Survival effect of PDGF-CC rescues neurons from apoptosis in both brain and retina by regulating GSK3β phosphorylation

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Platelet-derived growth factor CC (PDGF-CC) is the third member of the PDGF family discovered after more than two decades of studies on the original members of the family, PDGF-AA and PDGF-BB. The biological function of PDGF-CC remains largely to be explored. We report a novel finding that PDGF-CC is a potent neuroprotective factor that acts by modulating glycogen synthase kinase 3β (GSK3β) activity. In several different animal models of neuronal injury, such as axotomy-induced neuronal death, neurotoxin-induced neuronal injury, 6-hydroxydopamine–induced Parkinson's dopaminergic neuronal death, and ischemia-induced stroke, PDGF-CC protein or gene delivery protected different types of neurons from apoptosis in both the retina and brain. On the other hand, loss-of-function assays using *PDGF-C* null mice, neutralizing antibody, or short hairpin RNA showed that PDGF-CC deficiency/inhibition exacerbated neuronal death in different neuronal tissues in vivo. Mechanistically, we revealed that the neuroprotective effect of PDGF-CC was achieved by regulating GSK3β phosphorylation and expression. Our data demonstrate that PDGF-CC is critically required for neuronal survival and may potentially be used to treat neurodegenerative diseases. Inhibition of the PDGF-CC–PDGF receptor pathway for different clinical purposes should be conducted with caution to preserve normal neuronal functions.

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Abbreviations used: 6-OHDA, 6-hydroxydopamine; CSF, cerebrospinal fluid; EB, Evan's blue; GSK3β, glycogen synthase kinase 3β; HUVSMC, human umbilical vein smooth muscle cell; INL/ONL, inner/outer nuclear layer; MAPK, mitogen-activated protein kinase; MCAO, middle cerebral artery occlusion; NMDA, *N*-methyl-D-aspartic acid; ONC, optic nerve crush; PDGF-CC, platelet-derived growth factor CC; PDGFR-α, PDGF receptor α; RGC, retinal ganglion cell; SN, substantia nigra; ST, striatum; TH, tyrosine hydroxylase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Neurodegeneration caused by neuronal death occurs in different types of neurodegenerative diseases and leads to severe morbidity and mortality in humans. Glaucoma is a common optic neuropathy in which loss of retinal ganglion cells (RGCs) occurs because of apoptosis, resulting in loss of vision. Current treatment for glaucoma has only limited efficacy. Parkinson's disease involves progressive death of dopaminergic neurons in the brain and is the most common neurodegenerative movement disorder worldwide, with no satisfying cure currently. Ischemic stroke, in which cortical neurons die because of ischemia insult, represents one of the most challenging diseases clinically. Currently, thrombolytic therapy is the only available treatment and is limited to <10% of total stroke patients, with potentially deleterious side effects. With the promise offered by the studies on Alzheimer's disease (Reisberg et al., 2003; Lipton, 2006) and amyotrophic lateral sclerosis (Nirmalananthan and Greensmith, 2005), neuroprotection achieved by neuroprotective factors to enhance neuronal survival has emerged to be a potentially promising general strategy to treat different

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types of neurodegenerative diseases (Schwartz, 2005). Therefore, identifying such novel neuroprotective molecules is highly warranted.

Platelet-derived growth factor CC (PDGF-CC) was discovered more than two decades after the initial studies on PDGF-AA and PDGF-BB as the third member of the PDGF family (Kazlauskas, 2000; Li et al., 2000; Heldin et al., 2002). The biological function of PDGF-CC remains largely to be explored. PDGF-CC protein is produced as a secreted homodimer that needs to be proteolytically processed to allow receptor binding (Li et al., 2000; Fredriksson et al., 2005). PDGF-CC binds to and activates both PDGF receptor α (PDGFR-α) and PDGFR-β (Li et al., 2000; Gilbertson et al., 2001; Li and Eriksson, 2003). PDGF-CC is critically required for embryonic development, as PDGF-CC deficiency in mice led to postnatal lethality because of developmental defects (Ding et al., 2004). In addition, the protein structure of PDGF-CC is predicted to be more similar to vascular endothelial growth factor than to the PDGFs, indicating its potential functional uniqueness (Reigstad et al., 2005). PDGF-CC is abundantly expressed in different types of neuronal tissues, including the brain (Ding et al., 2000; Li et al., 2000; Aase et al., 2002), eye (Aase et al., 2002; Lei et al., 2007), and spinal cord (Hamada et al., 2000, 2002), indicating a role of PDGF-CC in the neural system. However, direct evidence has been lacking thus far.

In this study, we used several different animal models and approaches to investigate the neuronal effect of PDGF-CC. We also investigated the potential effect of PDGF-CC on blood vessel permeability in both normal and pathological conditions in mouse retina and brain, because it was recently reported that intraventricular injection of PDGF-CC into normal mouse brain increased cerebrovascular permeability (Rieckmann, 2008; Su et al., 2008). We found that PDGF-CC is a potent neuroprotective factor and rescued neurons from apoptosis in both injured retina and brain in vivo. We further revealed that the neuroprotective effect of PDGF-CC was achieved by regulating glycogen synthase kinase 3β (GSK3β) phosphorylation. Thus, PDGF-CC is critically required for neuronal survival and may have a therapeutic value in treating neurodegenerative diseases. Suppression of the PDGF–PDGFR pathway for various therapeutic purposes should be conducted with caution to avoid neuronal damage.

RESULTS

PDGF-CC protects RGCs from axotomy-induced neuronal death

In situ hybridization detected *PDGF-C* expression in the RGC layer and inner/outer nuclear layer (INL/ONL; Fig. 1 A) in the retina. Western blotting revealed PDGF-CC protein in the retina as several forms because of differential proteolytic processing (Fig. 1 B). Real-time PCR revealed increased expression levels of *PDGF-C* and its receptors, PDGFR-α and PDGFR-β, in the retinae after optic nerve crush (ONC) injury ($n = 6$; $P < 0.05$ or 0.01; Fig. 1, C–E), indicating a potential role of PDGF-CC in injured retina. Indeed, *PDGF-C* deficiency led to >40% fewer viable RGCs in the retina 2 wk after ONC injury ($n = 9$ or 11; $P < 0.01$; Fig. 1, F, G, and L). *PDGF-C* shRNA intravitreal injection, which decreased *PDGF-C* transcript to <50% of normal level ($n = 8$; $P < 0.01$; Fig. 1 M), led to fewer viable RGCs in the retina compared with those of control group 2 wk after ONC injury ($n = 7$; $P < 0.05$; Fig. 1, H, I, and L). On the contrary, intravitreal injection of active rhPDGF-CC protein (500 ng/eye; Li et al., 2000) increased the number of viable RGCs in the retina by ∼1.7-fold 2 wk after treatment ($n = 8$; $P < 0.01$; Fig. 1, J–L). Thus, both loss- and gain-of-function assays showed that PDGF-CC is critically required for RGC survival after retina injury.

It is recently reported that intraventricular injection of PDGF-CC into normal mouse brain increased cerebrovascular permeability 1 h after injection (Su et al., 2008). We therefore tested whether PDGF-CC affected retinal permeability. First, *PDGF-C* deficiency did not affect Evan's blue (EB) extravasation in the retina in *PDGF-C* null mice ($n = 14$–16; $P > 0.05$; Fig. 1 S). Second, intravitreal injection of PDGF-CC protein (500 ng/eye; Li et al., 2000) did not significantly affect retinal permeability at different time points tested even though intravitreal injection of the same amount of BSA increased retinal permeability transiently ($n = 8$–10; $P > 0.05$; Fig. 1 T), confirming a negligible role of PDGF-CC in inducing retinal blood vessel permeability. Thus, PDGF-CC increased RGC survival without affecting retinal blood vessel permeability.

PDGF-CC regulates expression of apoptotic/cell death–related and survival/neurotrophic genes

We next performed a genome-wide gene expression profiling assay using a whole mouse genome microarray to investigate the molecular mechanisms underlying the neuroprotective effect of PDGF-CC. PDGF-CC treatment up- and down-regulated the expression of numerous survival/neurotrophic and apoptotic/cell death–related genes, respectively, in ONC-injured retinae (Tables I and II). Microarray data have been deposited in the Gene Expression Omnibus under accession no. GSE19207. We validated the microarray data using real-time PCR both in vivo and in vitro. PDGF-CC treatment (500 ng/eye) inhibited the expression of numerous apoptotic/cell death–related genes in ONC-injured retinae at different time points ($n = 8$; Fig. 1, N and O), and also in normal retinae ($n = 8$; Fig. 1 P). Further, PDGF-CC treatment up-regulated the expression of many neurotrophic/survival genes in the retinae with ONC ($n = 8$; Fig. 1, Q and R). The microarray data were further validated in other types of PDGF-CC–treated neuronal tissues or cells, including *N*-methyl-D-aspartic acid (NMDA)–injured retinae (see Fig. 2, D and E), 6-hydroxydopamine (6-OHDA)–injured substantia nigra (SN) in the brain (see Fig. 3, L and M), ischemia-injured brain cortex (see Fig. 4, G and I), and isolated primary cortex neurons (see Fig. 4 H). Thus, the neuroprotective effect of PDGF-CC is achieved, at least partially, by regulating the expression of numerous apoptotic/cell death–related and survival/neurotrophic genes in neuronal tissues.

