Netrin-1/DCC-mediated PLCγ1 activation is required for axon guidance and brain structure development

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Abstract

Coordinated expression of guidance molecules and their signal transduction are critical for correct brain wiring. Previous studies have shown that phospholipase C gamma 1 (PLCγ1), a signal transducer of receptor tyrosine kinases, plays a specific role in the regulation of neuronal cell morphology and motility in vitro. However, several questions remain regarding the extracellular stimulus that triggers PLCγ1 signaling and the exact role PLCγ1 plays in nervous system development. Here, we demonstrate that PLCγ1 mediates axonal guidance through a netrin-1/DCC complex. Netrin-1/DCC activates PLCγ1 through Src kinase to induce actin cytoskeleton rearrangement. Neuronal progenitor-specific knockouting of Plcg1 in mice causes axon guidance defects in the dorsal part of the mesencephalon during embryogenesis. Adult Plcg1-deficient mice exhibit structural alterations in the corpus callosum, substantia innominata, and olfactory tubercle. These results suggest that PLCγ1 plays an important role in the correct development of white matter structure by mediating netrin-1/DCC signaling.

Keywords: axon guidance; DCC; mesencephalon; neural development; PLCγ1

Subject Categories: Cell Adhesion, Polarity & Cytoskeleton; Neuroscience

Introduction

During development, several axon guidance molecules act as key regulators of neuronal wiring by inducing cytoskeleton rearrangement [1]. These guidance cues are perceived by specific receptors that are associated with diverse types of signal transducers that generate secondary messengers, thereby inducing axons to grow toward their proper destinations [2]. Netrin-1, a ligand for the deleted in colorectal cancer (DCC) receptor, functions as a guidance cue for migrating neuronal progenitors and axons in nervous system development by recruiting intracellular signaling complexes. To the best of our knowledge, DCC has not been proposed to function as a receptor tyrosine kinase (RTK), because DCC does not contain an intracellular catalytic domain, but contains three highly conserved protein-binding domains termed P1, P2, and P3 [3,4]. These domains mediate the assembly of various combinations of multiple signaling components such as the non-catalytic region of tyrosine kinase adaptor protein 1 (NCK1), and Src family kinases [5–8], which are necessary for the integration of axon guidance cues. In particular, the dimerized P3 domain is important for recruiting focal adhesion kinase (FAK) and Src to the DCC complex [6,9]. These signaling components may contribute to cell motility by regulating the dynamics of the actin cytoskeleton [10,11]. Despite advances in the study of netrin-1/DCC signaling, little is known about how the intracellular DCC signaling complex is organized or how the cells translate the complicated instructions transmitted by this complex into actions. Recently, several in vitro studies have suggested the possibility that the netrin-1/DCC, a guidance cue, may be linked to PLCγ1 signaling. Xie et al [12] have reported that netrin-1 can hydrolyze PI(2) in a DCC-dependent manner. This study showed that PLCγ1 may be a potential messenger of netrin-1/DCC signaling; however, there is no direct evidence of a relationship between the DCC receptor and PLCγ1 because receptor DCC does not contain an intracellular catalytic domain. Thus, it is unclear whether PLCγ1 may be a downstream effector of netrin-1/DCC signaling, and if it is, how the netrin-1/DCC complex may regulate PLCγ1 activity.

PLCγ1 functions as a signal transducer that converts an extracellular stimulus into intracellular signals by generating secondary messengers, such as diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3) [13]. These messengers enable cells to respond to growth factors in a polarized manner, resulting in activities such as chemotactic migration [14]. Importantly, the transient calcium level...
embryonically lethal vasculogenesis defect in mice. Limited because homozygous deletion of the nervous system development by regulating neuronal cell morphol-

gogy and motility. Therefore, the regulation of PLC allows for the spatiotemporal regulation of PLC in vivo studies on the developmental role of PLC in many neurological disorders [21]. In this regard, RTK-mediated PLC1 signaling is thought to play a pivotal role in the central nervous system development by regulating neuronal cell morphology and motility. Therefore, the regulation of PLC1 activity by a specific ligand–receptor complex is an attractive model because it allows for the spatiotemporal regulation of PLC1 activity. However, in vivo studies on the developmental role of PLC1 have been limited because homozygous deletion of the Plcg1 gene causes an embryonically lethal vasculogenesis defect in mice.

