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Histone Deacetylase Inhibition–Mediated Differentiation of RGC-5 Cells and Interaction with Survival

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Abstract

PURPOSE—The acetylation state of histones is modulated by histone deacetylase (HDAC) and histone acetyltransferase and is an important component in regulating gene transcription, including neuronal differentiation. The authors studied the relationship between histone acetylation and the differentiation and survival of the RGC-5 cell line and compared it with nontranscriptional-dependent differentiation with staurosporine.

METHODS—The retinal ganglion cell line RGC-5 was treated with trichostatin A (TSA), other HDAC inhibitors, and staurosporine; differentiation, neuritogenesis, neurotrophic factor dependence, and dependence on RNA transcription were assessed.

RESULTS—TSA caused significant differentiation and neuritogenesis. Differences between HDAC inhibition and staurosporine differentiation included the proportion of differentiated cells, cell viability, cell morphology, and transcriptional dependence. HDAC inhibition, but not staurosporine differentiation, resulted in RGC-5 cells that were neurotrophic factor dependent.

CONCLUSIONS—These results implicate two different mechanisms for RGC-5 differentiation, with a common downstream effect on neurite outgrowth but a differential effect on neurotrophic factor dependence.

The differentiation of progenitor cells is an important step in the repopulation of neurons from stem cells. Progenitor cells may be differentiated in vivo after transplantation or differentiated in vitro before transplantation.[1] In both cases, the eventual functional replacement of the absent neuronal population requires appropriate differentiation signals. We used as a model system the retinal ganglion cell line RGC-5, which was immortalized from a committed RGC progenitor cell at postnatal day (P)1.[2] We have previously shown that the nonspecific kinase inhibitor staurosporine can induce the mitotically active RGC-5 cell line to stop proliferation, extend neurites, and express many of the electrophysiological and histochemical markers characteristic of primary RGCs.[3] However, staurosporine-differentiated RGC-5 cells differ in significant ways from primary cultured RGCs. Staurosporine differentiation is transcription independent and results in cells that are viable in the absence of any neurotrophic factor support, unlike normally differentiated RGCs. Neurotrophic factor dependence would be a necessary component of reproducing functional connectivity of neurons to central nervous system targets,[4] which is the goal of in vivo application of neuronal stem cells.

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Here we report that histone deacetylase (HDAC) inhibition differentiates RGC-5 cells in a manner that is transcription dependent and that results in neurotrophic factor-dependent cells. We focused on trichostatin A (TSA), a potent, specific, and well-characterized class 1 and class 2 HDAC inhibitor[5,6] reported to induce differentiation in rat hippocampal neural progenitor cells and Neuro 2a cells.[7,8] We found that TSA induces neurite outgrowth from RGC-5 cells that are morphologically, quantitatively, and mechanistically different from the differentiation induced by staurosporine, suggesting that HDAC inhibition holds promise as a method for differentiating RGC progenitors in vitro.

MATERIALS AND METHODS

Materials

TSA was purchased from A.G. Scientific (San Diego, CA); α-amanitin from AXXORA (San Diego, CA); brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) from PeproTech (Rocky Hill, NJ); insulin, forskolin, bromodeoxyuridine (BrdU), monoclonal mouse anti–BrdU (clone Bu 33), rabbit anti–actin, and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO); staurosporine (from *Streptomyces staurosporeus*; 98% or greater purity; catalog number 380–014) from Alexis Biochemicals (San Diego, CA); monoclonal mouse anti–tau-1 (catalog number MAB3420), polyclonal rabbit anti–MAP2 (catalog number AB5622), polyclonal rabbit anti–Brn-3a (catalog number AB5945), horseradish peroxidase (HRP)–conjugated donkey anti–goat IgG, and HRP-conjugated goat anti–rabbit IgG antibodies from Chemicon (Temecula, CA); polyclonal goat anti–Brn-3b antibody (catalog number sc-31,987) from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal mouse anti–H3 (catalog number 05-499) and polyclonal rabbit anti–acetyl-H3 (catalog number 06-599) antibodies from Upstate (Lake Placid, NY); goat anti–mouse IgG and goat anti–rabbit IgG from LI-COR (IRDye 800 and IRDye 680, respectively; Lincoln, NE); and Alexa Fluor 594 goat anti–mouse IgG, Alexa Fluor 488 goat anti–rabbit IgG antibodies, and propidium iodide (PI) from Molecular Probes (Eugene, OR). Cell culture reagents, unless otherwise noted, were from BioWhittaker (Rockland, ME).

Cell Culture

RGC-5 cells were cultured as previously described.[3] For proliferation and immunohistochemical experiments, cells were replated on 12-mm round coverglass in 24-well plates at a density of 80 cells/mm^2 in 500 μL media 24 hours before pharmacologic treatment. In experiments in which pharmacologic treatments were withdrawn, the original media were replaced under all conditions to control for conditioned media effects. All conditions were tested in duplicate.

Immunohistochemistry for Tau and MAP2

RGC-5 cells plated on coverslips were fixed with ice-cold glycine-ethanol (150 mM glycine, 70% EtOH [pH 2.0]) for 30 minutes at −20°C. The wells were washed with Tris-buffered saline (TBS; 100 mM Tris [pH 7.6], 0.9% NaCl), incubated with blocking buffer (0.3% Triton X-100, 5% normal goat serum in TBS) for 30 minutes at room temperature, and incubated overnight at 4°C with monoclonal mouse anti–tau-1 and polyclonal rabbit anti–MAP2 antibodies at 4 μg/mL in blocking buffer. After washing, cells were incubated for 1 hour at room temperature with goat anti–mouse IgG (Alexa Fluor 594; Molecular Probes) and goat anti–rabbit IgG (Alexa Fluor 488; Molecular Probes) at 2 μg/mL in TBS. Coverslips were then transferred and mounted on microscope slides. The resultant tau fluorescence was detected with a Texas Red filter set (excitation 560 ± 20 nm, dichroic 595-nm long-pass, emission 630 ± 30 nm), and MAP2 fluorescence was detected with an FITC filter set (excitation 470 ± 20 nm, dichroic 505 nm long-pass, emission 540 ± 20 nm). Digital photomicrographs were taken at 400× total magnification.

Cell Viability

RGC-5 cells were assayed for cell death with PI and visualized by incubation with 2 μM PI for 30 minutes at 37°C. To exclude the possibility of removing nonadherent dead cells, media were not aspirated until after imaging. PI fluorescence was detected with a Texas Red filter set, with digital photomicrographs taken at a final magnification of 200×. Dead cells were identified by superimposition of PI fluorescence and phase images of the same fields.

Cell Proliferation

RGC-5 cells were assayed for mitotic activity by staining for BrdU incorporation, as previously described.[3] Proliferating cells were identified by superimposition of BrdU fluorescence and phase images of the same fields.

Quantitative Morphology Assessment

Digital photomicrographs were taken at 200× total magnification various times after pharmacologic treatment. Six photomicrographs were taken, three from each duplicated condition, from different locations around the center of each well. Photomicrographs were batch analyzed offline to assess the development of neurites. The proportion of differentiated cells, defined by the appearance of three or more neurites longer than the soma, was calculated as a percentage of the total number of cells present in the photomicrographs. The three cells with the longest neurites per photomicrograph (18 total per condition) were selected for each experimental condition and time point for quantitative morphologic analysis. Longest neurite length and number of neurites longer than the soma were measured (NeuronJ plug-in for NIH ImageJ software; National Institutes of Health, Bethesda, MD).[9] Descriptive statistics were calculated for each condition with the use of a spreadsheet (Excel; Microsoft, Redmond, WA). Photomicrographs used to generate quantitative data are not presented here. Rather, photomicrographs from cells plated onto a coverglass in 24-well plates and fixed with 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) in PBS (pH 7.2) for 20 minutes at room temperature are shown (see Figs. 1, 10-12).

Immunoblot Analysis

Relative HDAC inhibition activity of staurosporine was assayed by immunoblotting for total H3 and acetylated H3 in control, TSA-, and staurosporine-treated RGC-5 cells. Cells were plated at a density of 80 cells/mm^2 in 100-mm tissue culture plates and were harvested 24 hours after pharmacologic treatment. Lysates were collected, underwent electrophoresis, and were transferred to nitrocellulose membrane as previously described.[3]

After transfer, the membrane was incubated for 1 hour (Odyssey Blocking Buffer; LI-COR), followed by incubation for 1 hour with monoclonal mouse anti–H3 and polyclonal rabbit anti–acetyl-H3 at 0.5 μg/mL in blocking buffer with 0.1% Tween-20. The membrane was washed four times for 5 minutes each in PBS with 0.1% Tween-20 (Fisher Scientific), followed by incubation for 1 hour with goat anti–mouse IgG (IRDye 800; LI-COR) and goat anti–rabbit IgG (IRDye 680; LI-COR) at 0.5 μg/mL in blocking buffer with 0.1% Tween-20. The membrane was again washed four times for 5 minutes each in PBS with 0.1% Tween-20, imaged on an infrared scanner (LI-COR), and analyzed for band density (Odyssey software; LI-COR).

The expression of Brn-3a and Brn-3b was assayed at 48 hours after pharmacologic treatment as described here, with ECL detection as previously described.[3] Brn-3a, Brn-3b, and actin antibodies were used at 1 μg/mL, 0.1 μg/mL, and 0.2 μg/mL respectively, with secondary antibodies used at 1:10,000 dilution.

