# Histone Deacetylases Regulation by δ-Opioids in Human Optic Nerve Head Astrocytes

Syed A. H. Zaidi,[1] Nakul Thakore,[1] Sudha Singh,[1] Wendy Guzman,[1] Shikhar Mehrotra,[2] Vamsi Gangaraju,[3] and Shahid Husain[1]

[1]Hewitt Laboratory of the Ola B. Williams Glaucoma Centre, Department of Ophthalmology, Storm Eye Institute, Medical University of South Carolina, Charleston, South Carolina
[2]Department of Surgery, Hollings Cancer Center, Medical University of South Carolina, Charleston, South Carolina
[3]Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina

Correspondence: Shahid Husain, Storm Eye Institute, Room 713, Medical University of South Carolina, 167 Ashley Ave, Charleston, 29425, South Carolina; husain@musc.edu.

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**Purpose.** We determined whether δ-opioid receptor agonist (SNC-121) regulates acetylation homeostasis via controlling histone deacetylases (HDACs) activity and expression in optic nerve head (ONH) astrocytes.

**Methods.** ONH astrocytes were treated with SNC-121 (1 µM) for 24 hours. The HDAC activity was measured using HDAC-specific fluorophore-conjugated synthetic substrates, Boc-Lys(Ac)-AMC and (Boc-Lys(Tfa)-AMC). Protein and mRNA expression of each HDAC was determined by Western blotting and quantitative real-time PCR. IOP in rats was elevated by injecting 2.0 M hypertonic saline into the limbal veins.

**Results.** Delta opioid receptor agonist, SNC-121 (1 µM), treatment increased acetylation of histone H3, H2B, and H4 by 128 ± 3%, 45 ± 1%, and 68 ± 2%, respectively. The addition of Garcinol, a histone-acetyltransferase inhibitor, fully blocked SNC-121–induced histone H3 acetylation. SNC-121 reduced the activities of class I and IIb HDACs activities significantly (17 ± 3%) and this decrease in HDACs activities was fully blocked by a selective δ-opioid receptors antagonist, naltrindole. SNC-121 also decrease the mRNA expression of HDAC-3 and HDAC-6 by 19% and 18%, respectively. Furthermore, protein expression of HDAC 1, 2, 3, and 6 was significantly ($P < 0.05$) decreased by SNC-121 treatment. SNC-121 treatment also reduced lipopolysaccharide-induced TNF-α production from ONH astrocytes and glial fibrillary acidic protein immunostaining in the optic nerve of ocular hypertensive animals.

**Conclusions.** We provided evidence that δ-opioid receptor agonist activation increased histone acetylation, decrease HDACs class I and class IIb activities, mRNA, and protein expression, lipopolysaccharide-induced TNF-α production in ONH astrocytes. Our data also demonstrate that SNC-121 treatment decrease glial fibrillary acidic protein immunostaining in the optic nerves of animals with ocular hypertension.

Keywords: optic nerve head astrocytes, histone deacetylases, protein acetylation, glaucoma

Optic nerve head (ONH) astrocytes, while providing consistently cellular support to the axons, are considered to be "quiescent" under physiologic conditions.[1] However, often they become reactive in response to various forms of insults including glaucomatous injury.[2,3] Reactive astrocytes release a host of molecules ranging from proinflammatory cytokines, chemokines, and nitric oxide, which establish a hostile microenvironment that is detrimental for retinal ganglion cells (RGCs).[4] Conflicting reports are available for the role of nitric oxide in RGC death. For example, nitric oxide regulates IOP[5] and small quantities could be beneficial to maintain metabolites and blood circulation.[6] In contrast, a sustained level of nitric oxide may result in direct tissue toxicity and contribute to neuronal degeneration[7] and agents that lower the level of nitric oxide provide RGC neuroprotection.[8,9] Under glaucomatous condition, various signaling pathways, including homoeostasis of acetylation, are altered. The maintenance of acetylation homeostasis is critical not only for RGCs, but also for glial cells. Thus, a pharmacologic approach that can modulate the homeostasis of acetylation and regulate the activity and expression of HDACs and their downstream signaling pathways that may be involved in maintaining the physiologic quiescent state of astrocytes is highly needed.

Regulation of signaling events through acetylation is one of the most pervasive modes of post-translational protein modification, which is next to phosphorylation in ranking. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate a variety of gene expression in a delicate homeostatic equilibrium, and an imbalance in this equilibrium can lead to dysregulation of certain genes that may result in cell death. Studies have shown that

aberrant increases in HDAC activity and decreased histone acetylation may be a major causative factor in aging and neurodegenerative diseases.[10,11] In the eye, studies have shown that a broad range HDAC inhibitors (e.g., trichostatin A [TSA] and valproic acid) protect RGCs by decreasing the HDAC activity in both retina acute ischemia/reperfusion and chronic glaucoma models.[12–15]

Earlier, we have shown that activation of δ-opioid receptor by a selective ligand (e.g., SNC-121) provides RGC neuroprotection as measured by pattern ERG and counting of RGC.[8,16,17] Additionally, δ-opioid receptor activation in primary cultures of human ONH astrocytes suppressed p38 mitogen activated protein kinase and nuclear factor-κB signaling.[16,18,19] However, it remains unknown if δ-opioid receptor activation regulates acetylation homeostasis via regulation of HDACs activity in ONH astrocytes. In this study, we explored whether SNC-121 can regulate the activity and expression of HDACs and histone acetylation in the primary cultures of human ONH astrocytes. We provide substantial evidence in this manuscript that SNC-121 increased histone H3, H4, and H2B acetylation, reduced class I and class IIb HDAC activity, and expression of HDACs in ONH astrocytes. We also have shown that toll-like receptor 4 agonist lipopolysaccharide (LPS)-induced TNF-α is reduced by SNC-121 treatment in ONH astrocytes. Additionally, in vivo studies using a chronic rat glaucoma model also shows that SNC-121 treatment decreased astrocyte activation as measured by glial fibrillary acidic protein (GFAP) immunostaining in the optic nerve of animals with ocular hypertension. To the best of our knowledge, this report is the first in which we have shown that δ-opioid receptor activation regulates protein acetylation homeostasis via regulation of histone acetylation and HDACs expression in ONH astrocytes. We also provided evidence that SNC-121 treatment decreased astrocyte activation as measured by GFAP immunostaining in the optic nerves of ocular hypertensive animals.

Overall, we hypothesized that protein acetylation may play a crucial role in astrocytes that can reduce the levels of detrimental factors (e.g., proinflammatory cytokines), which are potentially involved in RGC death during glaucomatous injury.

## Materials and Methods

### Materials

The catalogue numbers and sources of all the reagents, chemicals, and antibodies are provided in Table 1.

### Animals

Equal numbers of adult male and female Brown Norway rats (2–5 months of age; weighing 150–350 g) were used in this study. These animals were obtained from Charles River laboratory (San Diego, CA). Rats were maintained under a cycle of 12-hours of light and 12-hours of dark throughout the studies. Animals were treated with SNC-121 (1 mg/kg body weight) intraperitoneally once a day for 7 days. All animal handling was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study protocol was approved by the Animal Care and Use Committee at the Medical University of South Carolina.

### Development of Glaucoma Model by Hypertonic Saline Injection

The baseline IOP was recorded using a Tonolab tonometer before hypertonic saline injection. The IOP was increased by injecting 50 µL of 2 M hypertonic saline into the limbal veins as described elsewhere.[8,16,20] Animals that had an elevation in IOP of more than 25% were included in the experimental design.

