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CCN3 overexpression inhibits growth of callosal projections via upregulation of RAB25

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ABSTRACT

The cysteine-rich 61/connective tissue growth factor 3 (CCN3) is a member of the CCN family of secreted multifunctional proteins involved in a variety of cellular processes including migration, adhesion, and differentiation. Previous studies have shown that CCN3 is expressed in the developing rat central nervous system, and enhanced CCN3 expression is highly correlated with tumorigenesis. However, the expression pattern and influence of abnormal CCN3 expression during mouse cortical development remains to be elucidated. Here, we show that CCN3 expression in mice is first detectable at embryonic day 15 and increases until postnatal day 21. We overexpressed CCN3 in mouse cortical neurons using uni- and bilateral electroporation. Our in vivo overexpression experiments showed that elevated CCN3 expression inhibited the axonal outgrowth of callosal projection neurons. Moreover, we identified the small GTPase RAB25 as a downstream effector molecule of CCN3 using transcriptomic analysis with CCN3 overexpressed in cortical tissue. In vivo ectopic expression of RAB25 or the dominant-negative RAB25-T26N also revealed that the GTPase activity of RAB25 is involved in the CCN3-mediated regulation of neuronal outgrowth. Taken together, our results suggest that tight regulation of CCN3 expression is necessary for normal cortical neuronal connectivity during development, and RAB25 negatively regulates neuronal differentiation as a downstream effector of CCN3.

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1. Introduction

The corpus callosum is a well-characterized commissural projection in the mammalian brain that connects the 2 cerebral hemispheres, mainly incorporating projections originating from layer II–IV pyramidal neurons [1,2]. Unusual corpus callosum development has been linked to neurodevelopmental and psychiatric disorders such as autism spectrum disorder, Opitz syndrome, and dyslexia [3–6]. The structure is formed based on intrinsic genetic factors and cellular responses to various extrinsic cues during cortical development. To date, several important factors for corpus callosum development have been identified including the special AT-rich sequence-binding protein 2 transcription factor (SATB2), which is thought to be crucial for callosal projection neuron specification [7], the cytoskeletal remodeling factor Midline-1 [6], and multiple Eph receptors (B1-3 and A4) and ephrins (B1-3), which act as axon guidance molecules [8].

However, despite the numerous advances in the field, it remains unclear how callosal projection neurons are properly positioned and extended into the contralateral hemisphere. One interesting protein implicated in this neurodevelopmental process is the cysteine-rich 61/connective tissue growth factor 3 (CCN3, also known as nephroblastoma overexpressed), which is involved in several fundamental biological processes [9,10]. Although a few studies have reported regulatory roles for CCN3 in neuronal development [11–13], none has specifically investigated its role in cortical pyramidal neurons.

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CCN3 is a member of the CCN family of proteins composed of 4 conserved domains (an insulin-like growth factor binding protein-like domain, a von Willebrand factor C repeat domain, a thrombospondin-1 repeat domain, and a C-terminal domain containing a cysteine knot) [14]. The Ccn3 gene was identified from a chick nephroblastoma, and high CCN3 protein expression was found to induce abnormal proliferation and differentiation in the avian kidney [15,16]. CCN3 protein levels are highly regulated by extracellular and environmental stimuli, including various growth factors such as platelet-derived growth factor, fibroblast growth factor 2, and transforming growth factor β1 [10]. However, the effects of dysregulation of intracellular levels of CCN3 on neuronal development in the central nervous system and on the downstream molecules influenced by CCN3 are poorly understood.

In this study, we demonstrate that CCN3 is expressed during cortical development and that its overexpression diminishes neurite growth in neuro2a cells. Our results also show that overexpression of CCN3 in the developing mouse cortex using a focal gene delivery method is sufficient to disturb normal callosal projection by the induction of RAB25 expression.

2. Materials and methods

2.1. Animals

Timed-pregnant ICR mice were used in this study; 12 pm on the day when a vaginal plug was found was designated as embryonic day 0.5 (E0.5), and the day of birth was defined as postnatal day 0 (P0). All experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Asan Medical center.

