

Letter to the Editor

Intracellular sortilin expression pattern regulates proNGF-induced naturally occurring cell death during development

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Dear Editor,

Neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) can support the survival and differentiation of neural cells during development and regeneration. The control of cell survival by neurotrophins is mediated by two types of transmembrane glycoprotein, the trk tyrosine kinase (Trk) receptors and the neurotrophin receptor p75 (p75^{NTR}). Neurotrophins affect neural cell survival by activating Trk receptors, whereas NGF binding to p75^{NTR} can activate an intracellular pathway similar to that activated by death receptors.¹ We previously documented that p75^{NTR} is expressed in postmitotic mouse retinal ganglion cells (RGCs) and induces programmed cell death before embryonic day (E) 15.² This function may contribute to the appropriate RGC number control and optic nerve formation. However, despite its stable expression, p75^{NTR}-dependent RGC death is inhibited after E17 by as yet unknown mechanisms. Recent studies have shown that the unprocessed precursor form of neurotrophin family members (pro-neurotrophins), such as proNGF and proBDNF, can act through a co-receptor system of p75^{NTR} and sortilin to mediate cell apoptosis.^{3–5} Although the expression and function of sortilin *in vivo* remain unclarified, it might be involved in the programmed RGC death that is observed only in the early phase of retinal development. Thus, we were interested in the expression and functions of p75^{NTR}/sortilin receptor complex in the developing retina.

The majority of sortilin (80–90%) resides in intracellular membranes, particularly in the Golgi apparatus, and the remaining is localized on the cell surface.⁶ However, in E13 and E15 retina, most sortilin immunopositive (-ip) cells (red in Figure 1a) were double-labeled with p75^{NTR} (green in Figure 1a) that is expressed on the cell surface. Coexpression of sortilin and p75^{NTR} was observed in the inner retina including RGCs. However, in the postnatal retina, sortilin expression changed into the intracellular region and did not occur concomitantly with p75^{NTR} (arrowheads in Figure 1a). To determine further this fact, we employed an antibody for 58K Golgi protein. Sortilin expression was detected in the Golgi apparatus whereas p75^{NTR} was negative for the antibody at postnatal day (P) 10 (Figure 1b). We also prepared cultured RGCs from P6 retina⁷ and confirmed that sortilin (red in Figure 1c) is negative for pan-cadherin antibody, a plasma membrane marker (green in Figure 1c). We examined further sortilin expression in both purified

plasma membrane and non-plasma membrane fractions by immunoblot analysis (Figure 1d). In E15 retina, sortilin was mainly detected in the plasma membrane fraction bound to anti-pan-cadherin antibody, but shifted to non-plasma membrane fraction in P6 retina. On the other hand, p75^{NTR} was mainly detected in the plasma membrane fraction in both E15 and P6. These results suggest that the intracellular sortilin expression pattern may regulate programmed cell death in the developing retina. To determine this possibility, we prepared cultured RGCs from E15 or P6 mice, and examined the effect of proNGF on RGC death (Figure 1e). ProNGF enhanced the death of E15 RGCs approximately two-fold compared with unstimulated control, but such an increase was inhibited by neurotensin that blocks the interaction between proNGF and sortilin.⁴ ProNGF did not cause the death of E15 RGCs from p75^{NTR} knockout (KO) mice and P6 RGCs from wild-type (WT) mice. On the other hand, misexpression of sortilin in P6 RGCs increased proNGF-dependent death (162 ± 12%; *n* = 3; *P* < 0.05) compared with GFP transfection. We also examined whether p75^{NTR} and sortilin physically associate on the retinal cell surface as well as HEK293 cells.⁴ Dissociated retinal cells from E15 and P6 mice were incubated in the absence or presence of proNGF, followed by treatment with a membrane-impermeable reducible crosslinker, lysis and immunoprecipitation using anti-p75^{NTR} antibody. No co-precipitated sortilin was observed without proNGF at both E15 and P6, whereas it was clearly detectable in the presence of proNGF at E15, but not at P6 (arrowhead in Figure 1f). Taken together, these results suggest that sortilin and p75^{NTR} receptors form receptor complexes on the cell surface via proNGF, which is required for proNGF-induced RGC death in the developing retina.²

p75^{NTR} and proNGF are recently involved in neural cell death under pathological conditions including Alzheimer's disease.^{10,11} So, we next examined the role of the p75^{NTR}/sortilin receptor system after ischemic injury, a common pathological condition that occurs in retinal artery occlusion and glaucoma, which lead to RGC death.^{7,9} In adult retina, sortilin was expressed in the intracellular region of RGCs and not overlapped with p75^{NTR} (arrows in Figure 1g). Immunoblot analysis revealed that proNGF expression was clearly increased 24 h after ischemic injury, whereas sortilin expression level was unchanged (Figure 1h). p75^{NTR}/sortilin expression pattern was stable even in the ischemic retina

