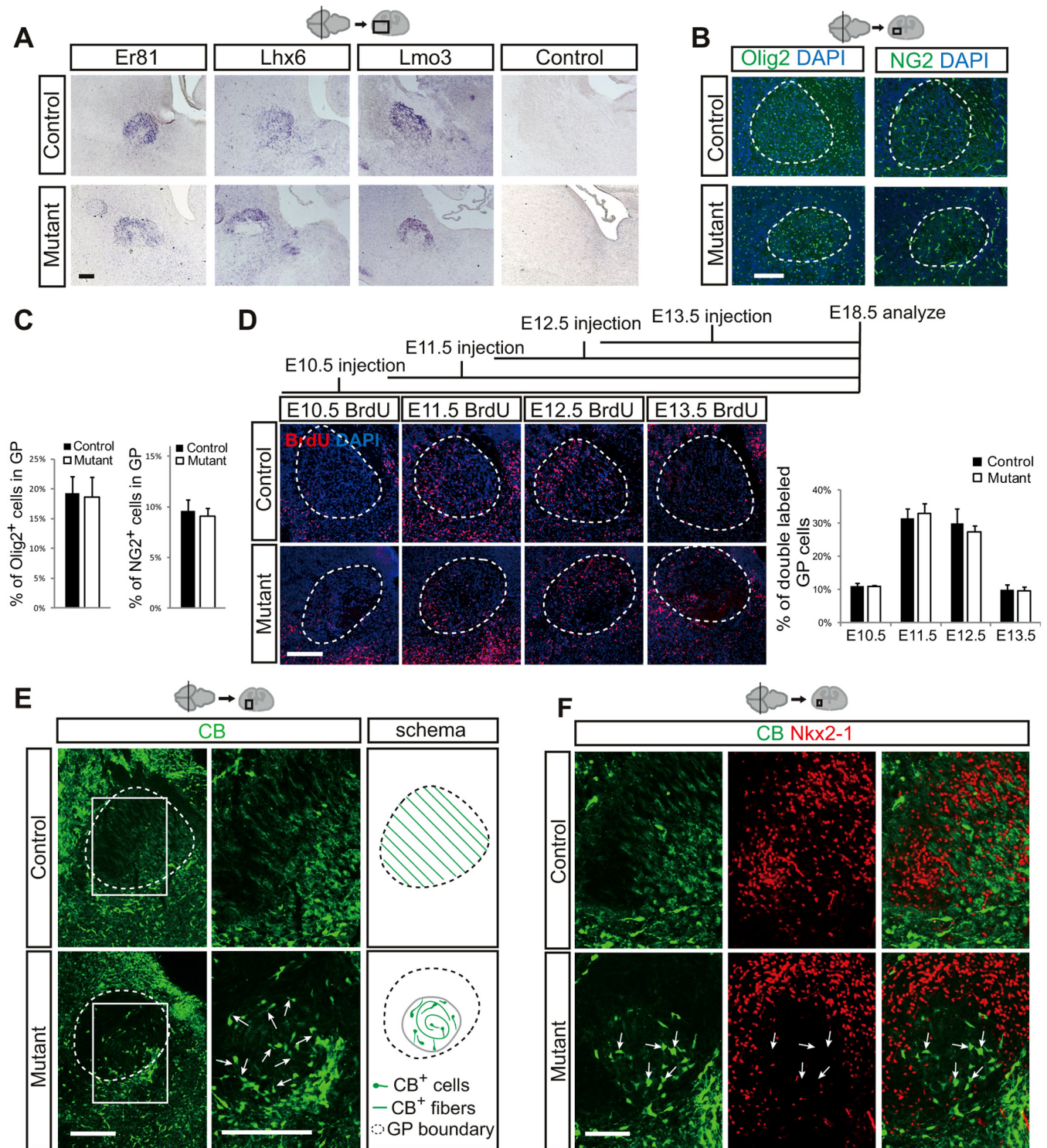


FIG 5 Aberrant CB⁺ cells in the mutant ventral GP. (A) *In situ* hybridization of *Er81*, *Lhx6*, and *Lmo3* with a sense probe as the control. (B and C) Olig2 and NG2 staining (B) and quantification (C) of the oligodendrocytes in the GP. (D) Birth dating of the GP cells with BrdU injected at E10.5, E11.5, E12.5, and E13.5. (E) CB staining and the schema of the E18.5 control and mutant GP. Abnormal CB⁺ cells are indicated by arrows. (F) CB and Nkx2-1 staining of GP. Note that CB and Nkx2-1 are not colocalized (arrows). Data are means ± SD (*n* = 3 mice). Scale bars, 200 μm (A to E) and 100 μm (F).