2.2. Plasmid constructs

To express Ccn3 and reporter genes, full-length complementary DNA sequences encoding mouse Ccn3 (BC003774) tagged with a V5 epitope, green fluorescent protein (Gfp), or red fluorescent protein (Rfp) were inserted into the pCAGEN (Addgene, Cambridge, MA, USA) mammalian expression vector containing a cytomegalovirus immediate early enhancer with a modified chick β-actin promoter, which enabled high and long-lasting neural tissue expression. For Rab25 and Rab25-T26N expression vector, full-length complementary DNA sequences encoding mouse Rab25 (BC006624) tagged with a V5 epitope was inserted into the pCAGEN vector and subjected to mutagenesis using overlapping PCR methods. To construct Rab25-T26N, a 92 bp fragment was amplified with primers 5′- ATGGGGAATCGAACAGATGA -3′ (Forward) and 5′- CGGGACAGCAGATTGTTCTTGCCCACGCCT -3′ (Reverse), and a 577 bp fragment was amplified with primers 5′- AGGCGTGGGCAAGAACAATCTGCTGTCCCG -3′ and 5′- GAGGCTGATGCAACAGGC -3′. The two partially complementary PCR fragments thus generated were annealed and used as the templates in another PCR with primers 5′- ATGGGGAATCGAACAGATGA -3′ and 5′- GAGGCTGATGCAACAGGC -3′. The resulting 642-bp product was digested with SpeI and subcloned into the pCAGEN vector including V5 epitope sequences.

2.3. Cell culture and western blotting

Neuro2a cells were obtained from American Type Culture Collection (Manassas, VA, USA). Neuro2a cells were cultivated in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum (Hyclone, Logan, UT, USA). They were differentiated by treatment with 5 μM retinoic acid (RA) (Sigma, St. Louis, MO, USA) for 48 h. Cells of about 90% confluence were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were lysed in radio-immunoprecipitation (RIPA) buffer (Thermo Scientific, Waltham, MA, USA) with protease inhibitor cocktail (Sigma). For differentiation, medium was replaced with DMEM supplemented with 1% fetal bovine serum. Total proteins was loaded onto 4–12% sodium dodecyl sulfate-polyacrylamide gels (Invitrogen) and transferred to polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). The filters were immunoblotted with anti-V5 (1:3000, Invitrogen) and anti-actin (I-19) antibodies (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.4. Uni- and bilateral in utero electroporation

In utero electroporation was performed as described previously [17,18] with a few modifications. Briefly, E14.5–15.5 timed-pregnant ICR mouse (Orientbio, Seongnam, South Korea) were anesthetized with ketamine/xylazine (80/10 mg/kg, Sigma), and the uterine horns were exposed by laparotomy. DNA (1–2 μg/μL in distilled water) was injected through the uterine wall into one of the lateral ventricles (cortex) using a mouth-controlled glass capillary micropipette under a fiber optic light source. After soaking the uterine horn with a prewarmed saline solution, the embryo's head was carefully held between the electrodes. Five electrical pulses (amplitude, 45 V; duration, 50 ms/pulse; interval, 950 ms) were delivered with an ECM 830 electro square porator (BTX, Harvard Apparatus, Holliston, MA, USA). For bilateral electroporation experiments, DNA filling in both ventricles was achieved with a single monolateral injection. For all experiments, the uterine horns were repositioned into the abdominal cavity after electroporation, and the abdominal wall was sutured. In some experiments, a reported plasmid encoding RFP (2 μg/μL) was also injected.

2.5. Fluorescence image acquisition

For neuronal morphology analysis, neuro2a cells were fixed with 4% paraformaldehyde (PFA) and washed with phosphate-buffered saline (PBS) three times for 10 min each at room temperature. The nuclei were stained with 4′, 6-diamidino-2-pheylindole dihydrochloride (DAPI, Sigma), and the cells were mounted on slides using VECTASHIELD mounting medium (Vector Labs, Burlingame, CA, USA). Fluorescence images were obtained using an ECLIPSE Ti microscope (Nikon, Tokyo, Japan). Neurites were defined as a process with lengths equivalent to three or more cell body diameters. Forty cells were randomly chosen for neurite-bearing cell quantification. Brains from postnatal stages 0 were dissected and fixed in 4% PFA overnight at 4 °C and sectioned using a vibratome (Leica, Wetzlar, Germany). The slices were directly mounted in VECTASHIELD mounting medium containing DAPI and analyzed fluorescence signals with confocal microscopy.