Statistical Analysis

Comparisons among conditions for longest neurite and number of neurites per differentiated cell were performed using two-sided unpaired *t*-tests. Two-sample F-tests were used to establish whether equal or unequal variance was present. Comparisons between conditions for proportions of differentiated or PI-positive cells were performed with two-sample proportion tests. $P < 0.05$ was considered significant for all test statistics. Values stated in text are expressed as mean ± SEM, and error bars in figures are ± SEM.

RESULTS

HDAC Inhibition Causes RGC-5 Cell Differentiation, Which Differs Morphologically and Quantitatively from Differentiation Induced by Staurosporine

Both TSA (500 nM) and staurosporine (316 nM) induced the differentiation of RGC-5 cells, as defined by the presence three or more neurites longer than the soma (Fig.1). Differentiation was not observed at any time point from untreated control cells or from control cells treated with the RGC survival promoting the combination[10] of BDNF (50 ng/mL), CNTF (10 ng/mL), insulin (5 μg/mL), and forskolin (5 μM; Fig. 1); each group became confluent by 72 hours. RGC-5 cells differentiated with TSA had significantly longer longest neurites than staurosporine-differentiated cells at 24 hours (78 ± 5 μm vs. 63 ± 4 μm; $P = 0.017$), whereas by 120 hours staurosporine differentiation induced significantly longer longest neurites than TSA (108 ± 4 μm vs. 64 ± 5 μm; $P < 0.0001$; Fig. 2A). Primary neurite counts per differentiated cell were greater for staurosporine than for TSA differentiation at 24 hours (4.00 ± 0.20 neurites vs. 3.22 ± 0.10 neurites; $P = 0.0017$) and 120 hours (9.11 ± 0.29 neurites vs. 3.24 ± 0.12 neurites; $P < 0.0001$; Fig. 2B). Furthermore, TSA induced a significantly lower proportion of cells to differentiate than staurosporine at 24 hours (0.39 ± 0.04 vs. 0.97 ± 0.02; $P < 0.0001$) and 120 hours (0.07 ± 0.02 vs. 0.86 ± 0.03; $P < 0.0001$; Fig. 2C). The observed large decrease in TSA proportion differentiated between 24 and 120 hours was accompanied by a significantly greater proportion of TSA-than staurosporine-treated cells becoming PI positive at 120 hours (0.78 ± 0.04 vs. 0.14 ± 0.03; $P < 0.0001$; Fig. 2C).

RGC-5 cells differentiated with TSA were more sensitive to plating density than those differentiated with staurosporine. Plating densities of 20 and 160 cells/mm^2 resulted in fewer TSA-differentiated cells than did plating densities of 80 cells/mm^2 (Fig. 3B). This effect was not observed with staurosporine. A similar effect was observed when cells were plated at the same density (80 cells/mm^2) with different volumes of media. TSA induced fewer cells to differentiate when plated with 50 and 300 μL/well media compared with the standard 100 μL/well, but this effect was not observed with staurosporine (Fig. 3A). Except for a large increase in the proportion of PI-positive cells with TSA differentiation at 50 μL/well, no significant effects were observed in the proportion of PI-positive cells with TSA or staurosporine differentiation when the plating density or media volume was varied (Figs. 3A, 3B). These data collectively suggest that the observed increase in TSA differentiation sensitivity to plating density is caused by a conditioned media effect and not by direct cell– cell contact. The proportion of TSA-differentiated cells was similar between homologous conditions (i.e., 50 μL/well media over 80 cells/mm^2 approximates the media/cell ratio of 100 μL/well media over 160 cells/mm^2), indicating that cell– cell contacts are not necessary to explain the observed plating density sensitivity. However, the increase in the proportion of PI-positive cells at 50 μL/well media over 80 cells/mm^2 compared with 100 μL/well media over 160 cells/mm^2 (0.72 ± 0.03 vs. 0.13 ± 0.02; $P < 0.0001$) suggests that cell– cell contacts may be important not for differentiation but for survival after differentiation.

RGC-5 cells had a bimodal response to differentiation with TSA, unlike the graded response observed with staurosporine. Frequency distribution of the number of neurites per cell at 0, 24,

72, and 120 hours after treatment with TSA and staurosporine illustrated the progression of neurite outgrowth (Figs. 4A, 4B). The initial pool of undifferentiated cells treated with TSA shifted from a unimodal, undifferentiated population at 0 hours to a bimodal distribution, consisting of distinct differentiated and undifferentiated populations, by 24 hours after treatment. The two populations appeared to be distinct because the proportion of cells with two neurites was significantly lower than the proportion of cells with one neurite or three neurites. Furthermore, the response was stable because the frequency distribution was not observed to shift to the right (to greater neurite numbers per cell) at 120 hours after treatment. This observation of two distinct populations also validated the criterion of classifying cells as differentiated if three or more neurites longer than the soma were present. In contrast to the bimodal response to TSA, the differentiation induced by staurosporine was graded, with the distribution of undifferentiated cells at 0 hours progressively shifting to the right between 24 and 120 hours after treatment.

Dose–Response of RGC-5 Cells to HDAC Inhibition

The concentration dependence of differentiation with TSA was parabolic, whereas the effect on the proportion of PI-positive cells was exponential (Fig. 5). TSA caused neurite outgrowth from RGC-5 cells at concentrations greater than 125 nM. Cells treated with TSA at concentrations of 125 nM or less resulted in RGC-5 cells morphologically indistinguishable from untreated controls, dividing and becoming confluent by 72 hours. At concentrations between 125 and 500 nM, progressively greater fractions of RGC-5 cells exhibited neurite outgrowth. Concentrations of TSA greater than 500 nM did not result in greater differentiation at 24 hours; by 120 hours, the fraction of differentiated cells was smaller and the fraction of PI-positive cells was increased. Because of the decrease in fraction differentiated at higher concentrations, TSA at 500 nM was used for subsequent experiments. The effect of added RGC survival factors was only evident at TSA concentrations of 500 and 1000 nM. At 500 nM TSA, the inclusion of RGC survival factors significantly increased the proportion that was differentiated (0.23 ± 0.04 vs. 0.08 ± 0.02; $P = 0.001$) and decreased the proportion that was PI positive (0.59 ± 0.04 vs. 0.77 ± 0.04; $P = 0.0023$), whereas at 1000 nM only the PI-positive proportion was significantly reduced (0.71 ± 0.03 vs. 0.83 ± 0.03; $P = 0.0053$).

HDAC Inhibition–Differentiated RGC-5 Cells Are Mitotically Inactive

An important criterion for classifying a cell as differentiated is that it must become postmitotic on differentiation. To investigate whether RGC-5 cells differentiated with TSA became irreversibly postmitotic, cells were incubated with BrdU, followed by immunohistochemical staining for incorporation into chromatin. Mitotic activity was investigated under conditions in which TSA (500 nM) and staurosporine (316 nM), with and without RGC survival factors, were maintained or withdrawn from media between 24 and 120 hours after initial treatment. Although control cells, with and without RGC survival factors, were mitotically active at 24 hours (68% ± 5% and 69% ± 5% BrdU positive, respectively), cells differentiated and maintained in TSA or staurosporine were postmitotic at 24, 72, and 120 hours, with less than 2% BrdU positivity in all cases (Fig. 6).

Withdrawal of TSA or staurosporine from the culture at 24 hours had differential effects on mitotic activity measured at 72 hours (Fig. 6B). TSA-differentiated cells became mitotically active and undifferentiated, and the continued presence of RGC survival factors did not prevent the resumption of proliferation (31%–33% [±5%] BrdU positive in all cases) or dedifferentiation. This effect was not observed with staurosporine induced differentiation, whereby cells remained postmitotic. However, when TSA was withdrawn at 72 hours and mitotic activity was assayed at 120 hours (Fig. 6C), cells remained mitotically inactive, the proportion of cells differentiated was the same as cultures in which TSA was not withdrawn, and the proportion of PI-positive cells was significantly decreased (0.41 ± 0.04 [TSA

withdrawn] vs. 0.75 ± 0.04 [TSA maintained]; $P < 0.0001$). These results indicate that for RGC-5 cells, differentiation with TSA becomes irreversible somewhere between 24 and 72 hours. However, as early as 72 hours, the continuous presence of TSA caused elevated cell death without increasing the proportion differentiated. In contrast, the removal of staurosporine at 24 and 72 hours caused a significantly higher proportion of PI-positive cells at 72 and 120 hours, respectively, compared with cells maintained with staurosporine. Together, these observations suggest that the withdrawal of TSA did not cause dedifferentiation after 72 hours, whereas the withdrawal of staurosporine caused rapid dedifferentiation and death.

