A Comparison of Differentiation Protocols for RGC-5 Cells

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Purpose. Although the RGC-5 cell line is widely used in retinal ganglion cell (RGC) research, recent data have raised questions about the nature of these cells. The authors performed a systematic analysis of RGC-5 cells to determine which RGC or neuronal markers are expressed after treatment with known differentiating agents, thus providing further insight into the nature of these cells and assisting in defining their future use.

Methods. RGC-5 cells were treated for 5 days with staurosporine (STSN; 316 nM), trichostatin A (TSA; 500 nM), or succinyl-concanavalin A (sConA; 50 μg/mL), after which they were assayed for specific marker antigen/mRNA expression. Treated cells were also assayed for excitotoxic responsiveness.

Results. Neither treated nor untreated RGC-5 cells expressed any specific RGC marker mRNAs or proteins (Brn-3, neurofilaments, Thy-1) or calbindin, calretinin, synaptophysin, PKCα, or glial fibrillary acidic protein. However, control RGC-5 cells did express the neuronal markers tau, βIII-tubulin, microtubule-associated protein (MAP)-1b, MAP2, and PGP9.5. Although treatment with sConA had no effect on the expression of these markers, STSN and (dose dependently) TSA increased their expression and induced excitotoxic responsiveness. All cells, treated or not, expressed high levels of nestin but no other progenitor cell markers. All cells also expressed cone-specific, but not rod-specific, opsin indicative of cone photoreceptor lineage.

Conclusions. RGC-5 cells expressed neuronal, but not RGC-specific, markers that were dose dependently upregulated by TSA. Hence, TSA provided the best tested means to terminally differentiate the cells to a neuronal phenotype from a precursor-like lineage. (*Invest Ophthalmol Vis Sci.* 2010;51:3774–3783) DOI:10.1167/iovs.09-4305

The finding that the rat retinal ganglion cell-line RGC-5 was in fact derived from the mouse[1] has led researchers to question the nature and worth of these cells. Given that many researchers retain this cell line, it was deemed important to undertake a systematic analysis into what the cells express before and after treatment with previously published differentiation agents. This information was garnered to provide further insight into the nature of these cells and to assist in defining their future use.

Glaucoma comprises a family of ocular diseases characterized by intraocular pressure-associated optic neuropathy and death of retinal ganglion cells (RGCs), including loss of somata and axons.[2–4] RGC structure and function is ideally investigated in human glaucoma, but this approach has inherent limitations; hence, models of RGC injury provide a convenient research tool. It is, however, critical that the limitations of a model be well recognized so that conclusions drawn from hypothesis testing in the laboratory can be used appropriately to predict and explain phenomena in the real world. The ideal in vitro model has an environment in which cells are intact, have molecular and morphologic similarities to those found in vivo, and are separate from other retinal neurons.[5] In most cases, the establishment of such an RGC model has involved isolating a mixed population of retinal cells and purifying ganglion cells by methods such as immunopanning[6–8] and selective propagation.[9] Such procedures are relatively time consuming and produce limited numbers of cells; moreover, those that are isolated often lack functional processes.

The RGC-5 cell line, therefore, was favorably received by researchers when first characterized in 2001.[10] This cell line was described as derived from mixed rat retinal cells that had been transformed with the Ψ2 E1A adenovirus, that displayed certain characteristics of ganglion cells, such as expression of the specific cell-type markers Thy-1, Brn-3C, neuritin, NMDA receptor, $GABA_B$ receptor, and synaptophysin, and that displayed sensitivity to neurotrophin withdrawal.[10] Furthermore, differentiating treatment with the nonagglutinating lectin succinyl concanavalin A (sConA) rendered the cells sensitive to glutamate excitoxicity, reinforcing the RGC-like nature of these cells and explaining their perceived value to the research community.[10] Consequently, in the past 8 years, more than 100 research reports have been published with data derived from experiments performed on RGC-5 cells, including characterization of cell death induced, for example, by growth factor withdrawal,[11] hydrostatic pressure,[12,13] endoplasmic reticulum stress,[14,15] light exposure,[16] optineurin,[17] oxidative stress,[18,19] and calcium ionophore.[20]

Recently, questions have been raised regarding the true nature of these cells. One such study has pinpointed the fact that though originally characterized as *rat* derived, RGC-5 cells are in fact of *mouse* origin.[1] Furthermore, in the same study, it was pointed out that the apparent sensitivity of RGC-5 cells to glutamate, as induced by sConA treatment, has become reduced in more recent publications. Although 1 mM glutamate was sufficient to kill 50% of sConA-treated cells in the initial study, this concentration of glutamate killed less than 5% of cells in the latter study.[1] These data led researchers to a single conclusion, namely, that the RGC-5 cell line is not, as had originally been characterized, a rat ganglion cell line but is instead a mouse line of unknown phenotype.