2.6. RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from mice at different developmental stages (E13.5, E15.5, E18.5, P0, P7, P14, and P21) using RNeasy Mini Kit (QIAGEN, Venlo, The Netherlands). cDNA synthesis was performed using HiSenScript RH RT PreMix Kit (Intronbio, Seongnam, South Korea) according to the manufacturer's instructions. For Ccn3 cDNA, a 100-bp stretch was amplified with 5′-CTGAGATGAGACCCTGTGAC-3′ and 5′- TTGTCTCCCTCTGGAACCAT -3′ primers. For Gapdh cDNA, a 153-bp stretch was amplified with 5′-ATCACTGCCACCCAGAAGAC-3′ and 5′-CATGCCAGTGAGCTTCCCGT-3′ primers. Polymerase chain reaction (PCR) was performed using HotStarTaq DNA polymerase (QIAGEN) with 25 cycles of amplification using a 1-min denaturation step at 95 °C, a 0.5-min

annealing step at 60 °C, and a 1.5-min polymerization step at 72 °C. The PCR products were separated in 2% agarose gels.

2.7. Microarray

Total RNA was isolated from freshly dissected cortical tissues which were cotransfected with Gfp and Ccn3 expression vectors by using the TRIzol (Invitrogen) reagent and RNeasy Plus Kit (QIAGEN, Venlo, The Netherlands). Dissection of cortical tissues performed under fluorescence microscopy at P0. The quantity and quality of total RNA was determined by the Bioanalyzer (Agilent Technologies, Santa Clara, CA). Whole mouse genome microarray was performed by the Affymetrix GeneChip Mouse Gene 430 (Santa Clara, CA). After scanned the arrays, analyzed using Affymetrix Expression console DAVID. Criteria used to select genes were significantly up-regulated or down-regulated (>1.5 fold) in CCN3 overexpressed as compared to empty vector electroporated cortices.

2.8. Statistical analysis

Quantitative data are presented as the mean ± standard deviation (SD) from representative experiments (n = 3). Statistical analyses were carried out using one-tailed Student's t-tests. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Expression pattern of Ccn3 during cortical development

Previous studies have revealed that CCN3 is highly and age-dependently expressed in various postnatal mammalian neural tissues including the rat cortex and cerebellum [12,19]. We established its temporal expression pattern in embryonic and postnatal mouse brain using reverse-transcriptase-PCR. Ccn3 mRNA was expressed at very low levels on embryonic day 15 (E15.5), when neurons destined for the upper cortical layer are generated (Fig. 1A and B). Consistent with previous reports, Ccn3 mRNA was strongly expressed in the cortex (particularly the upper layers) at similar levels from postnatal day 7 (P7) to P21 (Fig. 1A and B). These findings indicate that Ccn3 expression slightly overlaps with cortical neurogenesis (E12.5–18.5), although its expression was more abundant at postnatal stages.

3.2. CCN3 overexpression inhibits RA-induced neurite outgrowth of neuro2a cells

To investigate the role of CCN3 overexpression in neurite outgrowth in neuro2a cells, we upregulated CCN3 expression in neuro2a cells by cotransfection with a pCAG-GFP reporter vector. First, we performed western blotting to assess whether the