In this study, to determine the function of PLC1 in the developing brain, we used Nestin-Cre (Nes-Cre) transgenic mice to ablate Plcg1 in developing neuronal precursors. We found that netrin-1/DCC signaling, a mediator of chemoattractant guidance cues, activates the lipase activity of PLC1 as a specific ligand–receptor complex is an attractive model because it allows for the spatiotemporal regulation of PLC1 activity. However, in vivo studies on the developmental role of PLC1 have been limited because homozygous deletion of the Plcg1 gene causes an embryonically lethal vasculogenesis defect in mice.

Our results indicate that PLC1 is a crucial molecule mediating the directional movement of axons that are regulated by netrin-1/DCC signaling during brain development.

Results and Discussion

PLC1-deficient mouse embryos show a deficit in mesencephalic axon guidance

Plcg1-deficient mice were generated by crossing a B6.Cg-Tg (Nes-Cre) 1 Kln/J mouse with a Plcg1-floxed mouse (Fig EV1). Unexpectedly, the brains of the Plcg1f/f;Nes-Cre embryos showed a severe axon guidance defect in the dorsal region of the mesencephalon, suggesting that PLC1 may be involved in axon guidance in midbrain dopaminergic (mDA) neurons. This deficit persisted into adulthood, with structural alterations observed in the olfactory tubercle (OT) and substantia innominata (SI). Moreover, Plcg1-deficient mice exhibited diffused axon fibers in the corpus callosum (CC), suggesting that PLC1 plays a role in axon extension and guidance by mediating netrin-1/DCC signaling and that disruption of PLC1 signaling adversely affects nervous system development.

Our results indicate that PLC1 is a crucial molecule mediating the directional movement of axons that are regulated by netrin-1/DCC signaling during brain development.

Netrin-1/DCC regulates mesencephalic axon guidance via PLC1

To establish the relationship between DCC and PLC1, we tested whether netrin-1/DCC could affect the phosphorylation of PLC1 on Y783 in primary mesencephalic neurons. We found that the level of PLC1 phosphorylation (pY783) increased 30 min after treatment with recombinant netrin-1 protein (NTN1; Fig 2A). Furthermore, when DCC was knocked down with siRNA, we found that the Plcg1 pY783 level was decreased to the basal levels and this decrease was not attenuated when DCC-depleted mesencephalic neurons were treated with netrin-1 (50 ng/ml; Fig 2B). To test whether PLC1 is involved in the netrin-1-mediated chemotraction of axons, the ventral mesencephalon (VM, A9) was micro-dissected from Plcg1f/f (Control) and Plcg1f/f;Nes-Cre mouse brains and co-cultured with 293T cells that had been transiently transfected with a netrin-1-expressing vector (Fig 2D). We confirmed that netrin-1 was detected in both the cytoplasm and culture media soup of the 293T cells (Fig 2C). Axons from the control mouse showed directionality toward the netrin-1-releasing 293T cells. By contrast, axons from the Plcg1f/f;
Nes-Cre mouse did not show straight directionality to the netrin-1-releasing 293T cells (Fig 2D), but displayed a more dispersed tendency (Fig 2E and F) \((N=4; n=200)\). Most axons in the mutant VM were TH-positive and extended for shorter distances than those in the control (Fig 2G). In addition, netrin-1-induced neurite outgrowth did not occur properly in Plcg1-deficient mDA neurons (Fig 2H). In culture, the mean length of the extending neurites from Plcg1f/f;Nes-Cre embryo was relatively short as compared to the control (Fig 2I–K). These results indicate that PLC\(_c\)1-deficient neurons have a significant defect in mDA axonal attraction by netrin-1.