cannot extend caudally at E13.5 and E14.5 (Fig. 9C), stages coinciding with those when TCA makes a sharp turn to the basal forebrain from the thalamus (42). The Nkx2-1⁺ cells in the caudal extension of GP send axons to the dorsal thalamus to guide the

TCA development and are the so-called IC cells (26–29, 45, 46). Finally, by inserting Dil in the E13.5 and E14.5 dorsal thalamus, we found no retrogradely labeled cells in the mutant basal forebrain, suggesting the absence of guidepost IC cells (Fig. 9D). To-

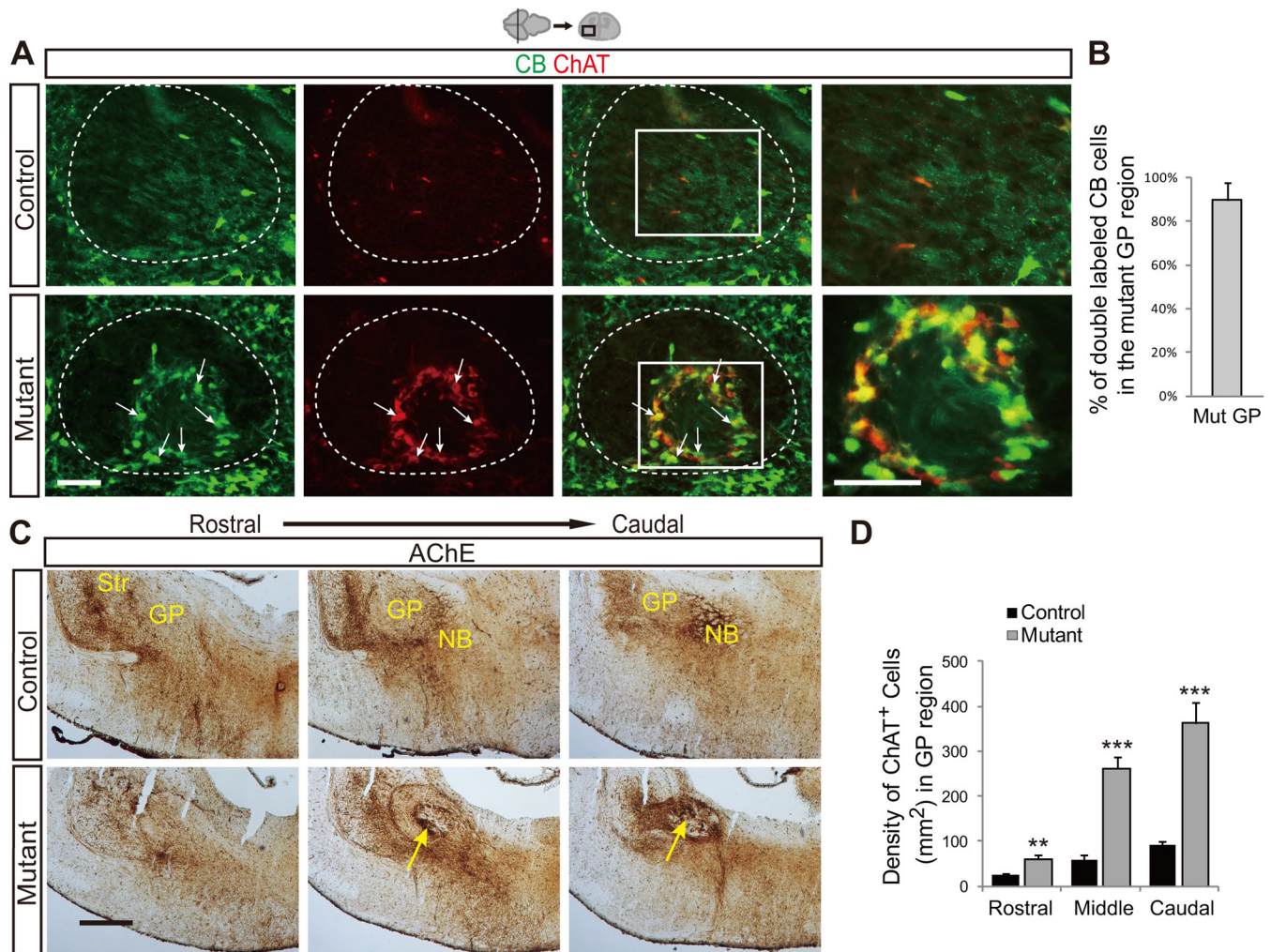


FIG 6 CB⁺ cells in the mutant GP are cholinergic neurons. (A) CB and ChAT staining of the E18.5 GP. Costained CB⁺ ChAT⁺ neurons are indicated by arrows. (B) Quantification of ChAT⁺ CB⁺ neurons in the mutant GP. (C) AChE labeling of the ventral telencephalon. Note the aberrant AChE signals in the mutant middle and caudal GP (arrows). (D) Quantification of the density of ChAT⁺ neurons in the rostral, middle, and caudal GP. NB, nucleus basalis. Data are means \pm SD ($n = 3$ mice). Significance was determined with Student's t test. **, $P < 0.01$; ***, $P < 0.001$. Scale bars, 100 μ m (A) and 400 μ m (C).

gether, these data lead us to propose that the absence of caudal GP IC cells underlies the TCA pathfinding defects in the *Celsr3* mutant mice.