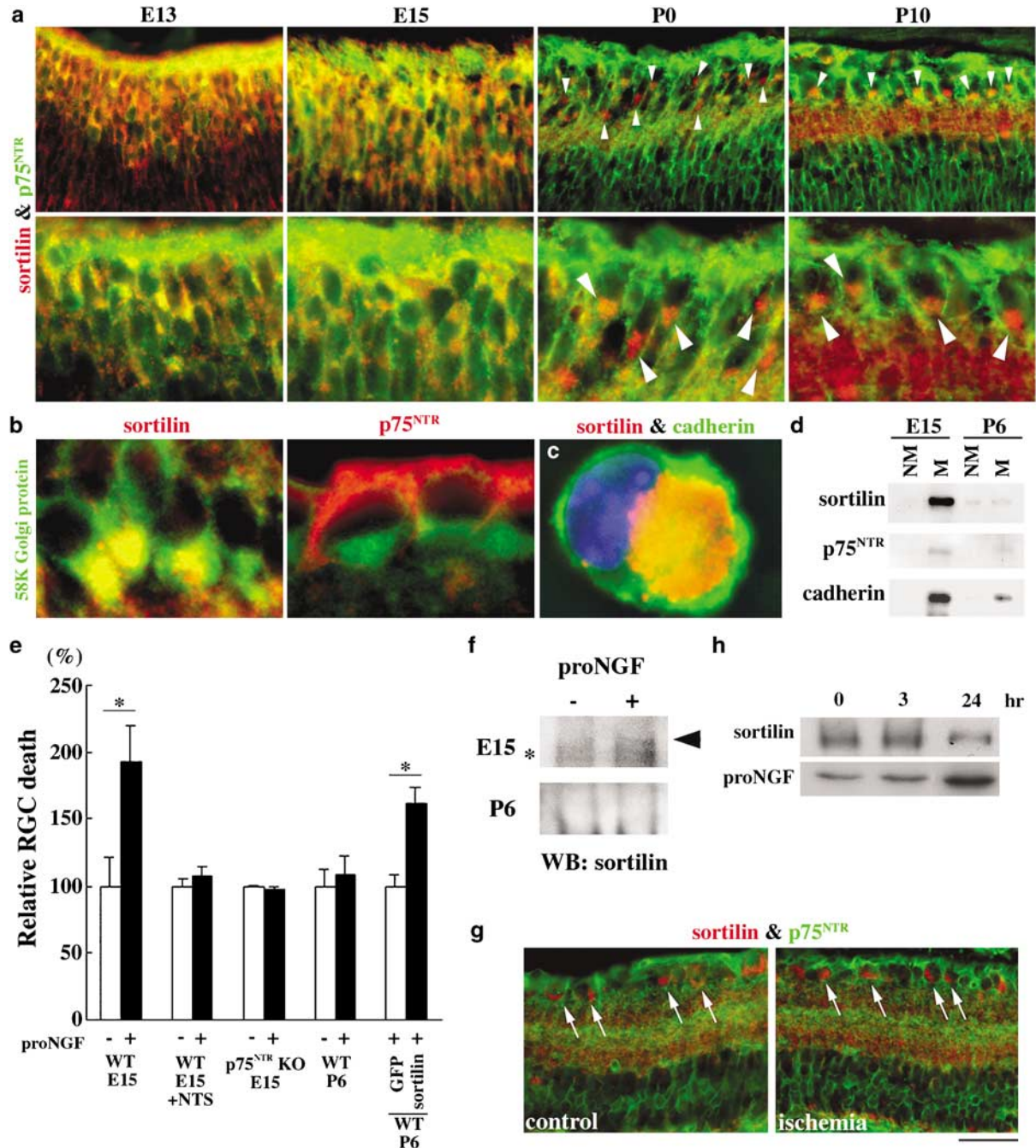


Figure 1 Expression of sortilin and p75^{NTR} regulates proNGF-induced retinal cell death. (a) Retinal sections from E13-P10 mice were incubated with rabbit polyclonal antibody against p75^{NTR} (Promega) and goat polyclonal antibody against sortilin (R&D System). (b) Retinal sections from P10 mice were incubated with anti-p75^{NTR} or anti-sortilin antibody with mouse monoclonal antibody against 58K Golgi protein (Abcam). (c) Isolated RGCs were prepared from P6 mice using two-step panning method⁷ and incubated with anti-sortilin antibody and mouse monoclonal antibody against pan-cadherin (Abcam). Nuclear staining was performed using Hoechst 33342 (Invitrogen). (d) Immunoblot and subcellular fractionation analysis of sortilin. Whole retinas from E15 or P6 mice were homogenized in PBS and centrifuged at 100 × g for 5 min. Dynabeads M-450 conjugated with a secondary antibody (Invitrogen) were added to the supernatants with anti-pan-cadherin antibody and recovered with a Magnetic Particle Concentrator (Invitrogen) as membrane fractions. Supernatants that failed to bind to Dynabeads were analyzed as non-membrane fractions. They were processed for immunoblot analysis using anti-sortilin, anti-p75^{NTR} or anti-pan-cadherin antibody as reported previously.⁷ NM, non-membrane fraction; M, membrane fraction. (e) Effect of proNGF (500 ng/ml; Alexis) on cultured RGCs isolated from E15 and P6 mice.⁷ E15 RGCs were treated with 20 μM neurotensin (NTS; Sigma). P6 RGCs were transfected with GFP or pcDNA3.1/zeo(-)-sortilin⁸ using the Nucleofector (Amaxa Biosystems). LDH activity was analyzed in culture medium using an LDH cytotoxic test kit (Wako), and the values relative to that of control are presented as mean ± S.E.M. Student's *t*-test was used to estimate the significance of difference in results. **P* < 0.05. (f) ProNGF-induced formation of heteromeric complexes comprising p75^{NTR} and sortilin. Cells from E15 and P6 retina were incubated for 2 h at 20°C with or without 1 μg/ml proNGF (Alexis) and treated for 30 min with 5 mM of reducible crosslinker DTSSP (Pierce) before lysis in 1% Triton-X 100 buffer containing protease inhibitors.⁴ Immunoprecipitation was performed using anti-p75^{NTR} antibody and detected by immunoblot with anti-sortilin antibody (arrow). Asterisk indicates non-specific bands. (g) Expression of sortilin and p75^{NTR} in ischemic retina. Ischemia was induced and the animals were treated essentially as previously described.