Netrin-1/DCC signaling induces PLC\(_c\)1 Y783 phosphorylation by Src kinase

To understand the molecular mechanism underlying the netrin-1/DCC catalytic activation of PLC\(_c\)1, we performed a co-immunoprecipitation assay. These results showed that PLC\(_c\)1 did not interact with the DCC receptor (Fig 3A). To identify the site-specific kinases responsible for the phosphorylation of Y783 on PLC\(_c\)1, we employed the LC-MS of anti-PLC\(_c\)1 Immunoprecipitates obtained from primary mesencephalic neurons treated with netrin-1. This assay initially identified 960 proteins. Among them, we found a substantial number of proteins sharing a common profile with the negative controls (untreated and nonspecific-binding samples). We excluded these proteins from the initial profile and selected only the tyrosine kinases to identify candidates that could potentially interact with PLC\(_c\)1. Two kinds of tyrosine kinase were identified: RET-proto-oncogene (NP_033076.2) and the neuronal proto-oncogene protein tyrosine kinase, Src (NP_033297.2). The Src kinase has been identified as a component of DCC [27]. To verify a direct interaction between PLC\(_c\)1 and Src, we performed co-immunoprecipitation assays for these two proteins in primary cortical neurons. Rac1, a
well-known interacting protein of PLCγ1 [11,28], was used as a positive control. Immunoprecipitation with a PLCγ1 antibody pulled down Src and Rac1 (Fig 3B). To test whether Src directly phosphorylated Y783, we performed an in vitro kinase assay with purified PLCγ1 and mouse Src protein. The product was resolved by SDS–PAGE and immunoblotted with an anti-PLCγ1 (pY783) antibody. The Src kinase directly phosphorylated the Y783 site of PLCγ1 (Fig 3C and D). To further determine which tyrosine residue of PLCγ1 was phosphorylated by Src, we tested the phosphorylation level of each residue in the presence of a Src-specific inhibitor (Src I). We found that only Y783, but not the other three tyrosine residues, was phosphorylated in response to netrin-1 treatment (Fig 3E). This result suggests...
specific regulation of Src at Y783. Taken together, these results show that netrin-1 induces phosphorylation of PLCγ1 at Y783 by Src kinase. Netrin-1/DCC regulates cellular motility by activating a variety of signaling molecules, including the small GTP-binding protein, Rac1, extracellular signal-regulated kinase (ERK), and Ca²⁺/calmodulin-dependent kinase IIα (CaMKII)α [7]. Therefore, we tested whether ablation of Plcg1 affects the activity of these netrin-1-induced signaling pathways. Using a Rac1 activity assay, we found that netrin-1 could activate Rac1 in control cells; however, Rac1 activity was significantly impaired in the PLCγ1-null neurons (Fig 3F and G). In addition, the netrin-1-induced phosphorylation of CaMKII and ERK was significantly reduced in PLCγ1-null neurons, whereas the phosphorylation of FAK and Src remained unaltered (Fig 3H). These results suggest that the recruitment of Src and FAK to DCC is a prerequisite for the PLCγ1 activation and it mediates the netrin-1/DCC-induced regulation of cell motility.

**PLCγ1-deficient mouse brain exhibits a CC size reduction and misrouted axon bundles of the OT**

During neural development, mDA neurons extend their axons toward the anteromedial and ventral parts of the striatum, and then innervate the limbic system and neocortex, where these neurons constitute the mesocorticobasal pathways. Based on our findings of an axonal guidance defect in Plcg1f/f;Nes-Cre embryos, we further characterized the structural changes in white matter tracts in the PLCγ1-deficient adult brain. The most discernible phenotype was manifested in abnormally dispersed neural projections into the OT and SI (Fig 4B and D). These two structures are formed through embryonic development. The OT and SI are noted for the being innervated by mDA neurons from the VTA (Fig 4A). At E9.5, subplate neurons initially extend pioneer axons through the internal capsule that provide paths for follower axons that begin to appear [29]. At E11.5, VM neurons begin to extend their axons along the pioneer axon pathways to reach their telencephalic targets [30,31]. At E13.5, the axons pass longitudinally through the midbrain and diencephalon to form the medial forebrain bundle [30,31]. From E14.5 to E18.5, axon bundles reach the telencephalon and striatum, followed by innervation of the limbic system and neocortex [30–33].

As we observed, partial tract fibers appeared non-directional in the SI (white arrows), and PLCγ1-depleted axons did not uniformly project toward the anterior portion of the cortex in the mutant brain (Fig 4B). In addition, the PLCγ1-deficient tract fibers exhibited higher variance than that of the controls (n = 3 per genotype: one-way ANOVA; F-value 118.57; ***p < 0.001; Fig 4C). To determine whether Plcg1 inactivation in dopaminergic neurons actually leads to the mDA projection defect, we generated dopaminergic neuron-specific Plcg1 knockout mice by crossing the Plcg1flox/flox;Ddclox/lox mouse with a Scl6a3 (DAT)-Cre transgenic line. In these mice, Plcg1 was specifically deleted in the TH-positive neurons (Fig 4F). Similar to the Plcg1flox/flox;Nes-Cre mouse brain, mDA neurons exhibited non-directional axon projections in the SI of Plcg1flox/flox;DAT-Cre mouse brains (yellow arrows; Fig 4D). To quantify the deviated axons, we divided the region of interest (ROI) into dorsal and ventral SI parts (> 0 to ≤ 1,350 and > 1,350 to ≤ 2,700) and measured fluorescence intensity in the ROI (red box). The axon fibers from Plcg1flox/flox;DAT-Cre mice were more widely distributed in the ventral part of the SI than in the control (n = 3 per genotype; one-way ANOVA; F-value 272.82, ***p < 0.001; Fig 4E). These observations suggest that the lack of response to netrin-1 signaling due to PLCγ1 deficiency causes a structural change in the mDA system of the mouse brain.