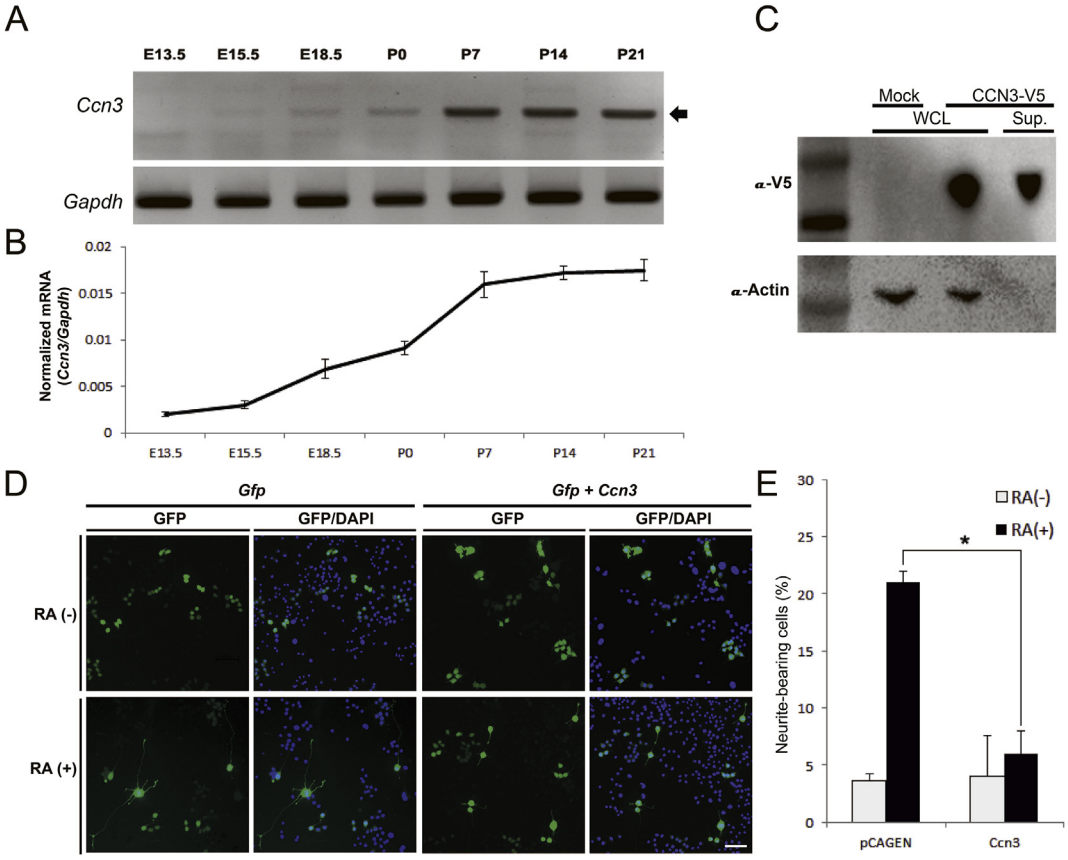

Fig. 1. CCN3 overexpression blocks retinoic acid (RA)-induced neurite outgrowth of neuro2a cells. (A) Temporal expression of Ccn3 mRNA during cortical development. (B) The relative mRNA expression level was normalized to that of Gapdh (n = 3, results are shown as mean ± SD). Ccn3 is upregulated from embryonic day 15 (E15.5), and a robust increase in expression is observed at postnatal day 0 (P0). (C) CCN3–V5 recombinant expression evaluated by western blot analyses with whole cell lysate (WCL) and supernatant (Sup.). Actin was used as a loading control. (D) Neuro2a cells were cotransfected with a Gfp reporter vector and either Ccn3 or empty vector as indicated. Neuro2a cells were treated with 5 μM RA for 48 h. Fluorescent images were analyzed with an inverted microscope (Scale bar = 100 μm). (E) Neurite-bearing cells were quantified from microscopic images. For each condition, 40 GFP-positive cells were counted. Cells with neurites longer than 3 cell body diameters were scored as morphologically differentiated (n = 3, results are shown as mean ± SD, $p < 0.05$ vs. control).

recombinant CCN3 tagged by V5 epitope was expressed and secreted (Fig. 1C). Neuro2a cells did not differentiate in the absence of retinoic acid (RA). However, after incubation in the presence of RA for 2 days, the cells began to grow neurites. Interestingly, CCN3-overexpressing RA-treated neuro2a cells had dramatically fewer neurites (Fig. 1D and E) relative to RA-treated controls. These results suggest that CCN3 overexpression inhibits RA-induced neurite outgrowth in neuro2a cells.

3.3. CCN3 overexpression inhibits midline crossing by callosal projection neurons

Next, to determine whether CCN3 overexpression affects callosal projections from upper layer pyramidal neurons in the cerebral cortex, we performed electroporation to overexpress certain genes in specific cortical neurons (Fig. 2A). In the control condition, GFP-expressing neurons successfully crossed the midline of the corpus callosum and then projected into the contralateral hemisphere (Fig. 2B, C and E). In contrast, electroporation with a CCN3-expressing construct dramatically decreased the number of GFP-positive axons in the midline compared with controls (Fig. 2B, D, and F). Interestingly, there was no significant difference in the extent of axonal fluorescence between control and CCN3-overexpressing neurons in the intermediate zone (IZ) of the ipsilateral cortex. Similarly, we did not find any ectopic axons extending into subcortical areas. These results suggest that CCN3 overexpression restricts the growth of callosal projections but does not result in ectopic subcortical projections.