Absence of GP axonal connections. In addition to the absence of IC cells causing TCA pathfinding defects, we found novel GP axonal connection defects with several brain nuclei. Specifically, staining with antibodies against CR, VGLUT2, TH, and SERT revealed no signals in the E18.5 mutant GP, suggesting dramatic defects of multitude afferent axonal projections to the GP from other brain nuclei such as the forebrain STN, midbrain SNc, and hindbrain dorsal raphe (Fig. 10A). To see whether it was caused by axonal pathfinding defects or axon degeneration after the connections had been established, we analyzed CR⁺, VGLUT2⁺, TH⁺, and SERT⁺ axonal fibers in the E15.5 and E13.5 GP. We found that the mutant GP is free of these axons at both stages (Fig. 10B and C), suggesting that *Celsr3* affects the early axonal pathfinding.

To confirm the axonal connectivity defects, we performed DiI tracing experiments by inserting DiI in GP or striatum. We found that DiI inserted in GP resulted in no signals in EP, STN, and SN in mutants compared with strong signals in controls (Fig. 10D). In

addition, DiI inserted in striatum resulted in no signals in the mutant GP (Fig. 10E). Taken together, these data indicate that there is no axonal connectivity between GP and other brain nuclei.

To investigate the underlying mechanism of GP axonal pathfinding defects, we stained sagittal sections with the TH antibody and found that TH axons from SNc cannot pass through the telencephalon-diencephalon boundary (TDB) and instead turn ventrally toward the hypothalamus (Fig. 10F). Western blot confirmed the absence of TH proteins in the telencephalon (Fig. 10G). In fact, CR⁺, VGLUT2⁺, and SERT⁺ axons all have similar defects (Fig. 10H). Thus, we propose that these axons projecting to GP from discrete brain nuclei are stalled at the TDB (Fig. 10I).

DISCUSSION

GP plays a central role in the neural circuits of BG, and its dysfunctions cause a repertoire of devastating heterogeneous neurological disorders (19, 47, 48). Here, we report that the protocadherin gene *Celsr3*, a member of core mammalian PCP genes, is regulated by transcription factors CTCF and NRSF and is required for GP development. We found that the ventral GP is occupied by