In addition, we further characterized the structural changes in the CC in the PLCγ1-deficient adult brain (Fig 4G and H). Previous studies have shown that mice lacking DCC exhibits severe neurodevelopmental defects in multiple central nervous system (CNS) commissures. In particular, netrin-1/DCC signaling exerts a significant influence on the development of the CC [1]. Based on our previous findings, we hypothesized that a deficiency in the netrin-1 response due to lack of PLCγ1 may have an adverse effect on CC development. To test this hypothesis, we analyzed the structural changes in the CC in the PLCγ1-deficient adult brain. We selected coronal sections from Plcg1flox/flox and Plcg1flox/flox;Nes-Cre brains at the same rostrocaudal point to compare CC morphology between the genotypes. The CC in the Plcg1flox/flox;Nes-Cre mouse had an oval shape owing to differences in CC size (Fig 4G). We acquired whole images of sagittal sections and delineated the CC to measure its area. The area of the CC in the Plcg1flox/flox;Nes-Cre mice was significantly smaller compared to that in the Plcg1flox/flox mice (control: 1.18 ± 0.07; Plcg1flox/flox;Nes-Cre mice: 0.71 ± 0.07). The proportion of the CC area in the Plcg1flox/flox;Nes-Cre mice compared to the wild type was significantly reduced (p < 0.001; Student’s t-test). These observations further suggest that PLCγ1 specifically mediates netrin-1/DCC signaling, which is involved in a variety of cellular and developmental processes.
This result suggests that the decreased CC track density might be attributable to the escape of the axon fibers from their proper tracks. Because the CC consists mainly of axon fibers rather than cell bodies, a decrease in its volume suggests a decrease in axon fibers.

**Diffuse axon fibers in the CC of Plcg1<sup>ff</sup>;Nes-Cre mice**

Based on the previous observations, we hypothesized that the axon fibers deviating from their proper tracks in Plcg1<sup>ff</sup>;Nes-Cre brains might result in a decreased CC track density. To test this hypothesis, we used fiber tractography, which can reflect the direction and connectivity of neuronal fibers. We estimated the diffusion tensors within the CC on MR images using diffusion tensor imaging (DTI). We compared control and Plcg1<sup>ff</sup>;Nes-Cre mice by extracting quantitative parameters from tensor calculations for the CC area in each case (Fig 5A, C, and E). First, the volume (µl) and fractional anisotropic (FA) values were extracted. An FA value of zero indicated that diffusion was isotropic in all directions, whereas a value of 1 indicated diffusion occurring in one dominant direction. To represent

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**Figure 3.** Netrin-1/DCC induces specific phosphorylation of phospholipase C gamma1 (PLCγ1) on Y783 by neuronal proto-oncogene tyrosine kinase Src.

A. Co-immunoprecipitation data showed that PLCγ1 did not interact with the DCC receptor (n = 2, N = 3).
B. Immunoprecipitation assay showed that Src kinase interacted with PLCγ1. Rac1 is a positive interacting control for PLCγ1 (N = 3).
C. In vitro kinase assay confirmed that Src kinase directly phosphorylated Y783 of PLCγ1.
D. Quantification of the band intensities in (C) (N = 3). Data are presented as the mean ± s.e.m.
E. Src kinase inhibitor reduced the specific phosphorylation of PLCγ1 on Y783 (arrow; Src I IC<sub>50</sub> conc = 44 nM; N = 3).
F. Rac1 activity assay (N = 3).
G. Quantification of Rac1 activity levels in (F). Data are presented as the mean ± s.e.m.
H. Representative immunoblot of cultured Plcg1<sup>ff</sup> (n = 2, N = 3) and Plcg1<sup>ff</sup>;Nes-Cre (n = 2, N = 3) mesencephalic neurons stimulated with conditioned media containing netrin-1.