To rule out the possibility of axonal expansion defects in CCN3-overexpressing neurons due to differences in electroporation timing or positioning [20], we also performed bilateral electroporation (Fig. 3A). The CCN3 overexpression construct with the GFP reporter was injected into 1 cortical hemisphere, and a control RFP construct was injected into the contralateral hemisphere. We then applied square electric pulses through the uterine wall to the embryos by physically holding them in parallel along the dorsoventral axis with the electrodes. As shown in Fig. 3A, both control RFP and GFP-containing Ccn3 expression vectors were almost symmetrically introduced into the cortex. An analysis of the callosal projections showed that CCN3-overexpressing GFP-positive axon fibers developed shorter branches and showed dramatically fewer midline crosses in the corpus callosum than RFP control axons. This finding is consistent with our data obtained from unilateral electroporation (Fig. 2) and neurite outgrowth assays of neuro2a cells (Fig. 1D and E). In contrast, no differences in the IZ were observed between the control (RFP-positive) and CCN3-overexpressing (GFP-positive) axon fibers, indicating that CCN3 overexpression did not affect initial axon outgrowth of cortical pyramidal neurons.

3.4. RAB25 overexpression recapitulates the effects of elevated CCN3 in vitro and in vivo

We next carried out microarray analysis to identify which molecules were regulated by overexpression of cortical CCN3. For this, we conducted electroporation experiments with Ccn3 expression vectors at E14.5, and then compared gene expression in cortical tissues microdissected at P0 from successfully

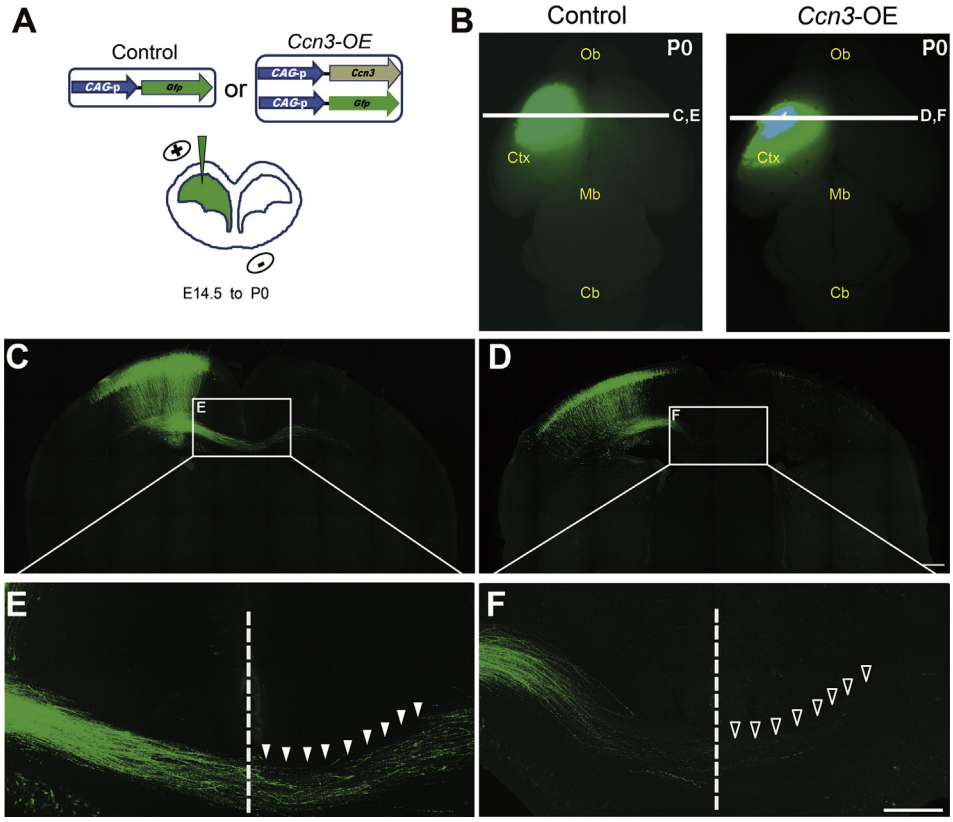

Fig. 2. CCN3 overexpression inhibits midline crossing in the corpus callosum. (A) Schematic representations of the expression vectors used for unilateral electroporation at embryonic day 14 (E14.5). (B) Representative fluorescent images of postnatal day 0 (P0) brains showing that expression vectors were electroporated into the desired cortical position. (C and E) In the control condition, GFP-positive axons projected to the contralateral hemisphere by crossing the corpus callosum midline (solid arrowheads). (D and F) In contrast, few CCN3-overexpressing neurons successfully crossed the midline (open arrowheads). Ob, olfactory bulb; Ctx, cortex; Mb, midbrain; Cb, cerebellum. (Scale bar = 500 μm).