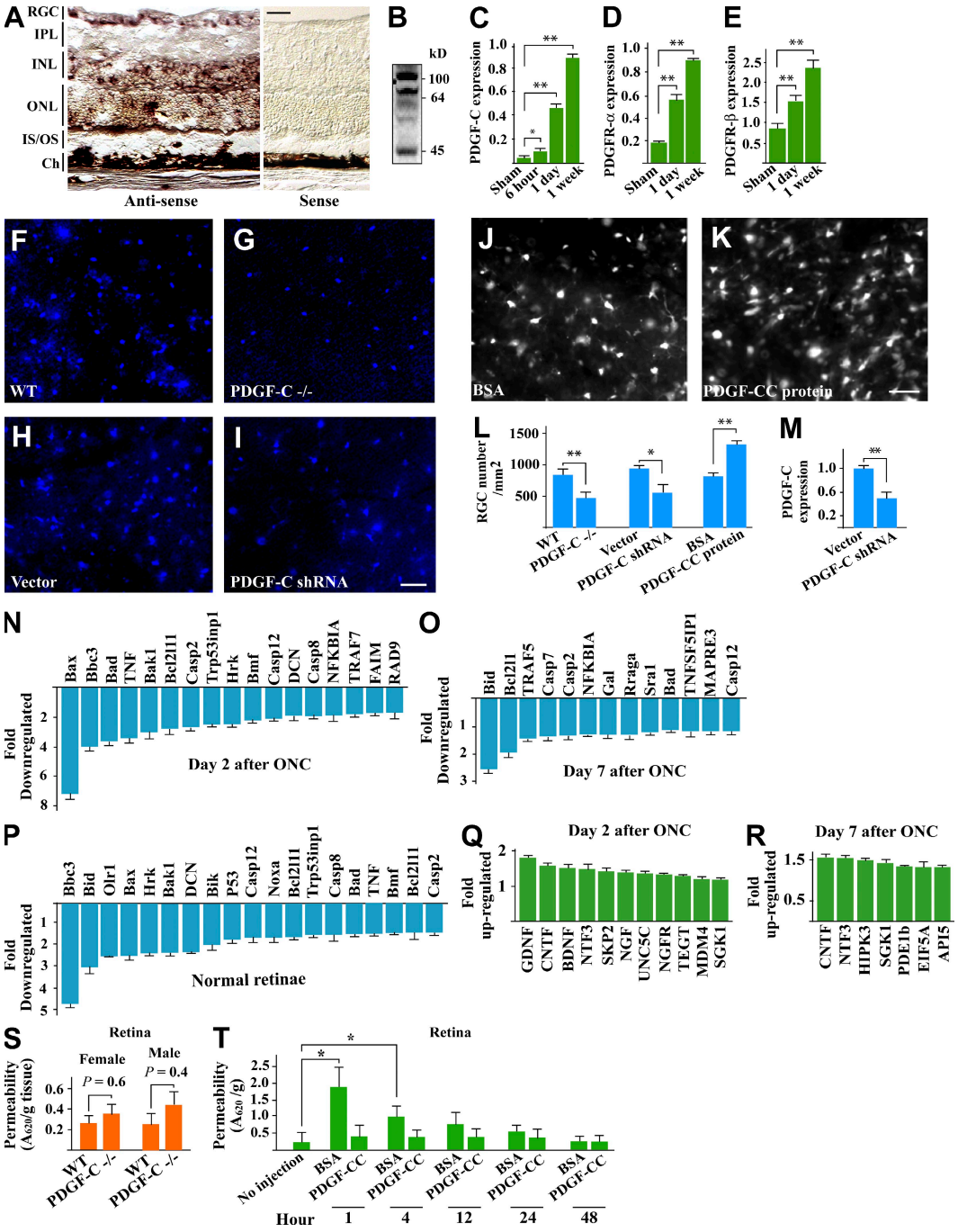

Figure 1. PDGF-CC protects RGCs from axotomy-induced neuronal death. (A) In situ hybridization assay detected abundant *PDGF-C* expression in the retina mainly in the RGC layer and INL/ONL. Bar, 50 µm. (B) Western blot assay detected PDGF-CC expression in the retina as several forms because of different proteolytic processing. (C–E) Real-time PCR showed up-regulated expression of *PDGF-C*, PDGFR-α, and PDGFR-β, in the retinae after ONC using β-actin as an internal control ($n = 6$ eyes). (F, G, and L) *PDGF-C* deficiency led to fewer viable RGCs (blue) after ONC ($n = 9$ or 11 eyes). (H, I, L, and M) *PDGF-C* shRNA intravitreal injection decreased *PDGF-C* transcript level to <50% of normal level (M, $n = 8$ eyes) and led to fewer viable RGCs (blue) compared with that of the control group (H, I, and L, $n = 7$ eyes). (J–L) PDGF-CC protein intravitreal injection increased the number of viable RGCs (white) after ONC ($n = 8$ eyes). (N–P) Real-time PCR validated the genome-wide gene expression profiling data. PDGF-CC treatment down-regulated the expression of numerous apoptotic/cell death–related genes in retinae after ONC at different time points (N and O, $n = 8$ eyes) and in normal retina (P). (Q and R) PDGF-CC treatment up-regulated the expression of many neurotrophic/survival genes in ONC-injured retinae at different time points ($n = 8$ eyes). (S) No difference was found in EB extravasation in the retina between *PDGF-C*–deficient and wild-type mice ($n = 14$–16 eyes). (T) Intravitreal injection of PDGF-CC protein did not affect retinal permeability at different time points, whereas the same amount of BSA increased retinal permeability transiently ($n = 8$–10 eyes). Bars: (F–K) 20 µm. *, $P < 0.05$; **, $P < 0.01$. The data are represented as means ± SEM of the number of determinations. All experiments were repeated independently once (A–E and M–R) or twice (F–L) with similar results. Representative images (A, B, and F–I) or pooled data (C–E and M–R) are shown. Ch, choroid; IS/OS, inner/outer segment.

PDGF-CC protects neurons from neurotoxin-induced apoptosis

Overstimulation of the NMDA receptor by glutamate leading to neuronal apoptosis is implicated in many neurodegenerative disorders. We therefore tested whether PDGF-CC could inhibit NMDA-induced apoptosis in mouse retina. Because NMDA induces only neuronal apoptosis without affecting the vascular system and PDGF-CC treatment in this model lasts only for 24 h (a time window too short for new blood vessels to grow), this model thus allowed us to verify the neuronal effect of PDGF-CC without implicating the vascular system. We injected NMDA (20 nmol/eye) with or without PDGF-CC protein (500 ng/eye) intravitreally into mouse eyes and analyzed apoptosis in the retina after 24 h. PDGF-CC protein treatment reduced the number of apoptotic cells in the RGC layer and INL/ONL (n = 8; $P < 0.01$ or 0.05; Fig. 2, A–C). Further, real-time PCR detected up- and down-regulated expression of many neurotrophic/survival (n = 8; Fig. 2 D) and apoptotic/cell death–related genes (n = 8; Fig. 2 E), respectively, in the NMDA-injured retina after PDGF-CC treatment. Thus, PDGF-CC protected NMDA excitotoxin-injured retinal neurons, at least partially, by regulating the expression of many neurotrophic/survival and apoptotic/cell death–related genes.

PDGF-CC protects dopaminergic neurons from apoptosis in SN

We next tested whether PDGF-CC affects dopaminergic neuron survival in the brain using a 6-OHDA–induced Parkinson's disease model. Western blotting detected PDGF-CC protein in both SN (where dopaminergic neurons reside) and striatum (ST; where the axons of dopaminergic neurons reside) as several forms because of different proteolytic processing (Fig. 3 A). *PDGF-C*–deficient mice displayed fewer tyrosine hydroxylase (TH; a marker for dopaminergic neurons)–positive cells in SN (Fig. 3 B) and fewer TH^+ fibers in ST (Fig. 3 C) 7 d after 6-OHDA injury. Indeed, *PDGF-C*–deficient mice displayed an increased contralateral rotation rate (n = 9–15; $P < 0.05$; Fig. 3 D), indicating that PDGF-CC is required for dopaminergic neuron survival. PDGF-CC recombinant protein or gene delivery into SN (0.5 μg/SN) increased TH^+ signals (dopaminergic neurons and axons) in both SN (n = 9–15; Fig. 3 E) and ST (n = 9–15; Fig. 3 F) in the 6-OHDA–injured mice measured by several different methods, including fluorescent staining (Fig. 3, E and F), real-time PCR (n = 6; $P < 0.001$ or 0.01; Fig. 3 G), and Western blotting (n = 6; Fig. 3 J). On the contrary, *PDGF-C* shRNA treatment, which decreased the *PDGF-C* expression level to <50% of normal (n = 8; $P < 0.01$; Fig. S1 A), decreased the TH expression level in the 6-OHDA–injured SN measured by real-time PCR (n = 6; $P < 0.05$; Fig. 3 G) and Western blotting (n = 6; Fig. 3 K). Moreover, PDGF-CC protein and gene delivery also increased expression level of c-fos (an inducible transcription factor used to determine neuron activation in response to dopaminomimetic compounds; Zhang et al., 2006) in 6-OHDA–injured SN, whereas *PDGF-C* shRNA treatment decreased c-fos expression (n = 6; $P < 0.01$; Fig. 3 H). Functionally, PDGF-CC protein or gene delivery into SN decreased the contralateral turning rate of the 6-OHDA–injured mice 2 wk after treatment (n = 9 or 15; $P < 0.01$; Fig. 3 I). PDGF-CC overexpression in SN after gene delivery was confirmed by fluorescent staining (Fig. S1 B). EB extravasation assay showed that PDGF-CC protein SN injection did not change blood vessel permeability in SN at different time points (n = 8; $P > 0.05$; Fig. S1 C). At the gene expression level, PDGF-CC treatment down- and up-regulated the expression of numerous apoptotic/cell death–related (Fig. 3 L) and neurotrophic/survival genes (Fig. 3 M), respectively, in the 6-OHDA–injured SN. Thus, both loss- and gain-of-function assays showed that PDGF-CC is required for the survival of dopaminergic neurons in the brain.

Table I. Survival/antiapoptotic genes up-regulated by PDGF-CC

Gene name	Accession no.	Fold up-regulated
SGK1	NM_011361	3.3
PEA15	NM_011063.1	2.2
DCTN1	NM_007835.1	2.2
UBQLN1	NM_152234.1	1.9
API5	NM_007466	1.9
RAF1	AK036317	1.7
UNC5C	NM_009472.1	1.7
SMPD1	NM_011421.1	1.7
SKP2	AK037002	1.7
MDM4	NM_008575.2	1.7
HIPK3	NM_010434.1	1.6
TAF10	NM_020024.3	1.5
FKBP8	NM_010223.1	1.5
EIF5A	NM_181582.2	1.5
PDE1B	NM_008800.1	1.5
MAPK8	AK030767	1.5
CNTF	NM_170786	1.5
NGFR	NM_033217	1.5

Nucleotide sequences are available from GenBank/EMBL/DDBJ under the accession numbers listed.

