Short Communication

Neuritogenic Activity of Trichostatin A in Adult Rat Retinal Ganglion Cells Through Acetylation of Histone H3 Lysine 9 and RARβ Induction

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Abstract. Like other CNS neurons, mature retinal ganglion cells (RGCs) cannot regenerate their axons after nerve injury due to loss of regenerative capacity. One of the reasons why they lose their capacity seems to be a dramatic shift in gene expression of RGCs under epigenetic modulation. In here, we found that levels of histone H3 lysine 9 acetylation decreased after birth in RGCs. This decrease showed good correlation with restriction of retinoic acid receptor β (RARβ) expression in RGCs after birth. Furthermore, we demonstrated that a histone deacetylase inhibitor, trichostatin A, induced axonal regeneration of adult rat RGCs through RARβ induction.

Keywords: CNS regeneration, epigenetic modulation, retinoic acid receptor β (RARβ)

Although mature CNS neurons in mammals are incapable of axonal regeneration, the newborn CNS neurons can retain the capacity of regeneration. This ability is lost during early development (1). In the rat retinal ganglion cells (RGCs), newborn RGCs can show a strong axonal regenerative capacity during the 1 – 2 weeks after birth, and then they rapidly lose their capacity (2, 3). This switch of restricted capacity during development is associated with a dramatic shift in the genetic program of the RGCs with normal development (4, 5). One of the changes in the genetic program leading to loss of regeneration capacity in RGCs seems to be a decrease of histone acetylation (6). In our previous paper, we reported that the loss of retinoic acid receptor β (RARβ) expression accompanying development restricted the permissible state of axonal regeneration in RGCs (7). Thus, a critical period exists in the developing rat retina in which RGCs start to restrict axonal regrowth. We also reported that an iridoid compound, a genipin derivative, indirectly induced RARβ through inactivating histone deacetylase (HDAC) type 2 by its S-nitrosylation and thereby induced optic nerve regeneration (7). However, whether an HDAC inhibitor also can be used to enhance axon regeneration through RARβ induction after injury has not been shown. Especially, although acetylation of histone H3 (AcH3) at lysine 9 (K9) is the most famous target for acceleration of transcriptional activity and correlates well with gene expression (8), there are no reports describing the pattern of AcH3K9 in RGCs after birth. Although, there is a report that an HDAC inhibitor, trichostatin A (TSA), facilitates axonal elongation of the RGC cell line (9), the mechanism is unclear. Therefore, we further examined the neuritogenic action of TSA in adult rat RGCs through the histone H3K9 acetylation and subsequent RARβ induction mechanism.