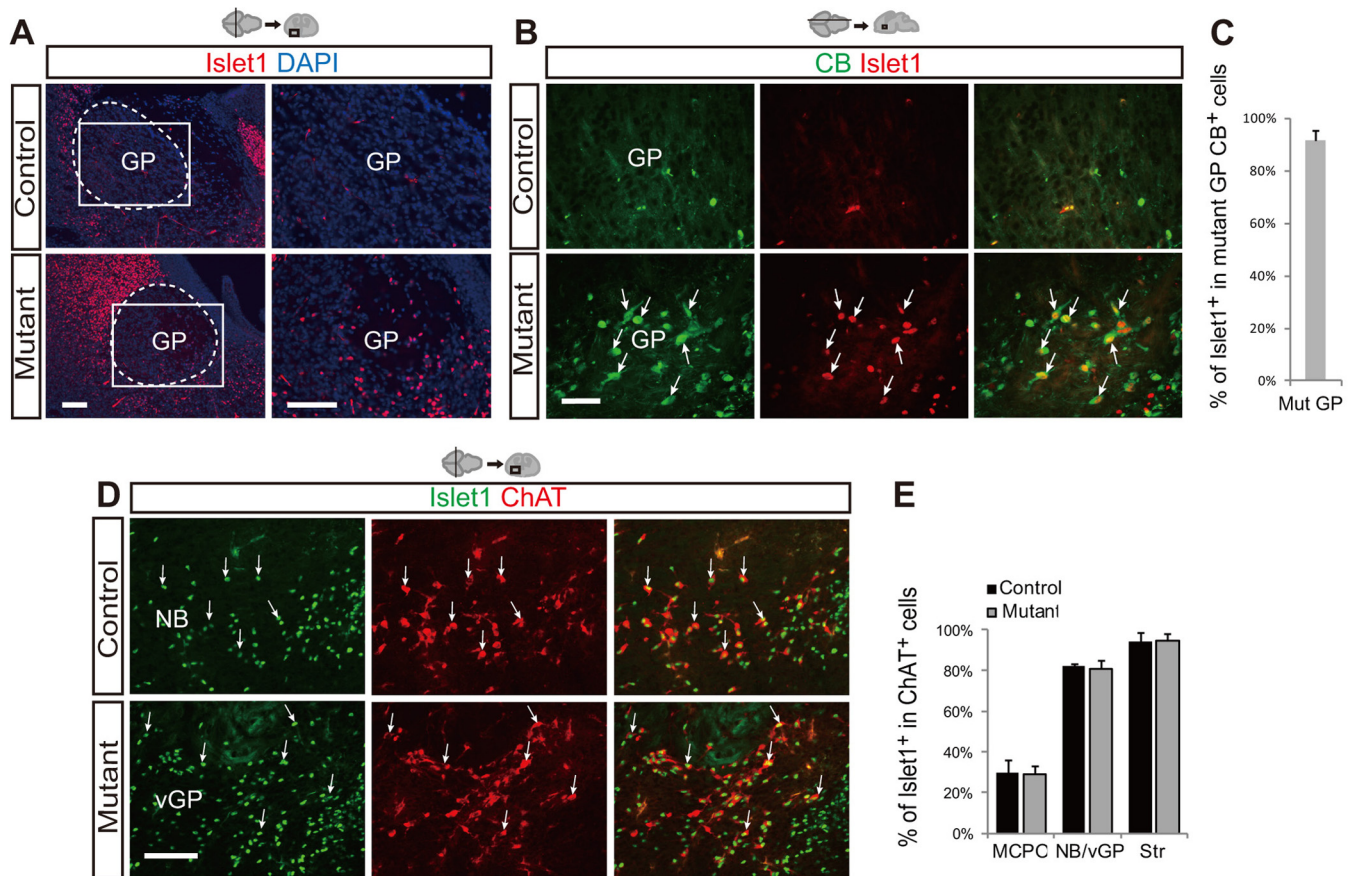


FIG 7 $CB^+ ChAT^+$ cells in the mutant are not endogenous GP cells. (A) Islet1 expression pattern in striatum and GP. Note the exclusion of Islet1⁺ cells in the control but not the mutant GP. (B) Costaining of CB and Islet1 (coexpressing cells are indicated by arrows). (C) Quantification of CB and Islet1 coexpression. (D) Coexpression of Islet1 and ChAT in the control NB and mutant ventral GP. (E) Quantification of the percentages of Islet1⁺ cells in different cholinergic neuron groups. MCPC, magnocellular preoptic nucleus; NB, nucleus basalis. Data are means \pm SD ($n = 3$ mice). Scale bars, 100 μ m.

aberrant cholinergic cells ectopic from NB. Furthermore, we provide evidence that *Celsr3* is required for the development of axonal projections between GP and the brain nuclei of the striatum, EP, STN, SN, and raphe. Taken together, our data reveal a new role of the protocadherin gene *Celsr3* in the morphogenesis of GP and provide mechanistic insights into the axonal guidance in the brain.

The *Pcdh* genes constitute a large subfamily of the cadherin superfamily that are expressed mainly in the central nervous system in a cell- or tissue-specific or developmental-stage-specific manner (49, 50). In particular, the clustered *Pcdh* genes are organized into variable and constant regions and generate enormous neural diversity by stochastic promoter choice combined with alternative pre-mRNA splicing (51–53). Recently, the neural expression of the clustered *Pcdh* genes was found to be controlled by both CTCF and NRSF (30, 38, 54, 55). In particular, the CTCF/cohesin complex plays a pivotal role in the promoter choice of the clustered *Pcdh* genes by mediating specific DNA looping interactions between enhancers and selective variable promoters (30). However, virtually nothing is known about the developmental regulation of members of the *Celsr* subfamily of nonclustered *Pcdh* genes. Here, we found that the *Pcdh Celsr3* gene is regulated by both the insulator CTCF and the repressor NRSF. Specifically, by a combination of bioinformatics analyses and biochemical exper-

