N-Methyl-D-Aspartic acid suppresses Akt activity through protein phosphatase in retinal ganglion cells

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Purpose: To investigate the relationship between Akt activity and retinal ganglion cell (RGC) death induced by N-Methyl-D-Aspartic acid (NMDA) in the rat retina.

Methods: Two microlitres of 1, 10, 50, 100, or 200 mM NMDA, or vehicle was injected into the vitreous cavity of Sprague-Dawley (SD) rats (n=125). Retinal damage was estimated by counting ganglion cells labeled with fluorochrome and retinal apoptosis was detected by TUNEL. Akt activity was determined by immunohistochemical analysis with a specific antibody to the activated (phosphorylated) form of Akt. To investigate the mechanism of dephosphorylation of Akt, Okadaic acid, a potent protein phosphatase inhibitor, was injected 1 h before NMDA injection and accessed the number of phosphorylated Akt positive cells 1 h after NMDA injection. To stimulate Akt activity in the retina, brain derived neurotrophic factor (BDNF) was injected into the vitreous 15 min before NMDA injection.

Results: Immunohistochemical analysis revealed a reduction in phosphorylated Akt in RGCs and amacrine cells one hour after NMDA injury. The RGCs and amacrine cells showed TUNEL positivity at 6 h and a decrease in cell number at 7 days after NMDA injury. No other cells in the retina stained positive with phosphorylated Akt antibody and TUNEL. Okadaic acid prevented the dephosphorylation of Akt by NMDA. The exogenous administration of BDNF prevented the dephosphorylation of Akt in N-Shc/ShcC-positive RGCs and significantly suppressed the NMDA-induced RGC death.

Conclusions: These observations suggest that Akt is one of the key signaling proteins in RGC death induced by NMDA, and that the presence of N-Shc/ShcC enhances BDNF-mediated neuroprotection via phosphorylated Akt. The regulation of phosphorylated Akt by growth factors and protein phosphatase activity may play an important role in cell fate following NMDA injury. Thus, an increase in phosphorylated Akt may have potential therapeutic implications in the treatment of glutamate-related disease.

Excitotoxicity-based neural degeneration by glutamate is linked to many neurodegenerative and ischemic diseases in the central nervous system (CNS) [1-3]. In the retina, excitotoxicity is believed to play an important role in retinal ischemia-reperfusion injury [4,5]. Glutamate is a major excitatory neurotransmitter in the retina and its effects are mediated by the ionotropic receptors, N-methyl-D-Aspartic acid (NMDA) receptor, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)/kinate receptor or by metabotropic receptors [6]. Among the glutamate receptors, the NMDA receptor’s role in cell death has been extensively studied; excessive doses of NMDA induce apoptotic cell death of retinal ganglion cells (RGC) and amacrine cells [7] and NMDA receptor antagonists are protective against ischemia-reperfusion induced cell death [8]. These reports underline the importance of better understanding NMDA-induced death signaling in preventing excitotoxic retinal disease.

A variety of growth factors, including brain-derived neurotrophic factor (BDNF) [9], ciliary neurotrophic factor (CNTF) [10], and interleukin-6 (IL-6) [11], have been reported to protect retinas from glutamate induced injury. However, the mechanistic functions of these growth factors on NMDA injury are unknown. These growth factors are known to activate mitogen activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathways in the neuron [12-14]. In addition, Akt protects neurons from apoptosis in the injured CNS [12,13,15,16], and the activation of Akt induces the phosphorylation of Bad, AFX, caspase 9, endothelial NOS, inhibitor of NF kappaB, and GSK3 β and suppresses their apoptotic effects [12-14]. We have previously shown that BDNF induces the phosphorylation of the adaptor protein, Shc (Src Homology and Collagen) C, in the axotomized RGCs [17]. In its function as an adaptor protein, ShcC binds to phosphorylated trkB receptor and subsequently activates Akt in the CNS [18,19]. The ShcC-Akt pathway downstream of BDNF may therefore be essential in the protection of retinas from injury [20].

Akt activity depends on its phosphorylation, which is positively regulated by PI3K [12-14] and negatively regulated by a class of protein phosphatases (PPs) in tissues [21]. We have recently demonstrated the localization of PPs, including PP-1, PP-2A, and PP-2B, in the retina and specifically to the RGCs [22]. However, the identity of Akt phosphatases in the retina...
remains unclear. Thus, identifying of the differential effect of Akt PPs in the retina may be important in better understanding the role of Akt.

