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# Regulatory expression and cellular localization of doublecortin in the rat retina following ischemia-reperfusion injury

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Doublecortin (DCX) is microtubule-associated protein and is required for neuronal migration, differentiation and plasticity. In the retina, it is highly expressed between embryonic day 18 (E18) and E20, and is poorly expressed postnatally. In this study, we investigated the expression and cellular localization of DCX in the rat retina following ischemia induced by transiently increasing the intraocular pressure. While DCX immunoreactivity in control retinas was restricted to the outer border of the inner nuclear layer, it appeared in horizontal cell somata and processes in affected retinas. Quantitative evaluation by immunoblotting confirmed that DCX expression continuously increased after ischemia-reperfusion and showed 370% of control protein levels at 4 weeks after ischemic insult. These results suggest that the DCX in horizontal cells might play a role in neurite remodeling or modulating other neurons in ischemic rat retinas.

**Keywords:** doublecortin; calbindin; horizontal cell; ischemia; rat; retina

## Introduction

Doublecortin (DCX) is 40 kDa microtubule-associated phosphoprotein containing a consensus AbI phosphorylation and other sites of potential phosphorylation (Francis et al. 1999; Gleeson et al. 1999). Differential phosphorylation and dephosphorylation of DCX by various kinases and phosphatases may be involved in intracellular signaling (Hannan et al. 1999). A recent study suggests that the signaling processes through nonreceptor tyrosine kinases are important for neuronal migration to the cerebral cortex, and it appears that DCX, homologous to the amino terminus of the nonreceptor tyrosine kinases, may play a role in this signaling pathway (Ware et al. 1997). It is also known that regions of DCX homologous to the $Ca^{2+}$/calmodulin-dependent protein kinase may be involved in neuronal migration through the $Ca^{2+}$-dependent signaling pathway (Sossey-Alaoui et al. 1998). High levels of DCX immunoreactivity are present in the cerebral cortex, lateral ganglionic eminence, thalamus, midbrain, hindbrain, cerebellum, spinal cord and retina of developing mouse nervous system (Gleeson et al. 1999). Strong DCX expression is seen in developing rat retinas from embryonic day 15 to postnatal day 15 (Lee et al. 2003). DCX immunoreactive cells have been also reported in the adult rostral migratory stream, and those cells formed by neuroblasts migrating from the subventricular zone to the olfactory bulb (Sossey-Alaoui et al. 1998; Gleeson et al. 1999), suggesting its importance in the migration and differentiation of special neuronal population.

Previous studies examining ischemia-reperfusion injury on rat nervous system revealed high levels of DCX immunoreactivity in the rostral migratory stream, rostral subventricular zone (SVZ) and subgranular zone (SGZ) of the dentate gyrus (DG) (Jin et al. 2001, 2003; Wada et al. 2003). Because very little is known about the expression pattern of DCX in rat retina after ischemia-reperfusion, we focused on the expression and cellular localization of DCX in rat retina after ischemia-reperfusion injury to obtain a better understanding of functional role of DCX using immunoblot analysis and immunohistochemistry. We also characterized DCX-expressing cells by double immunohistochemistry using an anti-calbindin antibody, a common marker for horizontal cells.

## Materials and methods

All experimental animal procedures were conducted with the approval of the Catholic Ethics Committee of the Catholic University of Korea, and were in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996).

In total, 35 adult male albino Sprague–Dawley rats weighing 200–250 g were used in this study. The

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animals were anesthetized with 4% chloral hydrate (1 ml/100 g body weight) and the pupils were dilated with 1% tropicamide drops. Intraocular pressure (IOP) was raised between 90 and 120 mmHg by cannulation of the anterior chamber with a 30-gauge needle connected to a hydrostatic pressure device, and maintained for 60 min. The animals were then sacrificed by an intraperitoneal overdose of 4% chloral hydrate at 1 day, 3 days, 1 week, 2 weeks, and 4 weeks after reperfusion. Animals in the control normal group received no treatment. The eyes of each animal were enucleated and the retinas were isolated.