All animal care and handling procedures were approved by the Animal Care and Use Committee of Kanazawa University. Sprague Dawley male rats (postnatal 1 day to adult, 300 g in weight) were used. Under anesthesia of sodium pentobarbital, TSA (Sigma-Aldrich, St. Louis, MO, USA) was injected into the eye ball with a Hamilton microsyringe needle (30G Hamilton Syringe; Hamilton, Whittier, CA, USA). The optic nerve was crushed 2 mm behind the eye with forceps (Dumont #5) for 10 s as previously described (7). Immunohistochemistry was performed as previously (7). After fixation, frozen sections of the eye (12 μm) were incubated with primary anti-AcH3K9 (1:500; Cell Signaling...
Technology, Tokyo), anti-RARβ (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-NeuN (1:500; Millipore, Billerica, MA, USA) antibodies. The sections were then incubated with AlexaFluoro anti-IgG (Molecular Probes, Eugene, OR, USA). Western blot analyses were performed as previously described (7). The proteins from whole retina were transferred to a nitrocellulose membrane and incubated with primary [AcH3K9 (1:500), RARβ (1:500)] and secondary antibodies (Santa Cruz Biotechnology). Protein bands were detected using a BCIP/NBT Kit (Funakoshi, Tokyo). Antibody against histone H4 (1:500, Cell Signaling Technology) was used as an internal standard. Protein bands isolated from retina were analyzed densitometrically using Scion Image Software (Scion Corporation, Frederick, MD, USA). Retinal explant cultures were performed as previously described (7). We observed neurite outgrowth in each explant from a total 30 – 40 explants per dish using phase contrast microscopy. Positive neurite outgrowth was defined on the basis of the length (> 200 μm) of the neurites. We showed the percentage of explants with positive neurites. As application of small interfering RNA (siRNA) for RARβ, transfection of siRNA (100 pmol) into retinal explants was carried out using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA) for 4 h before culture. siRNAs for the target region of RARβ mRNA were as follows: 5′-GCAGAACCUGUAUAAACCUCG-3′ (sense), 5′-AGGUAUUAACGCUUCGCA-3′ (antisense) (Sigma-Aldrich Japan, Tokyo); and a randomly shuffled sequence: 5′-AGUCGUCGUAAACGGAU AUC-3′ (sense), 5′-UAUACCUGUAAUCGACGUUC-3′ (antisense). All results were reported as the mean ± S.E.M. for 3 – 5 experiments. Differences between groups were analyzed using one-way ANOVA, followed by Dunnett’s multi-comparison test with PASW Software (SPSS, Inc., Chicago, IL, USA). P-values < 0.05 were considered statistically significant.

In our previous report, newborn rat retina at postnatal day 1 (P1) showed spontaneous axonal regeneration in explant cultures. In contrast, rat retinas at P14 and P60 lost the capacity for spontaneous axonal regeneration in 30% and 10% of the rats, respectively, as compared to the P1 rat, respectively. The levels of RARβ protein in the RGCs in P14 retina were less than 30% of P1 (7). In this study, to know the levels of AcH3 in rat retina after birth, we performed western blot analysis in total retina at P1, P14, and P60 (Fig. 1A). Newborn rat retina at P1 showed significant histone H3K9 acetylation. In contrast, rat retina at P14 and P60 exhibited decreased acetylation levels of histone H3K9 (51.3% ± 6.80% and 40.3% ± 13.3%, respectively) compared to that in the P1 rat. This decrease of AcH3 was localized in the RGCs at P1 (Fig. 1: B – D) and P60 (Fig. 1: E – G) by immunohistochemistry. These results showed the further correlation between axonal regeneration with RARβ expression and histone H3K9 acetylation in the developing rat RGCs. In our previous data, a specific siRNA for RARβ suppressed the neurite outgrowth to 63% (RARβ expression to 52%) compared with the vehicle control of P1 rat retina (7). These data further support that the neurotrophic activity of RARβ expression is strongly linked to histone H3K9 acetylation. Therefore, we confirmed whether acetylation of histone H3K9 by HDAC inhibitor could induce RARβ expression and axonal regeneration.

We tested TSA (Fig. 2A), an epigenetic modulator. It has been reported that TSA strongly induces histone H3 acetylation and neuritogenesis in RGCs (6, 9). TSA dose-dependently induced both acetylation of histone H3K9 and RARβ expression at 1 day after treatment (Fig. 2B). To determine the localization of histone H3K9 acetylation by TSA, we performed double-staining with anti-AcH3K9 and anti-NeuN antibodies (Fig. 2: C – H). In the vehicle control retina, weak histone H3 acetylation was seen in RGCs with anti-AcH3 and anti-NeuN antibodies (Fig. 2: C – E). Intraocular TSA [0.5 μM (2.5 pmol/eye)] increased acetylation of histone H3K9 in RGCs at 1 day post treatment (Fig. 2: F – H). Furthermore, intraocular injection of TSA strongly increased RARβ expression at 1 day after treatment (Fig. 2: L – N) compared to the vehicle control (Fig. 2: I – K). To test the involvement of RARβ expression on neurite outgrowth by TSA, we used siRNA to knockdown RARβ expression in adult rat retinal explant culture. siRNA for RARβ mRNA significantly suppressed the induction of RARβ protein level by TSA (Fig. 3A), whereas scrambled siRNA did not have any effect. Next, we checked the neurite outgrowth effect of TSA from adult rat retinal explant culture. TSA (0.5 μM) actually enhanced neurite outgrowth from retinal explant culture (Fig. 3C) compared to the vehicle control (Fig. 3B). The neurite outgrowth induced by TSA was also suppressed by siRNA for RARβ (Fig. 3D) but not by scrambled siRNA (Fig. 3E). siRNA for RARβ and scrambled siRNA alone had no effect on neurite outgrowth as compared to the vehicle control (Fig. 3E).