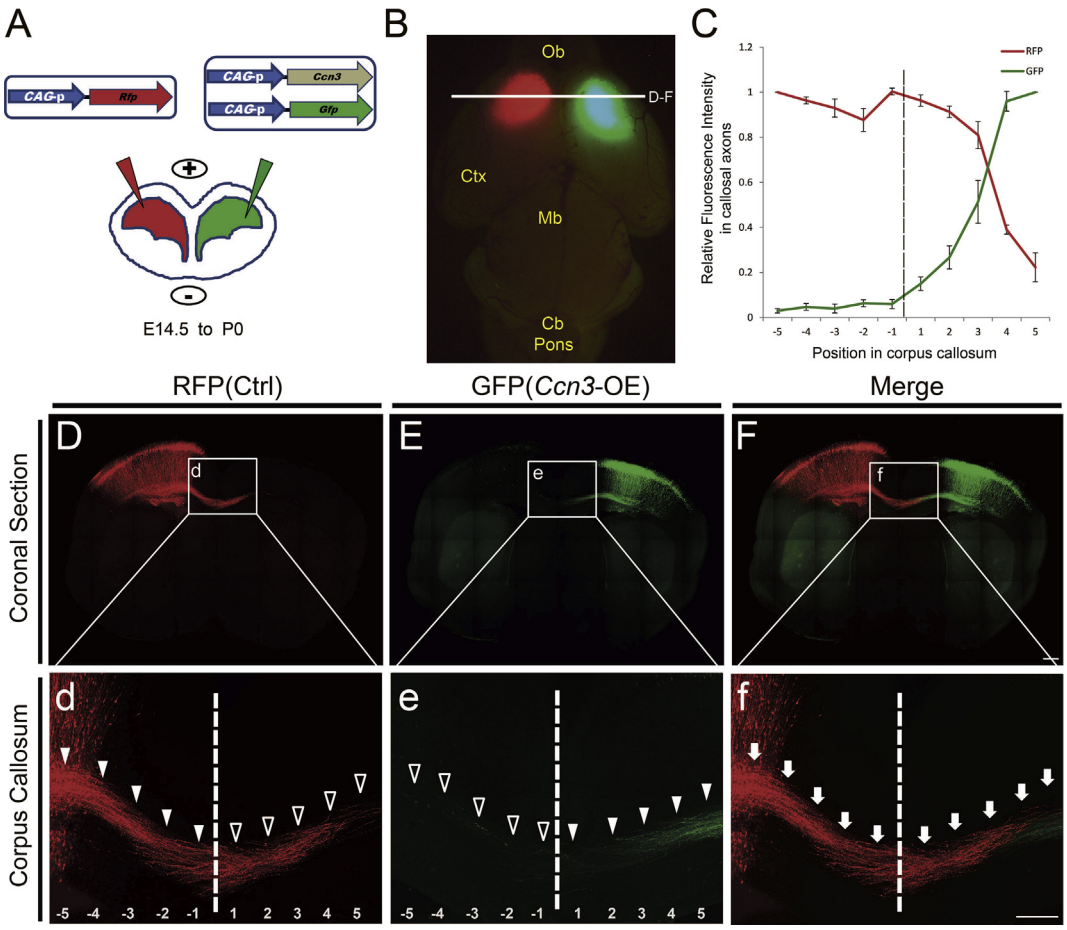

Fig. 3. Bilateral electroporation reliably overexpresses CCN3 in cortical neurons. (A) Schematic representations of the expression vectors used for bilateral electroporation at embryonic day 14 (E14.5). (B) Representative fluorescent images of P0 brains showing that both expression vectors were symmetrically electroporated into the desired cortical position. The coronal sectioning position is indicated with a white line. (C) Quantitative analysis of the fluorescence intensity index of callosal axons with adjacent sections (n = 3) using ImageJ software. The dashed line represents the corpus callosum midline (n = 3, results are shown as mean ± SD). (D) In the control hemisphere, projections from RFP-labeled upper layer pyramidal neurons extend to the contralateral hemisphere through the corpus callosum (D, d, F, f). Conversely, projections from GFP-positive neurons coexpressing CCN3 were dramatically decreased in the midline (dashed line) of the corpus callosum, and only a few fibers were detected on the contralateral side (E, e, F, f). Solid and open arrowheads indicate callosal axons of the ipsilateral and contralateral sides, respectively. The arrows indicate the paths of callosal axons (f). Scale bar = 500 μm.