Effects of Neurotrophic Factors on Differentiation Mediated by HDAC Inhibition

A combination of neurotrophic factors known to increase RGC survival[10] was tested to determine whether this would be additive with differentiation mediated by HDAC inhibition. RGC-5 cells were treated with BDNF (50 ng/mL), CNTF (10 ng/mL), insulin (5 μg/mL), and forskolin (5 μM), alone and in combination with TSA (500 nM) or staurosporine (316 nM). None of the RGC survival factors caused differentiation of RGC-5 cells alone or in combination. When RGC-5 cells were differentiated with TSA in the presence of RGC survival factors, there was a significant increase over TSA alone in the length of the longest neurite (101 ± 7 μm vs. 79 ± 6 μm; $P = 0.024$), the number of primary neurites per differentiated cell (3.94 ± 0.17 neurites vs. 3.39 ± 0.16 neurites; $P = 0.027$), and the proportion of differentiated cells (0.46 ± 0.04 vs. 0.23 ± 0.04; $P = 0.00017$) at 24, 72, and 120 hours (all statistics shown are for 72 hours unless otherwise indicated; Figs. 7A, 7C, 7E). The proportion of PI-positive cells was also significantly lower in the presence of RGC survival factors and TSA compared with TSA alone (0.31 ± 0.04 vs. 0.55 ± 0.05; $P = 0.00031$) at 72 and 120 hours (Fig. 7E). By studying each RGC survival factor individually in the presence of TSA, it appeared that the increase in longest neurite length could be accounted for by the effects of forskolin alone (110 ± 7 μm [TSA + forskolin] vs. 79 ± 6 μm [TSA]; $P = 0.0024$), whereas the increased number of neurites could be accounted for by BDNF alone (4.39 ± 0.22 neurites [TSA + BDNF] vs. 3.39 ± 0.16 neurites [TSA]; $P = 0.00082$; Figs. 8A, 7C). In addition, forskolin alone could account for the significant increase in proportion differentiated (0.46 ± 0.04 [TSA + forskolin] vs. 0.23 ± 0.04 [TSA]; $P = 0.00018$) and the decrease in proportion of PI-positive cells (0.33 ± 0.04 [TSA + forskolin] vs. 0.55 ± 0.05 [TSA]; $P = 0.00048$; Fig. 8E).

The effects of neurotrophic factors on differentiation mediated by staurosporine were different from those mediated by HDAC inhibition. RGC survival factors plus staurosporine significantly increased the number of neurites per differentiated cell compared with staurosporine alone (7.00 ± 0.26 neurites vs. 5.89 ± 0.33 neurites; $P = 0.012$) at 72 and 120 hours (Fig. 7D) and the longest neurite length (130 ± 4 μm [120 hours] vs. 108 ± 4 μm [120 hours]; $P = 0.00017$) at 120 hours (Fig. 7B). The effect of RGC survival factors on the proportion differentiated was not apparent until 120 hours because differentiation was nearly 100% at 24 and 72 hours. At 120 hours, the inclusion of RGC survival factors significantly increased the proportion differentiated compared with staurosporine alone (0.95 ± 0.02 [120 hours] vs. 0.86 ± 0.03 [120 hours]; $P = 0.013$) and decreased the proportion that was PI positive (0.05 ± 0.02 [120 hours] vs. 0.14 ± 0.03 [120 hours]; $P = 0.013$; Fig. 7F). In contrast to what was seen with HDAC inhibition, no individual survival factor enhanced differentiation mediated by staurosporine, as observed at 72 hours (Fig. 8D).

HDAC Inhibition–Mediated Differentiation Induces Neuritogenesis

To assess the effect of TSA on neuritogenesis, RGC-5 cells were immunohistochemically stained for MAP2 and tau 24 hours after treatment with TSA (500 nM) and/or RGC survival factors (Fig. 9). Although TSA-differentiated cell somas were stained by tau, cells with tau-positive neurites had stained preferentially in a single neurite, and staining was localized to the most distal aspects of the neurite, primarily in the growth cone. In contrast, MAP2 stained

somas and neurites diffusely. A similar pattern has been reported in cultured rat hippocampal neurons.[11] In contrast, cells treated with only RGC survival factors did not exhibit this staining pattern (Fig. 9), with tau and MAP2 colocalized everywhere. These results suggest that the single neurites with localized tau staining in TSA-differentiated cells are likely axons.

HDAC Inhibition–Mediated Differentiation Is Transcription Dependent

To investigate the mechanism of action for TSA, cells were treated with α-amanitin, a potent RNA-polymerase II inhibitor,[12] at 10 μg/mL 4 hours before the addition of TSA (500 nM) or staurosporine (316 nM). Because staurosporine induces noticeable neurite outgrowth in minutes, cells were preincubated for 4 hours with α-amanitin to exclude the possibility that staurosporine would affect cell morphology before transcription was inhibited. Twenty-four hours after TSA and staurosporine were added, α-amanitin completely blocked neurite outgrowth in TSA-cotreated cells (Figs. 10A, 10B). Longest neurite length, primary neurite count, and proportion differentiated were not different from those values in untreated control cells. Compared with TSA alone, α-amanitin–cotreated cells had significantly shorter longest neurites (47 ± 4 μm vs. 79 ± 4 μm; $P < 0.0001$), fewer primary neurites (1.67 ± 0.11 neurites vs. 3.33 ± 0.13 neurites; $P < 0.0001$), and no differentiation (Figs. 10D–F). The proportion of PI-positive cells was significantly elevated compared with that found with TSA alone (0.33 ± 0.05 vs. 0.08 ± 0.02; $P < 0.0001$) but was statistically not different from that found with α-amanitin control cells (Fig. 10F).

In contrast to the observed total block of neuritogenesis with TSA, α-amanitin did not block staurosporine-mediated neurite outgrowth (Fig. 10C). Longest neurite length was statistically not different from staurosporine alone, but primary neurite counts were significantly reduced (3.33 ± 0.11 neurites vs. 4.39 ± 0.18 neurites; $P = 0.00026$), along with proportion differentiated (0.52 ± 0.05 vs. 0.96 ± 0.02; $P < 0.0001$; Figs. 10D–F). The proportion of PI-positive cells was similar among control, TSA-, and staurosporine-treated cells coincubated with α-amanitin, suggesting that survival was not differentially affected. To test whether TSA simply induced process formation by nontranscriptionally inhibiting cytoplasmic HDAC-6, which acetylates and stabilizes microtubules, cells were treated with sodium butyrate, an HDAC inhibitor that does not inhibit cytoplasmic HDAC-6.[13] Twenty-four hours after treatment, differentiation with 50 mM Na butyrate in the presence or absence of RGC survival factors was similar to that induced by TSA (500 nM; Fig. 11). These results together make it likely that HDAC inhibition–mediated differentiation is transcriptional and that the observed processes are true neurites.

To further confirm that HDAC inhibition–mediated differentiation of RGC-5 cells is transcriptional, cell lysates were immunoblotted for the Brn-3a and Brn-3b transcription factors 48 hours after pharmacologic treatment. Cells treated with TSA and RGC survival factors were observed to have 1.4- and 2.0-fold increases in Brn-3a and Brn-3b (densitometry normalized to actin), respectively, compared with cells treated with RGC survival factors alone (Fig. 12). Interestingly, Brn-3b expression in cells treated with staurosporine and RGC survival factors was downregulated compared with control cells, with 0.6-fold normalized density (Fig. 12).

HDAC Inhibition and Staurosporine Are Synergistic with Respect to Neurite Outgrowth

Staurosporine alone does not induce differentiation or neurite outgrowth below a threshold of approximately 100 nM.[3] We treated RGC-5 cells with TSA (500 nM) and a subthreshold dosage of staurosporine (1 nM; Fig. 12D) and found significantly longer longest neurites (121 ± 10 μm [120 hours] vs. 64 ± 5 μm [120 hours]; $P < 0.0001$) at 24 and 120 hours and increased primary neurite counts (4.56 ± 0.12 neurites vs. 3.24 ± 0.12 neurites; $P < 0.0001$) at 120 hours compared with TSA alone (Figs. 13A, 13B). No statistical difference was observed in proportion differentiated or PI positive between cells treated with TSA alone and with 1 nM staurosporine (Fig. 13C).

Staurosporine Does Not Inhibit HDAC

Although differentiation with HDAC inhibition was quantitatively and qualitatively different from that seen with staurosporine, it was possible that the effect of staurosporine could partly be attributed to HDAC inhibition. To test this possibility, lysates from cells treated with TSA (500 nM), staurosporine (316 nM), or controls were immunoblotted for total and acetylated class I histone H3. As expected, staurosporine showed no increase in H3 acetylation compared with control cells, whereas TSA-treated cells were approximately 2.7-fold increased in H3 acetylation (Fig. 14). Similar results were obtained across all nuclear histones with cell-free assay in a fluorescent assay system (Fluor de Lys; Biomol, Plymouth Meeting, PA)[14] on HeLa nuclear extracts (data not shown).

DISCUSSION

HDAC inhibition–mediated differentiation of RGC-5 cells was synergistic with neurotrophic factors that increase RGC survival. This combination resulted in increased viability, differentiated proportion, longest neurite length, and number of neurites extending from the soma. Only 50% of differentiated and neurotrophin-treated RGC-5 cells were viable on day 3, similar to what was seen in primary P1 RGCs treated with neurotrophic factors but less than the 90% survival rate of neurotrophic factor–treated P8 RGCs.[10] The likely reason for this difference is that rat RGCs undergo target deprivation-induced cell death during P1 to P4[15] and RGC-5 cells are immortalized at P1, at the major peak of developmental cell death. Surprisingly, RGC neurotrophic factors also had noticeable effects on HDAC-inhibitor–treated cell neurite outgrowth as early as 24 hours (before significant cell death occurred). This finding strongly suggests that adding RGC survival factors to HDAC inhibition had an effect beyond simply increasing cell viability; it also had an effect on morphology, even though the survival factors themselves showed no differentiating activity when used alone. Unlike what was seen with staurosporine-differentiated RGC-5 cells, the length of the longest neurite and the primary neurite count did not change over time with HDAC inhibition, in the presence or absence of RGC survival factors. This lack of effect on neurite extension was similar to what others have observed for primary cultures of RGCs, where neurotrophic factors alone do not induce significant axon elongation in the absence of suitable chemoattractive cues, such as growth on laminin or localized gradients of attractive/repulsive neurotrophic factors.[16,17] Similarly, the fact that the number of differentiated cells had a bimodal distribution after HDAC inhibition but a graded distribution with staurosporine supports the notion that HDAC inhibition results in differentiation similar to the in vivo differentiation process. Cellular differentiation is similar to a switch, with cells passing rapidly from undifferentiated to differentiated states in a transcriptionally regulated process.[18,19] The finding of a bimodal distribution of cells, clustered in undifferentiated and differentiated states, is consistent with turning of an all-or-nothing differentiation switch, a pattern seen only with transcription-dependent HDAC inhibition.