Table II. Apoptotic/cell death–related genes down-regulated by PDGF-CC

Gene name	Accession no.	Fold down-regulated
TRAF7	NM_153792.1	−1.9
FAIM	NM_011810.2	−1.7
EEF1E1	NM_025380.1	−1.6
UACA	NM_028283	−1.6
AATK	NM_007377.1	−1.6
NFKBIA	NM_010907	−1.5
DIABLO	NM_023232.2	−1.5
PDCD4	NM_011050.1	−1.5
BAX	NM_007527.2	−1.5
GAL	NM_010253	−1.5

Nucleotide sequences are available from GenBank/EMBL/DDBJ under the accession numbers listed.

PDGF-CC protects cortical neurons from ischemia-induced neuronal death

We next tested whether PDGF-CC could rescue ischemia-induced cortex neuronal death using a middle cerebral artery occlusion (MCAO) stroke mouse model. Western blotting detected abundant PDGF-CC expression in brain cortex (Fig. 4 A), indicating a potential role of PDGF-CC in the brain. We injected active PDGF-CC protein (Li et al., 2000) or a PDGF-CC neutralizing antibody into mouse cerebrospinal fluid (CSF; 3 µl × 3 µM/mouse), performed MCAO 1 h after protein injection, and measured stroke volume after 24 h. PDGF-CC protein CSF injection decreased stroke volume (n = 11 or 13; P < 0.05; Fig. 4, B and C), whereas CSF injection of a neutralizing antibody against mouse PDGF-CC enlarged stroke volume (n = 9 or 11; P < 0.05; Fig. 4 C). Moreover, PDGF-CC protein cortex injection (0.15 µg/0.5 µl × three sites) decreased stroke volume by ~76% 7 d after protein injection (n = 10; P < 0.05; Fig. 4, D and E). Loss-of-function assay using *PDGF-C* null mice showed that *PDGF-C* deficiency led to significantly greater stroke volume (n = 8; P < 0.01; Fig. 4 F), further demonstrating a critical role of PDGF-CC in cortex neuron survival. Real-time PCR revealed increased expression of many neurotrophic/survival factors in both brain cortex (n = 6; Fig. 4 G) and isolated primary cortical neurons (n = 4; Fig. 4 H) after PDGF-CC treatment, and decreased expression of numerous apoptotic/cell death–related genes (n = 8; Fig. 4 I). Thus, PDGF-CC is critically required for cortex neuron survival in the brain.

Using *PDGF-C*–deficient mice as a genetic tool to test whether PDGF-CC affects cerebrovascular permeability, no significant difference in EB extravasation was found between *PDGF-C*–deficient and wild-type mice in either normal brains (n = 10; P = 0.4; Fig. 4 J) or brains with MCAO-induced ischemia (n = 8; P = 0.3; Fig. 4 K), suggesting a negligible role of PDGF-CC in inducing blood vessel permeability in the brain. Indeed, PDGF-CC protein CSF injection (3 µl × 3 µM/mouse) did not increase blood vessel permeability 1 h after injection in normal brain (n = 8; Fig. 4 L) or in brains with MCAO-induced ischemia (n = 8; Fig. 4 M).

Direct protective effect of PDGF-CC on cultured neurons

We cultured different types of neuronal cells derived from retina or brain to investigate whether PDGF-CC has a direct neuroprotective effect on them. We treated RGC5 cells (an RGC-derived cell line) with H_2O_2, which is known to induce oxidative stress–induced apoptosis. PDGF-CC treatment (50 ng/ml) significantly decreased H_2O_2-induced apoptosis in RGC5 cells and increased their survival at different time points tested (n = 6; P < 0.05; Fig. 5, A–C). PDGF-CC treatment (50 ng/ml) also increased the survival of primary retinal neuroprogenitor cells isolated from postnatal day 1 (P1) mice cultured in serum-free medium using islet1$^+$ as a marker (n = 6; P < 0.01; Fig. 5, D–F). Further, PDGF-CC treatment decreased hypoxia- and glucose deprivation–induced apoptosis in primary cortical neurons isolated from P1 mice by >50% (n = 6; P < 0.01; Fig. 5, G–I). Moreover, PDGF-CC treatment (50 ng/ml) increased survival of primary TH$^+$ neurons isolated from E13.5 mouse embryonic SNs when the neurons were cultured in serum-free medium (n = 6; P < 0.001; Fig. 5, J–L), or stressed by neurotoxin 6-OHDA treatment (n = 6; P < 0.05 or 0.01; Fig. 5, M–O). Thus, PDGF-CC displayed a direct neuroprotective effect on different types of neurons in culture.

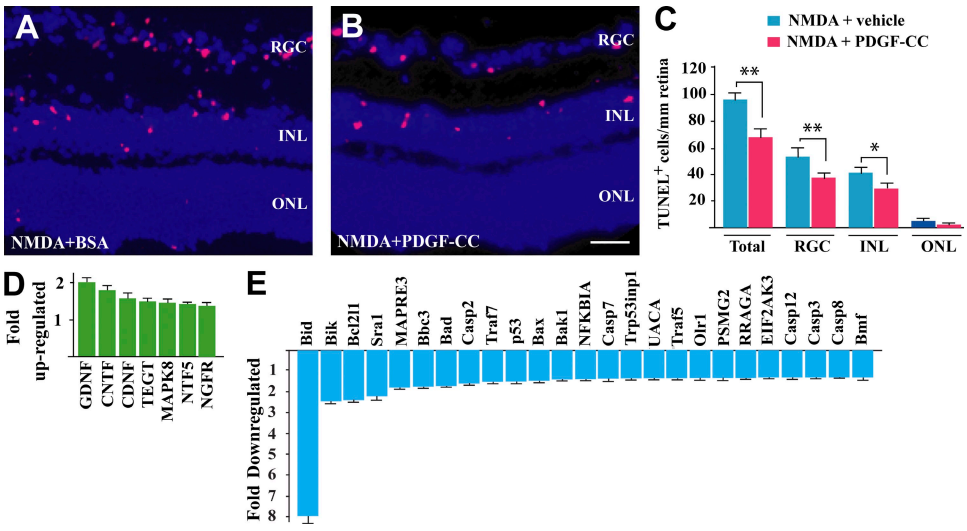

Figure 2. PDGF-CC inhibits NMDA-induced neuronal apoptosis in retina. (A–C) PDGF-CC treatment reduced the number of apoptotic cells (red) in the RGC layer and INL/ONL after NMDA injury measured by TUNEL staining (n = 8 eyes). Bar, 20 µm. (D and E) Real-time PCR revealed that PDGF-CC treatment up-regulated the expression of several neurotrophic/survival genes (D) and inhibited the expression of numerous apoptotic/cell death–related genes in the NMDA-injured retina (E; n = 8 eyes). *, P < 0.05; **, P < 0.01. The data are represented as means ± SEM of the number of determinations. All experiments were repeated independently once with similar results. Representative images (A and B) or pooled data (D and E) are shown.