In this study, we examined the roles of Akt in NMDA-induced retinal cell death. NMDA caused the dephosphorylation of Akt in the RGC and amacrine cell through PP activation, presumably through PP-2A activation. Furthermore, dephosphorylation of Akt was associated with TUNEL positivity. In addition, the application of exogenous BDNF prevented NMDA-induced dephosphorylation of Akt in RGC and also prevented RGC death. This protective effect was more evident in N-Shc/ShcC-positive neurons.

**METHODS**

**Animals and surgical procedures:** Male Sprague-Dawley (SD) rats weighing 300-350 g were used in this study. All animals were maintained and handled in accordance with the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal injury was induced by intravitreal injection of NMDA. Under general anesthesia with 50 mg/kg pentobarbital sodium, 2 µl of 1, 10, 50, 100, or 200 mM NMDA was injected with a Hamilton syringe attached to a 32 G needle at 1 mm away from the superior limbus over a 3 min interval. In a subset of animals assigned to study the effect of the drug, BDNF (1 µg in 3 µl of phosphate buffered saline [PBS]), okadaic acid, or FK-506 was injected into the vitreous cavity one hour before NMDA injection. PBS containing 5% dimethylsulfoxide (DMSO) was injected in control animals.

FK506 (Calbiochem, Darmstadt, Germany), an inhibitor of PP-2B, was dissolved in DMSO to a concentration of 0.02, 0.2, and 2 mM and Okadaic acid (Calbiochem), an inhibitor of PP-1 and PP-2A, was dissolved in DMSO to a concentration of 2, 20, or 200 µM. FK506 was subsequently diluted in PBS (1:19) to a final concentration of 1, 10, or 100 µM. Okadaic acid was diluted in PBS (1:19) to a final concentration of 0.1, 1, 10 µM. Two micro liters of the above final solution were injected in the vitreous cavity.

**Labeling and counting of retinal ganglion cells (RGC):** To examine the change in the number of RGCs following NMDA injection, RGCs were retrogradely labeled with a fluorescent tracer, fluorochrome (Fluorogold, Englewood, CO). Labeling was performed 3 days before animals were sacrificed. The animals were anesthetized with a ketamine/xylazine mixture containing 2% aqueous fluorochrome in 1% DMSO and then transferred onto a polyvinyl fluoride membrane (Bio-Rad). After blocking, the membrane was incubated in a blocking buffer containing rabbit anti-pan Akt antibody (dilution 1:1000; Cell Signaling Technology, Inc., Beverly, MA) overnight at 4 °C. Chemiluminescence was detected using an alkaline phosphatase-conjugated anti-rabbit IgG (dilution 1:20000; Promega Corp., Madison, WI) and with CDP-star as a substrate (Amersham Pharmacia Biotech, Buckinghamshire, UK). The blot was exposed to Medical X-ray film (RX-U, Fuji Film, Japan). After blocking the stripped membrane, the same membrane was incubated in the blocking buffer containing mouse anti-α-tubulin antibody (dilution 1:4000; Sigma) overnight at 4 °C. The density of each protein band was determined with the digital scanner and the Multi-Analyst software (version 1.0.2, Bio-Rad). To ensure that the density of each protein band was not saturated, the density of the bands was measured after incremental increases in exposure times, and the values were analyzed to determine an adequate exposure time (data not shown).

**Immunohistochemistry:** Immunohistochemistry was performed as described previously [17,20,24]. Briefly, surgically removed retinas were fixed with 4% PFA at 4 °C overnight and then cryoprotected with PBS (0.1 M phosphate buffer [pH 7.4]; 0.15 M NaCl) containing 20% sucrose. The cryo-sections (10 µm) were mounted onto slides and incubated with blocking buffer (PBS containing 10% goat serum, 0.5% gelatin, 3% BSA, and 0.2% Tween20), then were incubated with a primary antibody against phospho-Akt (CST, 1:100) or Akt-1 (Santa Cruz Biotechnology, Inc., clone B-1, 1:200). Normal rabbit serum (DAKO, Osaka, Japan) was used as the negative control. The sections were then incubated with fluorescent-conjugated secondary antibody for 1 h. These included goat anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG conjugated to either Alexa TM 546 or Alexa TM 488 (Molecular probes), all diluted 1:200 in blocking buffer. Sections were mounted with Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA). Photomicrographs were taken 1 mm from the center of the optic nerve with fluorescence microscopy (Qfluoro system, Leica Microsystems, Wetzlar, Germany) with a UV filter (DAPI) and a Rhodamine filter (TUNEL staining).