Source data are available online for this figure.
levels of dominant directionality, we divided the FA results into low, intermediate, and high directionality of tensor lines: < 0.35, ≥ 0.35 to < 0.75, and ≥ 0.75, respectively. FA values of < 0.35 do not reflect directionality, and tensors ≥ 0.75 were insufficient for statistical significance; therefore, we focused on the ≥ 0.35 to < 0.75 group. Volumes and FA values were extracted to evaluate changes in CC volume and the three levels of diffusion directionality between control and Plcg1f/Nes-Cre brains when compared with the controls (control: 8.59 ± 0.45; Plcg1f/+;Nes-Cre: 7.06 ± 0.35; Shapiro–Wilk and t-tests, *P < 0.05; Fig 5B). Furthermore, tensor lines with FA values were extracted to assess changes in tensor lines based on the three levels of diffusion directionality between control and Plcg1f/+;Nes-Cre mice (n = 4 per genotype). We found a significant reduction in the FA values in Plcg1f/+;Nes-Cre mice when compared with the controls (control: 5.573.50 ± 255.62; Plcg1f/+;Nes-Cre: 4.439.30 ± 255.62; Shapiro–Wilk and t-test, *P < 0.05; Fig 5D). Finally, we measured the lengths of the tensor lines to evaluate changes in tensor line length between Plcg1f/+ and Plcg1f/+;Nes-Cre mice (n = 4 per genotype). There was a significant reduction in tensor line length in Plcg1f/+;Nes-Cre mice when compared with Plcg1f/+ mice (control: 2.762.00 ± 307.33;
Recently, meaningful advances have been made toward revealing how neural networks are established during development; however, the precise signaling mechanisms that are involved in the formation of a typical wiring pattern are only partially understood. Our results have demonstrated that the regulation of neural development by netrin-1/DCC signaling may be mediated, at least in part, through the activation of PLCγ1, which controls key steps in the axonal projection of mDA neurons as well as CC structural formation. In particular, mDA neurons have been implicated in various kinds of neurological disorders such as addiction, parkinsonism, schizophrenia, sleep abnormalities, and ADHD, with some evidence indicating that structural changes in neuronal networks may in part underlie the pathogenesis of these disorders [35–39]. Although axon guidance events are crucial for the correct development of mDA

Figure 5. Axon tracts in the corpus callosum (CC) of the Plcg1<sup>fl/fl</sup>;Nes-Cre mice are more diffuse than those of control mice.

(A–F) (A, C, and E) Cumulative histograms based on fractional anisotropy (FA) values. (B, D, and F) show the CC volume and the number of tensors relative to FA values. As FA values increased, the structural (CC volume and the number of tensors) differences increased between the control and Plcg1<sup>fl/fl</sup>;Nes-Cre mice. Depending on the number of tensors, the length in the CC appeared to be shortened (E, F). (n = 4; Shapiro–Wilk and t-test; *P < 0.05). Data are presented as the mean ± s.e.m.
pathways, their precise role during the pathogenesis of mDA system-related disorders has not been established. Thus, further study assessing the causality between the structural defect in the PLCγ1-null mDA system and behavioral phenotypes is required to better understand their potential contribution of axon guidance mechanisms to neurological disorders.

Materials and Methods

Animals

All animal experiments were performed using 8-week-old age-matched, male C57/BL6 mice with neural progenitor-specific conditional knockout of Plcg1 (Plcg1f/f;Nes-Cre) or C57/BL6 mice expressing Cre recombinase under the control of the Slc6a3 (or DAT) promoter (Plcg1f/f;DAT-Cre); their floxed littermates were used as controls. All animal experiments were performed according to accepted international instructions for the use and implementation of such studies. All animal experiments were approved by the Institutional Animal Care and Use Committee at Ulsan National Institute of Science and Technology (UNIST-IACUC-14-005).

Conditioned medium

The conditioned medium used in Fig 3F–H was made as follows. 293T cells were transiently transfected with the netrin-1 expression vector (Origene, Rockville, MD 20850, USA) in 293T cells. After 16 h, the cells were washed three times with neurobasal media and then incubated for one more day in serum-free neurobasal media. The media was centrifuged and stored at −72°C.