### Immunohistochemistry

The optic nerves were collected on day 7 after injury and placed in 4% paraformaldehyde for 12 to 16 hours at 4°C. The optic nerves were then transferred to 30% sucrose for 24 hours followed by embedding the optic nerves in optimal cutting temperature (OCT) on dry ice. Cryosections were cut at –30°C and stored at –20°C until used for immunohistochemistry. Tissue sections were rinsed with Tris-buffered saline (TBS) three times at room temperature followed by permeabilization using 0.5% Triton-X-100 in TBS. The tissues were blocked with 5% BSA in TBS for 1 hour and then rinsed again with TBS followed by incubation with primary antibodies (anti-GFAP, dilution 1:500) for 16 hours at 4°C. After washing, the tissues were incubated with secondary antibody (Alexa Fluor 488, dilution 1:500) for 1 hour at room temperature. The sections were observed under a bright-field microscope equipped with epifluorescence, and digitized images were captured by a digital camera (Carl Zeiss Meditec, Jena, Germany) as described elsewhere.[8,16,21]

### ONH Astrocytes

Primary ONH astrocytes were isolated from cadaver human eyes (ages 60–90 years; NDRI, Philadelphia, PA) as described elsewhere.[18,20] Briefly, ONH freed of sclera tissue was used to grow ONH astrocytes and allow to grow 2 to 3 weeks in Dulbecco's Minimum Essential Medium F12 (HyClone, Logan, UT) containing 10% fetal bovine serum (HyClone, Logan, UT) along with antimycotics and antibiotics. ONH astrocytes were isolated and purified by immunopanning and grown in astrocytes growth medium as described elsewhere.[18,19] The purity of ONH astrocytes was determined by positive immunostaining for the astrocyte marker GFAP (Fig. 1). In this study, ONH astrocytes of passages two to six were used. ONH astrocytes were serum starved in astrocyte basal medium for 16 hours followed by treatment with δ-opioid agonist, SNC-121 (1 µM) for 24 hours as described elsewhere.[20] ONH astrocytes were pre-treated with δ-opioid receptor antagonist (naltrindole, 1 µM) or HATs inhibitor (Garcinol, 1 µM) 15 to 30 minutes before SNC-121 treatment.

### HDAC Activity Assay

The HDAC activity was measured using the peptidase, trypsin, and the fluorophore-conjugated synthetic substrates, Boc-Lys(Ac)-AMC and (Boc-Lys(Tfa)-AMC). Briefly, cell lysates were diluted to a concentration of 1.0 µg/µL using standard HDAC assay buffer (50 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 0.1 mg/mL BSA) and incubated with the conjugated fluorophore acetylated lysine substrate Boc-Lys(Ac)-AMC

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**Table 1.** List of Reagents, Vendors, and Catalog Numbers

| Reagents | Vendor/Company | Catalog Number |
|---|---|---|
| Antibodies | | |
| Anti-Acetyl Histone 3 Lys 9 (AcH3K9) | Cell Signaling Technology | 9649 |
| Anti-Acetyl Histone 4 Lys 8 (AcH4K8) | Cell Signaling Technology | 2594 |
| Anti-Acetyl Histone 2B Lys 5 (AcH2BK5) | Cell Signaling Technology | 12799 |
| Anti-Histone Deacetylase (HDAC)-1 | Millipore | 06-720 |
| Anti-Histone Deacetylase (HDAC)-2 | Millipore | 05-814 |
| Anti-Histone Deacetylase (HDAC)-3 | Millipore | 06-890 |
| Anti-Histone Deacetylase (HDAC)-4 | Abcam | ab12171 |
| Anti-Histone Deacetylase (HDAC)-5 | Millipore | 07-045 |
| Anti-Histone Deacetylase (HDAC)-6 | Cell Signaling Technology | 7558 |
| Anti-Histone Deacetylase (HDAC)-7 | Abcam | ab166911 |
| Anti-Histone Deacetylase (HDAC)-9 | Abcam | ab109446 |
| Anti-Histone Deacetylase (HDAC)-10 | Sigma Aldrich | H3413 |
| Anti-Histone Deacetylase (HDAC)-11 | Abcam | ab166907 |
| Anti-Beta-Actin | Sigma Aldrich | A5316 |
| Anti-rabbit IgG, HRP-linked | Cell Signaling Technology | 7074 |
| Anti-mouse IgG, HRP-linked | Cell Signaling Technology | 7076 |
| Anti-Glial Fibrillary Acidic Protein (GFAP) | Aves Labs | GFAP |
| Rhodamine Red-X (RRX) Anti-Chicken IgY (IgG) | Jackson Immunoresearch | 703-295-155 |
| Chemicals | | |
| Fetal Bovine Serum (Canada) | HyClone | SH3039603 |
| Astrocyte Growth Medium (AGM) | Lonza | CC3186 |
| TRIzol | Life Technologies | 15596-026 |
| SlowFade Gold Antifade Mountant with DAPI | Invitrogen | S36938 |
| SNC-121 dihydrochloride | Santa Cruz Biotechnology | SC-204291 |
| Naltrindole hydrochloride | Tocris | 0740 |
| Garcinol | Enzo Life Sciences | BML-GR343 |
| Trichostatin A (TSA) | Sigma Aldrich | T8552 |
| BOC-LYS(AC)-AMC | Aurum Pharmatech | K-6405 |
| BOC-LYS(TFA)-AMC | Bachem | I-1985 |
| Commercial kits | | |
| iScript cDNA Synthesis Kit | Biorad | 1708891 |
| SsoAdvanced Universal SYBR Green Supermix | Biorad | 1725274 |
| TNF alpha Human ELISA Kit, High Sensitivity | eBiosciences | BMS223HS |
| Pierce Rapid Gold BCA Protein Assay Kit | Thermo Fisher Scientific | A53226 |
| Amicon Ultra-4 Centrifugal Filter Unit | Millipore | UFC800396 |

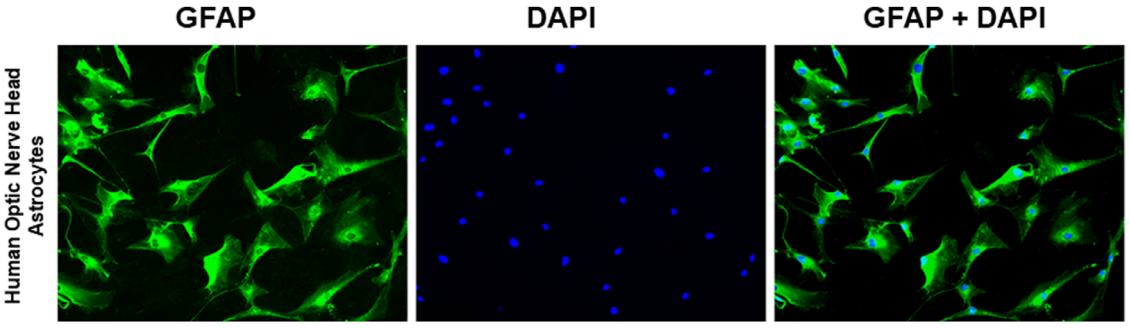


**Figure 1.** Immunostaining of ONH astrocytes using GFAP. ONH astrocytes were fixed by 4% paraformaldehyde followed by immunostaining with anti-GFAP antibodies. The signal was captured by using AlexaFluor 488 conjugated secondary antibodies. *Green* color indicates staining for GFAP and nuclear staining by DAPI was indicated by *blue*. Data shown are a representation of at least five independent experiments.

and Boc-Lys(Tfa)-AMC in 96-well black bottom plates at room temperature for 1 hour. Baseline fluorescence was measured, followed by treatment with the peptidase enzyme trypsin, freeing the fluorogenic 4-methylcoumarin-7-amide (AMC). The amount of fluorogenic AMC generated was then measured using an excitation wavelength of 355 nm and emission wavelength of 460 nm with a standard fluorospectrometer. The Boc-Lys(Ac)-AMC and Boc-Lys(Tfa)-AMC substrates are specific to HDAC 1, 3, 6, and 4, 5, 7, 8, 9, 10, and 11, respectively.