Regardless of its true origin, many researchers retain the RGC-5 cell line. Thus, it is pertinent to address the validity of its use in light of the recent data—that is, can the cells be differ-

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entiated into an RGC-like phenotype, regardless of their species of origin? Interestingly, a number of excellent research reports have appeared in the past few years that suggest alternative means of differentiating RGC-5 cells into a ganglion cell-like phenotype. Levin's group[21,22] has been instrumental in producing such studies and has shown that the nonselective protein kinase inhibitor staurosporine (STSN) can rapidly arrest RGC-5 cell proliferation, induce a neuronal-like morphology, establish outward rectifying channels, upregulate the neuronal markers Thy-1 and microtubule-associated protein (MAP)-2, and induce the development of axon-like neurites. Further analysis indicated that two other kinase inhibitors, H-1152 and H-89 (which primarily inhibit Rho-kinase and protein kinase A, respectively), caused elevations in the quantity of neurites, but the effects were mild compared with STSN.[21] It was concluded that the effect was brought about by inhibition of an as yet unidentified protein kinase.[21] After these studies, the same group went on to show that treatment with histone deacetylase (HDAC) inhibitors, such as trichostatin A (TSA) and sodium butyrate, also caused differentiation to a neuronal phenotype that was nonproliferative, had increased numbers of neurites, and became neurotrophic factor dependent but was morphologically distinct from that induced by STSN.[23]

With these data in mind, we decided to produce a detailed comparison of the effects of sConA, STSN, and TSA on the induction of differentiation in RGC-5 cells. In particular, we sought to monitor changes in expression of known neuronal, ganglion, and other retinal cell markers to validate and to further characterize these cells.

Materials and Methods

Materials

RGC-5 cells were generously donated by Neeraj Agarwal (Department of Pathology and Anatomy, The University of North Texas Health Science Center, Fort Worth, TX). Cell culture plasticware was from Sarstedt (Adelaide, Australia), and culture media, growth factors, and other reagents were from Invitrogen (Mount Waverley, Australia), as were PCR reagents, primers and chemicals. TSA and sConA, along with other general chemicals, were from Sigma Chemical Company (Castle Hill, Australia), whereas STSN was from Calbiochem (Merck Pty, Kilsyth, Australia). Antibodies used for the analysis of RGC-5 cells are detailed in Table 1.

Culture of RGC-5 Cells

RGC-5 cells were grown in normal growth medium (Dulbecco's modified Eagle medium; containing 5 mM glucose), supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 10% (vol/vol) heat-inactivated fetal bovine serum in a humidified incubator with 5% CO_2 at 37°C. Doubling time of these cells was approximately 20 hours under these conditions, and they were usually passaged at a ratio of 1:20. For viability assays, cells were plated onto positively charged 96-well plates (CellPlus; Sarstedt). For immunocytochemistry, cells were plated onto sterilized 13-mm diameter non–matrix-coated borosilicate glass coverslips in 24-well plates. For immunoblot and RT-PCR analyses, cells were harvested from 75-cm^2 filter-capped cell culture flasks.

Cell Treatment

After passaging into the appropriate vessel, cells were allowed to attach and proliferate in normal growth medium for 6 hours, at which juncture the medium was removed with three washes in sterile phosphate-buffered saline (PBS; 137 mM NaCl, 5.4 mM KCl, 1.28 mM NaH_2PO_4, 7 mM Na_2HPO_4; pH 7.4). Cells were then incubated in normal growth medium lacking serum for 24 hours, after which they were returned to normal growth medium with only 0.1% fetal bovine serum (to slow the proliferation rate of the cells) containing a putative differentiation-inducing agent—STSN (316 nM), TSA (50 nM-5 μM), sConA (50 μg/mL)—or the appropriate vehicle control. Cells were treated with agents for 5 days and then processed for analysis.

Immunocytochemical Analysis of RGC-5 Protein Expression

Cells to be processed for immunocytochemistry were fixed at the end of the treatment period with 10% neutral-buffered formalin containing 1% (vol/vol) methanol for 15 minutes at room temperature. After fixation and washing in PBS, coverslips were sequentially immersed in PBS containing 0.1% (vol/vol) triton X-100 (PBS-T) for 15 minutes and PBS containing 3% (vol/vol) neonatal horse serum (PBS-NHS) for 15 minutes; each step was preceded by washing. Cells were incubated with primary antibodies, diluted in PBS-NHS overnight at room temperature in humidified chambers (see Table 1 for antibodies and their dilutions). Subsequently, cells were subjected to sequential incubations in appropriate biotinylated secondary antibodies (1:500 in PBS-NHS) for 30 minutes and then with streptavidin-conjugated Alexa-Fluor 488 (1:500 in PBS-NHS) for 1 hour with a darkened background. Such cells were counterstained with the fluorescent nuclear binding label 4′,6-diamidino-2-phenylindole (DAPI; 500 ng/mL). Sometimes cells