More support for HDAC inhibition– differentiated RGC-5 cells behaving similarly to RGCs is the effect of conditioned media. RGCs in vitro exhibit conditioned media effects, whereby survival is significantly enhanced by plating at higher rather than lower densities.[10] Our results with HDAC inhibition (but not differentiated mediated by staurosporine) were similar in that differentiation was enhanced by increasing densities at all levels except the highest.

The observation that tau localizes predominantly to the distal portions of single neurites in RGC-5 cells differentiated by HDAC inhibition suggests that true neuritogenesis occurred, not process formation caused by actin stress fibers, as has been reported in v-*ras*–transformed NIH 3T3 cells treated with TSA.[20] Although tau staining of distal aspects of neurites is actin dependent, the localization of tau specifically to one neurite is actin independent.[11] In other words, actin stress fiber formation cannot explain the enrichment of tau observed localized to

one particular neurite, which is indicative of axons. Furthermore, the observed diffuse staining of MAP2 in all other neurites is consistent with these neurites being dendrites.

A likely mechanism for RGC-5 differentiation with HDAC inhibition is removal of the neuron gene–specific transcription repressor neuron-restrictive silencing factor (NRSF) from neuron-restrictive silencing element (NRSE). This process is a necessary, though not necessarily sufficient, component of in vivo central nervous system neuronal differentiation.[7] NRSEs are located in the promoter regions of many neuron-specific genes. NRSF, when bound to NRSE, sterically prevents transcription of these genes. TSA, however, inhibits the complexing of NRSE with NRSF.[21] NRSF recruitment to NRSE is mediated by the HDAC 1– and 2–containing transcription corepressor Sin3/HDAC1,2.[22,23] When HDAC activity is inhibited, NRSE is released from NRSF, permitting transcription factor access to genes downstream of NRSE-containing promoters. This mechanism would also explain why, in our preliminary screening of HDAC inhibitors, the class 3 HDAC inhibitor nicotinamide[24] did not induce differentiation in RGC-5 cells. Sin3/HDAC1,2 is composed of only TSA-sensitive class 1 and class 2 HDACs. Our observations that Brn-3a and Brn-3b, transcription factors necessary for RGC differentiation,[25] were upregulated on TSA treatment fits this model because both are regulated by Math5 and NRSF and are upregulated during in vivo RGC differentiation.[26] Although staurosporine-differentiation is not dependent on transcription, in contrast to HDAC inhibition-mediated differentiation, a subthreshold concentration of staurosporine (1 nM) caused longer longest neurites and increased the number of neurites in the presence of HDAC inhibition. These data imply that though these two mechanisms differ, some common downstream transduction processes likely take place.

In summary, we found that HDAC inhibition of a committed retinal neuronal cell line, RGC-5, results in differentiation similar to that occurring during development and different from that observed with staurosporine-mediated differentiation. HDAC inhibition could potentially be used to differentiate RGC progenitors for use in repopulating the retina in optic neuropathies, resulting in RGCs that are more physiologically neurotrophic factor dependent.

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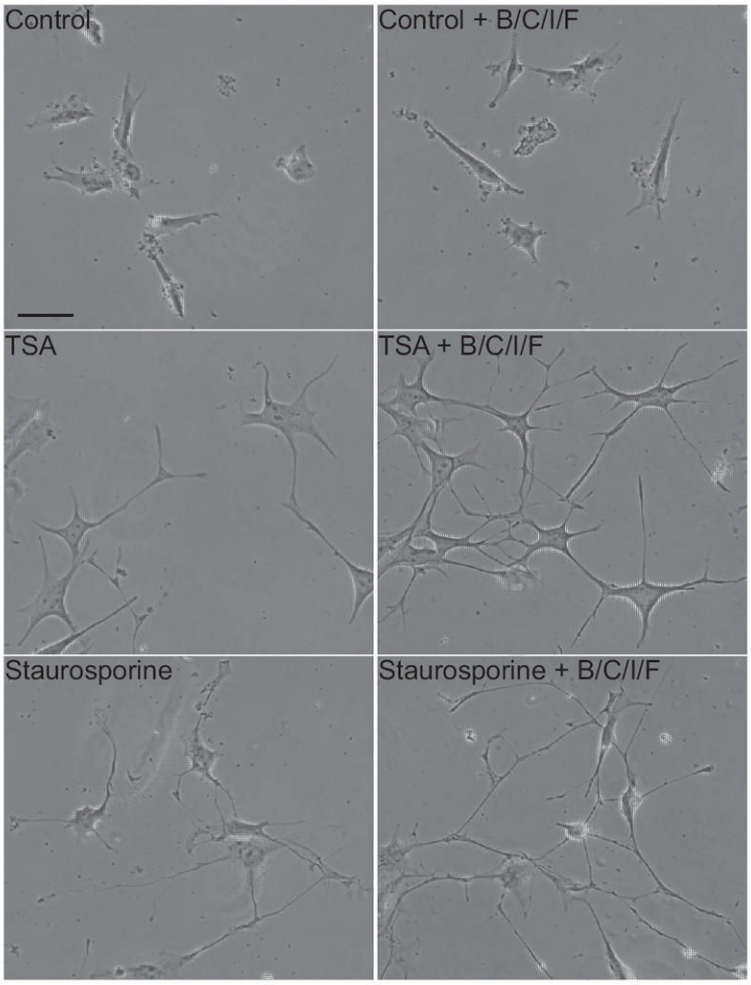

FIGURE 1.

HDAC inhibition differentiates RGC-5 cells. RGC-5 cells were treated with 500 nM TSA or 316 nM staurosporine, with or without the RGC survival-promoting combination[10] of BDNF (50 ng/mL), CNTF (10 ng/mL), insulin (5 μg/mL), and forskolin (5 μM; B/C/I/F). Photomicrographs were taken at 200× magnification 24 hours after treatment. Staurosporine and TSA induced neurite outgrowth greater than untreated (control) or B/C/I/F-treated cells. Cells with three or more neurites longer than the soma were classified as differentiated. B/C/I/F alone did not induce differentiation. Scale bar, 50 μm.

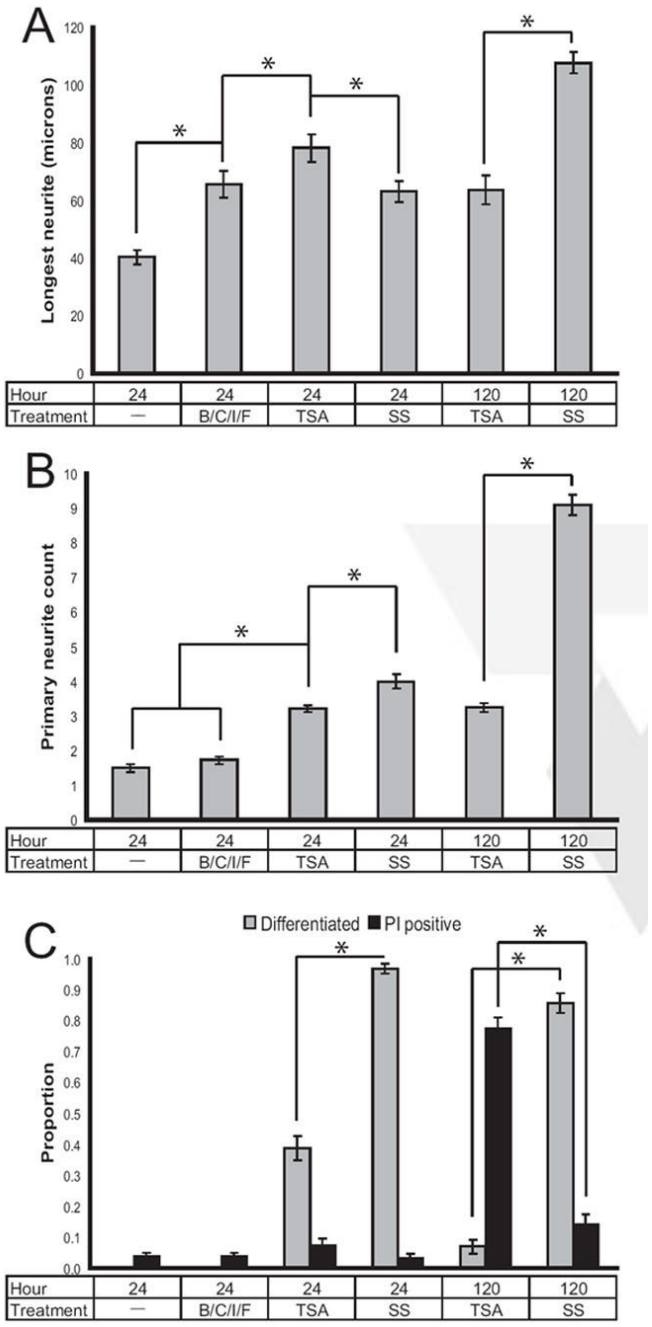

FIGURE 2.