electroporated mice. After CCN3 overexpression, 104 genes were upregulated and 35 genes were downregulated (fold change cut-off value 1.5, $p < 0.05$). Supplementary Table 1 gives the gene expression profiles for a selection of genes. One of the candidate genes is Rab25, which encodes for a member of the Ras superfamily of small GTPases [21,22]. Recent studies have demonstrated that some RAB proteins are localized in axons and involved in neurite outgrowth [21,23]. Therefore, we hypothesized that RAB25 may be a downstream effector of CCN3 acting as a negative regulator in axonal outgrowth.

To address this possibility, we examined the effects of RAB25 overexpression in neuro2a cells and developing mouse brains using cotransfection and in utero electroporation, respectively. Consistent with CCN3 overexpression, RAB25-overexpressing RA-treated neuro2a cells had dramatically fewer neurites (Fig. 4A–C). These results suggest that RAB25 overexpression negatively regulates RA-induced neurite outgrowth in neuro2a cells. Moreover, after RAB25 overexpression dramatically fewer midline crosses in the corpus callosum were observed (Fig. 4D). By contrast, electroporation-induced expression of the dominant-negative mutant RAB25-T26N, which is defective in GTP binding, failed to induce any defects in midline crossing by the callosal projections of cortical neurons (Fig. 4D). These results suggest that overexpressed CCN3 increases the expression of RAB25, resulting in the reduction of callosal projections from the mouse cortex.

4. Discussion

Understanding the precise regulation of neuronal differentiation processes is important for understanding normal brain function. Although various signaling and genetic factors have been proposed as mechanisms for regulating neuronal differentiation, they are not sufficient to explain the structural and functional complexity of the central nervous system. In recent years, deep-sequencing technologies have identified numerous novel factors that might be involved in cortical development, including CCN3 [24,25]. Transcriptomic analyses of developing human and mouse brains demonstrate that CCN3 is involved in neuronal differentiation and migration [24].

Although CCN3 is a secreted protein, several studies have also raised the possibility that it may have intracellular functions [26–28]. For instance, CCN3 can interact with various nuclear proteins to regulate transcriptional events underlying inflammatory processes [26]. Moreover, CCN3 overexpression significantly decreases matrix metalloproteinase (Mmp)-2 and Mmp-9 mRNA expression in atherosclerosis [29]. It is therefore possible that there