Explant culture

Ventral mesencephalic tissue (A9) from embryonic day 14.5 (E14.5) mice was dissected and embedded in low-melting-point agarose (LMA) gel (Sigma-Aldrich, St. Louis, MO, USA) and then sliced to 300 μm using a Leica LVT1000S vibrating-blade microtome (Leica Biosystems, St. Louis, MO, USA). Netrin-1-transfected 293T cells were aggregated by centrifugation and immobilized with matrigel (Sigma-Aldrich). The explants were cultured in a medium containing B-27 supplement and 10% fetal calf serum (Gibco, Waltham, MA, USA) and maintained in a humidified incubator at 37°C, 5% CO2 for 24 h. In explant experiment, netrin-1-secreting 293 T cell aggregate and the explant tissue were co-cultured with a distance of 150–200 μm in serum-free neuronalbasal media.

Immunohistochemistry

Mice were perfused transcardially with an ice-cold solution of 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). Fixed brains were embedded in an LMA gel and sliced using a vibrating blade microtome. Free-floating sections were rinsed with TBS and incubated with primary antibody. Antibody dilutions were as follows: mouse anti-neurofilament light chain (NF-L) [(clone 2H3), 1:500, (DSHB Hybridoma, Iowa, IA, USA)], rabbit anti-tyrosine hydroxylase (TH) antibody [(cat no. ab112), 1:1,000, (Abcam, Cambridge, UK)].

In situ hybridization

An antisense RNA probe was generated and labeled using the pGEM-T Easy Vector System (Promega, Madison, WI, USA) and the DIG RNA Labeling Kit (Roche Applied Science, Penzberg, Upper Bavaria, Germany). The coding sequence of PLCγ1 (NM_021280.3; 2,523–3,126) was used as a template for generating the RNA probe (603 nucleotides). In situ hybridization on brain slices was performed according to the manufacturer’s instructions (Roche Applied Science, Penzberg, Upper Bavaria, Germany).

Liquid chromatography–mass spectrometry (LC/MS) analysis

All mass analyses were performed using the LTQ Orbitrap (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray ion source. To separate the peptide mixture, we used a C18 reverse-phase high-pressure liquid chromatography (HPLC) column (150 × 75 mm i.d.). For MS/MS analysis, the precursor ion scan MS spectra (400–2,000 m/z) were acquired in the Orbitrap at a resolution of 60,000 at 400 m/z with an internal lock mass. The 20 most intensive ions were isolated and fragmented in the linear ion trap by collisional-induced dissociation (CID).

Magnetic resonance image (MRI) acquisition

Brains were extracted and embedded on LMA gels to prevent motion and susceptibility to artifacts during MRI. For the imaging, a 72-mm transmit and surface receive coil assembly was used with a Bruker 7.0T BioSpec system (Bruker Biospin MRI, Billerica, MA, USA). Two MRI pulse sequences were used. First, images were obtained using the rapid acquisition with refocused echoes (RARE) method to delineate the ROI in the corpus callosum (CC). The imaging parameters for the RARE sequence were as follows: TR = 2,500 ms, TE = 15 ms, RARE factor = 2, field of view (FOV) = 15.00 × 10.00 × 14.04 mm3, matrix size = 384 × 256 × 45 mm3, spatial resolution = 39.06 × 39.06 × 312 μm, number of signal averages (NSA) = 16, and scan time = 1 h 25 min 20 s. Second, diffusion tensor imaging (DTI) was used for neuronal tractography [40]. The imaging parameters for the DTI sequence were as follows: repetition time (TR) = 2,000 ms, echo time (TE) = 20 ms, B-value = 650 s/mm2 (30 directions and five reference scans), FOV = 15.00 × 10.00 × 14.04 mm3, matrix size = 192 × 128 × 45 mm3, spatial resolution = 78.125 × 78.125 × 312 μm, NSA = 8, and scan time = 19 h 43 min 40 s.

Mesencephalic neuron culture

Mesencephalic neurons were harvested and cultured as described previously [41]. Neuron preparations with a rate ≥ 95% on the trypan blue exclusion test were cultured further. The cells were seeded at a density of 4 × 105 cells per well on a poly-D-lysine-coated six-well plate and maintained in a humidified incubator at 37°C in an atmosphere of 5% CO2.

Microscope image

Fluorescence images were acquired using FV1000 (Olympus, Tokyo, Japan) and LSM780N (Carl Zeiss, Oberkochen, Germany) confocal
microscope. Images were analyzed by using ImageJ 1.52a (National Institutes of Health, Bethesda, MD, USA) and Imaris 8.1 (Oxford Instruments, Abingdon, UK).