**Table 2.** Primer Sequences Used in qRT-PCR

| Primer | Direction | Sequence |
|---|---|---|
| HDAC 1 Set-1 | Forward | GGAAATCTATCGCCCTCACA |
| HDAC 1 Set-1 | Reverse | TTGCCACAGAACCACCAGTA |
| HDAC 1 Set-2 | Forward | GGTCCAAATGCAGGCGATTCCT |
| HDAC 1 Set-2 | Reverse | TCGGAGAACTCTTCCTCACAGG |
| HDAC 2 Set-1 | Forward | ATGGCGTACAGTCAAGGAGG |
| HDAC 2 Set-1 | Reverse | TGCGGATTCTATGAGGCTTCA |
| HDAC 2 Set-2 | Forward | CTCATGCACCTGGTGTCCAGAT |
| HDAC 2 Set-2 | Reverse | GCTATCCGCTTGTCTGATGCTC |
| HDAC 3 Set-1 | Forward | TGGCTTCTGCTATGTCAACG |
| HDAC 3 Set-1 | Reverse | TTGATAACCGGCTGGAAAAG |
| HDAC 6 Set-1 | Forward | TATCTGCCCCAGTACCTTCG |
| HDAC 6 Set-1 | Reverse | GGACATCCCAATCCACAATC |
| GAPDH Set-1 | Forward | ATGGGAGTTGCTGTTGAAGTCA |
| GAPDH Set-1 | Reverse | CCGAGGGCCCACTAAAGG |

### Western Blotting

Equivalent amounts of cell lysates of ONH astrocytes (10–20 µg protein/lane) were loaded onto 10% Novex-Bis Tris gel, proteins separated, and proteins transferred to nitrocellulose membranes as described elsewhere.[16,18–20] The membranes were blocked with 5% BSA or nonfat dry milk followed by incubation with primary antibodies of acetyl histone H3 (AcH3K9), acetyl histone H4 (AcH4K8), or acetyl histone H2B (AcH2BK5) at (1:1000), HDAC 1, 2, or 6 (1:2000), HDAC 3, 4, 5, 7, 8, 9, 10, and 11 (1:1000), or $\beta$-actin (1:4000) dilutions overnight at 4°C. After washing, membranes were incubated with secondary antibodies (horseradish peroxidase–conjugated; dilution 1:5000) for 1 hour at room temperature. For chemiluminescent detection, the membranes were treated with enhanced chemiluminescent reagent, and the signal was captured using a Biorad Versadoc imaging system (Biorad, Hercules, CA).

### Quantitative Real-time PCR

Total RNA was isolated from ONH astrocytes using TRIzol reagent (Life Technologies, Grand Island, NY). cDNA was generated from 1 µg total RNA using iScript cDNA Synthesis Kit as per manufacturer's instructions (BioRad). Quantitative real-time PCR was performed using a SYBR Green mix (Biorad) in the CFX96 Detection System (BioRad). The relative gene expression was calculated based on the comparative threshold cycle method and normalized to housekeeping genes GAPDH. List of primers is provided in Table 2, which were obtained from Integrated DNA Technology (Coralville, IA).

### Enzyme-Linked Immunosorbent Assay

The levels of TNF-$\alpha$ in cell lysates and conditioned media of ONH astrocytes were measured by sandwiched-based ELISA kit (Human TNF-$\alpha$ ELISA, eBioscience, San Diego, CA) as described elsewhere.[19] Briefly, media collected from ONH astrocyte was concentrated using an ultrafiltration Amicon centrifugal filter units (3 kDa cut-off, Milipore-Sigma, Burlington, MA) and adjusted to a final volume of 250 µL as described elsewhere.[19] Equivalent amounts of cell lysate (50 µL) or conditioned media (100 µL) were incubated in a 96-well plate containing immobilized monoclonal TNF-$\alpha$ antibodies. Thereafter, the biotin-conjugated anti–TNF-$\alpha$ and streptavidin–horseradish peroxidase were added in each well with intermediate washing steps, as per the manufacturer's directions. Finally, the antibody–protein complex was detected by addition of the chromogenic substrate (tetramethylbenzidine) and absorbance was measured at 450 nm on a Synergy H1 multimode microplate reader (Biotek, Vinooski, VT). The amount of TNF-$\alpha$ in each sample was calculated using a standard curve prepared from the known concentrations of recombinant human TNF-$\alpha$ and extrapolated values were normalized to total cellular protein. To stimulate the TNF-$\alpha$ production, cells were treated with 10 µg/mL LPS for 6 hours at 37°C. To determine if $\delta$-opioid receptor activation attenuate LPS-induced TNF-$\alpha$ production, cells were pretreated with 1 µM SNC-121 for 24 hours at 37°C.

### Statistical Analysis

All data are presented as ± SEM from three or more independent experiments. Statistical analysis was performed by Student the *t* test for paired data. ANOVA Bonferroni post-test was used for multiple comparisons (GraphPad Software, Inc., San Diego, CA). A $P$ value of 0.05 or less was considered significant. Each experiment design consists of at least three "n", where "n" refers to biological replicates. Additionally, we performed each experiment in primary cultures obtained from at least two to three different donors.

## Results

### Human ONH Astrocytes

The purity of ONH astrocytes was assessed using astrocytes marker, GFAP. Immunostaining of GFAP along with DAPI (a nuclei marker) is shown in Figure 1. There were 34 DAPI-positive cells, which were all positively stained with GFAP, suggesting 100% purity of the ONH astrocytes used in the current study.

### Effect of $\delta$-Opioid Receptor Agonist (SNC-121) Treatment on Histone Acetylation

Addition of 1 µM SNC-121, a $\delta$-opioid receptor agonist, produced a time-dependent increase in histone H3 acetylation with the maximum acetylation occurring at 24 hours (145 ± 2% above control level; Figs. 2 and 3A, Supplementary Fig. 1). Based on these data, we chose a 24-hour time-point for all subsequent studies. To assess whether SNC-121 also affects the acetylation of other histones, we treated ONH astrocytes with 1 µM SNC-121 for 24 hours and acetylation of histone H3, H4, and H2B was determined by Western blotting using selective antibodies for each histone. As shown in Figures 3A to 3C, SNC-121 treatment for 24 hours increased acetylation of histone H3, H2B, and H4 by 128 ± 3% ($P = 0.002$), 45 ± 1% ($P = 0.005$), and 68 ± 2% ($P = 0.009$), respectively. It is evident from these data that histone H3 acetylation is the most robustly affected by SNC-121 treatment. Hence, we focused our studies on histone H3 acetylation in response to SNC-121 treatment for 24 hours in subsequent experiments using ONH astrocytes.