Table 1. Antibodies Used for Analysis of RGC-5 Cells

Target	Host	Clone/Catalog No.	Dilution	Source
Actin	Mouse	AC-15	1:20000	Sigma, Castle Hill, Australia
Brn-3	Goat	sc-6026	1:250	Santa Cruz Biotechnology, Santa Cruz, CA
Calbindin	Mouse	C9848	1:1000	Sigma
Calretinin	Rabbit	AB5054	1:1000	Millipore, North Ryde, NSW, Australia
Chx10	Sheep	AB9016	1:1000	Millipore
GFAP	Rabbit	Z0334	$1:1\times10^6$	Dako, Campbellfield, Victoria, Australia
Nestin	Mouse	MAB353	1:1000	Millipore
NFL	Mouse	NR4	1:1000	Sigma
NFM	Mouse	34-1000	1:1000	Zymed, South San Francisco, CA
Cone opsin	Rabbit	OPN1SW (H40)	1:1000	Santa Cruz Biotechnology
PCNA	Mouse	MAB4078	1:10000	Chemicon Australia Pty, Boronia, Victoria, Australia
PGP9.5	Mouse	RA95101	1:15000	Ultraclone; Wellow, IOW, UK
PKC-α	Mouse	Ab26318	1:500	Sapphire Biosciences Pty Ltd., Redfern, NSW, Australia
Rhodopsin	Mouse	RET-P1	1:2000	Sapphire Biosciences Pty Ltd.
Tau	Goat	C-17	1:500	Santa Cruz Biotechnology
Thy1.2	Rat	FF-10	1:200	Sapphire Biosciences Pty Ltd.
α-Tubulin	Mouse	MU121-UC	1:10000	BioGenex, St. Peter's, NSW, Australia
βIII-tubulin	Mouse	TU-20	1:1000	Millipore

TABLE 2. Primer Sequences for mRNAs Amplified by RT-PCR

mRNA	Primer Sequences	Product Size (bp)	Mg^{2+} Concentration (mM)	Annealing Temperature (°C)	Accession Number
Cyclophilin	5′-GAGAGAAATTTGAGGATGAGAAC-3′ 5′-AAAGAACTTCAGTGAGAGCAGAG-3′	374	5	59.5	NM_008907
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′ 5′-TCCACCACCCTGTTGCTGTA-3′	452	4	58.5	NM_008084
MAP-1b	5′-TGTTGGAAGGGAAGGCTCAGT-3′ 5′-AGCAGAGATGAACGGGGGTAG-3′	448	5	62	NM_008634
MAP-2*	5′-CTCCCAAGACCTTCCTCCATC-3′ 5′-TGGCAACTTTCTTCTCACTGG-3′	304	4.5	61	NM_001039934, NM_008632
Nestin	5′-GCGCTGGAACAGAGATTGG-3′ 5′-GATATAGGTGGGATGGGAGTG-3′	461	4	59	NM_012987
NFM	5′-CTGTGCCAAAATCACCCGTG-3′ 5′-TTCCTTCCACCTCCCCATTG-3′	402	4.5	58	NM_017029
Synaptophysin	5′-TCCTCAGCCCCTATCTGTTCC-3′ 5′-AACCCAAACCTGCCCACTC-3′	442	4.5	57	NM_009305
Tau*	5′-GAATGCCAAAGCCAAGACAGA-3′ 5′-CGAGCCACAAAAGCAGGTTAG-3′	347	5	58	NM_001038609, NM_010838
Thy1	5′-TGAGGGTGGCAGAAGAAGAC-3′ 5′-CTTTCCTCGGTGAGATGCTG-3′	360	4.5	61	NM_009382
βIII-Tubulin	5′-ATCTTCGGTCAGAGTGGTGCT-3′ 5′-TGTCGTAGAGGGCTTCATTGTC-3′	358	5	60	NM_139254

* Primers are homologous to transcript variant 1 mRNA and transcript variant 2 mRNA.

were labeled instead with a color reaction. In these cases, the procedure was identical to that described except that after washing with PBS-T, cells were incubated for 15 minutes in PBS containing 0.5% (vol/vol) H_2O_2 to inactivate endogenous peroxidase activity. Furthermore, conjugated Alexa-Fluor 488 was replaced by streptavidin-peroxidase conjugate (SPC; 1:1000 in PBS-NHS) for 1 hour. Color development was achieved with 3′-,3′-diaminobenzidine and counterstaining with hematoxylin.

For mouse tissue sections labeled as positive antibody controls, the procedure was similar to that described. Initially, however, fixed sections were deparaffinized, rinsed in 100% ethanol, and treated for 30 minutes with 0.5% H_2O_2 to block endogenous peroxidase activity. Antigen retrieval was achieved by microwaving sections in 10 mM citrate buffer (pH 6.0). Again, color development was achieved using 3′-,3′-diaminobenzidine, and sections were counterstained with hematoxylin. Specificity of antibody staining was confirmed by incubating adjacent sections in the absence of primary antibody.