Post-processing and tensor line calculation

Region of interests for the CC were drawn on each RARE image for post-processing. Spline algorithms were applied to create smooth CC borderlines for each slice [42]. A total of 45 ROIs from all slices were interpolated to have an isotropic spatial resolution for the CC by cubic interpolation (ImageJ, version 1.48, National Institutes of Health). Diffusion Toolkit and TrackVis programs were used to calculate, visualize, and quantify the tensor lines. During the tensor calculation, corresponding ROIs from RARE images were used to delineate areas of the CC.

Rac1 activity assay

For netrin-1 treatment, 1.5 \times 10^6 primary cortical neurons plated in 100-mm dishes. After 2 days of incubation, the cells were washed three times with serum-free neurobasal media. 8 h after serum starvation, the culture medium was changed to a conditioned medium with or without netrin-1. After 30 min, cells were lysed on ice in lysis buffer [50 mmol/l Tris (pH 7.2), 100 mmol/l NaCl, 5 mmol/l MgCl2, 1 mmol/l DTT, 10% glycerol, and 1% NP40] containing protease inhibitors, and Rac GTP pull-down assays were then performed according to the manufacturer’s instructions (Abcam, Cambridge, UK).

Western blotting

Whole brain tissues and mesencephalic neurons were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer [50 mM of Tris–HCl at pH 7.5, 0.25 M of sucrose, 1 mM of ethylenediaminetetraacetic acid (EDTA), 10 \mu g/ml of aprotinin, 10 \mu g/ml of leupeptin, and 1 mM of phenylmethylsulfonyl fluoride (PMSF)] for 5 min. The lysates were incubated on ice for 30 min and centrifuged at 20,800 \times g for 15 min. Primary antibodies used and their dilution factors were as follows: rabbit IgG against phospho-PLCγ1 [Y783 (clone: D6M9S), (1:3,000), (Cell Signaling, Danvers, MA, USA), Y1248 (clone: D25A9), (1:3,000), (Cell Signaling)], PLCγ1 [Y771, (cat no. ab131455), (1:3,000), (Abcam)], PLCγ1 [Y1253, (cat no. GTX32238), (1:1,000), (Genetex, CA, USA)], phospho-AKT [T308 (clone: 244F9), (Cell Signaling)], phospho-FAK [Y397 (cat no. 3283), (1:3,000), (Cell Signaling)], phospho-CamKII [T286, (cat no. ab5683), (1:3,000), (Abcam)], phospho-Erk [T202/Y204, (cat no. 4370s), (1:3,000) (Cell Signaling)], pRac1 [cat no. ab5482], (1:3,000), (Cell Signaling)], and phospho-Src [Y416, (clone: EP503Y), (1:3,000), (Cell Signaling)]; mouse IgG against PLCγ1 [(clone: EP1898Y), (1:3,000), (Abcam)] and goat IgG against DCC [(clone A-20), (1:1,000), (Santa Cruz Biotechnology, Dallas, TX, USA)], and Netrin 1 [(clone: EPR5428), (1:1,000), (Abcam)].

Statistics

All statistical tests used in this study were performed two-sided. Statistical data are expressed as means ± s.e.m. At least three biological replicates were performed for all studies. Independent two-tailed t-test was used for analysis of differences between two groups. When comparing more than two groups, ANOVA was followed by Holm–Sidak test or Bonferroni correction. For normal distribution, we applied the Shapiro–Wilk test. A P-value of less than 0.05 was considered statistically significant: *P < 0.05, **P < 0.005.

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Acknowledgements

We thank Dr. Jong Soon Kang, Il Hwan Kim, Ja-Hyun Baik, and In Koo Hwang for helpful discussions and/or valuable comments on the manuscript. We would also like to thank A Suzuki, il Shin Kim, Sooh Park, Kyung-Su Park, and Jin Hoe Hur who provided technical support and materials. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. 2017R1E1A1A01074510) and (No. 2014M3A9O8034459).

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. P-GS conceived the project. D-SK carried out all the steps from research design to manuscript preparation. BWP and HJC performed MR imaging and evaluated the data. JKS performed the proteomic analysis. YRY, CL, KILP, YKS, and CL performed the data evaluation and contributed to the manuscript preparation.

Conflict of interest

The authors declare that they have no conflict of interest.

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