iments, in conjunction with genome-wide ChIP-seq and 4C-seq, we found that NRSF binds to two sites and CTCF binds to one site in the *Celsr3* promoter region and that the *Celsr3* promoter interacts with several neighboring promoters through long-distance DNA looping. This suggests that CTCF/cohesin-mediated chromatin looping is involved in the regulation of the *Celsr3* gene. Interestingly, recent chromatin interaction analysis with paired-end tag sequencing (ChIA-PET) experiments revealed widespread CTCF-mediated promoter-promoter or promoter-enhancer interactions (56), and Hi-C experiments revealed largely invariant topologically associating domains (TADs) in different human and mouse cell types or tissues (57). Given the enrichment of CTCF and localization of the active histone mark H3K4me3 in the promoter regions of *Celsr3* and several neighboring genes (Fig. 2C and F), these genes may be coregulated in a TAD by CTCF. Finally, our data suggested that, in nonneural cells, NRSF represses the expression of *Celsr3* by binding directly to its promoter (Fig. 1). However, there is no NRSF binding site in the promoter regions of the *Celsr1* and *Celsr2* genes, and knockdown of CTCF in SK-N-SH results in no alteration of *Celsr2* gene expression (data not shown), suggesting that CTCF and NRSF specifically regulate *Celsr3* but not *Celsr2*.

In the basal forebrain, CB^+ cells are heterogeneous, and one subtype is cholinergic neurons (43). We found that $CB^+/ChAT^+$