Generally, histone acetylation is regulated by enzymes called HATs. To determine whether $\delta$-opioid receptor agonist (SNC-121)–mediated histone H3 acetylation is due to a direct activation of HATs, ONH astrocytes were pretreated with a

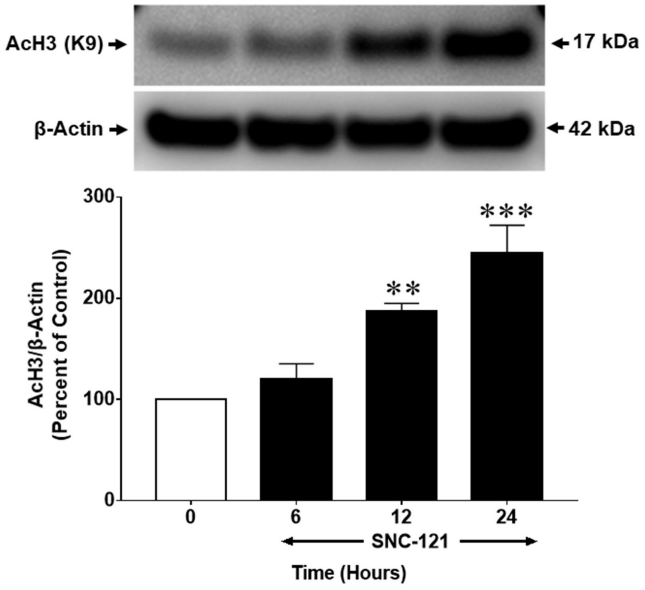


**Figure 2.** Time-dependent effects of δ-opioid receptor agonist, SNC-121, on the acetylation of histone H3. ONH astrocytes were starved in serum-free astrocyte basal medium for 16 hours. Cells were then treated with SNC-121 (1 µM) for the indicated time period. Cell lysates (20 µg) were analyzed by Western blotting using anti-acetyl histone H3 (Lys9) antibody followed by reprobing with anti–β-actin antibody as a loading control. The band intensities were measured using chemiluminescent reagent and Versadoc imaging system. Data are expressed as mean ± SE. $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 4$. Protein bands shown are representative of atleast 4 independent experiments.

selective HAT inhibitor, Garcinol, before SNC-121 treatment. As shown in Figure 4 and Supplementary Figure 1, 1 µM Garcinol before treatment fully blocked SNC-121–induced histone H3 acetylation, suggesting that the SNC-121–induced increase in H3 acetylation is via direct activation of HATs.

## Effect of δ-Opioid Receptor Agonist (SNC-121) Treatment on HDACs Activity

Protein acetylation homeostasis is maintained by the activities of crucial enzymes called HATs and HDACs. This homeostasis is disturbed under certain pathological conditions and drug treatments. To determine whether SNC-121 regulates HDACs activities, ONH astrocytes were treated with 1 µM SNC-121 for 24 hours followed by the measurement of HDACs activities. The activities of HDAC class I and IIb (HDAC 1–3, 6) and class IIa and IV (HDAC 4, 5, and 7–11) were measured using selective substrates as described in the method's section. As shown in Figure 5A, activation of the δ-opioid receptors by SNC-121 decreases the activities of class I and IIb HDACs activities significantly (17 ± 3%, $P = 0.009$). As a negative control in this assay, we have used a pan HDAC inhibitor (TSA). As expected, 1 µM TSA treatment inhibited 79 ± 2% ($P = 0.0001$) of class I and IIb HDACs activities (Fig. 5A). To further confirm that the SNC-121–mediated decrease in HDAC activity is due to δ-opioid receptors activation, we pretreated ONH astrocytes with a highly selective δ-opioid receptors antagonist, naltrindole, followed by SNC-121 treatment. As expected, the SNC-121–induced reduction in class I and class II b HDACs activities was fully blocked, suggesting that the SNC-121–induced decrease in HDACs activity is via activation of δ-opioid receptors. In contrast, we did not see any effects of SNC-121 and naltrindole on class IIa and IV HDACs activities (Fig. 5B).

## Effect of SNC-121 Treatment on mRNA and Protein Expression of HDACs

We determined whether δ-opioid receptor activation by SNC-121 also regulates gene and protein expression of HDACs in ONH astrocytes. To achieve this goal, we treated ONH astrocytes with 1 µM SNC-121 for 24 hours followed by measurement of mRNA by quantitative RT-PCR and protein expression of individual HDAC by Western blotting. As shown in Figures 6C to 6D, mRNA expression for HDAC 3 and HDAC 6 was reduced by 19% and 18%, respectively (HDAC 3, 0.81 ± 0.04 [$P = 0.002$] and HDAC 6, 0.82 ± 0.06 [$P = 0.03$]). In contrast, we did not see any changes in the mRNA levels of HDAC 1 and HDAC 2 by SNC-121 treatment (Figs. 6A–B). To confirm this finding, we have used another set of primers (Table 2) and we found similar results (data not shown). Interestingly, the protein expression pattern of HDAC 1, 2, 3, and 6 was significantly decrease by 35 ± 8% [$P = 0.001$], 20 ± 7% [$P = 0.02$], 20 ± 4% [$P = 0.0002$], and 14 ± 5% [$P = 0.014$], respectively, by SNC-121 treatment (Figs. 7 A–D and Supplementary Fig. 1). In contrast, protein expression of HDAC 4, 5, 6, 7, 9, 10, and 11 was not altered by SNC-121 treatment (Figs. 8A–F and Supplementary Fig. 2).

## Effect of SNC-121 Treatment on LPS-Induced TNF-α Production From ONH Astrocytes

We have shown that proinflammatory cytokines such as TNF-α are produced in response to acute ischemic retinal injury, chronic glaucomatous injury, and by LPS-induced activation of Toll like receptor-4.[16,19,21] To mimic the in vivo inflammatory conditions, we have used LPS to treat ONH astrocytes. Optimal timings and concentration of LPS treatment have been evaluated in our earlier studies.[19] As shown in Figures 9A and 9B, LPS (10 µg/mL) treatment significantly increased the production of TNF-α in cell lysates and conditioned media by 36.6 ± 0.4% ($P = 0.014$) and 210.5 ± 6% ($P = 0.013$), respectively, which was significantly inhibited by 1 µM SNC-121 treatment in ONH astrocytes.

## Effect of SNC-121 Treatment on Astrocytic Activity in the Optic Nerve of Ocular Hypertensive Animals

To further confirm that SNC-121 treatment decreased astrocytic activity, we measured changes in the GFAP expression in the nonmyelinated portion of optic nerve of normal, ocular hypertensive, and SNC-121–treated ocular hypertensive animals. To determine the effects of δ-opioids on astrocytes activation, we treated animals with SNC-121 (1 mg/kg body weight, intraperitoneal injections) daily for 7 days. The IOP increased to 24.3 ± 0.3 mm Hg in ocular hypertensive animals when compared with normal animals (16.8 ± 0.3 mm Hg) at day 7 after injury. The IOP was not changed by SNC-121 treatment (23.8 ± 0.9 mm Hg). As shown in Figure 10, a mild immunostaining of GFAP was seen in the normal optic nerve, which was remarkably increased in ocular hypertensive animals. Interestingly, GFAP immunostaining was remarkably decreased in