Electrophoresis/Immunoblot Analysis of RGC-5 Protein Expression

After treatment, RGC-5 cells were harvested from culture flasks by scraping into PBS. Cell pellets were then sonicated in freshly prepared 20 mM Tris/HCl buffer (pH 7.4) containing 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol (DTT), and the protease inhibitors phenylmethylsulfonyl fluoride (0.1 mM), leupeptin (50 μg/mL), aprotinin (50 μg/mL), and pepstatin A (50 μg/mL) as well as a phosphatase inhibitor cocktail (Sigma Chemical Company). An equal volume of sample buffer (62.5 mM Tris/HCl, pH 7.4, containing 4% sodium dodecyl sulfate, 10% glycerol, 10% β-mercaptoethanol, and 0.002% bromophenol blue) was added, and samples were boiled for 3 minutes. An aliquot was taken at this stage for determination of protein content by the method of Bradford.[24] Electrophoresis of samples was performed using 6% to 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Samples were transferred onto polyvinylidene difluoride membranes overnight and then were blocked with Tris-buffered saline containing 0.1% (vol/vol) Tween 20 and 5% (wt/vol) nonfat dried skimmed milk. Blots were probed with antibodies, as outlined in Table 1, for 3 hours at room temperature, appropriate secondary antibodies were conjugated to biotin, and SPC was subsequently and sequentially used. Blots were developed with a 0.016% solution of 3-amino-9-ethylcarbazole in 50 mM sodium acetate (pH 5) containing 0.05% Tween-20 and 0.03% H_2O_2. Images were acquired from labeled blots using a flatbed scanner (CanoLide; Canon, Lake Success, NY) and were analyzed for densitometry using Kodak image analysis software.

RT-PCR Analysis of mRNA Expression in RGC-5 Cells

After harvesting, cells were sonicated in reagent (TriReagent; Sigma Chemical Company), and total RNA was isolated according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg DNase-treated RNA. In brief, 100 ng oligo(dT)12–18 was annealed to the RNA, followed by the addition of a mix containing 200 U M-MLV reverse transcriptase, 0.5 mM each dNTP, 20 U RNaseOUT RNase inhibitor, and 2.0 mM DTT in 1× first-strand buffer. After incubation at 42°C for 1 hour, cDNA samples were diluted to a final volume of 200 μL with diethylpyrocarbonate-treated water, yielding a concentration equivalent to 10 ng/μL total RNA. To assess genomic DNA contamination, additional reactions were carried out in which the reverse transcriptase enzyme was omitted. PCR products were not observed for any of the primer pairs tested using these samples.

Primer pairs were designed from sequences contained in the GenBank database using the primer design software Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), selected to amplify sequences that spanned at least 1 intron. Primer sequences were analyzed for T_m (melting temperature), secondary structure, and primer-dimer formation with NetPrimer analysis software (http://www.premierbiosoft.com/netprimer), and verified for their specificity to the target sequence by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST). All primers were optimized for annealing temperature and $MgCl_2$ concentration before they were used in assays. Oligonucleotide primer sequences and their annealing temperatures and $MgCl_2$ concentrations are shown in Table 2. Primers were sequenced by Invitrogen.

Individual cDNA species were amplified in a reaction containing the cDNA equivalent of 20 ng total RNA, 1× PCR buffer, $MgCl_2$, 0.8 mM each dNTP, relevant sense and antisense primers (final concentration of 4 ng/μL), and 0.5 U polymerase (AmpliTaq Gold; Applied Biosystems, Melbourne, Australia). Reactions were initiated by incubation at 94°C for 10 minutes, and PCR (94°C, 12 seconds; annealing temperature, 30 seconds; 72°C, 30 seconds) was performed for a suitable number of cycles followed by final extension at 72°C for 3 minutes. Previous experiments had determined the linear phase of amplification for each set of primers. All the PCR products yielded single bands corresponding to the expected

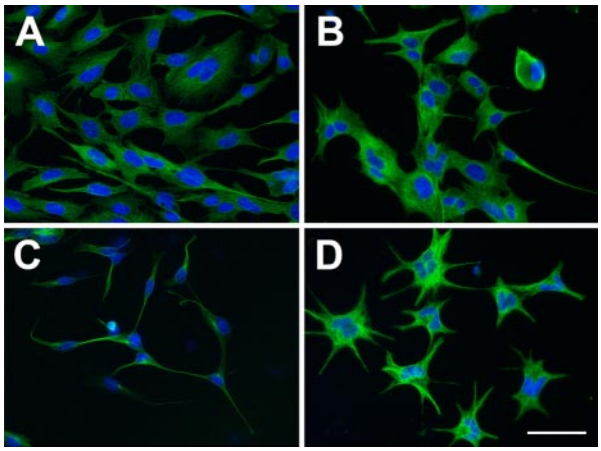

FIGURE 1. Effect of potential differentiating agents on cell morphology, as shown by α-tubulin immunolabeling and proliferation. RGC-5 cells (**A**) were morphologically altered when cells were treated with 50 μg/mL sConA (**B**), 316 nM STSN (**C**), or 500 nM TSA (**D**). Magnification, 200×. Scale bar, 10 μm. Nuclei were labeled with DAPI for visualization.