Western blot analysis was performed on the retinal extracts homogenized in 10 vols of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.02% sodium azide, 1 mM PMSF, and 5 μg/ml leupeptin. Protein concentration in each sample was assayed by the Lowry method (Lowry et al. 1951; Peterson 1979) in duplicate, and the average taken as the result. To estimate the protein content in the retinal extracts, duplicate sets of protein standards containing 0, 1, 3, 5, 10, 20, 40, or 60 μg bovine serum albumin were assayed by the same method, and the mean results were plotted on a graph to obtain a linear equation. Optical density of each sample was measured at 660 nm using a spectrometer (Spectronic 20; Bausch and Lomb, Rochester, NY, USA). Aliquots of tissue samples corresponding to 25 μg of total protein were heated at 100°C for 10 min with an equivalent volume of 2 × sample buffer (containing 4% SDS and 10% mercaptoethanol) and loaded onto 10% polyacrylamide gels. The protein was electrotransferred to a nitrocellulose membrane in Tris-glycine-methanol buffer, blocked for 1 h at room temperature in a solution containing 5% nonfat dry milk, 0.05% Tween-20, and phosphate-buffered saline (PBS; pH 7.4), and incubated for 15 h at 4°C with a guinea-pig polyclonal antiserum directed against DCX (Chemicon, Temecula, CA, USA; dilution 1:3000). It was rinsed for 30 min (three 10-min washes) with 0.05% Tween-20 in PBS and incubated for 1 h at room temperature in a 1:2000 dilution of biotinylated goat anti-guinea-pig IgG (Vector Laboratories, Burlingame, CA, USA). The blot was washed for 30 min (three 10-min washes) and analyzed using an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL, USA). The data were analyzed by analysis of variance (ANOVA) followed by Dunnett's $t$ test; $P < 0.05$ was regarded as significant.

For immunocytochemistry, the retinas were fixed by immersion in fixative (4% paraformaldehyde/0.2% picric acid in 0.1 M phosphate buffer [PB], pH 7.4) for 2 h. Following fixation, the retinas were rinsed in 0.1 M PBS (pH 7.4). Vibratome sections (50 μm thick) were incubated in 10% normal serum in PBS for 1 h at room temperature. The sections were then incubated overnight at 4°C in a solution of a polyclonal antibody against DCX (Chemicon, Temecula, CA, USA; dilution 1:3000). After washing in PBS for 45 min (three 15-min washes), the sections were incubated for 2 h in Cy3-conjugated goat anti-guinea-pig IgG (Jackson ImmunoResearch Labs, West Grove, PA, USA; dilution 1:500).

For the double-label studies, sections were incubated overnight in a mixture of a polyclonal antibody against DCX and a monoclonal antibody against calbindin (Sigma, St. Louis, MO; dilution 1:3000) at 4°C. After being washed in PBS for 45 min, the sections were incubated in a mixture of Cy3-conjugated goat anti-guinea-pig IgG (Jackson ImmunoResearch Labs, West Grove, PA, USA; dilution 1:500) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA, USA; dilution 1:50) for 2 h at room temperature. The sections were then rinsed in three changes of PB.

The immunolabeled retinal tissues were observed under a Bio-Rad Radiance Plus (Bio-Rad, Hemel Hempstead, UK) confocal scanning microscope (Nikon, Tokyo, Japan). FITC labeling was activated using the 488-nm line of the Argon ion laser, and detected by passing it through an HQ513/30 (Bio-Rad) emission filter. Cy3 signal was detected using the 543-nm line of the green HeNe laser along with the 605/32 (Bio-Rad) emission filter. The images were imported into Adobe Photoshop and printed on a film slide (Kodak Ektachrome 100; Eastman Kodak, Rochester, NY, USA).

## Results

We performed immunoblotting with specific antibodies against the DCX on protein extracts from the retinas of control and experimental rats. Western blot analysis demonstrated a DCX-immunoreactive single band of 40 kDa in control and ischemia-induced rat retinas. The intensity of those bands after ischemia-reperfusion was much stronger than that in the control one (Figure 1A). The densitometric analysis revealed that the DCX protein levels increased gradually after ischemia-reperfusion. At 1 day and 3 days after reperfusion, DCX protein levels increased to about 150% of controls, and DCX protein levels increased to about 370% of control levels at 4 weeks after reperfusion (Figure 1B).

The thickness of the treated retinas decreased with increasing reperfusion time after the ischemic insults (Figures 2 and 3). One week after reperfusion, a significant decrease in the thickness was observed especially in the inner nuclear layer (INL) and ganglion cell layer (GCL) compared with controls (Figure 3A). By 4 weeks after reperfusion, the thickness of the outer