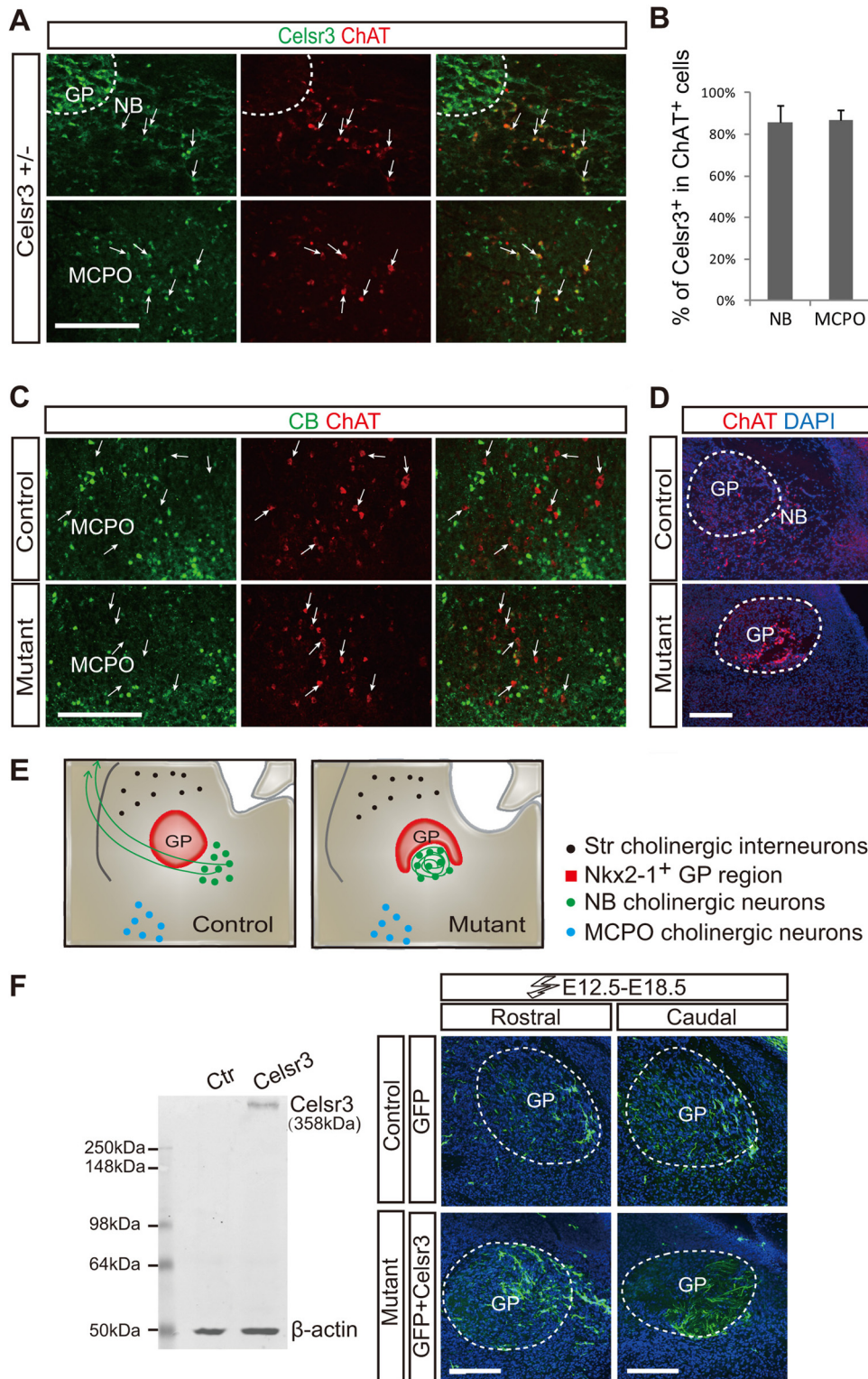


FIG 8 Aberrant CB⁺ ChAT⁺ cells are ectopic from NB. (A and B) Coexpression of Celsr3 and ChAT (A) and its quantification (B) in NB and MCPO cholinergic neurons. (C) Costaining of CB and ChAT. Note that most cholinergic neurons in MCPO are CB negative. (D) ChAT and DAPI staining showing relative positions of GP and NB. (E) Schema of the *Celsr3* mutant model. (F) Western blot confirmation of the recombinant Celsr3 expression and *in utero* electroporation of Celsr3 into the mutant GP. Data are means ± SD (*n* = 3 mice). Scale bars, 200 μm.

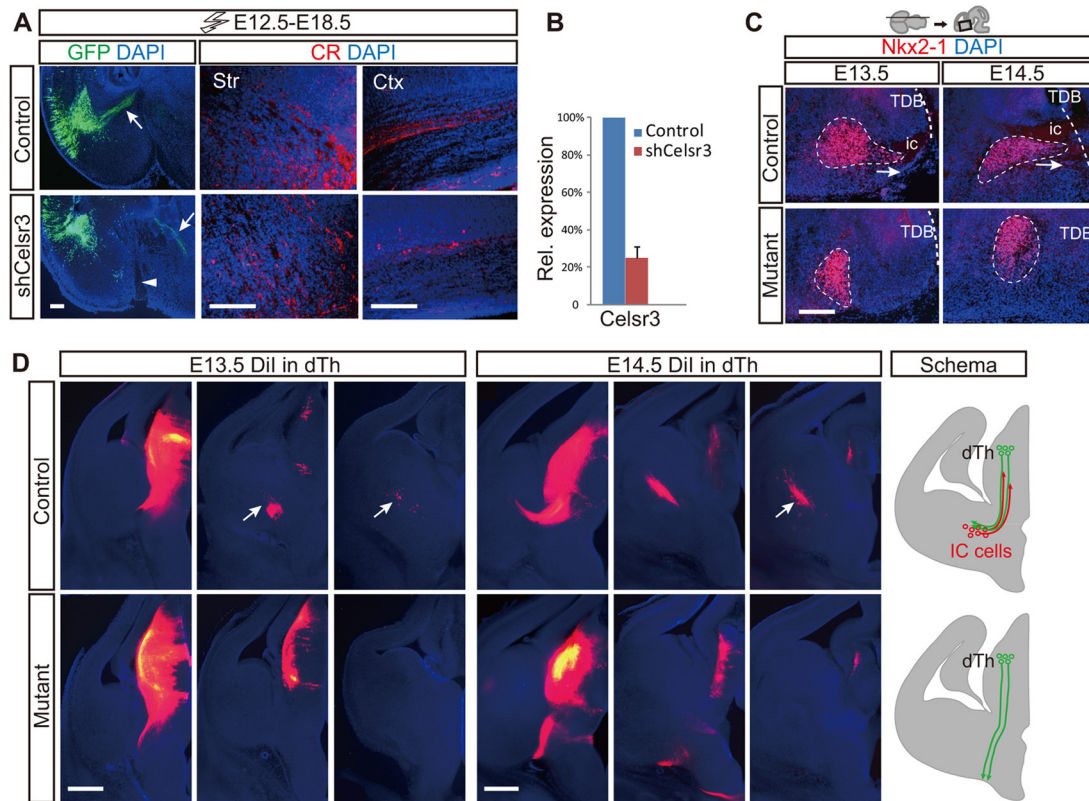


FIG 9 Absence of IC guidepost cells in the mutant ventral forebrain. (A) *Celsr3* knockdown in the basal forebrain by *in utero* electroporation. CR staining showed the decrease of CR⁺ TCA projections in the striatum and cortex. (B) Knockdown efficiency of sh*Celsr3*. (C) Nkx2-1 and DAPI staining of the E13.5 and E14.5 basal forebrain. (D) Retrograde tracing of IC cells (arrows) by inserting Dil in the E13.5 and E14.5 dorsal thalamus and sectioning rostrally, with a schema. TDB, telencephalon-diencephalon boundary; ic, internal capsule. Scale bars, 200 μ m (A and C) and 400 μ m (D). Data are means \pm SD ($n = 3$).

cells are mislocalized in the ventral GP of the *Celsr3* mutant forebrain. There are four groups of cholinergic neurons in the ventral telencephalon that originate from both subpallium and pallium (22, 58, 59). Ch1 neurons in the medial septum and Ch2 neurons in the vertical limb of the diagonal band project to the hippocampus. Ch3 neurons in the horizontal limb of the diagonal band project to the olfactory bulb. Ch4 neurons from the basal magnocellular complex project to the neocortex (40, 60). The basal magnocellular complex includes NB and MCPO. Abnormality of Ch4 neurons causes impairment in attention and memory, which are implicated in cognitive defects in Parkinson's and Alzheimer's diseases (61).