**TUNEL staining:** TUNEL staining was performed according to the manufacturer’s protocols (ApopTag Red kits, Intergen, Purchase, NY) to detect retinal cell death induced by NMDA [24]. The retinas, which were obtained 0, 1, 3, 6, and 24 h after injury (n=5 each), were fixed, cryoprotected, and mounted as mentioned above. After twice washing with PBS, sections were incubated with TdT enzyme at 37 °C for 1 h. The sections were washed 3 times in PBS for 1 min and incubated with anti-digoxigenin conjugate (Rhodamine) in a humidified chamber for 30 min at room temperature (RT), followed by three rinses with 0.1 M PBS at RT. Sections were mounted with Vectashield mounting media including DAPI.
for nuclear stain (Vector Laboratories). When we analyzed the number of TUNEL-positive cells on retinal slice, the number of cells in GCL was already different between the locations. To minimize the various in cell number, we chose the area of same distance from the optic disc. Photomicrographs were taken 1 mm from the center of the optic nerve with the QFluoro system (Leica Microsystems, Wetzlar, Germany) with a UV filter (DAPI) and a Rhodamine filter (TUNEL). TUNEL-positive cells in the INL and GCL were counted in an area 0.2 mm² that was 1 mm from the center of the optic nerve for each microscopic section (n=5). The data presented represent the mean number of TUNEL-positive cells per square millimeter.

Statistics: The data from the experimental and control groups were analyzed with unpaired t-test for comparison between two groups or Scheffes post hoc test following ANOVA in multi-groups analysis with StatView 4.11J software for Macintosh (Abacus Concepts Inc., Berkeley, CA). A significance level was set at p<0.05 (*) and p<0.01 (**). All values are expressed as the mean±standard deviation (SD).

RESULTS
Time course change of fluorochrome labeled RGCs following NMDA injury: Previous reports suggest that the intravitreal administration of glutamate is taken up in the retinal Müller cell and detoxified by glutamine synthetase [25,26]. NMDA was used in the experiment as an excitotoxic agent in the retina instead of glutamate, which induced retinal injury mainly in RGC and amacrine cells in retina [7,9,11,27]. Two hundred nmol of NMDA causes damage to the retina and decreases the number of cells in the GCL and thickness of the inner plexiform layer (IPL) [7,28]. Although the toxicity of NMDA is well characterized in the retina, the cell types affected by NMDA treatments in the GCL have not been identified. In this study, we examined the NMDA-induced damage to RGCs and investigated the molecular mechanisms underlying NMDA toxicity. In our study, it was difficult to identify RGC in the GCL in the presence of displaced amacrine cells, which looked similar in size to RGC in transverse retinal sections. To overcome this problem, the retrograde fluorochrome (Fluorogold) labeling method, which specifically labels RGCs, was utilized to identify surviving RGCs [11]. We defined the surviving RGCs as the cells capable of transporting the fluorochrome in a retrograde fashion. We injected 3 µl of 2% fluorochrome into the optic nerve three days before sacrificing the animals. The density of fluorochrome-labeled RGCs was 2122±237 cells/mm² (n=15) for the vehicle-treated control animals. The above findings are similar to those observed following the labeling of RGCs by the injection of fluorochrome in the superior colliculus [20,24,29]. Five days after NMDA injection, the