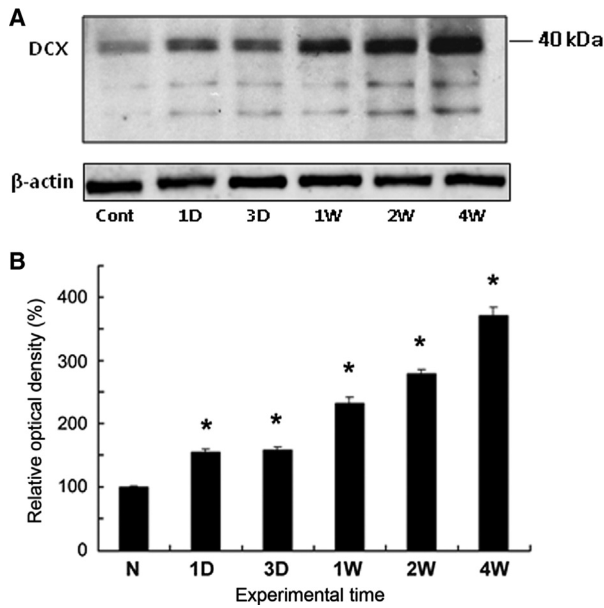


Figure 1. Western blot analysis of DCX protein levels in control and ischemic rat retinas. (A) Immunoblot stained for DCX demonstrating a signal band at 40 kDa for all time points tested: control retina (lane 1), 1 day (lane 2), and 3 days (lane 3), 1 week (lane 4), 2 weeks (lane 5), and 4 weeks (lane 6) after reperfusion. (B) Densitometric analysis of immunoblotting of the DCX. The data are expressed as a percentage of controls. $*P < 0.05$.

nuclear layer (ONL) was markedly reduced compared with controls and 1 week after reperfusion (Figure 2C).

Immunohistochemistry was used to study the expression and cellular localization of the DCX in the control and ischemic retinas. In control rat retina immunostained for the DCX, weak labeling was confined in the outer border of the INL (Figure 2A). Three days after reperfusion, DCX immunoreactivity was visible in individual somata located in the outer border of the INL (Figure 2B). One week after reperfusion, DCX immunoreactivity became strong enough to be clearly detectable in the somata and processes of cells in the outer part of the INL (Figure 3A). Four weeks after reperfusion, strong DCX immunoreactivity observed in the individual somata located in the outer part of the INL and processes ramified mainly in the inner plexiform layer (IPL), close to the INL (Figure 2C and 3C). As shown in Figure 2C, these processes emerge from the labeled somata located in the outer part of the INL.

To define the phenotype of cells expressing DCX in the outer part of the INL, double-labeling was performed using calbindin, a specific marker for horizontal cells (Röhrenbeck et al. 1987). In double-labeling immunofluorescence of DCX and calbindin, DCX immunoreactivity (Figure 3A, C) was localized to the same cells that showed calbindin immunoreactivity (Figure 3B, D) at 1 and 4 weeks after reperfusion. These results demonstrated that DCX immunoreactive cells are horizontal cells.

## Discussion

We have investigated the expression and cellular localization of DCX in the rat retina after ischemia-reperfusion using Western blot analysis and immunohistochemistry. DCX protein levels were gradually increased after ischemia-reperfusion, while very little DCX expression was detected in control retinas. DCX was expressed in the differentiating and migrating cells of the rat retina during embryonic development (Lee et al. 2003). Very weak DCX immunoreactivity in normal adult retina is consistent with the suggestion that DCX exerts its effect during early development (Francis et al. 1999).

The thickness of the treated retinas decreased with increasing reperfusion time after the ischemic insult, as previously reported (Kim et al. 1998; Ju et al. 2000). One week after reperfusion, a significant decrease in the

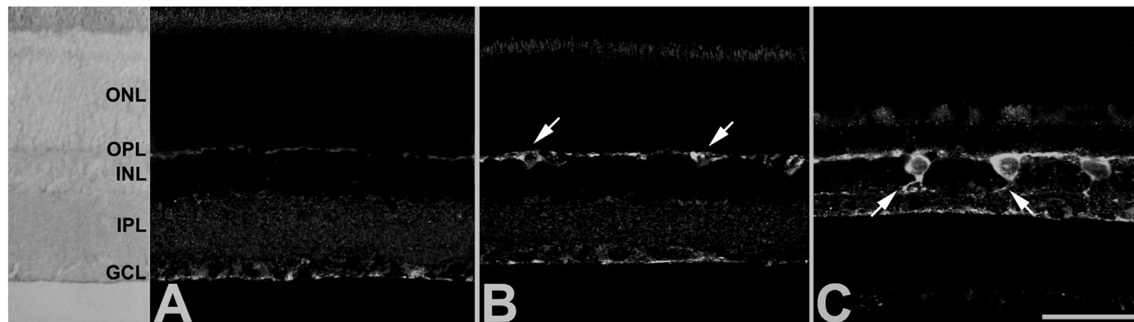


Figure 2. Light microphotographs showing 40-μm-thick vertical vibratome sections processed for DCX immunoreactivity in normal and ischemic retinas. (A) Control retina; weak DCX immunoreactivity is visible on the outer border of the INL. (B) On day 3 following reperfusion; much stronger DCX immunoreactivity is seen in the somata and processes (arrows). (C) At 4 weeks after reperfusion; DCX labeled cell bodies and processes are clearly visible. Note DCX labeled processes (arrows) descending into the inner plexiform layer (IPL). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar = 50 μm.