Without *Celsr3*, ectopic cholinergic cells are aberrantly localized in the ventral GP. These ectopic Ch4 neurons appear to be specifically from NB but not from MCPO. First, although Ch4 neurons in both NB and MCPO express *Celsr3* (Fig. 8A and B), the Ch4 neurons in MCPO are not altered by the loss of *Celsr3* (Fig. 8C). Second, in contrast to NB cells, MCPO cells do not coexpress CB and ChAT (Fig. 8C) (43). Finally, while about 80% of cholinergic neurons in NB express *Islet1* (Fig. 7D and E) (41), only about 30% of ChAT⁺ cells express *Islet1* in the MCPO (Fig. 7E) (41). Thus, these CB⁺ ChAT⁺ *Islet1*⁺ neurons in the mutant ventral GP are ectopic from NB but not MCPO.

The ventral telencephalon is central for axonal projections of TCA and corticothalamic axon (CTA) (62). For example, the GP in the mantle zone of the MGE constitutes a critical decision region for the early projection of TCA (25, 29, 62). The

Nkx2-1 gene is a specific marker for GP, and the Nkx2-1⁺ cell domain in the mantle region of the MGE is equivalent to that of the GP (22, 27, 28).

The Nkx2-1⁺ GP region transiently extends a caudal cell domain associated with the internal capsule (IC) at the early embryonic stage of E13.5 to E14.5 (Fig. 9C) (27). This Nkx2-1⁺ cell domain, referred to as VTel1 (27), is required for TCA to cross the TDB and reach the internal capsule of the ventral telencephalon (27–29, 46, 63). The TCA then traverses the internal capsule corridor in close proximity to GP en route to the cortex (42, 64). Their projections are thought to be guided by the so-called guidepost cells, aka Nkx2-1⁺ IC cells, to pass through TDB and the *Islet1*⁺ corridor (27–29, 42, 46). The phenotype of absence of caudal extension of Nkx2-1⁺ IC cell domain in the *Pcdh Celsr3* mutant is very similar to that of the *Pcdh10* mutant (28). Due to this defect, TCA cannot cross the crucial TDB (28; also this study). Thus, our data demonstrate a critical role of *Celsr3* in patterning axonal guidance cues and provide a mechanistic insight into the function of *Celsr3* in TCA pathfinding.

Both clustered *Pcdh* genes and nonclustered *Pcdh* genes play roles in diverse cellular functions in neurodevelopment, such as cell adhesion (65), neuronal migration (11, 13), dendrite development (9, 66, 67), spine morphogenesis (66), axon outgrowth (8, 10, 68, 69), and synaptogenesis (70). The molecular mechanisms underlying these diverse cellular processes are not known; however, they may regulate actin cytoskeletal dynamics through molecular switches of small GTPase proteins (66). In particular,