HDAC inhibition–mediated differentiation differs quantitatively from staurosporine differentiation. RGC-5 cells were treated with 500 nM TSA, 316 nM SS, or RGC survival factors (B/C/I/F), and photomicrographs were taken at 24, 72, and 120 hours after initial treatment. Longest neurite length (microns; **A**) and number of neurites longer than the soma (**B**) per differentiated cell were quantified (NeuronJ; NIH). The proportion of differentiated cells for each condition (**C**) was quantified using the photomicrographs used to generate (**A**) and (**B**). TSA induced significantly shorter longest neurites (**A**) and fewer primary neurites (**B**) 120 hours after treatment. Furthermore, at 120 hours, TSA-mediated differentiation induced a significantly lower proportion of RGC-5 cells to differentiate and a significantly

greater proportion of cells to become PI-positive compared with staurosporine (**C**). However, compared with untreated (–) and B/C/I/F-treated cells, TSA induced significantly longer longest neurites (**A**) and greater primary neurite counts (**B**). In addition, no untreated or B/C/I/F-treated cells were classified as differentiated because none had three or more neurites longer than the soma, whereas a significant proportion of TSA-treated cells met these criteria (**C**). *Statistically significant ($P < 0.05$) difference between treatments.

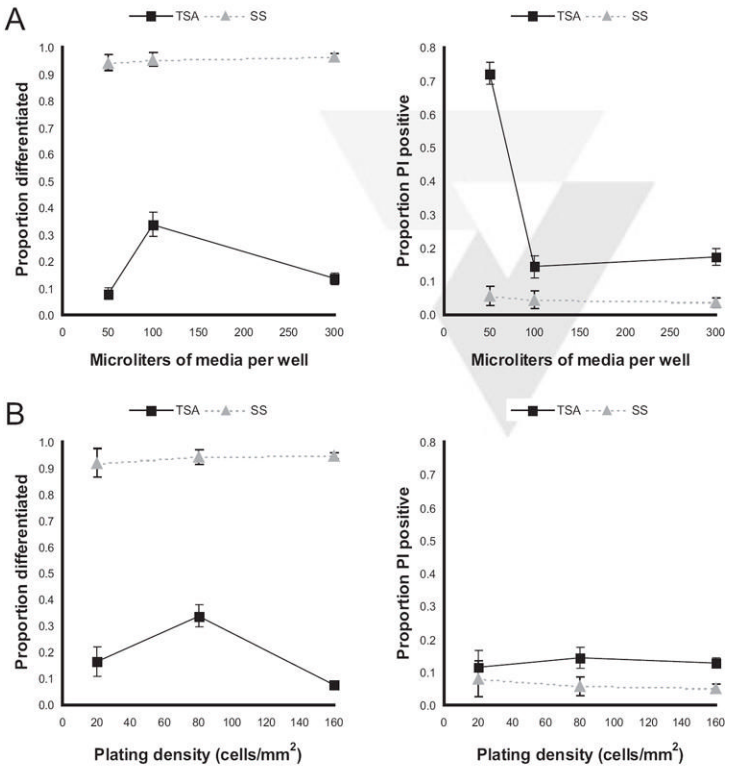

FIGURE 3.

HDAC inhibition–mediated differentiation of RGC-5 cells is sensitive to plating density. RGC-5 cells were plated in 50, 100, and 300 μL media at a plating density of 80 cells/mm^2 (**A**) or at plating densities of 20, 80, and 160 cells/mm^2 in 100 μL media (**B**) and were treated with 500 nM TSA or 316 nM SS 24 hours after plating. Twenty-four hours after treatment, a significantly greater proportion of cells differentiated with TSA at 80 cells/mm^2 in 100 μL media than at greater or lower media volumes (**A**) or plating densities (**B**). This result was not observed for staurosporine-mediated differentiation, and there was no significant effect on the proportion of PI-positive cells, except with HDAC inhibition differentiation at 50 μL media.

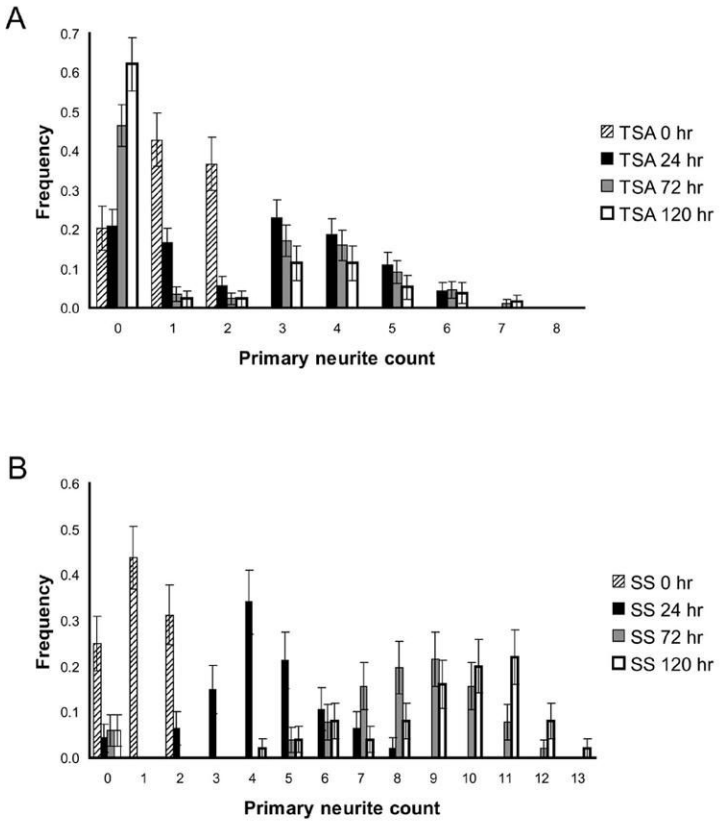

FIGURE 4.

HDAC inhibition causes a bimodal differentiation response, and SS causes a graded response. RGC-5 cells were treated with 500 nM TSA or 316 nM SS, with photomicrographs taken at 0, 24, 72, and 120 hours after initial treatment. The frequency distribution of neurite counts from all completely visible RGC-5 cells was constructed for TSA (**A**) and SS (**B**) for the four time points from photomicrographs. (**A**) HDAC inhibition converted the population of undifferentiated cells at 0 hours to a bimodal distribution of neurite counts that was stable from 24 to 120 hours. (**B**) SS-differentiated cells had a unimodal distribution of neurite counts that shifted to the right over time (i.e., increased number of neurites).

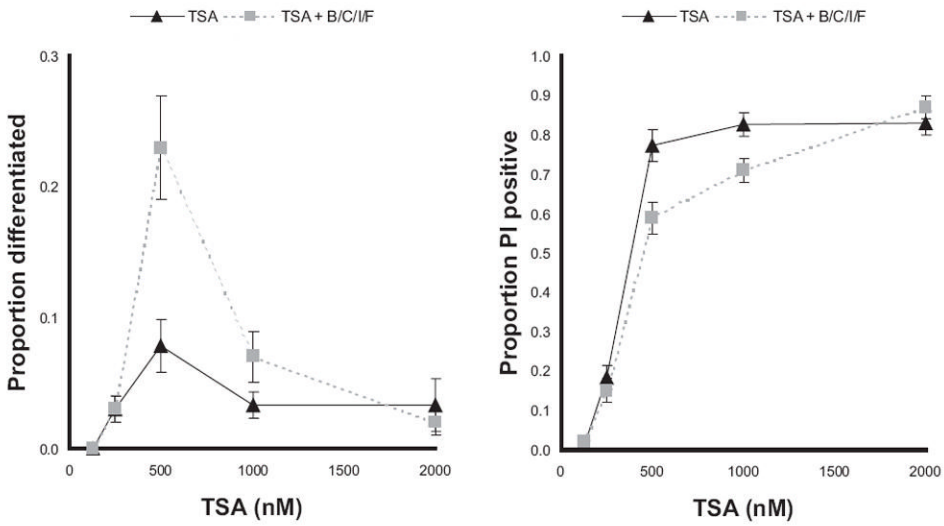

FIGURE 5.

Dose–response of RGC-5 differentiation to HDAC inhibition. RGC-5 cells were treated with or without RGC survival factors (B/C/I/F) at 125, 250, 500, 1000, and 2000 nM TSA. The proportions of differentiated and PI-positive cells were determined from photomicrographs taken at 120 hours after initial treatment. Concentrations above 500 nM enhanced dedifferentiation, whereas those below 500 nM caused reduced differentiation. Furthermore, the proportion of PI-positive cells increased from 125 to 500 nM TSA and remained approximately the same from 500 to 2000 nM TSA. The most significant effects of B/C/I/F addition were evident at 500 nM TSA, with the proportion of cells differentiated or PI positive significantly increasing and decreasing, respectively.