Figure 1. Time course change and dose response curve of fluoro-chrome labeled RGCs following NMDA injury. Representative photographs of flat mounted fluorochrome labeled RGCs at various time points following 200 nmol NMDA injection (A-E) and quantitative data of time course of surviving RGCs (F) and the dose-response curve 7 days after NMDA injection (E). A: Vehicle control. B: 5 days. C: 7 days. D: 10 days. E: 14 days. The density of RGC decreased significantly 5 days after NMDA injection and there was no further RGC loss from 7 to 14 days. 2, 20, and 200 nmol of NMDA induced significant loss of RGC compared to vehicle control (unpaired t-test) and there was no significant difference between 20 and 200 nmol of NMDA.
RGC density decreased significantly to 1714±368 cells/mm² (n=13, p<0.0001), compared to vehicle-control RGC density (Figure 1F). The RGC density at 7, 10, and 14 days after NMDA injection decreased to 706±116 (n=6, p<0.0001), 591±65 (n=10, p<0.0001), and 696±106 (n=6, p<0.0001). Examinations over longer intervals (7 to 14 days after NMDA treatment) did not increase RGC loss. A dose-response curve following NMDA-injury to RGCs at different concentrations of NMDA (2, 20, 200 nmol) 7 days following NMDA treatment is shown in Figure 1G. The density of RGC was significantly decreased to 1811±192 (2 nmol, n=8, p=0.0049), 488±118 (20 nmol, n=7, p<0.0001), and 480±132 (200 nmol, n=15, p<0.0001). There was no significant difference between 20 and 200 nmol of NMDA treatment.

**Immunohistochemical analysis of Akt-1 and phosphorylated Akt in the retina one hour after NMDA injury:** To investigate the relationship between Akt activation and NMDA injury in RGCs, the phosphorylation of Akt was examined by immunohistochemistry with anti-phosphorylated Akt antibody, which specifically recognizes the phosphorylated site (Ser473) of Akt. Phosphorylated Akt has previously been reported to be the active form of Akt [12]. Without NMDA injection, im-

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Figure 2. Akt and phosphorylated Akt positive cells in NMDA-treated retina.

A-H: Representative photographs showing the immunoreactivity of Akt (AKT; A-D) and phosphorylated Akt (p-Akt; E-H) in rat retina treated with vehicle control, phosphate buffer (PB; A,B,E,F) or 200 nmol NMDA (C,D,G,H) 1 h after injection (PB1h, NMDA1h). B,D,F,H show DAPI nuclear staining corresponding images to A,C,E,G, respectively. Arrows in E,G indicate the bipolar cell layer. NMDA induced dephosphorylation of Akt in GCL and inner margin of INL (G, arrowhead). The ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), outer segment (OS), retinal pigment epithelium (RPE) are identified. J: Representative photographs of western-blot of rat retina treated with different doses of NMDA (2 to 400 nmol) 1 h after injection, untreated control (Control) and vehicle control with phosphate buffer (PB). The intensity of band with Akt antibody (Akt) was not changed by different doses of NMDA. I: Bar chart shows the quantitative data of p-Akt positive cells in GCL 1 h after different doses of NMDA. The number represents the percentage of p-Akt positive cells in all DAPI-positive cells in the GCL. P-Akt positive cells decreased significantly after 20 nmol or more of NMDA injection. Asterisk indicates statistically significant difference compared to the vehicle control (ANOVA followed by Scheffe post hoc test).
Figure 3. Double staining with phosphorylated Akt antibody and TUNEL in NMDA-treated retina. A-T: Representative photographs of double staining with phosphorylated Akt antibody (p-Akt) and TUNEL 1 h after vehicle control (PB 1h) or 200 nmol NMDA injection at different time points. A-D: 1 h after phosphate buffer injection (PB 1h). E-E: 1 h after NMDA injection (NMDA 1h). F-F: 3 h after NMDA injection (NMDA 3h). G-G: 6 h after NMDA injection (NMDA 6h). H-H: 12 h after NMDA injection (NMDA 12h). I-I: Immunoreactivity of p-Akt. J-J: Merged images of immunoreactivity of p-Akt antibody and TUNEL (p-Akt+Tunel). K-K: TUNEL. L-L: Merged images of triple staining, immunoreactivity of p-Akt antibody, TUNEL, and DAPI nuclear staining (p-Akt+Tunel+DAPI). NMDA induced dephosphorylation of Akt within 1 h. TUNEL positive cells are detected at 6 and 12 h after NMDA injection. TUNEL and immunoreactivity of p-Akt are never colocalized. U: Time course analysis of p-Akt positive cells and TUNEL positive cells in GCL. Y-axis of the green bar indicates the percentage of p-Akt positive neurons in all DAPI positive cells and the red bar indicates the percentage of TUNEL positive cells in all DAPI positive cells in GCL. The percentage of p-Akt positive cells is significantly decreased between 1 h (1h) to 12 h (12h) after 200 nmol NMDA treatment and the level is constant. Asterisk indicates statistically significant difference compared to the vehicle control (ANOVA followed by Scheffe post hoc test). V: Quantitative data of total neurons in GCL. The cell numbers are not changed within 12 h after NMDA administration. The data show that the decrease of p-Akt immunoreactivity is due to dephosphorylation, not protein degradation.
munoreactivity of phosphorylated Akt was detected in the GCL, the inner nuclear layer (INL), and weakly detected in the outer nuclear layer (ONL; Figure 2E). With NMDA injection, phosphorylated Akt was decreased markedly in the GCL and amacrine cell layer (inner margin of INL; Figure 2G, arrowhead) and increased between the GCL and IPL, and putative bipolar cells layer in the middle to outer margin of INL (Figure 2E,G, arrow). On the other hand, NMDA treatment did not cause significant changes in the cellular distribution of Akt (Figure 2A,C) and the total level of Akt protein did not change with any concentration of NMDA by immunoblot analysis with antibody against Akt (Figure 2J).