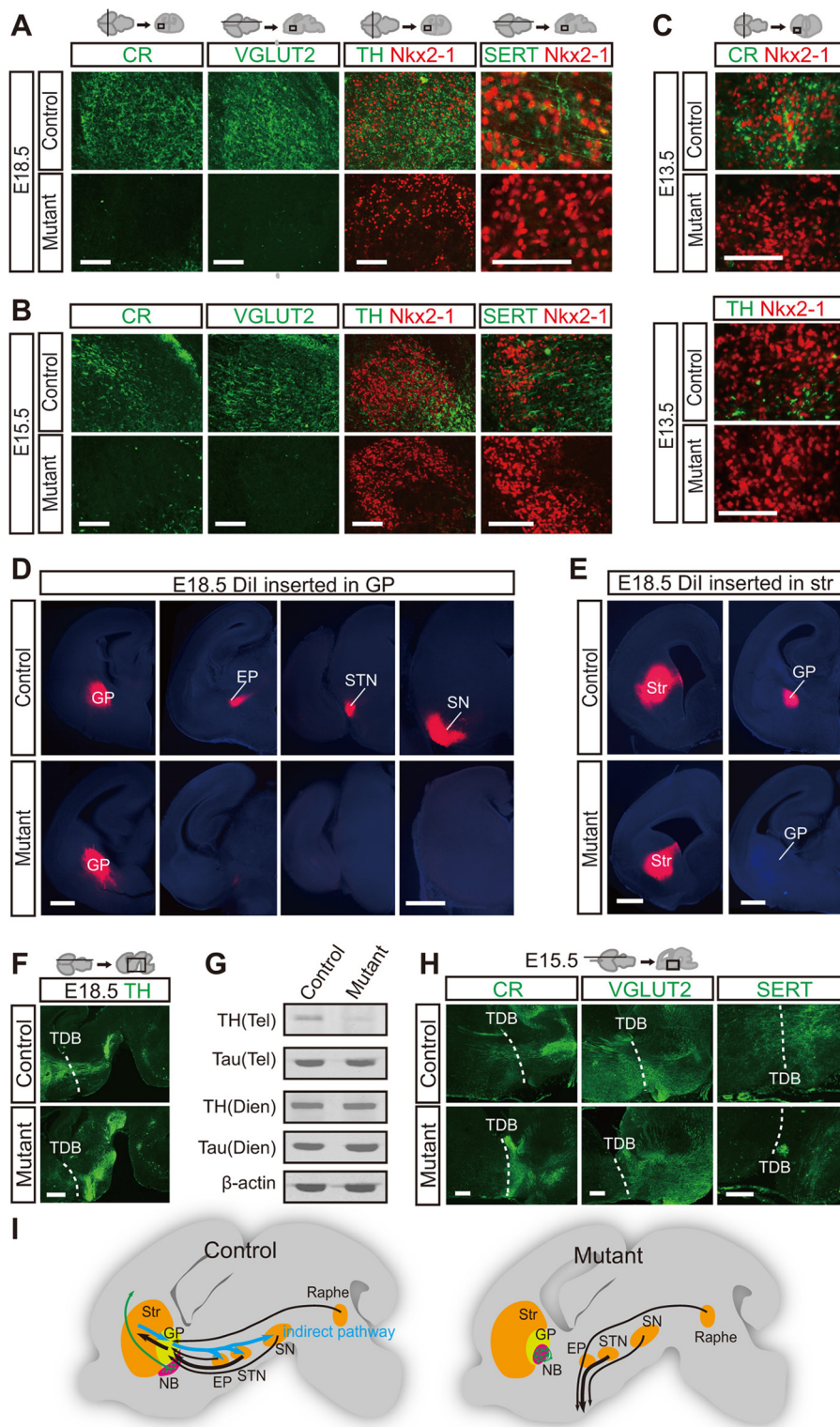


FIG 10 Absence of GP axonal connections in the *Celsr3* mutant. (A and B) CR, VGLUT2, TH, and SERT staining of the mutant and control E18.5 (A) and E15.5 (B) GP. (C) CR and TH staining of the E13.5 GP. (D) Axonal tracing with DiI inserted in the E18.5 GP and observed at caudal levels ($n = 3$ mice). (E) Axonal tracing at E18.5 by inserting DiI in the rostral striatum and sectioning to GP levels ($n = 3$ mice). (F) TH staining of forebrain sagittal sections. (G) Western blot of TH and Tau in the telencephalon and diencephalon with β -actin as a control. (H) CR, VGLUT2, and SERT staining of sagittal sections. (I) Schema depicting the GP axonal connectivity defects. EP, entopeduncular nucleus; STN, subthalamic nucleus; SN, substantia nigra; Dien, diencephalon; Tel, telencephalon; TDB, telencephalon-diencephalon boundary. Scale bars, 100 μ m (A to C), 400 μ m (D to F), and 200 μ m (H).

recent studies identified ~120 diverse membrane-associated proteins, including numerous protocadherins with a conserved WAVE regulatory complex (WRC)-interacting receptor sequence (WIRS) motif that may regulate the actin dynamics during cell migration through the pentamer WRC (69, 71). Interestingly, we found that the *Celsr3* cytoplasmic domain contains a perfect WIRS motif (LASFNS) that may stimulate actin dynamics during cell migration. This is consistent with our previous finding of interneuron migration defects in the *Celsr3* deletion mice (11). Similar defects may occur for the migration of NB cells; in view of the increased apoptosis in GP, we favor the idea that GP structure is altered due to cell loss and the NB simply fills the space left by the dead cells. Strikingly, *Pcdh10* also contains WIRS motifs that stimulate activity of the WRC complex (71). Given the high similarity of the IC cell domain defects in the caudal GP, it is tempting to speculate that both *Celsr3* and *Pcdh10* regulate actin dynamics in the caudal GP at the ventral forebrain through the common downstream WRC complex.

In summary, we found that *Celsr3* is essential for axonal connections between GP and other brain nuclei. Dopaminergic, serotonergic, CR⁺, and glutamatergic axons all have pathfinding defects through the TDB to the GP in the *Celsr3* mutant mice. The defects of GP connectivity cause diverse human diseases (19). *Celsr3* mutant mice may be a useful model for investigating normal GP development and its malfunctions in human neurological diseases.

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