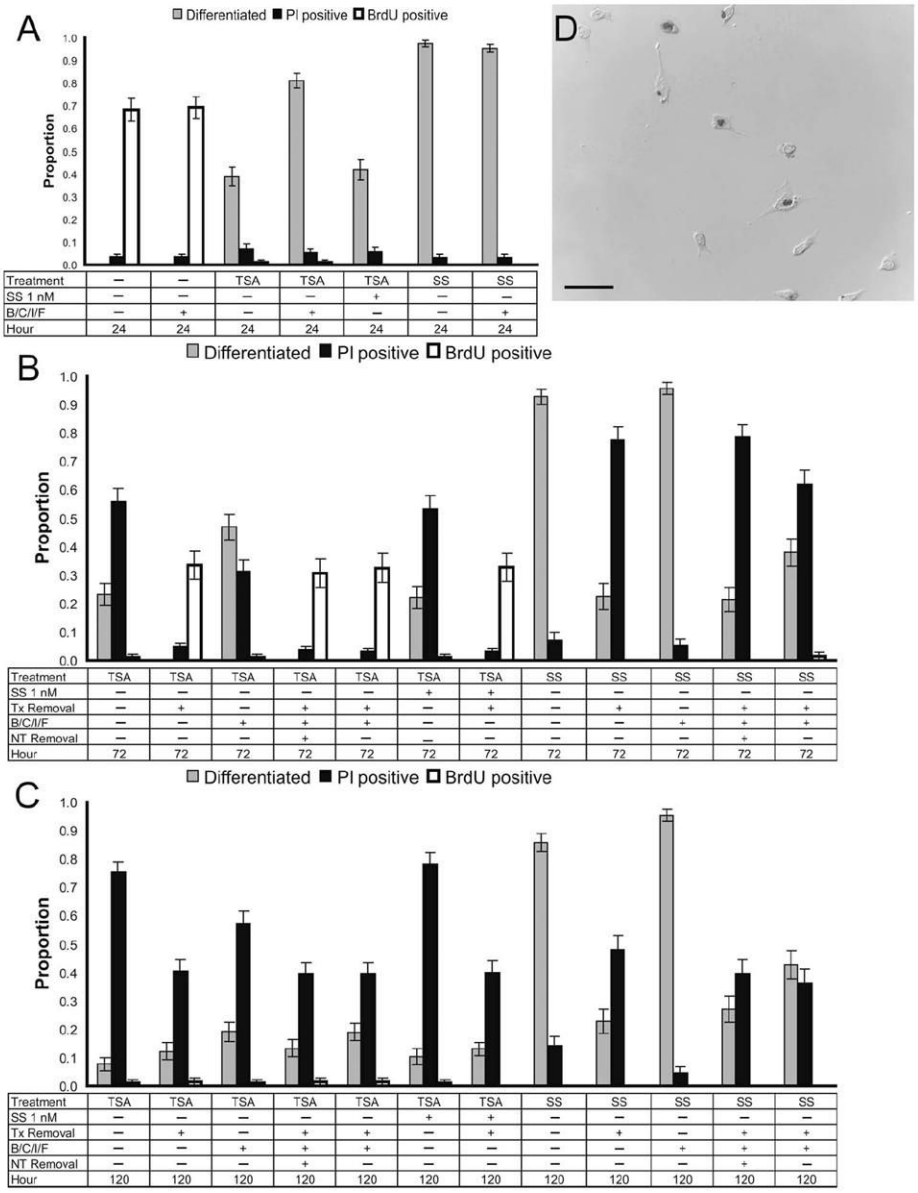

FIGURE 6.

HDAC inhibition–differentiated RGC-5 cells are postmitotic. RGC-5 cells were treated with 500 nM TSA or 316 nM SS with or without RGC survival factors (B/C/I/F) or a subthreshold dose of SS (1 nM). Cells were maintained in their initial treatment conditions, and photomicrographs were taken 24 hours (**A**) after treatment. Alternatively, various combinations of the differentiation-inducing drugs and neurotrophic factors (NT; i.e., B/C/I/F) were removed at 24 and 72 hours, with photomicrographs taken 48 hours later, at 72 (**B**) and 120 hours (**C**), respectively. These conditions were performed simultaneously in 24-well plates. BrdU (100 μM) was administered 4 hours before the end of the test period, and immunohistochemical staining was performed for incorporation into chromatin to examine the mitotic state of cells. Although untreated control cells were mitotically active at 24 hours (**A**, **D**; 68% ± 5% BrdU positive), cells differentiated with TSA or staurosporine were observed to be mitotically inactive, with less than 2% BrdU positive at all time points (**A**–**C**). The exception

was when TSA was removed at 24 hours, with at least 31% of cells becoming BrdU positive regardless of maintenance in neurotrophic factors (**B**). This indicated that HDAC inhibition for 24 to 72 hours is necessary for these cells to become irreversibly postmitotic.

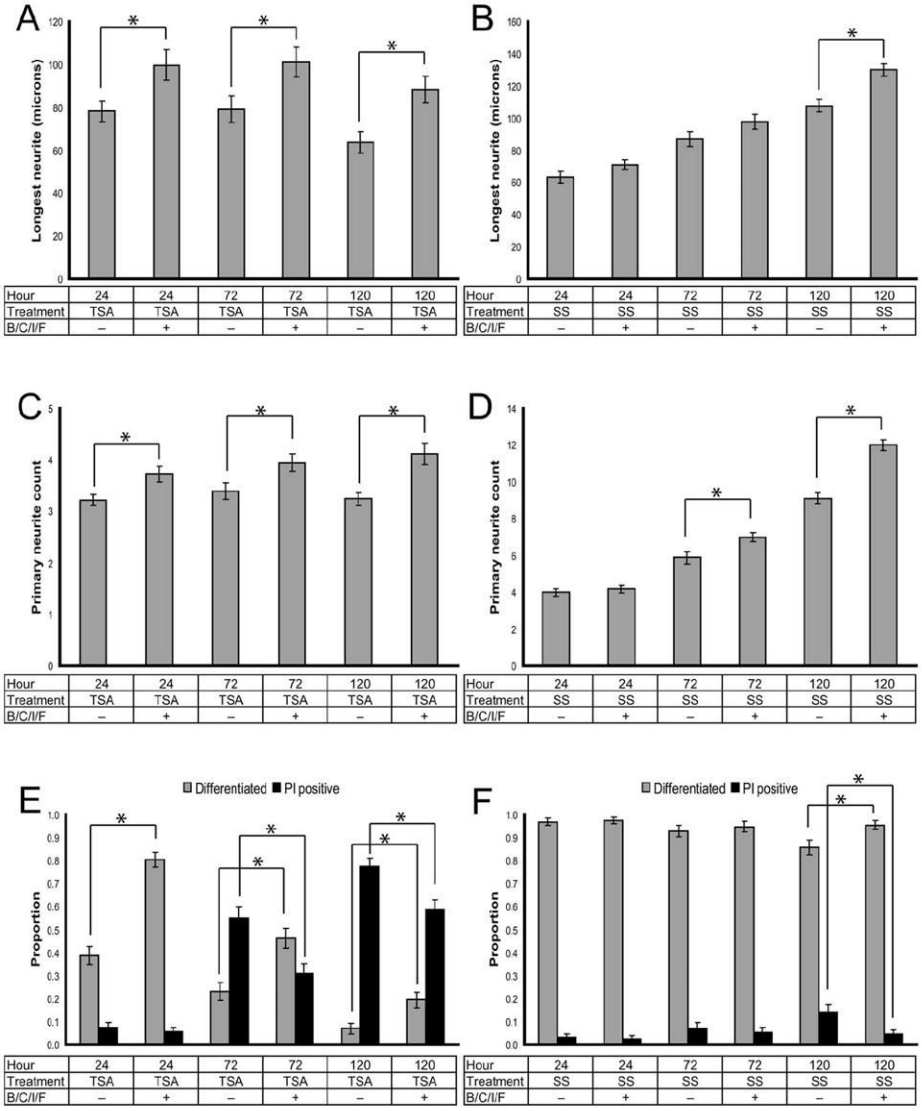

FIGURE 7.

RGC survival factors enhance HDAC inhibition- and staurosporine-mediated RGC-5 cell differentiation. RGC-5 cells were treated with 500 nM TSA or 316 nM SS with and without RGC survival factors (B/C/I/F). Longest neurite length (microns; **A**, **B**) and number of neurites longer than the soma (**C**, **D**) were determined at 24, 72, and 120 hours after initial treatment from photomicrographs, along with the proportion of cells differentiated (**E**, **F**). Inclusion of B/C/I/F augmented the longest neurite length (**A**), primary neurite count (**C**), and proportion differentiated (**E**) at all time points for HDAC inhibition–mediated differentiation of RGC-5 cells. RGC survival factors also significantly reduced cell death at 72 and 120 hours (**E**), as measured by the proportion of PI-positive cells. Inclusion of B/C/I/F with staurosporine increased longest neurite length at 120 hours (**B**) and primary neurite counts at 72 and 120 hours (**D**). In contrast to what was seen with TSA, SS had little effect on proportions of differentiated or PI-positive cells before 120 hours (**F**) because differentiation was close to 100% at 24 and 72 hours. *Statistically significant ($P < 0.05$) difference between treatments.