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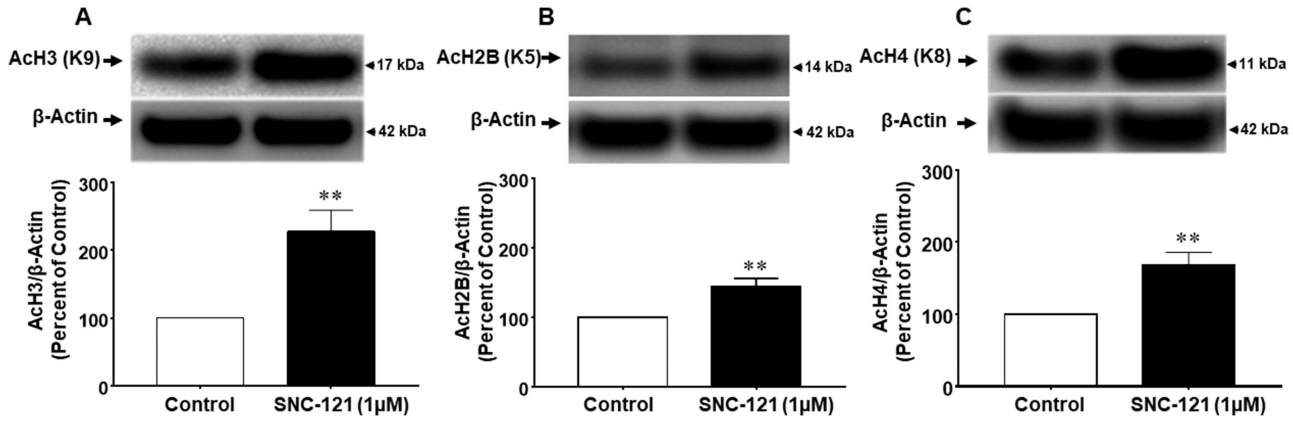


**Figure 3.** SNC-121–induced acetylation of histones (**A**) H3, (**B**) H2B, and (**C**) H4 in ONH astrocytes. Cells were starved in serum-free astrocyte basal medium for 16 hours followed by treatment with SNC-121 (1 μM) for 24 hours. Cell lysates (20 μg) were analyzed by Western blotting using anti-acetyl histone H3 (Lys9), anti-acetyl histone H2B (Lys5), anti-acetyl histone H4 (Lys8), or anti–$\beta$-actin antibodies. The band intensities were measured using chemiluminescent reagent and Versadoc imaging system. Data are expressed as mean ± SE. $^{**}P < 0.01$; $n = 7$–11. Protein bands shown are representative of atleast 7 independent experiments.

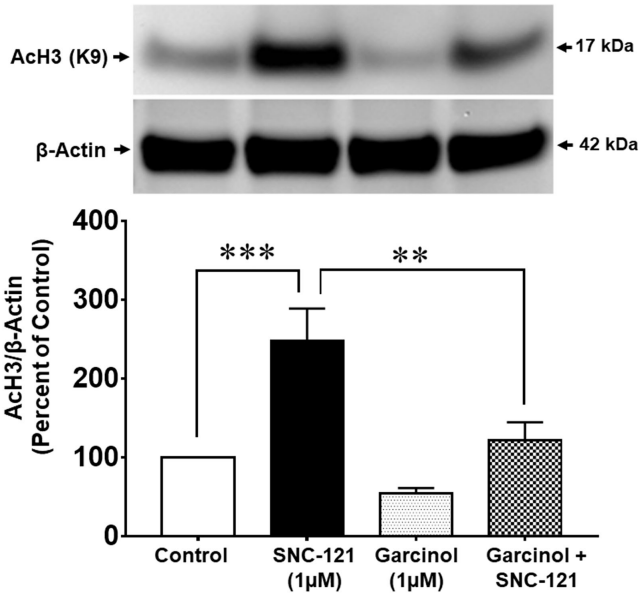


**Figure 4.** Effects of HATs (p300/HAT) inhibitor, garcinol, on SNC-121–induced acetylation of histones H3 in ONH astrocytes. Cells were starved in serum-free astrocyte basal medium for 16 hours followed by treatment with SNC-121 (1 μM), Garcinol (1 μM), or with both drugs for 24 hours. Briefly, ONH astrocytes were pretreated with Garcinol for 15 minutes before SNC-121 treatment. Cell lysates (20 μg) were analyzed by Western blotting using anti-acetyl histone H3 (Lys9) or anti–$\beta$-actin antibodies. The band intensities were measured using chemiluminescent reagent and Versadoc imaging system. Data are expressed as mean ± SE. $^{**}P = 0.005$; $^{***}P = 0.0009$; $n = 6$. Protein bands shown are representative of atleast 6 independent experiments.

the optic nerves of SNC-121–treated ocular hypertensive animals.

## Discussion

The process of deacetylation and acetylation is reversible in nature and it involves two evolutionary conserved classes of enzymes: HDACs and HATs. HDACs remove acetyl groups from histone proteins thereby allowing the histones to wrap DNA more tightly resulting in repression of transcriptional activity and gene expression. In contrast, HATs (via increase in acetylation) enhance transcription activity and gene expression. Based on their regulatory actions on the gene expression, HDACs and HATs are attractive therapeutic targets for the drugs/agents that can provide neuroprotection. There are 18 HDACs that have been reported in mammals based on their homology to yeast deacetylases. HDACs are classified into four categories: class I (HDAC 1, 2, 3, and 8), class IIa (HDAC 4, 5, 7 and 9), class IIb (HDAC 6 and 10), class III (Sirtuins 1–7), and class IV (HDAC 11).[22–25] Class I, class IIa, class IIb, and class IV require zinc as a cofactor, whereas class III requires nicotinamide adenine dinucleotide ($NAD^+$) as a cofactor for their activation. The regulation of HDACs has been shown to be associated with cellular function including gene expression, cell differentiation, apoptosis, and wound healing.[26,27] In glial cells, HDAC inhibitors suppressed the expression of TNF-$\alpha$, IL-6, IL-1$\beta$, iNOS, Cox-2, and heat shock protein 70 in response to different stimuli.[28–32]

In the last decade, histone modification via lysine acetylation has gained much attention because of their active role in neurodegenerative diseases, such as Alzheimer's disease,[33] Parkinson's disease,[34] AMD,[35] and glaucoma.[36] Studies have also shown that administration of HDAC inhibitors promotes neuronal survival in models of Huntington's disease,[37,38] amyotrophic lateral sclerosis,[39] spinal muscular atrophy,[40] Parkinson's disease,[41] Alzheimer's disease,[42] and stroke.[43,44] In the eye, we and others have shown hypoacetylation of retinal proteins in acute ischemia,[14] glaucoma,[13] and photoreceptor degeneration (rd1 mouse) models.[45] The protein hypoacetylation has been shown to be associated with an increase in HDAC activity, caspase activation, and retina degeneration.[12,13,45] Additionally, studies have shown that HDAC inhibitors such as TSA, sodium butyrate, and valproic acid, can protect RGCs in acute ischemia, optic nerve crush injury,[14,46] and chronic glaucoma models.[12,13,47–49]

Although protein acetylation and alterations in HDACs expression have been reported in the retina of acute