molecular weights (Table 2), and some PCR products were sequenced to ensure their validity. PCR reaction products were separated on 1.5% agarose gels using ethidium bromide for visualization.

Glutamate Sensitivity Determination

Cells were plated in 96-well plates at 10,000 cells per well in growth medium plus serum. After 24 hours, they were subjected to treatment with differentiating agents, as described. For the final period of 24 hours, L-glutamate or N-methyl-D-aspartic acid (NMDA) was added to media at a range of concentrations. Cell viability was determined using the 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay modified from that of Mosmann,[25] exactly as described previously.[26]

Statistical Analyses

All data are presented as mean ± SEM for the indicated number of individual experiments (minimum, $n = 4$). To determine significant differences between groups of data after experimental treatments, values were analyzed by one-way ANOVA followed by a post hoc Bonferroni test. $P < 0.05$ was considered significant.

RESULTS

Cell Morphology

Control cells proliferated rapidly, at a doubling time of approximately 20 hours in normal medium (with supplemented 10% fetal bovine serum). With only 0.1% serum present, the doubling rate was extended to approximately 100 hours. Untreated cells in these studies had a heterogeneous appearance (Fig. 1A), but most appeared ovular or glial-like and had elongated central axes with no distinct neurites. On treatment with sConA, cells developed a more rounded appearance, and α-tubulin labeling became more intense as a result (Fig. 1B). Some neurites were discernible. STSN, on the other hand, caused cells to shrink such that they had perikarya only slightly larger than the nuclei; many elongated and branch-like neurites, morphologically resembling neurons, were observed (Fig. 1C). TSA caused cells to attain a "star-like" or interneuron-like morphology, with very intense α-tubulin labeling. Neurites were plentiful but often did not branch after their initial outgrowth from the perikaryon (Fig. 1D). All treatments led to decreased culture density, in contrast to control cells. Numbers of cells after treatment were in the following order: sConA > TSA > STSN.

Expression of Markers Characteristic of Retinal Ganglion Cells

Figure 2 shows that none of the cells, regardless of treatment, were labeled with the Brn-3 antibody. Positive control labeling, however, was seen in the mouse retina (Fig. 2A). RT-PCR and immunoblot analyses of mRNAs (Fig. 2F) and proteins (Fig.