This study has four salient findings. Levels of histone H3K9 acetylation decrease after birth in RGCs; this decrease shows a good correlation with loss of RARβ and/or regenerative capacity of RGCs; intraocular treatment of TSA promotes axonal regeneration in adult RGCs through RARβ induction; acetylation of histone H3K9 is one of the important residues for TSA-induced RARβ expression and subsequent axonal regeneration in adult RGCs. It has been reported that TSA induced...
Fig. 1. Histone H3K9 acetylation in rat retina after birth. A) Quantification of AcH3K9 expression in rat retina during development. Levels of immunoreactive AcH3K9 in the retina were decreased during development. *P < 0.01 vs. P1 (n = 3). B – G) Immunohistochemistry of AcH3K9 and NeuN in rat RGCs. AcH3-positive RGCs were decreased by 60 days after birth (E – G) compared to P1 (B – D). B and E: Immunoreactivity of AcH3K9. Scale = 50 μm. C and F: NeuN staining. D and G: Merged images.

Fig. 2. Correlation between RARβ expression and histone H3K9 acetylation by TSA in rat retina. A) Chemical structure of TSA. B) Levels of AcH3K9 and RARβ expression after treatment of TSA in the adult rat retina. *P < 0.01 vs. vehicle control (n = 3). C – H) Immunohistochemistry of AcH3K9 and NeuN in rat RGCs. Levels of AcH3K9 were increased 1 day after TSA treatment (F – H) compared to the vehicle control (C – E). C and F: Immunoreactivity of AcH3K9. Scale = 50 μm. D and G: NeuN staining. E and H: Merged images. I – N) Immunohistochemistry of RARβ and NeuN in rat RGCs. Levels of RARβ expression were increased 1 day after TSA treatment (L – N) compared to the vehicle control (I – K). I and L: Immunoreactivity of RARβ. J and M: NeuN staining. K and N: Merged images.
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However, the detailed mechanism for neurite outgrowth was unclear. Gaub et al. reported that histone H3 lysine18 (K18) acetylation, another epigenetic marker, was rather increased after birth in RGCs until day 21 and returned to the control level in adulthood (6). The pattern of histone H3K18 acetylation in RGCs during development was totally different from that of RARB expression and axonal regeneration. In this study, decrease of AcH3K9 shows good correlation with loss of RARB induction and/or capacity of axonal regeneration of RGCs after birth. In the present study, TSA facilitated neuritogenic action on RGCs through histone H3K9 acetylation and RARB expression even after the critical period for regeneration in the adult stage of rat retina. There are many reports that RARB expression is involved in axonal regeneration in CNS neurons, including the spinal cord dorsal root ganglia (10–12), and that TSA induced RARB expression (13, 14). We could here show the important role of RARB expression on neuritogenic action in adult RGCs through histone H3K9 acetylation.

The concurrent neuritogenic actions of TSA by RARB induction could provide a new therapeutic tool for CNS disorders, including RGC degenerative diseases, spinal cord injury, and brain ischemia.

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Conflicts of Interest

The authors have declared that no competing interests exist.

References

2 Chen DF, Jhaveri, S, Schneider, GE. Intrinsic changes in developing retinal neurons result in regenerative failure of their