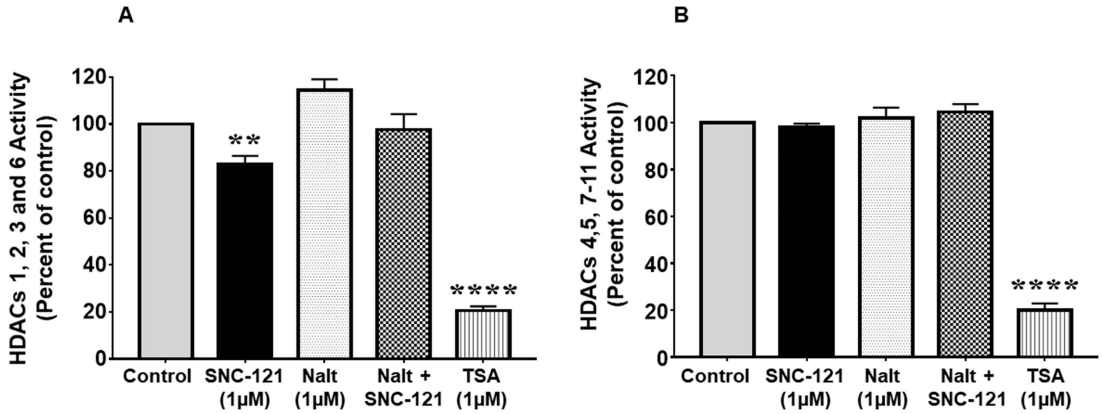


**Figure 5.** Effect of δ-opioid receptor agonist (SNC-121) and antagonist (naltrindole) on HDACs activity in ONH astrocytes. (**A**) Enzyme activity of class I and IIb HDACs (HDACs 1-3, 6) was measured using fluorophore-conjugated substrate, Boc-Lys(Ac)-AMC. (**B**) Enzyme activity of class IIa and IV (HDACs 4, 5, and 7- 11) was measured using fluorophore-conjugated substrate, Boc-Lys(Tfa)-AMC. Cells were starved in serum-free astrocyte basal medium for 16 hours followed by treatment with SNC-121 (1 μM), naltrindole (1 μM), or with both drugs for 24 hours. Cells were also treated with broad-spectrum HDAC inhibitor, TSA (1 μM) for 24 hours. Equal amount of cell lysates (1 μg) from each treatment group was incubated with the substrate for one hour at room temperature. The HDAC activity was measured by detection of fluorescent amino-methoxy cumarin (AMC) released at excitation/emission of 355/460 nm using spectrofluorometer. Data are expressed as mean ± SE. $^{**}P < 0.01$; $^{****}P < 0.0001$; $n = 3$–5.

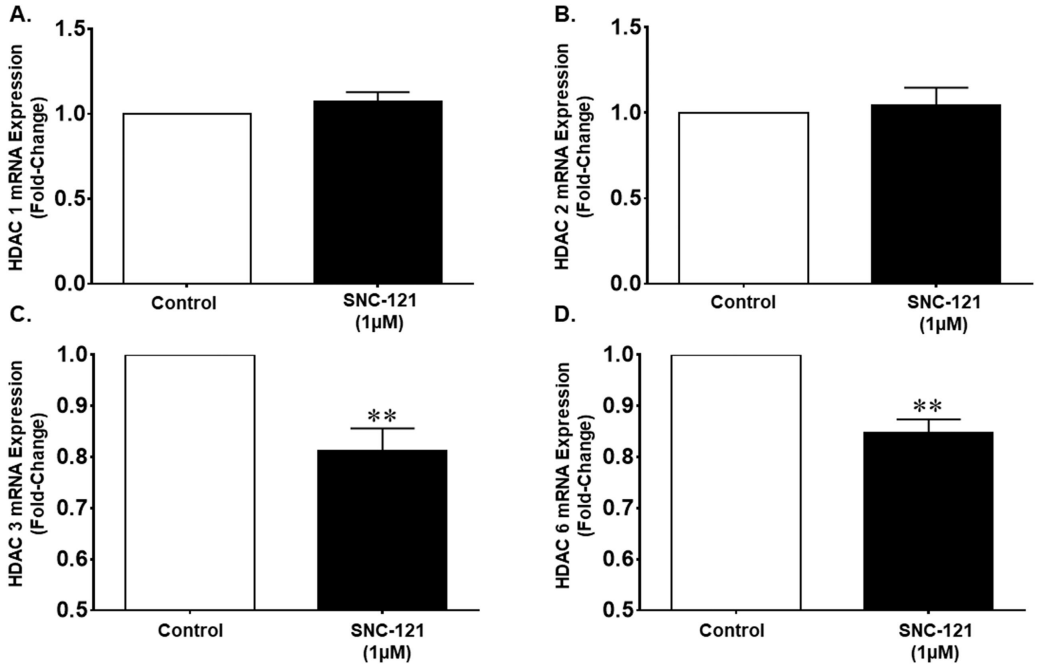


**Figure 6.** Changes in mRNA expression of (**A**) HDAC 1, (**B**) HDAC 2, (**C**) HDAC 3, and (**D**) HDAC 6 in response to SNC-121 treatment. ONH astrocytes were starved in serum-free astrocyte basal medium for 16 hours followed by treatment with SNC-121 (1 μM) for 24 hours. Total RNA was extracted using TRIzol reagent and 1 μg RNA was used to synthesize cDNA. The mRNA levels were examined by quantitative RT-PCR and relative gene expression was calculated based on the comparative threshold cycle (Ct) method and normalized to housekeeping genes, GAPDH. Data are expressed as mean ± SE. $^{**}P < 0.01$; $n = 4$–8.

ischemia and chronic glaucoma models,[13,14] their expression and δ-opioids–induced changes in glial cells, specifically in ONH astrocytes, remains largely unknown. The current study provides significant insights about protein acetylation homeostasis, HDACs activity and their expression (mRNA and protein), and their regulation by δ-opioid receptor activation in ONH astrocytes. Despite the extensive research to understand glial–neuron interactions and their behavioral changes in response to glaucomatous injury we are still unaware about the mechanisms involved in RGCs death during glaucomatous injury. Under physiologic conditions, quiescent astrocytes provide a variety of functional and structural support to the neurons, such as meeting metabolic demands, regulating cerebral blood flow, and scavenging free radicals.[50] Under pathophysiologic conditions, biomechanical injury or stress-stimulated reactive astrocytes produce proinflammatory cytokines (e.g., TNF-α and interleukins), nitric oxide, and matrix metallo-