We further analyzed the ratio of the number of phosphorylated Akt positive neurons to total cells stained with DAPI in GCL (Figure 2I). In the untreated controls, 91.4±7.4% (n=8) of DAPI positive cells in GCL were positive for phosphorylated Akt immunoreactivity and the vehicle controls were not significantly different from the untreated controls (92.1±2.7%, n=8). NMDA administration caused a significant reduction of phosphorylated Akt positive cells in the GCL (compared to vehicle control) to 72.2±7.4% (n=8, 2 nmol/eye, p=0.0075), 64.4±7.7% (n=8, 20 nmol/eye, p=0.0001), 54.1±7.3% (n=8, 100 nmol/eye, p<0.0001), 49.6±7.0% (n=8, 200 nmol/eye, p<0.0001), and 50.4±5.5% (n=8, 400 nmol/eye, p<0.0001). These data indicate that the decrease in number of cells positive for phosphorylated Akt was due to dephosphorylation of Akt but not degradation of Akt protein, because the level of total Akt protein and the number of DAPI positive cells did not change during the period of this experiment.

**Time course of double staining of phosphorylated Akt and TUNEL:** Akt was dephosphorylated one hour after NMDA injection (Figure 2G) without any significant changes in numbers of Akt-positive cells or DAPI-positive cells (Figure 2C,D). To determine the subsequent fate of cells with dephosphorylated Akt, double staining of anti-phosphorylated Akt antibody and TUNEL was performed 1 h, 3 h, 6 h, and 12 h after NMDA treatment. NMDA treatment (200 nmol/eye) led to a significant loss of phosphorylated Akt-positive cells: 49.6±7.0% (1 h, n=8, p<0.0001), 41.9±8.1% (3 h, p<0.0001), 39.1±7.0% (6 h, p<0.0001), and 41.3±9.7% (12 h, p<0.0001, Figure 3U). On the other hand, TUNEL staining to detect apoptotic cells revealed TUNEL positive neurons in the GCL and the amacrine cell layer 6 h after NMDA administration and an increase at 12 h in GCL. At 6 and 12 h after NMDA treatment,
there was minimal co-labeling of TUNEL positivity and immunostaining of phosphorylated Akt (Figure 3N,R). This suggests that Akt dephosphorylation precedes the detection of apoptosis in the RGCs and amacrine cells.

Akt was dephosphorylated by PP-1/-2A: A correlation between Akt dephosphorylation and apoptotic cell death was observed in NMDA-treated GCL in the retina (Figure 3). We examined the effect of PP inhibitor FK-506 (PP-2B) and okadaic acid (PP-1/-2A) to identify specific Akt phosphatases in GCL cells of the retina. The proportion of cells containing

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<th>PBS+NMDA</th>
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<td>488 ± 118</td>
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The data show the density of fluorochrome labeled RGCs (RGCs/mm²). The table indicates that BDNF prevented cell loss in the GCL 7 days after NMDA injury.