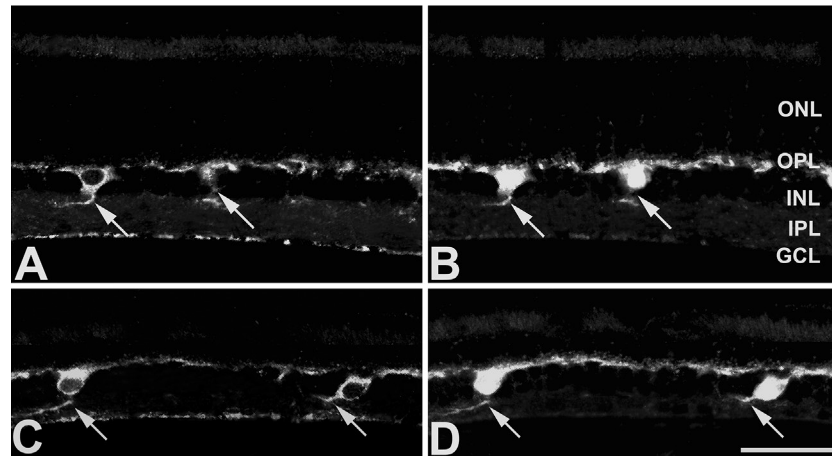


Figure 3. Confocal micrographs taken from a vertical 40-μm vibratome section processed for DCX (A, C) and calbindin (B, D) immunoreactivities at 1 week (A, B) and 4 weeks (C, D) after ischemia-reperfusion. DCX immunoreactive cells (arrows) also express calbindin immunoreactivity (arrows). Scale bar = 50 μm.

thickness was observed especially in the inner retina. At this time, DCX immunoreactivity appeared in the outer part of the INL. Strong DCX expression appeared in the somata and cell processes on the outer border of the INL at 4 weeks after ischemic insults. In this stage, the thickness of the ONL was markedly decreased with little changes of the inner retina. It has been reported that in early stages (up to 72 hours) after reperfusion, cellular degeneration takes place mainly in the INL and GCL, only appearing in the ONL at later stages (1–4 weeks) (Kim et al. 1998). These data suggest that enhanced DCX expression at the later stages after reperfusion might be involved in the maintenance of INL. DCX upregulation has been reported in the ischemic penumbra of the cerebral cortex striatum following focal cerebral ischemia, and plays a functional role in pathological process after cerebral ischemic injury (Jin et al. 2003; Tonchev et al. 2003; Wada et al. 2003). Taken together, it suggests that the DCX expression is upregulated in response to stress insults, such as ischemic injury, in an attempt to protect the retina.

Ischemia triggers the release of glutamate and other substances producing neuronal damage, mainly through a massive intracellular influx of $Ca^{2+}$ (Siesjö and Smith 1991; Choi 1994, 1995). Because rat horizontal cells have been shown to express glutamate receptors (Brandstätter et al. 1998), they are thought to be susceptible to the excitotoxicity of glutamate liberated from degenerated photoreceptor cells after ischemia-reperfusion (Chun et al. 1999). In the present study, all DCX-labeled cells showed strong immunoreactivity for anti-calbindin antibody, a marker for horizontal cells in the mammalian retina after ischemia-reperfusion. This suggests that rat horizontal cells would be more resistant to neurodegenerative processes due to their greater capacity to buffer intracellular calcium levels induced by excitotoxicity. The colocalization of DCX with calbindin was also observed in mouse cerebellum Purkinje cells during the periods of migration (Gleeson et al. 1999). It was detected in rat retinas during early development that DCX immunoreactive cells transformed into calbindin-labeled horizontal cells (Lee et al. 2003). This implies that DCX might be correlated with $Ca^{2+}$-dependent signaling pathways in the developing retina. Biochemical data from a previous study suggest that $Ca^{2+}$/calmodulin-dependent kinase in horizontal cells of the fish retina has an important function in the spinule plasticity at dendrites of the cells (Weiler et al. 1996). The presence of $Ca^{2+}$/calmodulin-dependent kinase has also been detected in rat horizontal cells using immunocytochemistry (Ochiishi et al. 1994). DCX contains several potential phosphorylation sites that may regulate the transduction of signals within the neurons, thereby controlling migrational activity during the development of the nervous system (Sobel 1991). It also participates in microtubule reorganization (Francis et al. 1999; Gleeson et al. 1999), which is recessing for the formation of new neurites (Gordon-Weeks 1991; Williamson et al. 1996). Therefore, DCX may play a role not only in neuronal migration but also in other 'plastic' events such as neurite outgrowth or synaptogenesis in the adult central nervous system (CNS), as suggested by Nacher et al. (2001). Thus DCX may play an important role in the survival of horizontal cells to ischemic injury by putative $Ca^{2+}$/calmodulin-dependent protein kinase, probably involving microtubule reorganization, and in the maintenance of the INL after ischemia-reperfusion.

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