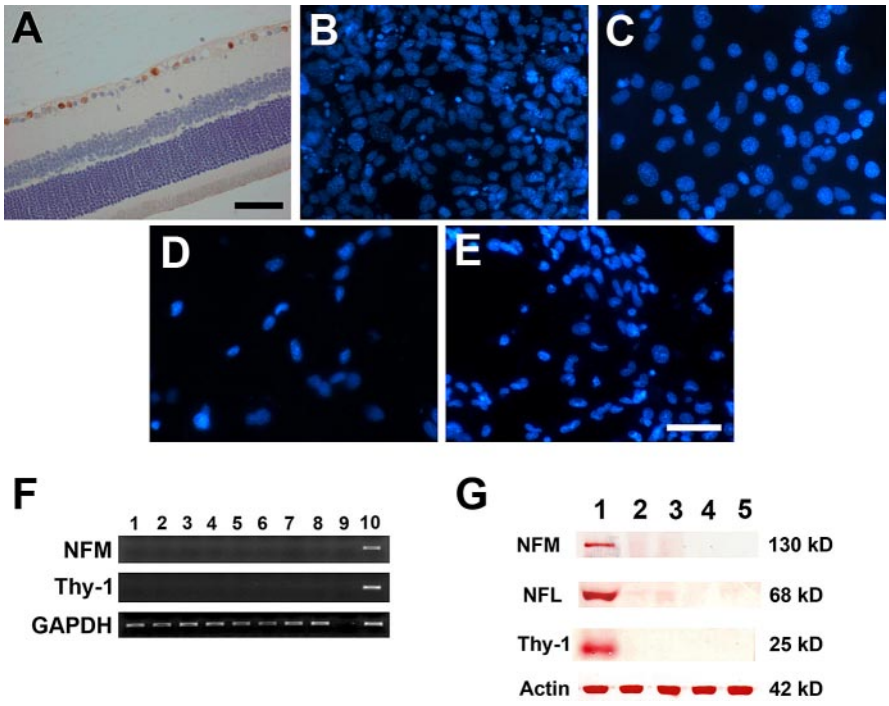

FIGURE 2. Expression of ganglion cell-specific markers after treatment of RGC-5 cells with potential differentiating agents. (**B–E**) Brn-3 immunolabeling in RGC-5 cells compared with positive control labeling in mouse retinal ganglion cells (**A**). No labeling for this ganglion cell-specific marker was present in control cells (**B**) or cells treated with sConA (**C**), STSN (**D**), or TSA (**E**). Magnification, 100×. Scale bars, 20 μm. Nuclei labeled with DAPI for visualization. (**F**) Analysis of mRNA expression for NFM and Thy-1 in RGC-5 cells compared with GAPDH (housekeeping gene product). *Lanes 1, 2*, control RGC-5 cells; *lanes 3, 4*, cells treated with sConA; *lanes 5, 6*, cells treated with STSN; *lanes 7, 8*, cells treated with TSA; *lane 9*, mouse retina negative RT; *lane 10*, mouse retina positive control sample. RGC-5 cells did not express mRNA for NFM or Thy-1 either before or after any of the treatments. (**G**) Western immunoblot labeling of ganglion cell protein markers in extracts from mouse retina (*lane 1*, positive control) and RGC-5 cells (*lane 2*, control cells; *lane 3*, 316 nM STSN; *lane 4*, 50 μg/mL sConA; *lane 5*, 500 nM TSA). Neither NFM, NFL, nor Thy-1 was expressed in RGC-5 cells regardless of treatment.

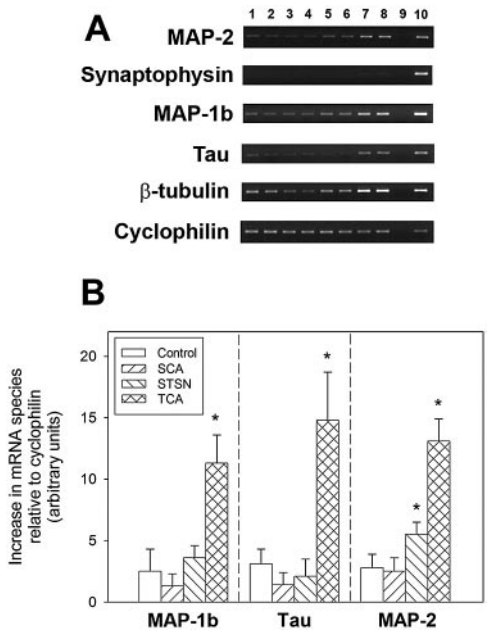

FIGURE 3. (**A**) Expression of mRNAs for neuron-specific MAP-2, synaptophysin, MAP-1b, tau, and βIII-tubulin compared with housekeeping gene cyclophilin nRNA expression in cultured RGC-5 cells. *Lanes 1, 2*, control RGC-5 cells; *lanes 3, 4*, cells treated with sConA; *lanes 5, 6*, cells treated with STSN; *lanes 7, 8*, cells treated with TSA; *lane 9*, mouse retina negative RT; *lane 10*, mouse retina positive control sample. MAP-2, MAP-1b, tau, and βIII-tubulin were expressed at notable levels in control cells. Synaptophysin mRNA, however, was not expressed in any RGC-5 cells. Interestingly, expression levels of all mRNAs investigated were markedly increased in cells after treatment with 500 nM TSA for 5 days. (**B**) Quantification of effects of differentiator treatments on RGC-5 cells for mRNAs for MAP-1b, tau, and MAP-2. (**A**) TSA caused an elevation in all three mRNAs investigated. In addition, STSN caused a significant increase in mRNA for MAP-2 alone. $^*P < 0.05$, one-way ANOVA followed by a post hoc Bonferroni test ($n = 4$).

2G), respectively, showed that neither control cells nor cells treated with any of the test agents expressed neurofilaments or showed any expression of Thy-1. Mouse retinal extracts were positive for proteins and mRNA species for all these markers (Figs. 2G, 2H).

Expression of Neuronal Markers

Figures 3 and 4 document the analyses of more general neuronal marker proteins and mRNAs in RGC-5 cells. Control cells expressed mRNAs for MAP-2, MAP-1b, tau, and βIII-tubulin (Fig. 3A), and, interestingly, treatment with 500 nM TSA caused upregulation in all four mRNAs. This proved to be significant when quantified (Fig. 3B showing, e.g., effects on MAP-2, MAP-1b, and tau mRNAs) and was approximately fourfold in each case. STSN also caused a more modest (approximately twofold), but significant, increase in MAP-2 mRNA. None of the cells expressed synaptophysin mRNA.