^{7,9} (h) Expression of sortilin and proNGF in ischemic retina. Whole retinas were removed 0, 3 and 24 h after ischemic injury, then 3 μg protein was analyzed by immunoblot using anti-sortilin or anti-proNGF (Alomon Labs) antibody. Scale bar = 30 μm (a, upper panel); 15 μm (a, lower panel); 15 μm (b); 6 μm (c); 35 μm (g)

(Figure 1g). Consistently, ischemic injury in p75^{NTR} KO mice was nearly identical to that in WT (date not shown). These results imply that p75^{NTR}/sortilin receptor system has no effect on ischemia-induced RGC death in the adult retina.

There are two periods of cell death in the developing retina.¹² The first period occurs during E15–E17 that is the main onset of neurogenesis, neural migration and initial axon growth. In this period, at least part of retinal apoptosis is regulated by the p75^{NTR}, and most dying cells are observed in the neuroepithelium of the central retina, close to the optic nerve exit.^{2,13} In this study, we first documented that sortilin plays a role in retinal apoptosis by functioning as a p75^{NTR}/sortilin receptor complex in this first period. On the other hand, despite the increased proNGF expression level, this receptor system had no contribution for RGC death after ischemic injury. As well as during development, this may be partially due to the absence of sortilin from the RGC surface. We recently demonstrated that ischemia-induced RGC death is at least partly regulated by apoptosis signal-regulating kinase 1 (ASK1)-p38 pathway.⁷ Thus different cell death pathways seem to be working on one cell type at different times.¹⁴

In parts of the central nervous system other than the retina, p75^{NTR} and proNGF may be involved in neurodegeneration.^{10,11} Together with our present results, a therapeutic approach that inactivates sortilin on the cell surface or alters the sortilin expression pattern may be effective for neurodegenerative diseases in which the p75^{NTR}/sortilin receptor complex is involved. Further studies determining the detailed functions of this receptor system may provide important

information that lead to the development of new therapeutic methods for neural cell protection, as well as the better understanding of central nervous system development.¹⁴

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