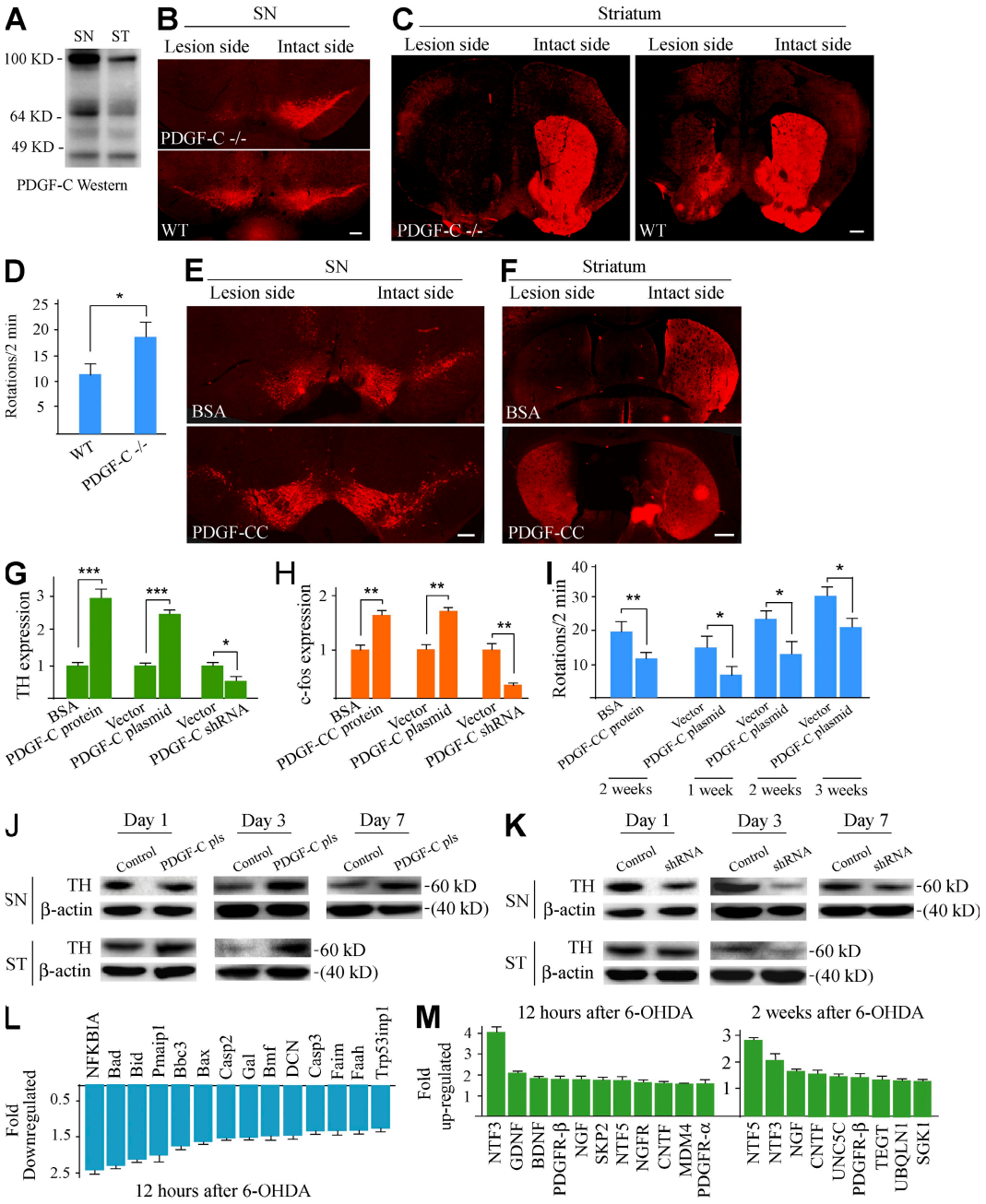

Figure 3. PDGF-CC protects dopaminergic neurons from apoptosis. (A) Western blotting detected PDGF-CC expression in the SN and ST in the brain as several forms because of differential proteolytic processing. (B and C) *PDGF-C* deficiency led to fewer TH⁺ cells in SN (B) and fewer TH⁺ fibers in ST (C) in the lesion side of the brain 7 d after 6-OHDA injury. The images shown in C represent a mosaic of several individual images. (D) *PDGF-C*–deficient mice displayed an increased contralateral rotation rate 1 wk after 6-OHDA injury ($n = 9$ or 15 mice). (E and F) PDGF-CC protein SN injection increased TH⁺ signals (dopaminergic neurons) in both SN (E) and ST (F) in 6-OHDA–injured brain measured by immunofluorescent staining ($n = 9$ or 15 mice). (G and H) PDGF-CC protein treatment increased expression levels of TH (G) and c-fos (H) in SN, whereas *PDGF-C* shRNA treatment decreased their expression levels (G and H) measured by real-time PCR. *PDGF-C* gene delivery (*PDGF-C* plasmid) into SN increased TH and c-fos expression levels ($n = 6$ mice). (I) PDGF-CC protein or gene delivery into SN decreased contralateral turning rate in the 6-OHDA–injured mice at different time points ($n = 9$ or 15 mice). (J) *PDGF-C* plasmid gene delivery (*PDGF-C* pls) increased TH expression level in both SN and ST at different time points measured by Western blotting ($n = 6$ mice). (K) PDGF-C shRNA treatment decreased TH expression level in both SN and ST in 6-OHDA–injured SN at different time points measured by Western blotting ($n = 6$ mice). (L) PDGF-CC protein treatment inhibited expression of numerous apoptotic/cell death–related genes in 6-OHDA–injured SN at different time points measured by real-time PCR. (M) PDGF-CC protein treatment up-regulated expression of many neurotrophic/survival factors in 6-OHDA–injured SN at different time points measured by real-time PCR. Bars: (B and E) 200 µm; (C and F) 400 µm. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The data are represented as means ± SEM of the number of determinations. All experiments were repeated independently once (A, G, H, and J–M) or twice (B–F and I) with similar results. Representative images (A–C, E, F, J, and K) or pooled data (G–I, L, and M) are shown.

Both PDGFR-β and PDGFR-α mediate the neuroprotective effect of PDGF-CC

PDGF-CC binds to and activates PDGFR-α and PDGFR-β (Li et al., 2000; Gilbertson et al., 2001; Cao et al., 2002; Li and Eriksson, 2003). Real-time PCR detected PDGFR-α and PDGFR-β expression in both the retina (Fig. 6 A) and primary cortical neurons (Fig. 6 B). PDGF-CC protein treatment up-regulated PDGFR-α and PDGFR-β expression (Fig. 6 B). Immunoprecipitation followed by immunoblotting assay showed that PDGF-CC stimulation led to PDGFR-β and PDGFR-α activation in different types of neuronal cells and tissues, including RGC5 cells, primary cortical neurons, and SN (Fig. 6, C and D). Immunofluorescent staining using antibodies against phosphorylated PDGFR-β or PDGFR-α detected activated PDGFR-β and PDGFR-α mainly in the RGC layer in the retina after PDGF-CC (500 ng/eye) intravitreal injection (Fig. 6 E), and also in Map2^{+} (neuronal marker) primary cortical neurons after PDGF-CC protein treatment (Fig. S1, D and E). In cultured primary cortical neurons, PDGF-CC protein treatment (50 ng/ml) inhibited the expression of several proapoptotic genes ($n = 6$; $P < 0.01$; Fig. 6 F), and up-regulated the expression of many neurotrophic/

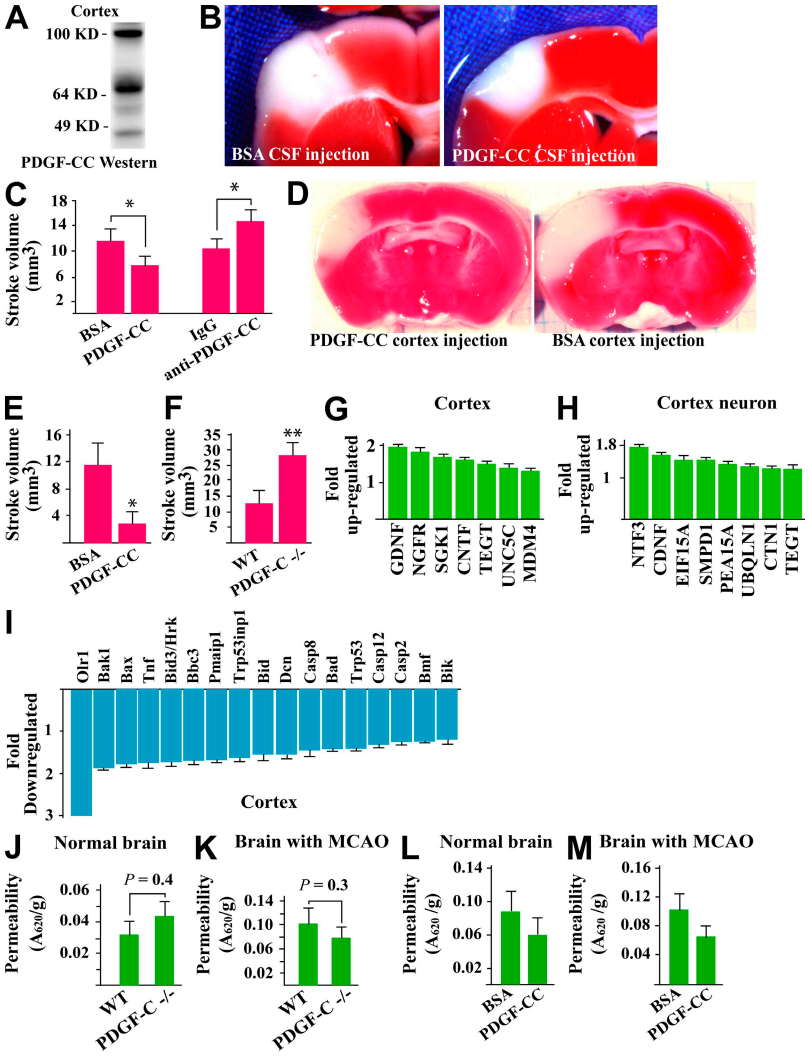

Figure 4. PDGF-CC protects brain cortical neurons from ischemia–induced neuronal death. (A) Western blotting detected PDGF-CC expression in brain cortex as several forms because of differential proteolytic processing. (B and C) PDGF-CC protein CSF injection decreased stroke volume, whereas CSF injection of PDGF-CC neutralizing antibody (anti–PDGF-CC) enlarged stroke volume ($n = 11$ or 13 mice). (D and E) PDGF-CC protein pretreatment by cortex injection decreased stroke volume significantly ($n = 10$ mice). (F) *PDGF-C*–deficient mice displayed greater stroke volume than that of wild-type mice ($n = 8$ mice). (G and H) Real-time PCR showed up-regulated expression of many neurotrophic/survival factors in brain cortex (G, $n = 6$ mice) and in isolated primary cortical neurons (H, $n = 4$ mice) after PDGF-CC treatment. (I) PDGF-CC treatment inhibited the expression of numerous apoptotic/cell death–related genes in brain cortex ($n = 8$ mice). (J and K) No difference in EB extravasation was found between *PDGF-C*–deficient and wild-type mice in normal brains (J, $n = 10$ mice) or brains with MCAO (K). (L and M) PDGF-CC CSF injection did not increase blood vessel permeability in normal brain (L, $n = 8$ mice) or in brains with MCAO (M, $n = 8$ mice). *, $P < 0.05$; **, $P < 0.01$. The data are represented as means ± SEM of the number of determinations. All experiments were repeated independently one to three times with similar results. Representative images (A, B, and D) and experiments are shown.

survival genes (n = 6; P < 0.05, 0.01, or 0.001; Fig. 6 G). However, this regulatory effect of PDGF-CC on gene expression in cortical neurons was largely abolished by both PDGFR-α and PDGFR-β neutralizing antibodies (n = 6; P < 0.05, 0.01, or 0.001; Fig. 6, F and G). Functionally, PDGFR-α or PDGFR-β neutralizing antibody treatment largely abolished the survival effect of PDGF-CC on RGCs in the ONC injury model in vivo (n = 8; P < 0.01; Fig. 6, H and I). In addition, PDGF-CC protein treatment led to significant Akt activation in cultured RGC5 cells (Fig. 7 A). Thus, the neuroprotective effect of PDGF-CC is mediated by both PDGFR-β and PDGFR-α.