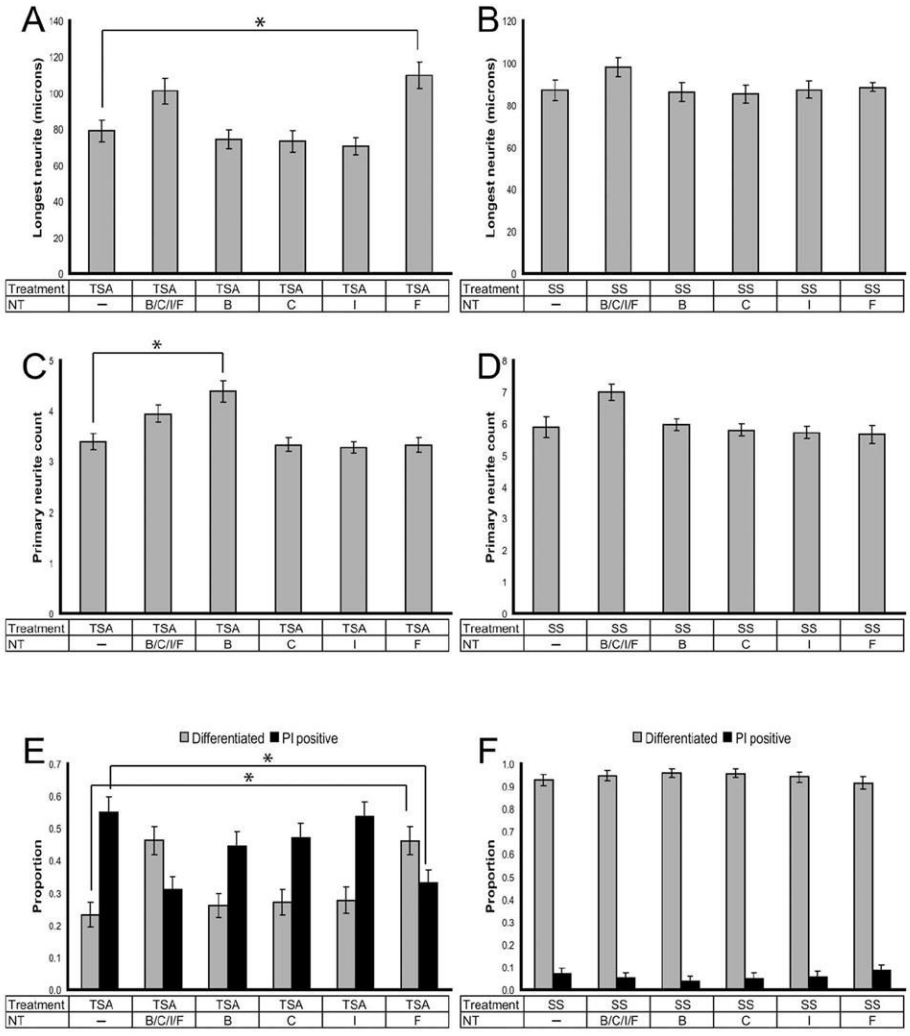

FIGURE 8.

Individual components of the RGC survival factor mixture differentially affects HDAC inhibition–mediated differentiation. RGC-5 cells were treated with TSA and RGC survival factors (BDNF, CNTF, insulin, forskolin; B/C/I/F) or the individual components BDNF (B), CNTF (C), insulin (I), and forskolin (F) and were compared with TSA control (−). The longest neurite length (microns; **A**) and the number of neurites longer than the soma (**C**) were determined 72 hours after initial treatment from photomicrographs, along with the proportion of cells differentiated (**E**). The same experiment was performed with SS (**B**, **D**, **F**). RGC survival factors augmented each of these differentiation measures. Forskolin and BDNF alone could account for the increases in longest neurite length and number of neurites per TSA-differentiated cell, respectively. The increased proportion of differentiated cells and the decreased proportion of PI-positive cells observed with B/C/I/F could also be explained by forskolin inclusion alone. In contrast, no component individually could account for the enhanced differentiation observed in SS differentiated RGC-5 cells cotreated with RGC survival factors. *Statistically significant ($P < 0.05$) difference between treatments.

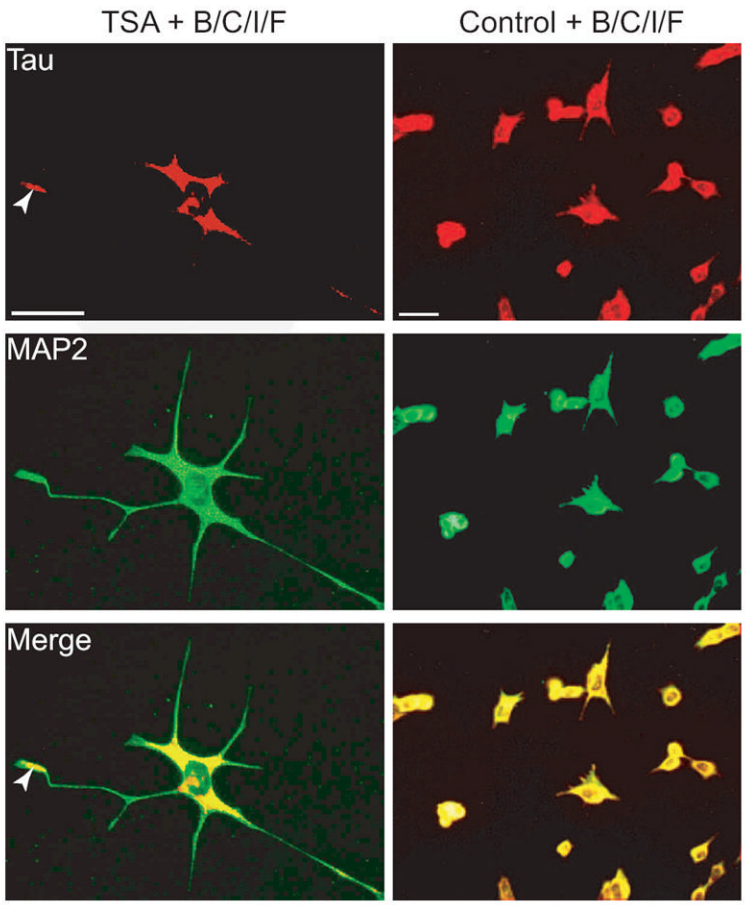

FIGURE 9.

HDAC inhibition differentiation induces true neurite outgrowth. RGC-5 cells were treated with 500 nM TSA and/or RGC survival factors and were immunohistochemically stained for tau (*red*) and MAP2 (*green*) 24 hours after treatment. Contrast was enhanced, and *red* and *green* images were merged to compare focal and diffuse staining. Moreover, the TSA photomicrographs were cropped to show enhanced detail. Although in TSA-differentiated cells MAP2 was observed diffusely throughout the soma and all neurites, tau was observed to be localized primarily to the soma and a single neurite (*arrows*). Furthermore, tau-positive neurites showed a distal gradient, with tau staining localized primarily to presumed growth cones. This pattern was not observed in cells treated solely with RGC survival factors because tau and MAP2 diffusely stained all projections from the soma. Scale bar, 50 μm.

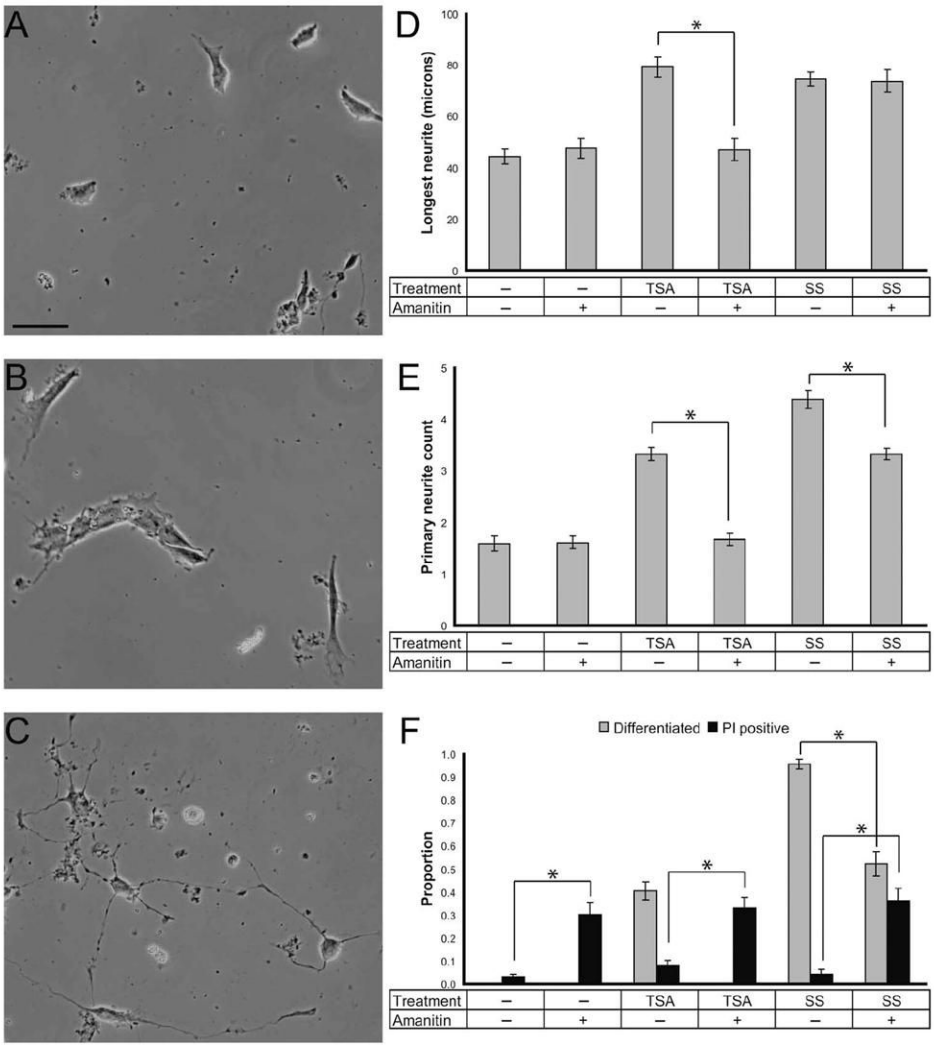

FIGURE 10.