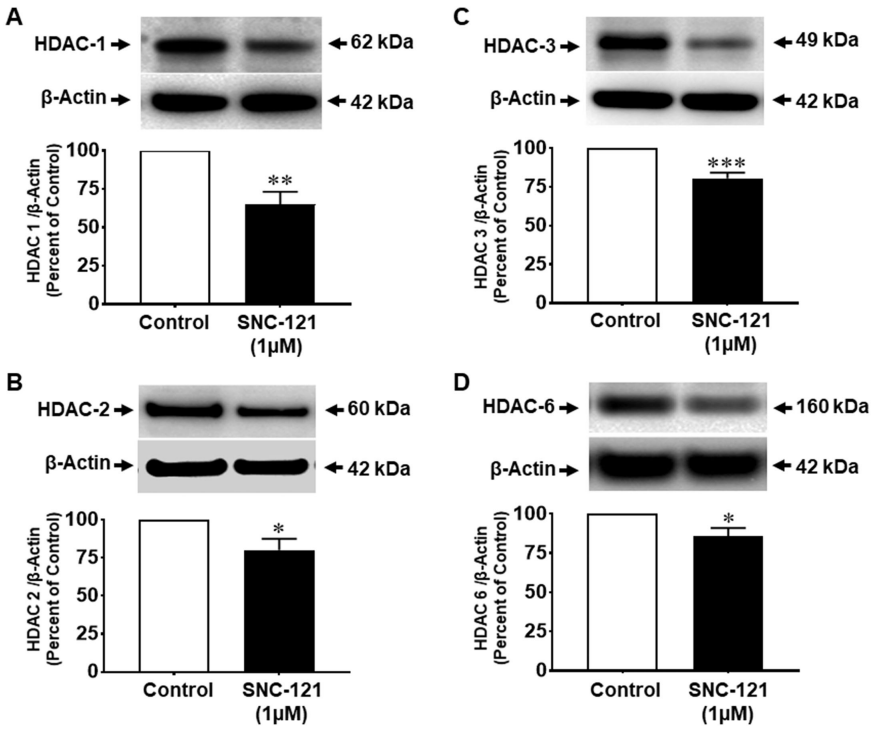


**Figure 7.** Changes in protein expression of (**A**) HDAC 1, (**B**) HDAC 2, (C) HDAC 3, and (**D**) HDAC 6 by SNC-121 treatment. ONH astrocytes were starved in serum-free astrocyte basal medium for 16 hours followed by treatment with SNC-121 (1 μM) for 24 hours. Cell lysates (20 μg) were analyzed by Western blotting using anti-HDAC 1, anti-HDAC 2, anti-HDAC 3, anti-HDAC 6, or anti–$\beta$-actin antibodies. The band intensities were measured using chemiluminescent reagent and Versadoc imaging system. Data are expressed as mean ± SE, $n$ = 11–14. Protein bands shown are representative of atleast 11 independent experiments.

proteinases that may subsequently cause direct or indirect injury to the axons and RGCs.[2,8,16,51]

Data presented in this report provide first evidence that $\delta$-opioid receptor activation by SNC-121 treatment significantly increases the protein acetylation of histone H2B, H3, and H4. We found the most robust acetylation of histone H3 in response to SNC-121 treatment. We further confirmed that SNC-121 increases histone H3 acetylation by HATs activation because pretreatment of cells with Garcinol (i.e., a selective HATs inhibitor) fully blocked SNC-121–induced histone H3 acetylation. Garcinol is a highly selective inhibitor of histone acetyl transferases, principally p300 and p300/CBP-associated factor, which is known to inhibit HATs activity in other systems.[52–54] Furthermore, $\delta$-opioid receptor activation by SNC-121 treatment significantly decreases the activities of class I HDACs (e.g., HDAC 1, 2, and 3) and class IIb HDAC (HDAC 6). In contrast, we did not see any inhibitory effect of SNC-121 on class IIa and IV HDACs (HDAC 4, 5, 7, and 9–11) activities in ONH astrocytes, suggesting that $\delta$-opioid receptor agonist selectively regulates the class I and class IIb HDACs activities in ONH astrocytes. This SNC-121–induced decrease in HDACs activities is due to $\delta$-opioid receptor activation because a selective $\delta$-opioid receptor antagonist (e.g., naltrindole) fully blocked SNC-121–induced reduction in HDAC activities. Moreover, we found that SNC-121 treatment attenuated the mRNA expression of HDAC 3 and 6 and decreases the protein expression levels of HDAC 1, 2, 3, and 6. Interestingly, mRNA expression of HDAC 1 and 2 was not inhibited by SNC-121 treatment. These data further suggest that $\delta$-opioid receptor agonist is targeting upstream transcription factors that are regulating the transcription of specific HDACs (i.e., HDAC 3 and 6), whereas it can regulate other HDACs (i.e., HDAC-1 and 2) post-transcriptionally. To confirm this observation, we have used another set of HDAC 1 and 2 primers (Table 2) and we did not see any changes in mRNA expression of HDAC 1 and 2 in response to SNC-121 treatment (data not shown), suggesting that $\delta$-opioids differentially regulate HDACs mRNA expression at transcriptional level. Additionally, we did not see any decrease in protein expression of class IIa (4, 5, 7, and 9) and IV (11) HDACs in response to SNC-121 treatment. In other systems, studies have shown that TNF-$\alpha$ treatment suppressed HDAC 1 protein expression owing to proteasomal degradation without affecting its mRNA levels in ZR-75-1 cells.[55] Based on our observation and previously published work in other systems, we speculate that SNC-121–induced reduction in HDAC 1 and 2 protein expression are due to ubiquitination and/or proteosomal degradation process and not due to decrease in transcriptional activity. We also believe that SNC-121–induced increase in histone acetylation is due to both activation of HATs and decrease in HDACs expression in ONH astrocytes. However, this claim requires further experimental support, which will be tested in our future studies. In this study, we did not measure the effects of SNC-121 on HDAC class III (sirtuins 1–7); however, this work is under progress and it will be published in future studies. We also determined if $\delta$-opioid receptor activation regulates the production of proinflammatory cytokines (e.g., TNF-$\alpha$) and astrocyte activation. We provided evidence that LPS-induced TNF-$\alpha$ is fully blocked by SNC-121 treatment. LPS activates TLR-4 receptors and it has been frequently used to mimic

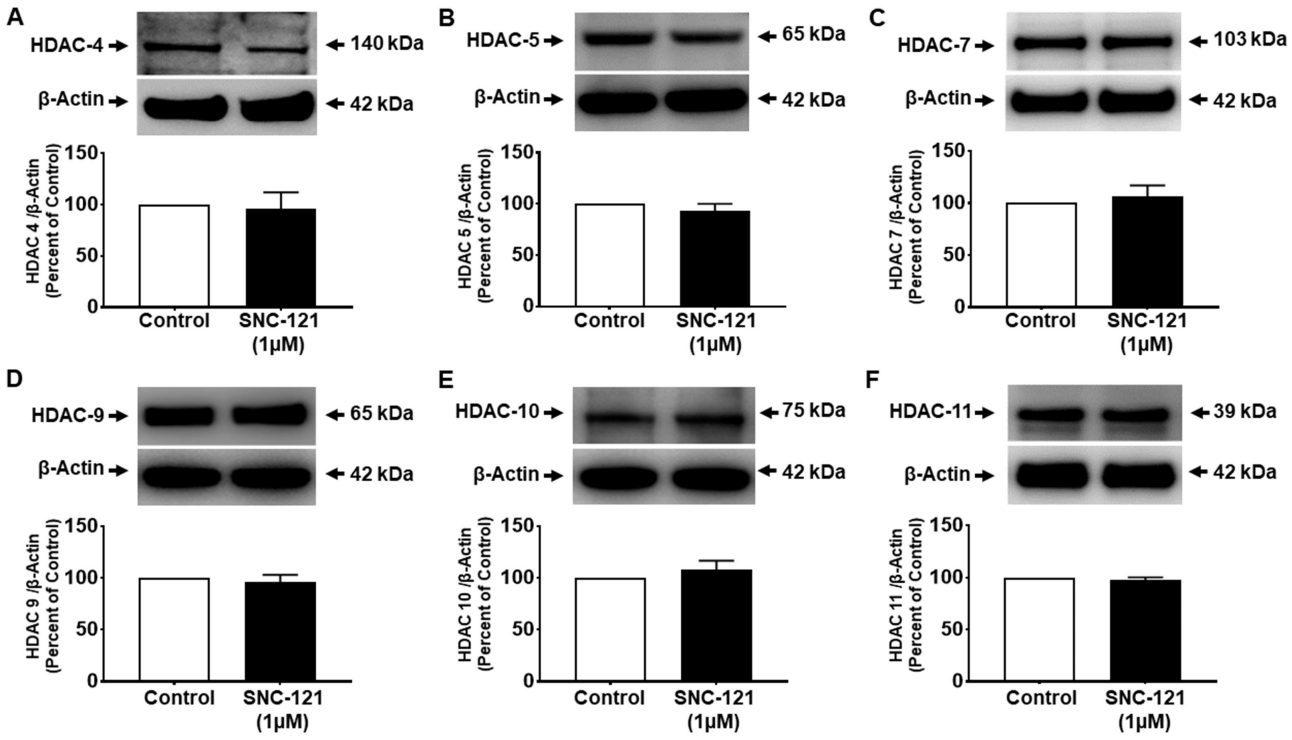


**Figure 8.** Effects of SNC-121 on the expression of (**A**) HDAC 4, (**B**) HDAC 5, (**C**) HDAC 7, (**D**) HDAC 9, (**E**) HDAC 10, and (**F**) HDAC 11 in ONH astrocytes. Cell lysates (20 µg) were analyzed by Western blotting using anti-HDAC 4, anti-HDAC 5, anti-HDAC 7, anti-HDAC 9, anti-HDAC 10, anti-HDAC 11, or anti–$\beta$-actin antibodies. The band intensities were measured using chemiluminescent reagent and Versadoc imaging system. Data are expressed as mean ± SE, $n = 4$–10. Protein bands shown are representative of atleast 4 independent experiments.