PDGF-CC induces and inhibits GSK3β Ser^{9} and Tyr^{216} phosphorylation, respectively

To further investigate the intracellular signaling pathways induced by PDGF-CC, we screened a phospho–mitogen-activated protein kinase (MAPK) array to pinpoint the down-

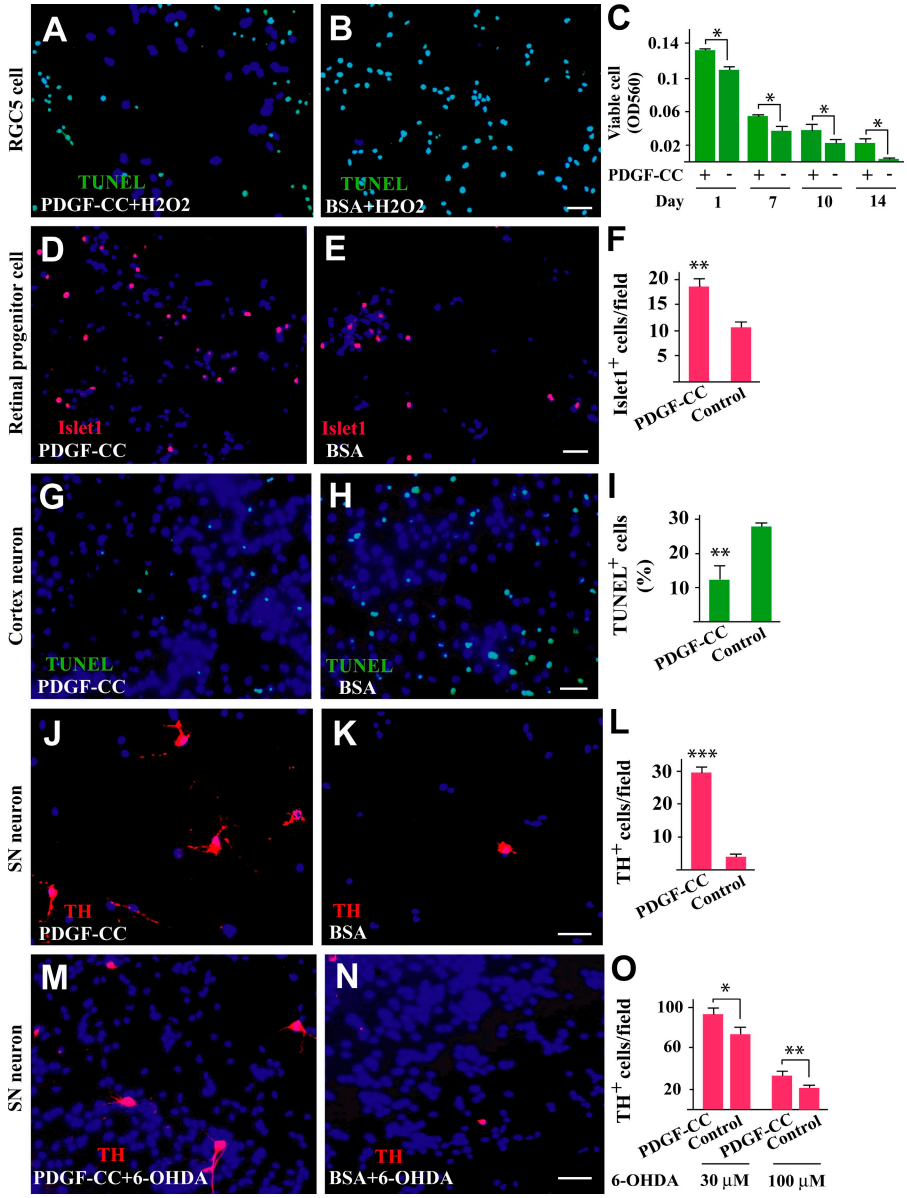

Figure 5. Direct protective effect of PDGF-CC on cultured neurons. (A–C) PDGF-CC treatment significantly decreased H_2O_2-induced apoptosis of the RGC5 cells measured by TUNEL staining (green; A and B) and increased their survival when the cells were cultured in serum-free medium (C). (D–F) PDGF-CC treatment increased survival of retinal neural progenitor cells (islet1$^+$; red) when cultured in serum-free medium. (G–I) PDGF-CC treatment decreased hypoxia- and glucose deprivation–induced apoptosis in primary cortical neurons measured by TUNEL staining (green). (J–L) PDGF-CC treatment increased survival of TH$^+$ neurons cultured in serum-free medium measured by TH staining (red). (M–O) PDGF-CC treatment increased survival of TH$^+$ neurons measured by TH staining (red) when the neurons were stressed with neurotoxin 6-OHDA. Bars, 10 μm. *, P < 0.05; **, P < 0.01; ***, P < 0.001. The data are represented as means ± SEM of the number of determinations. All experiments were repeated independently twice with similar results. Representative images and experiments are shown.

stream effectors. Out of many signaling molecules screened, PDGF-CC protein treatment (50 ng/ml for 10 min) increased GSK3β Ser^9 phosphorylation specifically in human umbilical vein smooth muscle cells (HUVSMCs; Fig. 7 B, top, arrows). It is known that GSK3β plays important roles in neuronal apoptosis (Liang and Chuang, 2007). GSK3β phosphorylation

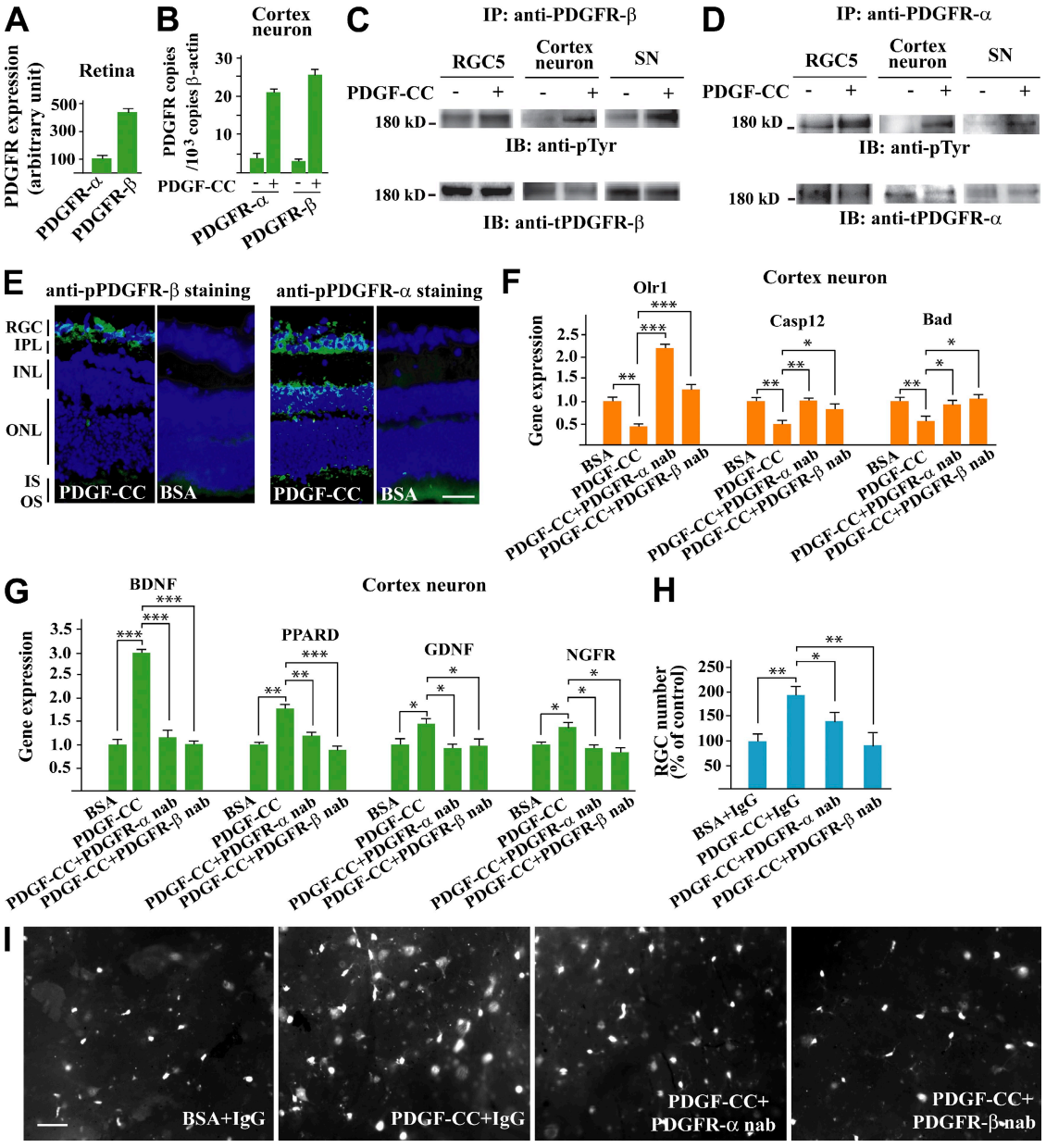

Figure 6. Both PDGFR-β and PDGFR-α mediate the neuroprotective effect of PDGF-CC. (A) PDGFR-α and PDGFR-β were expressed in the retina, with the PDGFR-β expression level being more abundant as measured by real-time PCR. (B) Real-time PCR detected PDGFR-α and PDGFR-β expression at a similar level in primary cortical neurons. PDGF-CC protein up-regulated their expression. (C and D) Immunoprecipitation followed by immunoblotting (antiphosphotyrosine antibody [anti-pTyr]) detected PDGFR-β (C) and PDGFR-α (D) activation by PDGF-CC in RGC5 cells, primary cortical neurons, and SN using total PDGFR-α/β as controls (anti-tPDGFR-α/β). (E) Immunofluorescent staining using antibodies against phosphorylated PDGFR-β and PDGFR-α detected activated PDGFR-β and PDGFR-α (green) mainly in the RGC layer in the retina after PDGF-CC treatment. Bar, 50 µm. (F) PDGF-CC treatment inhibited expression of several proapoptotic genes in cortical neurons. The effect of PDGF-CC was largely abolished by both PDGFR-β and PDGFR-α neutralizing antibodies ($n = 6$ mice). (G) PDGF-CC treatment up-regulated the expression of many neurotrophic/survival genes in cortical neurons. This effect of PDGF-CC was abolished by both PDGFR-β and PDGFR-α neutralizing antibodies ($n = 6$ mice). (H and I) In the ONC model, both PDGFR-α and PDGFR-β neutralizing antibodies abolished the survival effect of PDGF-CC on RGCs (white; $n = 8$ eyes). Bar, 20 µm. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The data are represented as means ± SEM of the number of determinations. All experiments were repeated independently once (A, B, and E–G) or twice (C, D, H, and I) with similar results. Representative images (C–E and I) and experiments are shown. IB, immunoblotting; IP, immunoprecipitation; IPL, inner plexiform layer; IS/OS, inner/outer segment; nab, neutralizing antibody.