Figure 5. Phosphorylated Akt and N-Shc/ShcC positive cells in NMDA-treated retina. **A-L**: Representative photograph of double immunohistochemistry with phosphorylated Akt (p-AKT) and N-Shc/ShcC antibody (ShcC). **A-D**: Untreated control (Control). **E-H**: 1 h after 20 nmol NMDA injection pretreated with PBS (PBS+NMDA). **I-L**: 1 h after 20 nmol NMDA injection pretreated with 1 µg BDNF (BDNF+NMDA). **A,E,I**: Immunoreactivity of ShcC (ShcC). **B,F,J**: Merged images of immunoreactivity of ShcC and p-Akt (ShcC+p-AKT). **C,G,K**: Immunoreactivity of p-Akt (p-AKT). **D,H,L**: Merged images of immunoreactivity of p-Akt and DAPI nuclear staining (p-AKT+DAPI). BDNF prevents NMDA-induced dephosphorylation of Akt. **M**: The table shows the quantitative data of the percentage of p-Akt positive (+) neurons in all neurons, p-Akt (+) neurons in ShcC positive (+) neurons, and ShcC (+) neurons in all neurons. Asterisk represents significant decrease compared to PBS group (*) or PBS+NMDA group (**). Pretreatment with BDNF reverses NMDA induced dephosphorylation of Akt but it is not fully reversed compared to the PBS group. However, if we exclude the cells which lack a BDNF response (ShcC negative cells), there is no significant difference between PBS and BDNF-treated groups.
phosphorylated Akt was 96.1±3.7% (n=6) for untreated-control and 96.6±2.0% (n=6) for phosphate buffered control of DAPI-positive cells (Figure 4A,E). Pretreatment with 5% DMSO following NMDA injection resulted in a decrease in Akt-containing cells to 64.4±12.7% of total DAPI-positive cells (Figure 4B,E). FK506, a specific inhibitor of PP-2B, did not prevent NMDA-induced dephosphorylation of Akt significantly (n=6, p<0.0001, Figure 4C,E). Okadaic acid strongly prevented dephosphorylation of Akt at all dose used (0.2 pmol: p=0.0006, 2 pmol: p=0.006, 20 pmol: p<0.0001, n=9, respectively, Figure 4D,E). These data demonstrate that the dephosphorylation of Akt by NMDA depends on PP-1 and/or PP-2A rather than PP-2B inhibition.

Exogenous BDNF prevents dephosphorylation of Akt by NMDA in the N-Shc/ShcC positive cells in GCL: We previously reported that BDNF had neuroprotective effects on axotomized RGCs through phosphorylation of Akt [20]. In addition, we have demonstrated a correlation between dephosphorylation of Akt and apoptotic cell death (Figure 3). Therefore, we examined the effect of exogenous administration of BDNF on NMDA-induced cell death 7 days after NMDA treatment. To identify the RGC in the retina, RGCs were specifically labeled using fluorochrome by retrograde transport [11,23,27]. Three micro litter of 0.1% bovine serum albumin in PBS, followed by 20 nmol/eye of NMDA administration led to the loss of fluorochrome labeled RGCs in the retina (488±118 cells/mm², n=7). The RGC density was significantly decreased compared to the vehicle control (2220±212 cells/mm², n=6, p<0.0001). However, the administration of BDNF prevented significantly the NMDA-induced reduction of RGCs (774±163 cells/mm², n=6, p=0.005; Table 1). These results indicate that BDNF has RGC neuroprotective effect.

The protective effects of BDNF on NMDA-induced dephosphorylation of Akt were evaluated by double staining with phosphorylated Akt and ShcC antibody in order to exclude cells, which lack of a BDNF response in the GCL. The number of cells containing phosphorylated Akt in the GCL was decreased significantly by NMDA (Figure 5C) and BDNF significantly prevented NMDA-induced Akt dephosphorylation in the GCL (Figure 5K). However, the protective effects of BDNF did not reverse fully the NMDA-induced Akt dephosphorylation (Figure 5M, p=0.0052). The protective effects of BDNF on NMDA-induced dephosphorylation of Akt were evaluated by double staining with phosphorylated Akt and ShcC antibody in order to exclude cells, which lack of a BDNF response in the GCL. We have demonstrated previously the potential involvement of BDNF-TrkB-ShcC signaling pathway in the GCL [17]; furthermore, ShcC is involved in Akt signaling in other tissues and cells [18]. In the vehicle control, 97.0±3.5% (n=6) of ShcC positive neurons in the GCL contained phosphorylated Akt. These data indicate that ShcC and phosphorylated Akt are well co-localized in the GCL. NMDA treatment reduced the population of phosphorylated Akt neurons to 51.2±10.2% (n=6) in the GCL. The NMDA-induced reduction of phosphorylated Akt-positive neurons was suppressed significantly in ShcC positive neurons in the GCL (86.5±12.6%, n=6) in the presence of BDNF. There was no significant difference between the control and BDNF-treated group (Figure 5M, p=0.2013) in the ShcC positive neurons in the GCL. Significant changes in the number of ShcC-positive neurons were not observed in the GCL during the experiments (vehicle control; 67.5±11.4%, NMDA; 60.9±8.5%, BDNF+NMDA; 57.0±11.6%, n=6, respectively, Figure 5M). These results together with our previous report [17] indicate a
role for BDNF-ShcC-Akt signaling in RGCs, and suggest that the BDNF-ShcC pathway may have an important role in preventing the dephosphorylation of Akt induced by NMDA injury.