Positive immunolabeling was discerned in all cells for βIII-tubulin (Figs. 4A–D). Similarly, obvious immunolabeling was observed for tau in all cells (Figs. 4E–H). There were no discernible increases at the cellular level in either βIII-tubulin or tau labeling as a result of treatment with sConA, STSN, or TSA. Levels of two other proteins were investigated in cell extracts by Western immunoblot analysis (Fig. 4I). No synaptophysin protein was detected in any of the cell samples; labeling was very clear in mouse retinal extracts. Neuron-specific PGP9.5, however, was expressed only at low levels in control cells but was markedly and significantly elevated in cells treated with either STSN or TSA (Fig. 4J). Elevations in the levels of PGP9.5 protein were approximately fivefold after STSN treatment and 10-fold after TSA treatment.

To investigate this result further, some cells were treated with three different concentrations of TSA (50 nM, 500 nM, 5 μM). The effect of these three concentrations of TSA on cell morphology was obvious (Fig. 5A–D), with 50 nM TSA having only a slight effect but 500 nM and 5 μM having drastic effect. In fact, cells treated with the highest concentration of TSA (5 μM) showed lower density and greater degree of neurite-branching than at the 500 nM concentration (the concentration used throughout the rest of this study). The effect of TSA was also easily discernible on expression of PGP9.5 by immunolabeling (Figs. 5E–H) and immunoblotting (Figs. 5I, 5J). The effect was concentration-dependent, with the highest concentration of TSA inducing the most significant increase in the level of PGP9.5 expression.

Sensitivity to Excitotoxic Challenge

MTT reduction viability assay was used for determination of the sensitivity of both treated and untreated cells to glutamate (Fig. 6A) and the ionotropic glutamate receptor agonist, NMDA (Fig. 6B). Neither control cells nor cells treated with sConA or STSN were significantly killed by either glutamate or NMDA up to concentrations of 25 mM. In contrast, cells treated with 500 nM TSA were significantly reduced in measurable viability by 10 mM, or higher, glutamate or 25 mM NMDA.

Expression of Other Retinal Markers

None of the treated cells expressed either the ON-bipolar cell marker PKCα, the inner retinal neuronal calcium-binding protein calretinin, the horizontal cell-specific marker calbindin, or the astrocytic glial fibrillary acidic protein (GFAP), in contrast to mouse retina (Fig. 7A).

Interestingly, although the retinal neural progenitor marker CHX10 was not expressed in RGC-5 cells, the neural precursor intermediate protein nestin was present in both treated and untreated cells (Fig. 7B). RT-PCR analysis confirmed that nestin mRNA was present in all extracts; none of the test agents caused levels of this protein to be significantly altered. However, no mRNAs were detected for the precursor cell-associated Musashi-1 or doublecortin.

When analyzed for expression of photoreceptor-specific cell markers, positive labeling for the short-wavelength (blue) cone opsin was observed. This was determined in all (treated or untreated) cells by immunolabeling (Fig. 8A–D) and by immunoblotting (Fig. 8E). Conversely, there was no positive expression of rod-specific rhodopsin in RGC-5 cells (Fig. 8E).

DISCUSSION

The RGC-5 cell-line has become a valuable tool for studying RGC responses to pathologic and protective situations. Recent questioning regarding the origin of these cells[1] will likely have extensive ramifications for the retinal research community because many research laboratories now use them. It seemed pertinent, therefore, to reevaluate their worth. It is already clear that such cells are not derived from the rat, as was originally stated, but from the mouse.[1] The focus of the present study was to address their potential ganglion cell-like nature and to determine which previously published treatment for inducing terminal differentiation in mitotically active RGC-5 cells provides the optimal RGC-like phenotype. In addressing these issues, a detailed investigation was undertaken into expression of ganglion cell-specific or neuron-specific proteins/mRNAs after treatment with compounds previously described