at Ser[9] inhibits its apoptotic activity, whereas phosphorylation at Tyr[216] promotes its apoptotic activity (Liang and Chuang, 2007). We validated the phospho-MAPK screening data both in vitro and in vivo. In cultured PC12 and RGC5 neuronal cells, PDGF-CC protein stimulation led to GSK3β Ser[9] phosphorylation and GSK3β Tyr[216] dephosphorylation, respectively, in a time-dependent manner (Fig. 7, C and D). This observation was confirmed in vivo. PDGF-CC protein intravitreal injection (500 ng/eye) increased Ser[9] phosphorylation (Fig. 7 E) and decreased Tyr[216] phosphorylation (Fig. 7 F) in the retina, respectively, both of which are known to inhibit the apoptotic activity of GSK3β (Liang and Chuang, 2007). On the contrary, loss-of-function assay showed that PDGF-CC neutralizing antibody intravitreal injection led to increased GSK3β Tyr[216] phosphorylation in the retina (increasing the apoptotic activity of GSK3β; Fig. 7 G). Further, PDGF-CC protein treatment also down-regulated GSK3β expression in different types of neuronal tissues, including SN, retina (normal or with ONC/NMDA injury), RGC5 cells, and brain cortex with MCAO-induced ischemia, as measured by real-time PCR (Fig. 7 H).

To further verify whether the neuroprotective effect of PDGF-CC was achieved by inducing GSK3β Ser[9] phosphorylation, we expressed the wild-type or mutant human GSK3β, in which Ser[9] was mutated to alanine (GSK3β-WT or GSK3β-A9, respectively; Stambolic and Woodgett, 1994) in RGC5 cells. PDGF-CC protein treatment protected the RGC5 cells from H_2O_2-induced cell death in the control cells as well as in cells expressing GSK3β-WT ($n = 6$; $P < 0.001$ or 0.01; Fig. 7 I). However, the protective effect

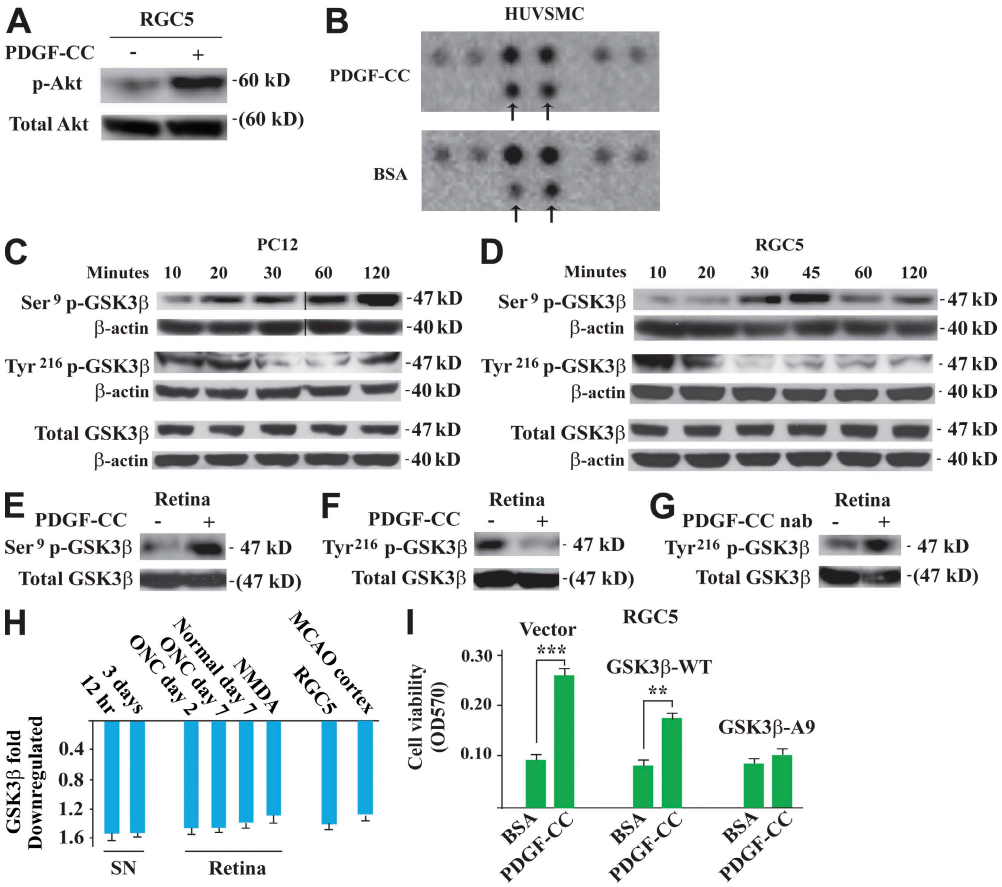

Figure 7. Neuroprotective effect of PDGF-CC is achieved by regulating GSK3β phosphorylation. (A) PDGF-CC protein treatment activated Akt significantly in cultured RGC5 cells. (B) In a phospho-MAPK array screening assay, PDGF-CC treatment increased GSK3β Ser[9] phosphorylation specifically (top, arrows) in HUVSMCs, whereas the phosphorylation of the other molecules remained unchanged. (C and D) In cultured PC12 (C) and RGC5 (D) neuronal cells, PDGF-CC protein treatment increased GSK3β Ser[9] phosphorylation and decreased GSK3β Tyr[216] phosphorylation, respectively, in a time-dependent manner. Black lines indicate that intervening lanes have been spliced out. (E and F) PDGF-CC protein treatment increased GSK3β Ser[9] phosphorylation (E) and decreased GSK3β Tyr[216] phosphorylation (F), respectively, in the retina in vivo. (G) PDGF-CC neutralizing antibody treatment increased GSK3β Tyr[216] phosphorylation in the retina in vivo. (H) PDGF-CC protein treatment inhibited GSK3β expression in different types of neuronal tissues/cells, including SN, retina (normal or with ONC/NMDA injury), RGC5 cells, and brain cortex with MCAO as measured by real-time PCR. (I) PDGF-CC protein treatment protected RGC5 cells from H_2O_2-induced cell death in the control cells and cells expressing wild-type GSK3β (GSK3β-WT). The protective effect of PDGF-CC diminished in the RGC5 cells expressing mutant GSK3β, in which Ser[9] was mutated to alanine (GSK3β-A9). **, $P < 0.01$; ***, $P < 0.001$. The data are represented as means ± SEM of the number of determinations. Experiments, except B, were repeated independently twice with similar results. Representative images (A and C–G) and experiments are shown. nab, neutralizing antibody.

of PDGF-CC diminished in the cells expressing GSK3β-A9 (lacking Ser^9 phosphorylation; $n = 6$; Fig. 7 I), demonstrating that PDGF-CC–induced GSK3β Ser^9 phosphorylation was required for its neuroprotective effect.

PDGF-CC at a dose effective for neuroprotection did not induce angiogenesis

Our previous work showed that PDGF-CC at a higher dose (4.5 μg/mouse/day × 7 d) promoted angiogenesis in pathological conditions in the heart (Dimmeler, 2005; Li et al., 2005). We therefore investigated whether PDGF-CC protein treatment at a dose effective for neuroprotection induced angiogenesis in neuronal tissues. CD31 (endothelial cell marker) and smooth muscle cell α-actin (smooth muscle cell marker) staining showed that 7 d after cortex injection of PDGF-CC protein (0.15 μg/0.5 μl PBS × three sites), there was no difference in blood vessel density in the stroke zone or the border zone surrounding the stroke region between PDGF-CC– and BSA-treated samples ($n = 5$; $P > 0.05$; Fig. S2, A–C). In the SN region, 2 wk after PDGF-CC protein injection (500 ng/SN), no difference in blood vessel density was found between PDGF-CC– and BSA-treated SN samples measured by CD31 staining ($n = 5$; $P > 0.05$; Fig. S2, D and E). Similarly, in the retina, 2 wk after PDGF-CC protein injection (500 ng/eye), no difference in blood vessel density was found between PDGF-CC– and BSA-treated retinae measured by collagen IV staining ($n = 6$; $P > 0.05$; Fig. S2, F–H). Thus, PDGF-CC at a dose effective for neuroprotection did not induce angiogenesis.

DISCUSSION

The PDGF family is best known as mitogens and chemoattractants for various types of mesenchyme cells, and for their critical roles during development (Andrae et al., 2008). However, their neuronal effects have been less explored. In this study, using several neuronal injury animal models and loss- and gain-of-function assays, we showed, for the first time to our knowledge, that PDGF-CC is a potent neuroprotective factor for various types of neurons by regulating GSK3β phosphorylation. PDGF-CC treatment rescued both retina and brain neurons from apoptosis in different animal models in vivo, and inhibited oxidative stress–, neurotoxin-, and serum deprivation–induced neuronal apoptosis in vitro. We further revealed a novel molecular mechanism underlying the neuroprotective effect of PDGF-CC. Both in vitro and in vivo, PDGF-CC treatment increased and decreased GSK3β Ser^9 and Tyr^{216} phosphorylation, respectively. Thus, because of its potent neuroprotective effect, PDGF-CC may potentially be used to treat neurodegenerative diseases. Suppression of the PDGF–PDGFR pathway for different clinical purposes should be conducted with caution to maintain normal neuronal function.