HDAC inhibition–mediated differentiation is transcription dependent. RGC-5 cells were treated with 500 nM TSA and 316 nM SS, with and without the RNA polymerase II inhibitor α-amanitin (10 μg/mL; TSA and SS with α-amanitin (**B**) and (**C**), respectively), and were analyzed from photomicrographs taken 24 hours after treatment. HDAC inhibition–mediated differentiated cells were statistically not different from untreated control or α-amanitin–treated control cells (**A**) in terms of longest neurite length (microns; **D**), number of neurites longer than the soma (**E**), and proportion differentiated (**F**), indicating that transcription is necessary for HDAC inhibition differentiation. These results were not observed with SS differentiation (**D**–**F**) because significant neurite outgrowth and differentiation occurred in the presence of α-amanitin. *Statistically significant ($P < 0.05$) difference between treatments. Scale bar, 50 μm.

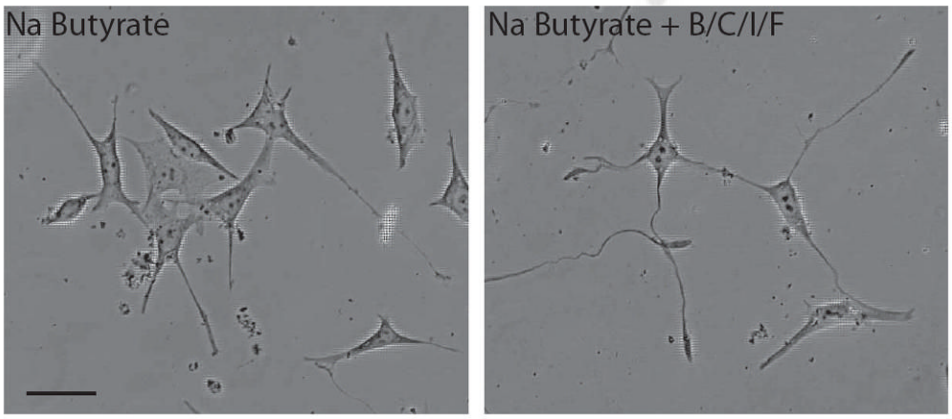

FIGURE 11.

HDAC inhibition induces neuritogenesis independently of cytoplasmic HDAC-6. RGC-5 cells were treated with 50 mM Na butyrate with and without RGC survival factors. Na butyrate is an HDAC inhibitor that does not inhibit cytoplasmic HDAC-6.[13] Photomicrographs were taken at 200× magnification 24 hours after treatment. Neuritogenesis was seen, suggesting that HDAC inhibition differentiates RGC-5 cells by acting on nuclear HDACs. Scale bar, 50 μm.

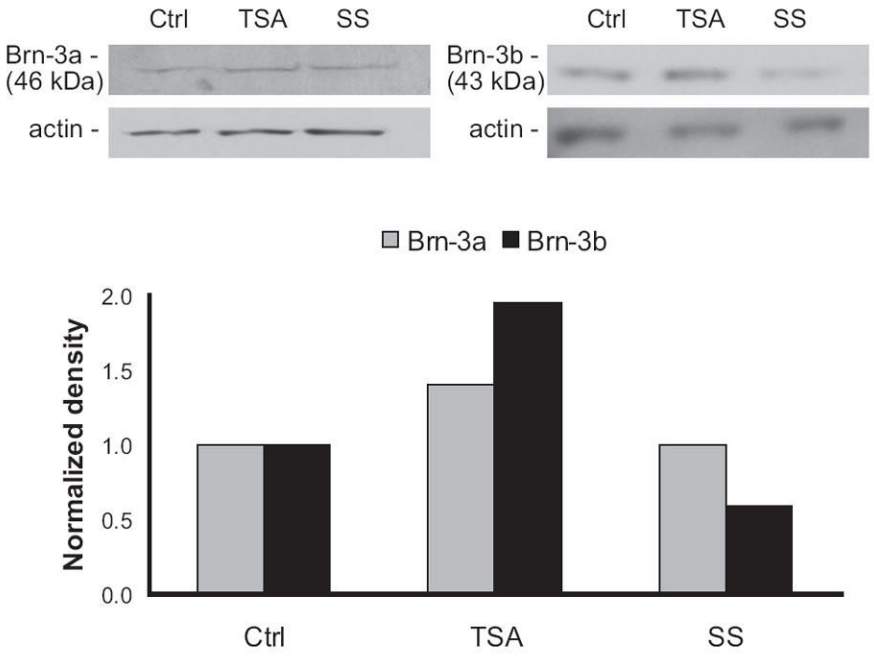

FIGURE 12.

HDAC inhibition upregulates Brn-3a and Brn-3b expression. RGC-5 cells were treated with 500 nM TSA, 316 nM SS, or medium alone (Ctrl) in the presence of RGC survival factors. Cell lysates were immunoblotted for Brn-3a and Brn-3b 48 hours after treatment. Band density was normalized to actin. TSA treatment resulted in Brn-3a and Brn-3b levels 1.4- and 2.0-fold those of control cells, respectively, whereas staurosporine treatment resulted in the downregulation of Brn-3b to 0.6-fold that of control cells.

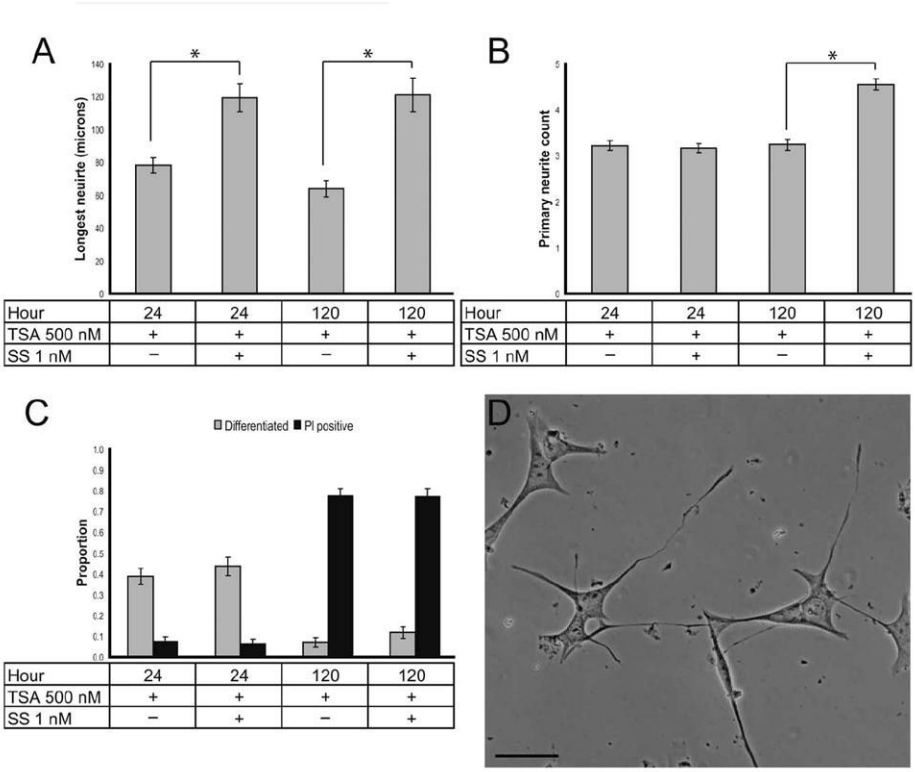

FIGURE 13.

HDAC inhibition and SS are synergistic with regard to the induction of neurite outgrowth. RGC-5 cells were treated with 500 nM TSA with (**D**) and without a subthreshold dose of SS at 1 nM. Photomicrographs were taken 24 and 120 hours after initial treatment to quantify longest neurite length (microns; **A**), number of neurites longer than the soma (**B**), and proportion of differentiated cells (**C**). The number of neurites in the TSA plus subthreshold SS condition was significantly larger than TSA alone 120 hours after treatment (**B**). Longest neurite length was also significantly augmented by subthreshold SS inclusion at 24 and 120 hours (**A**). However, the proportion differentiated was not significantly different from that of TSA alone at either time point (**C**). *Statistically significant ($P < 0.05$) difference between treatments. Scale bar, 50 μm.

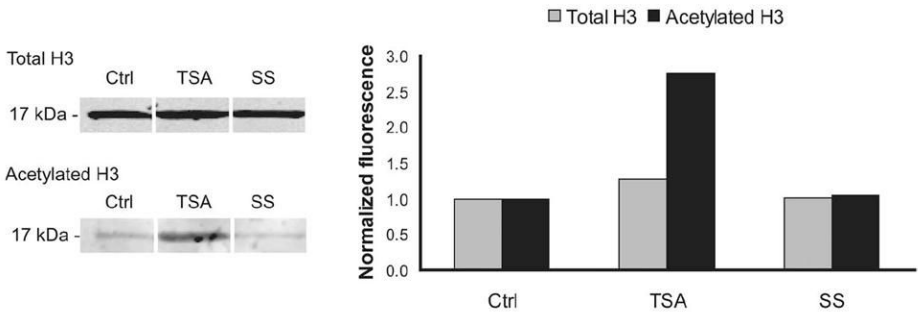

FIGURE 14.

Staurosporine does not inhibit HDAC. RGC-5 cells were treated with 500 nM TSA or 316 nM SS, and cell lysates were immunoblotted for total and acetylated histone H3 24 hours after treatment. Total and acetylated H3 fluorescence was normalized to that of untreated control cells. HDAC inhibition caused a 2.7-fold increase in H3 acetylation over control cells, whereas staurosporine H3 acetylation was unchanged compared with control cells.