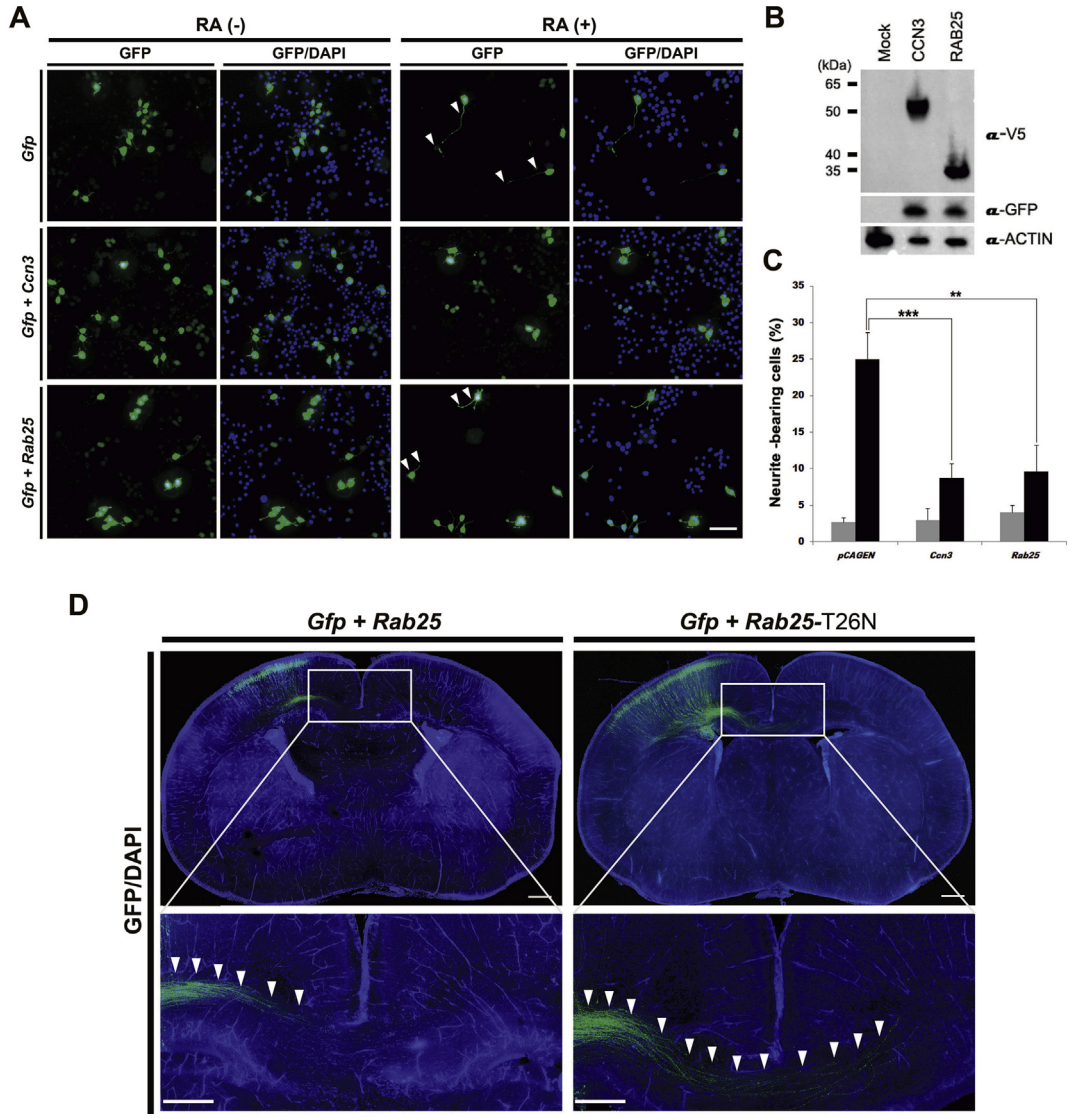

Fig. 4. RAB25 overexpression inhibits neurite outgrowth in vitro and in vivo. (A) Neuro2a cells were cotransfected with a Gfp reporter vector and either Ccn3 or Rab25 as indicated. (B) CCN3-, RAB25-V5 recombinant expression evaluated by western blot analyses with whole cell lysate. (C) Neurite-bearing cells were quantified from microscopic images. For each condition, 40 GFP-positive cells were counted. Cells with neurites longer than 3 cell body diameters were scored as morphologically differentiated ($n = 3$, results are shown as mean ± SD, **, $p < 0.05$; ***, $p < 0.005$ vs. control [pCAGEN]). (D) After RAB25 overexpression, GFP-positive axons were not found projecting to the contralateral hemisphere by crossing the corpus callosum midline (solid arrowheads). In contrast, GFP-positive axons that expressed the dominant-negative RAB25-T26N successively projected to the contralateral hemisphere (solid arrowheads). Slices were counterstained with DAPI. (Scale bar = 500 μm).

is an intracellular function of dysregulated CCN3 expression in the central nervous system.

The present results show that CCN3 was spatiotemporally expressed in a tightly regulated fashion during cortical development (Fig. 1A and B). Both in vitro and in vivo, CCN3 overexpression was sufficient to inhibit neurite outgrowth (Figs. 1D and 2). Bilateral electroporation with 2 different DNAs clearly showed the neurological effect of CCN3 overexpression (Fig. 3). Moreover, we found that overexpression of CCN3 induces upregulation of Rab25 gene expression. Further, overexpression of RAB25 protein negatively regulates neurite outgrowth in vitro and in vivo, suggesting it serves as an effector protein downstream of CCN3 (Fig. 4). Previous research using several extracellular stimuli has implicated a role of RAB proteins in neuronal differentiation and other functions, such as mediating downstream effects of the binding of neurotrophins to receptors [21]. Interestingly, elevation of intracellular CCN3 levels has also been implicated in regulation of the actin cytoskeleton by increasing the activity of the small GTPase RAC1 in breast cancer cells [25]. Here, for the first time, we suggest that CCN proteins can also regulate the intracellular activity of RAB25 by controlling the expression.

In conclusion, our findings demonstrate that tight regulation of CCN3 expression is critical for normal neuronal differentiation and cortical development. Additionally, maintaining normal intracellular levels of RAB25 is also necessary for normal formation of neuronal connectivity.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.016.

Transparency document

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