PDGF-CC is abundantly expressed in different types of neural tissues, including the brain, spinal cord, cochlea, etc. (Li et al., 2000; Aase et al., 2002; Hamada et al., 2002; Lee et al., 2004; Lei and Kazlauskas, 2008). In the inner ear neuronal cells, PDGF-CC and PDGF-AA, but not PDGF-BB, are expressed (Lee et al., 2004). Accumulating data have thus indicated a potential role of PDGF-CC in the neural system. However, direct evidence has been lacking thus far. In this study, we found that PDGF-CC was highly expressed in both retina and brain. *PDGF-C* deficiency by genetic gene deletion or shRNA gene knocking down exacerbated neuronal death in both retina and brain under pathological conditions. On the contrary, PDGF-CC protein treatment or gene delivery protected both retinal and brain neurons from apoptosis in different pathological conditions in vivo. It is worth noting that the neuroprotective effect of PDGF-CC is on several different types of neurons in different tissues, including RGCs, neuronal cells in the INL/ONL in the retina, dopaminergic neurons in SN, and brain cortical neurons. Thus, PDGF-CC is a broad-acting neuroprotective factor for different types of neurons. Further studies on the therapeutic potential of PDGF-CC protein, gene, and cell therapy to treat different types of neurodegenerative diseases are therefore warranted.

In this study, we found that both PDGFR-α and PDGFR-β are expressed in different types of neurons. PDGF-CC protein stimulation activated both PDGFR-α and PDGFR-β in the retina and brain in vivo, and in cultured neuronal cells in vitro. Furthermore, PDGFR-β and PDGFR-α neutralizing antibodies largely abolished the neuroprotective effect of PDGF-CC on RGCs in vivo and the regulatory effect of PDGF-CC on gene expression in vitro. Thus, the neuroprotective effect of PDGF-CC was mediated by both PDGFR-β and PDGFR-α. We further explored the intracellular signaling pathway induced by PDGF-CC. Using a phospho-MAPK array, we found that out of numerous different signaling molecules screened, PDGF-CC specifically induced GSK3β Ser^9 phosphorylation. It is known that GSK3β is a proapoptotic gene playing important roles in neuronal apoptosis (Liang and Chuang, 2007). GSK3β phosphorylation at Ser^9 inhibits its apoptotic activity, whereas phosphorylation at Tyr^{216} promotes its apoptotic activity (Liang and Chuang, 2007). Indeed, we validated the phospho-MAPK screening data both in vitro and in vivo. In cultured neuronal cells and in mouse retinae, PDGF-CC protein stimulation increased and decreased GSK3β Ser^9 and GSK3β Tyr^{216} phosphorylation, respectively, indicating that the neuroprotective effect of PDGF-CC was achieved by regulating GSK3β phosphorylation. Indeed, this observation was confirmed by a loss-of-function assay. When the GSK3β Ser^9 was mutated to an alanine and the mutant GSK3β-A9 was expressed in RGC5 cells, the protective effect of PDGF-CC on RGC5 cells was abolished, whereas PDGF-CC protected the RGC5 cells expressing GSK3β-WT from H_2O_2-induced cell death, demonstrating that the neuroprotective effect of PDGF-CC was achieved by regulating GSK3β phosphorylation.

It was recently reported that intraventricular injection of PDGF-CC protein increased cerebrovascular permeability in normal mice (Su et al., 2008). It was therefore hypothesized that blocking the PDGFR–PDGF-CC pathway may extend the therapeutic time window of tissue plasminogen activator,

which can activate the latent form of PDGF-CC (Fredriksson et al., 2004; Rieckmann, 2008; Su et al., 2008). This study (Su et al., 2008) is of significant importance as it may lead to a therapeutic breakthrough in stroke treatment. Based on this study, a clinical trial was planned to treat stroke patients with imatinib (Gleevec) to block the PDGFR pathways (Rieckmann, 2008; Su et al., 2008). In our current study, using *PDGF-C*–deficient mice as a genetic tool, no difference in EB extravasation was found between *PDGF-C*–deficient and wild-type mice in the retinae, normal brains, or brains with MCAO-induced ischemia, suggesting that PDGF-CC has a negligible role in inducing blood vessel permeability in these tissues. In support of this, PDGF-CC protein injection into vitreous, SN, normal brain, or brain with MCAO-induced ischemia did not change EB extravasation in the eye or brain. Instead, PDGF-CC protein or gene delivery into these tissues decreased neuronal death in the retina and reduced stroke volume in the brain. Our observations were further confirmed by loss-of-function assays, because CSF injection of a neutralizing antibody against mouse PDGF-CC enlarged stroke volume. In summary, using both genetic and pharmacological tools, our data showed that PDGF-CC has a negligible role in inducing blood vessel permeability while having a potent neuroprotective effect. On the other hand, it is known that differences in the degree and timing of PDGFR activation can lead to different biological effects. Thus, we cannot exclude the possibility that the difference between our results and the previous ones (Su et al., 2008) on the role of PDGF-CC in inducing blood vessel permeability may relate to different controls used, experimental conditions, animal models, or proteins used. Further studies are needed to verify this.

Our previous work showed that PDGF-CC at a relatively high dose (4.5 μg/mouse/day for 7 d) promoted angiogenesis in pathological conditions in the heart (Dimmeler, 2005; Li et al., 2005). There is therefore a possibility that the neuroprotective effect of PDGF-CC could be an indirect effect via its vascular effect by improving blood supply. In this study, using an acute neural toxin NMDA-induced retinal neuron injury model in which only neurons were injured and the vascular system was not affected, and in which PDGF-CC treatment lasted only for 24 h (a time period too short for new blood vessels to grow), PDGF-CC treatment rescued retinal neurons from apoptosis, suggesting a direct neuroprotective effect of PDGF-CC on neurons. This notion was further supported by neuronal culture experiments in which PDGF-CC treatment rescued cultured primary neurons from apoptosis when the neurons were challenged by hypoxia, glucose/serum deprivation, or neurotoxin injury. In addition, although genome-wide gene profiling revealed a regulatory effect of PDGF-CC on the expression of numerous apoptotic/cell death–related and survival/neurotrophic genes, it did not significantly affect the expression profile of angiogenic/proangiogenic genes (unpublished data). Thus, PDGF-CC at a dose effective for neuroprotection did not induce angiogenesis in the brain or retina.

In summary, using different techniques and approaches, we showed, for the first time to our knowledge, that PDGF-CC is a potent neuroprotective factor and is critical for neuronal survival. We further revealed a novel molecular mechanism underlying the neuroprotective effect of PDGF-CC. That is, PDGF-CC achieves its neuroprotective effect by regulating GSK3β phosphorylation. Thus, PDGF-CC may have therapeutic values in treating neurodegenerative diseases. Interference of the PDGFR pathway for different therapeutic purposes should be conducted with caution.

MATERIALS AND METHODS

ONC and NMDA retina injury models. All animal experiments were approved by the Animal Care and Use Committee at the National Eye Institute, National Institutes of Health (NIH; animal study protocols 06-553, 06-570, and 07-608), and were performed according to NIH guidelines and regulations on animal studies. The ONC and NMDA retinal injury models were performed and results were analyzed as described previously (Li et al., 2008). *PDGF-C*–deficient mice were described previously (Ding et al., 2004) and were bred onto a C57BL/6 background for more than six generations, and littermates were used for experiments. For PDGF-CC protein or shRNA treatment in the ONC injury model, 0.5 μg/μl × 1 μl of active rhPDGF-CC protein (Li et al., 2000) or shRNA (1 μg/eye; Open Biosystems) was injected into mouse vitreous once a week for 2 wk. In the NMDA injury model, 0.5 μg/μl × 1 μl of PDGF-CC protein was injected into mouse vitreous. For PDGFR blocking assay, PDGFR-α or PDGFR-β neutralizing antibody (5 μg/eye; R&D Systems) was injected into mouse vitreous once a week for 2 wk.

Permeability assay. EB extravasation permeability assay in different tissues was performed as previously described (Su et al., 2008). For brain permeability assay, anesthetized C57BL/6 mice were placed onto a stereotactic frame. Active rhPDGF-CC core domain protein (3 μl of 3 μM; Li et al., 2000) or the same amount of BSA was intraventricularly injected into CSF at a position of bregma −2 mm, mediolateral 0 mm, and dorsoventral 2 mm. Cerebrovascular permeability was determined at different time points as previously described (Su et al., 2008). In brief, mice were intravenously injected with 100 μl of 4% EB (Sigma-Aldrich) at different time points after MCAO. 1 h after EB injection, animals were perfused with PBS for 4 min, and brains were removed and separated into hemispheres ipsilateral and contralateral to the MCAO. Each hemisphere was then homogenized in N,N-dimethylformamide (Sigma-Aldrich), and EB extravasation was measured as previously described (Su et al., 2008). For retina permeability assay, active rhPDGF-CC protein (500 ng/eye; Li et al., 2000) or the same amount of BSA was injected into mouse vitreous, and retina permeability was measured at different time points as previously described (Su et al., 2008).

Parkinson's disease model. The neurotoxin-induced Parkinson's disease model was performed using 8–10-wk-old female mice as described previously with some modifications (Lindholm et al., 2007). 2 μl 6-OHDA (4 μg/μl in 0.2 mg/ml ascorbate-saline; Sigma-Aldrich) was injected into the right medial forebrain bundle at 1.9 mm posterior to the bregma, 1 mm lateral to the midline, and 4.4 mm below the dura. 1–3 wk after 6-OHDA injection, mice received intraperitoneal injection of D-apomorphine (0.05 mg/kg) and were allowed to habituate in a box with a flat round bottom (24 cm in diameter) for 5 min, and the contralateral turning was counted during the following 2 min. To test the effect of PDGF-CC, PDGF-CC protein or BSA control (500 ng/mouse) was injected into the right SN at 3.3 mm posterior to the bregma, 1.35 mm lateral to the midline, and 4.4 mm below the dura 30 min before 6-OHDA injection. For *PDGF-C* gene or shRNA delivery, a *PDGF-C* expression construct (PDGF-CC core domain cloned into a pLenti-6/V5 vector; Invitrogen) or shRNA (2 μg of plasmid in 0.5 μl PBS) together with the in vivo transfection reagent (Jet-PEI; Polyplus-transfection) were injected into SN 1 wk before 6-OHDA injection. Brains were harvested at different time points (3 d to 3 wk after

6-OHDA injection) and subjected to subsequent analysis. TH staining using a commercial anti-TH antibody (Abcam) in both SN and ST was visualized using AxioVision software (Carl Zeiss, Inc.).