**DISCUSSION**

To develop effective treatments and prevention for excitotoxicity-based diseases including diabetic retinopathy [30], vessel occlusion [8], and possibly glaucoma [1,31], the molecular mechanisms underlying the NMDA-induced retinal cell loss must be established. In this study, we demonstrated for the first time that one of the critical mechanisms of NMDA-induced RGC death in rat retina is regulated by specific PP activation and dephosphorylation of Akt. In addition, exogenous administration of BDNF could prevent the dephosphorylation of Akt induced by NMDA especially in N-Shc/ShcC-positive RGCs.

NMDA signals involve both Akt and p38 MAPK activation in the retina [27]. In whole retina, we have also detected by immunoblot analysis the increased Akt phosphorylation after NMDA administration (data not shown). Surprisingly, Akt was dephosphorylated in RGCs and amacrine cells (Figure 2, Figure 3). In addition, the cell population containing phosphorylated Akt was constant 12 h after NMDA treatment (Figure 3U). TUNEL positive cells, however, were observed 6 h after NMDA treatment and most of those cells lacked phosphorylated Akt (Figure 3). These data may suggest that NMDA-induced Akt dephosphorylation is one of the causes to induce apoptotic cell death.

PP-1, PP-2A, and PP-2B regulate the dephosphorylation of Akt in various cell types [21]. Our results suggest that okadaic acid prevented NMDA-induced dephosphorylation of Akt in the GCL (Figure 4). Okadaic acid is a specific inhibitor of PP-2A at the lower concentration used in this experiment (2 nM in the vitreous, PP-1: IC50=10-15 nM, PP-2A: IC50=0.1 nM) [32]. PP-2A is widely expressed throughout the retina [22]. PP-2A is believed to be always active [21], allowing the regulation of their activities endogenous inhibitors, such as I-1, DARPP-32 and G-substrate [33-36]. One of these endogenous inhibitors, G-substrate, which preferentially inhibits PP-2A, is localized to RGC, and amacrine cells [22]. Further study of the mechanism of regulation of PP induced by NMDA is therefore needed.

Several types of neurotoxic injuries increase the Akt phosphorylation, including optic nerve clamping [24] and episcleral vein cauterization [37]. In the GCL, optic nerve clamping, but not axotomy, increases the endogenous expression of BDNF and that leads to the increase of phosphorylated Akt within 6 h [38]. Presumably, phosphorylated Akt works as a self-defense mechanism through the growth factor cascade in response to injury. In this study, we specifically used NMDA-injury to clarify the involvement of the Akt pathway in the retina and demonstrated the dephosphorylation of Akt through the activation of PP within 1 h of NMDA administration. The data suggest that the dephosphorylation of Akt was a unique response of NMDA-induced injury.

BDNF has a significant neuroprotective effect on NMDA injury, however, the protective effect on NMDA injury was weaker than that on other models such as optic nerve axotomy [20]. The results suggest that NMDA-induced cell death may have one or more alternative pathways in addition to Akt signaling, as the administration of BDNF was not sufficient to fully reverse the effect of NMDA injury. Extensive research will be necessary to clarify this issue.

In conclusion, we found a co-localization between Akt dephosphorylation, and apoptotic cell death caused by NMDA-induced injury in RGCs, and Akt dephosphorylation was suppressed by BDNF. Therefore, the BDNF-induced increase in phosphorylated Akt and the PP-induced dephosphorylation of Akt may play an important role in the setting of NMDA-induced RGC death (Figure 6). A better understanding of these molecular pathways might provide new targets for the development of therapies in retinal disease.

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