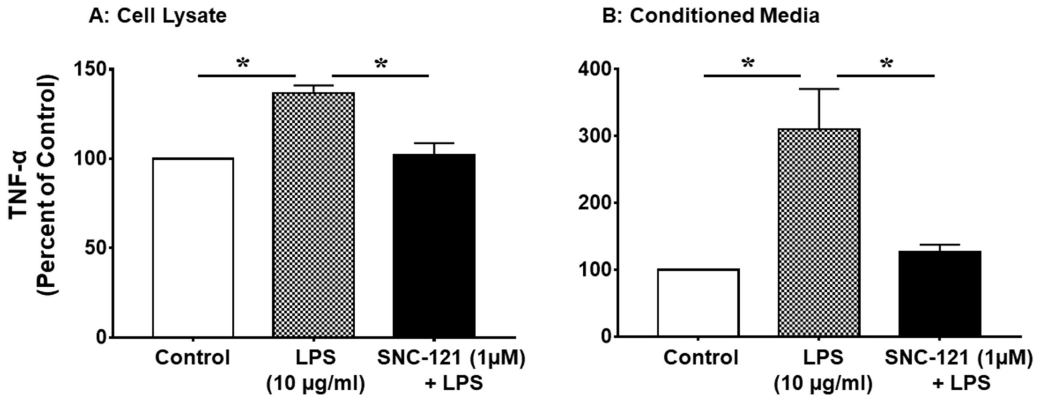


**Figure 9.** Effects of SNC-121 on LPS-induced TNF-$\alpha$ production in (**A**) cell lysates and (**B**) conditioned media of ONH astrocytes. Cells were starved in serum-free astrocyte basal medium for 16 hours followed by treatment with SNC-121 (1 µM) for 24 hours followed by treatment with LPS (10 µg/mL) for 6 hours at 37°C. The equivalent amounts of cell lysate (50 µL) and conditioned media (100 µL) were analyzed for TNF-$\alpha$ by ELISA as per manufacturers' instructions. The amount of TNF-$\alpha$ was measured by detection of absorbance at 450 nm using multimode plate reader. Data are expressed as mean ± SE. *$P < 0.05$; $n = 3$.

stress/inflammatory conditions in numerous cell types.[19,56] In addition, in vivo studies herein showed that SNC-121 treatment decrease the astrocytes activation as measured by GFAP immunostaining, suggesting that $\delta$-opioid receptor activation lowers inflammation and astrocytes activation under glaucomatous condition.

In summary, we provide evidence that $\delta$-opioid receptor agonist, SNC-121, increases histone H3, H4, and H2B acetylation, and reduces HDACs class I and class IIb activities, mRNA, and protein expression in ONH astrocytes. We further confirmed that SNC-121–induced histone H3 acetylation is mediated via HATs activation.

Interestingly, $\delta$-opioids reduce mRNA expression of HDAC 3 and HDAC 6, whereas they blocked protein expression of HDAC 1, 2, 3, and 6 in ONH astrocytes. Our data suggest that $\delta$-opioids differentially regulate HDACs at transcriptional level. We also have shown that LPS-induced TNF-$\alpha$ production is fully blocked by SNC-121 pretreatment. Finally, we have shown that GFAP immunostaining (an indicator of astrocytes reactivity) is remarkably decreased in

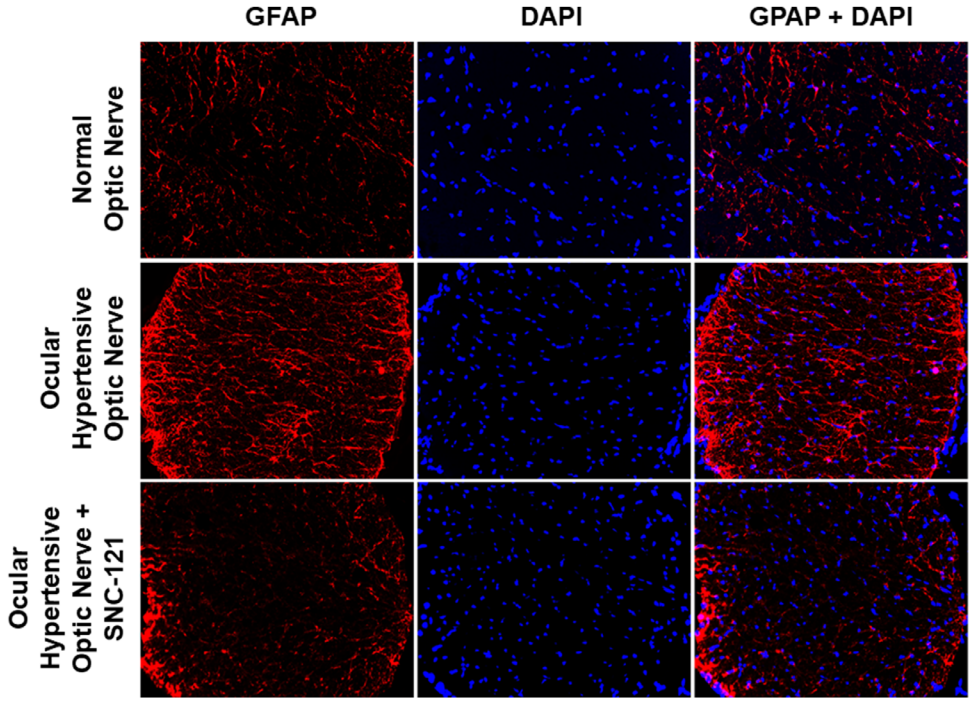


**Figure 10.** Effects of SNC-121 treatment on GFAP immunostaining in ocular hypertensive animals. The optic nerves of Brown Norway rats were removed 7 days after hypertonic saline injection. Cryosections were immunostained by anti-GFAP antibodies. *Red* color indicates staining for GFAP and *blue* nuclei for DAPI. There was no positive staining when primary antibodies were omitted (not shown). Data shown in this figure are a representation of at least four independent experiments.

the optic nerves of ocular hypertensive animals that were treated with 1 mg/kg SNC-121 for 7 days. Taken together, our data support the idea that the reactivity of ONH astrocytes under pathologic conditions may be regulated by enhancing protein acetylation either via HATs activation, decrease HDACs activation, or by both. Overall, this study provides novel insight for the role of $\delta$-opioid receptors in regulating protein acetylation and HDAC expression in ONH astrocytes. However, the molecular mechanisms by which $\delta$-opioids suppresses HDACs and induce epigenetic changes requires further investigation.

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