Cerebral ischemic stroke model. Focal cerebral ischemia was produced by permanent MCAO as described previously (Li et al., 2008). For PDGF-CC protein treatment, 3 µl of 3 µM active rhPDGF-CC protein or the same amount of BSA in PBS was injected into CSF. For PDGF-CC neutralizing antibody treatment, 3 µl of 0.4 µg/µl of neutralizing antibody against mouse PDGF-CC or the same amount of IgG in PBS was injected into CSF. 1 h after protein or neutralizing antibody injection, MCAO was performed and stroke volume was measured 24 h after MCAO. For PDGF-CC protein injection into cortex, a skin incision was made at the top of the head of the anesthetized mouse. Three small openings (1 mm in diameter) were made in the skull at −1, 0, and 1 mm from the bregma to the lambda and 1 mm left from bregma. A fine needle (33 gauge) was inserted to a depth of 1.2 mm from the skull surface, and 0.3 µg of rhPDGF-CC core domain protein (Li et al., 2000) or BSA in 0.5 µl PBS was injected over 5 min into each opening and the skin was sutured. 1 wk after PDGF-CC protein injection, MCAO was performed and stroke volume was measured 24 h after MCAO. We chose to inject the PDGF-CC protein 7 d before MCAO because it allowed us to compare the neuroprotective effect of PDGF-CC with that of PDGF-BB, which was shown to be effective when administered 7 d before MCAO (Sakata et al., 1998).

Microarray analysis. 7 d after the ONC injury and PDGF-CC protein treatment, retinae were harvested and total RNA was isolated using TRIzol reagent (Invitrogen) followed by RNeasy Mini kit (QIAGEN) purification according to the manufacturer's instructions. Microarray assay was performed using Mouse-6 Expression BeadChips (Illumina Inc.) containing ~48,000 mouse transcripts. Three biological repeats were included in the microarray assay. A two-tailed Student's *t* test was used for statistical analysis of gene expression data. Functional grouping of the differentially expressed genes was performed using several different tools, including the WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt) and the Ingenuity Pathways Analysis (https://analysis.ingenuity.com/pa/login/login.jsp) programs. Microarray data have been deposited in the Gene Expression Omnibus under accession no. GSE19207.

Cell culture, isolation of primary neurons from brain and retina, and survival assay. Rat RGC-derived cell line RGC5 cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO_2. For cell survival/viability assay, RGC5 cells were starved in serum-free medium for 3 d. Cells were seeded in a 96-well tissue culture plate (BD) and allowed to attach for 24 h in serum-free medium. The medium was replaced with 100 µl of serum-free medium supplemented with 50 ng/ml of rhPDGF-CC core domain protein (Li et al., 2000). Serum-free medium with 50 ng/ml BSA was used as a negative control. Cell-culture medium with 10% FBS was used as a positive control. Cell viability was evaluated using the methylthiazolyldiphenyl-tetrazolium bromide method according to the manufacturer's protocol (Invitrogen) at different time points. Or, RGC5 cells were stressed with 2.5 mM H_2O_2 for 1 h with or without 50 ng/ml of PDGF-CC protein treatment. Apoptosis was investigated after 24 h using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Cortical neurons were isolated from 1-d-old neonatal mice as previously described (Li et al., 2008) and collected into Neurobasal A media (Invitrogen) with 0.1% EDTA and digested with trypsin (Invitrogen) for 30 min at 37°C, followed by density gradient centrifugation with 7, 9.4, 11.9, and 16.4% Optiprep (Axis-Shield). Neurons positioned between the second and third layers were collected and cultured in Neurobasal A for subsequent analysis. Neuronal survival experiments were performed within 10 d of culture. For neuronal survival assay, neurons were cultured in serum-free medium without glucose in 1% oxygen at 37C° for 6 h. TUNEL assay was performed as previously described (Li et al., 2008).

Neural cells from SN and retina were isolated from E13.5 and P3 mice, respectively. Tissues were treated with 12 U/ml papain with 2 U/ml DNase I (Sigma-Aldrich) in artificial CSF (124 mM NaCl, 5 mM KCl, 3.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose) for 20 min and washed with culture medium (1:1 of DMEM/F12, supplemented with 10% FBS and 100 U/ml penicillin/streptomycin; Invitrogen). The cells were plated into polyornithine-coated dishes at 5×10^5 cells/cm^2. After 3 h, medium was changed to Neurobasal A with or without B27 (Invitrogen). The neural progenitor cells were cultured in serum-free medium with or without 50 ng/ml of PDGF-CC protein for 7 d and subjected to anti-TH or TUNEL staining. Or, the SN-derived cells were treated with 30 or 100 µM 6-OHDA for 24 h and subjected to anti-TH staining. Images were taken from eight randomly selected microscopic fields and TH^+ cells were counted. All of the experiments were performed in triplicate and were repeated at least once.

Real-time PCR, in situ hybridization, and immunofluorescent and TUNEL staining. For real-time PCR assay, total RNA was isolated using the RNeasy Mini kit according to the manufacturer's instructions. 3 µg of total RNA was used for cDNA synthesis using the SuperMix kit (Invitrogen) and was used for the real-time PCR reaction using a sequence detection system (ABI Prism 7500 HT; Applied Biosystems). All experiments were performed in triplicate and repeated at least once. Primers used are listed in Table S1. Immunofluorescent staining and in situ hybridization were performed as previously described (Li et al., 2000). TUNEL assay was performed according to the manufacturer's protocol (Roche). Antibodies used for immunofluorescent staining were anti-islet1 (clone 39.4D5; Developmental Studies Hybridoma), anti-TH, anti–phosphorylated PDGFR-β (Santa Cruz Biotechnology, Inc.), anti–phosphorylated PDGFR-α (Santa Cruz Biotechnology, Inc.), rabbit anti–mouse collagen IV polyclonal antibody (AbD Serotec), and anti–PDGF-CC (Li et al., 2000). For in situ hybridization, a mouse *PDGF-C* antisense riboprobe and 12-µm frozen tissue sections were used. A sense riboprobe was used as a negative control. The riboprobes were prepared using T7 and T3 RNA polymerases and digoxigenin-11-dUTP (Roche) according to the manufacturer's instructions.

PDGFR-α, PDGFR-β, and Akt activation/expression assay and Western blotting. PDGFR and Akt activation assays were performed as previously described (Li et al., 2000). In brief, for in vitro receptor and Akt activation assays, cultured cells were stimulated with the active form of rhPDGF-CC protein (Li et al., 2000) at 50 ng/ml for 10 min and cell lysate was subjected to further analysis. For in vivo receptor activation assays, PDGF-CC protein was injected into mouse vitreous or SN (500 ng/µl × 1 µl), and tissues were harvested after 20 min to 2 h for receptor activation assay. For immunoprecipitation assay, cell lysates were incubated with anti–PDGFR-α (Santa Cruz Biotechnology, Inc.) or anti–PDGFR-β antibody (Santa Cruz Biotechnology, Inc.) overnight at 4°C and were precipitated with immobilized protein G (Thermo Fisher Scientific). Immunoprecipitated samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane and were incubated with an antiphosphotyrosine antibody (PY99; Santa Cruz Biotechnology, Inc.). Other antibodies used for Western blot assays were anti–mouse PDGF-CC (R&D Systems), anti-TH, and monoclonal anti–β-actin conjugated with horseradish peroxidase (Sigma-Aldrich).

Phospho-MAPK antibody array screening and GSK3β phosphorylation and expression assays. For phospho-MAPK antibody array screening, HUVSMCs were cultured as instructed (American Type Culture Collection), serum starved overnight, and treated with 50 ng/ml of the active form of rhPDGF-CC protein for 10 min. The cells were then processed for the human phospho-MAPK array assay according to the manufacturer's protocol (R&D Systems). The processed array membrane was scanned and quantified using an image reader (LAS-3000) and Multi Gauge software (Fujifilm). For in vivo GSK3β phosphorylation assay, 500 ng/ml of rhPDGF-CC protein was injected into mouse vitreous, and the retinae were harvested after 1–24 h for Western blot assay. The GSK3β-WT and GSK3β-A9 expression constructs were a gift from J.S. Gutkind (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD). Antibodies used for Western blotting were anti-GSK3α/β (R&D Systems), anti–phospho-GSK3α/β (S21/S9; R&D Systems), and anti–phospho-GSK3α/β (Tyr279/Tyr216; Millipore).

Statistics. A two-tailed Student's *t* test was used for statistical analysis. Differences were considered statistically significant at $P < 0.05$. The data are represented as means ± SEM of the number of determinations. Assays using cultured cells were performed in triplicate.

Online supplemental material. Fig. S1 shows that *PDGF-C* shRNA treatment decreased *PDGF-C* expression level in the SN (A), *PDGF-C* gene delivery into SN led to *PDGF-C* overexpression (B), PDGF-CC protein SN injection did not change blood vessel permeability (C), and immunofluorescent staining detected phosphorylated PDGFR-α and PDGFR-β colocalized with $MAP2^+$ cortical neurons (D and E). Fig. S2 shows that PDGF-CC treatment did not induce angiogenesis in the stroke zone or the border zone surrounding the stroke area (A–C), in the SN (D and E), or in the retinae (F–H). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091704/DC1.

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