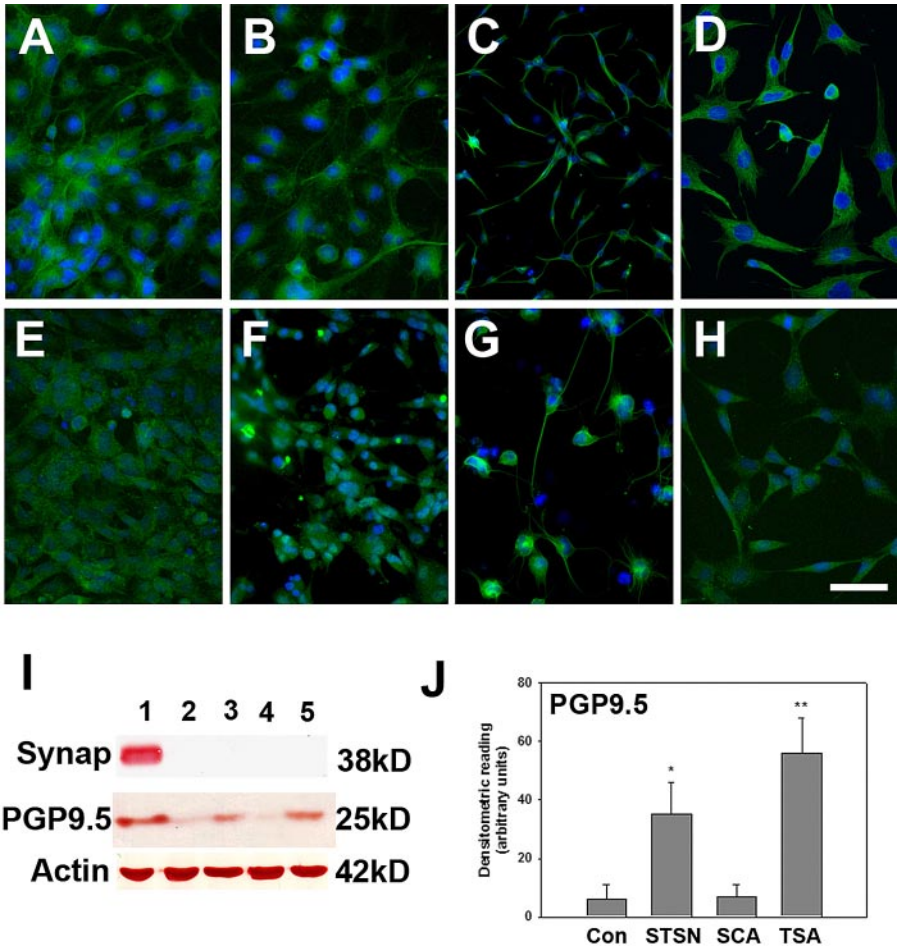

FIGURE 4. Expression of neuron-specific marker proteins in RGC-5 cells. (**A–D**) βIII-tubulin immunolabeling in cells after treatment with sConA (**B**), STSN (**C**), and TSA (**D**) compared with controls (**A**); (**E–H**) tau immunolabeling in cells after treatment with sCon A (**F**), STSN (**G**), and TSA (**H**) compared with controls (**E**); labeling was present in all cells. Magnification, 200×. Scale bar, 10 μm. Nuclei labeled with DAPI for visualization. (**I**) Western immunoblot labeling of neuron-specific PGP9.5 and synaptophysin in extracts from mouse retina (*lane 1*, positive control) and RGC-5 cells (*lane 2*, control RGC-5 cells; *lane 3*, cells treated with 316 nM STSN; *lane 4*, cells treated with 50 μg/mL sConA; *lane 5*, cells treated with 500 nM TSA). Synaptophysin was not expressed in cultured RGC-5 cells, but PGP9.5 was expressed at trace levels and was markedly increased when cells were treated with either STSN or TSA. Effect was normalized for actin levels in each sample and was quantified (**J**), and it was obvious that treatment with TSA or STSN (to a lesser degree) led to significant increases in the level of PGP9.5 protein present in RGC-5 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, one-way ANOVA followed by post hoc Bonferroni test ($n = 4$).

to exert clear influences on nonterminally differentiated cells. Three treatments were thus tested: sConA, STSN, and TSA.

In the present study it was clear that the RGC-5 cells tested did not express mRNA or proteins for any of the (relatively) selective ganglion cell markers examined (Thy-1, NFM, NFL, Brn-3). In the case of Brn-3, RT-PCR analysis was not used and so it cannot be definitively stated whether the cells expressed mRNA for Brn-3; mRNA for Brn-3c has been previously detected in these cells.[10] Furthermore, it is possible that the particular antibody used did not react with antigens on RGC-5 cells under the conditions used, but this seems unlikely given that positive and selective ganglion cell immunolabeling was detected in mouse retinal sections. Furthermore, a *pan*-Brn antibody was used that shows no selectivity for Brn-3a, Brn-3b, or Brn-3c. It was further surprising that no protein or mRNA for Thy-1 was detected in the cells because it has previously been stated that even undifferentiated cells express mRNA for Thy-1.[10] It was thus concluded that the RGC-5 cells from the laboratory in which the present studies were carried out were not ganglion cells and could not be altered to a true ganglion cell-like phenotype with any of the differentiating treatments tested. Because RGC-5 cells were originally derived from mixed retinal cells, we were interested to learn whether the cells expressed markers characteristic of other retinal cell types. The results showed, as expected, that RGC-5 cells were negative for calbindin (horizontal cells), calretinin (predominantly amacrine cells), PKCα (ON-bipolar cells), and GFAP (astrocytes).

The original research report describing RGC-5 cells stated that although the untreated cells expressed neuronal markers characteristic of retinal ganglion cells, such as Thy-1, Brn-3c, and NMDA receptors, they were still mitotically active, did not express typical ion channels, and were morphologically similar to glial cells.[10] Application of sConA to the cells, however, "…induced differentiation and rendered the cells responsive to glutamate toxicity."[10] This nonagglutinating lectin had previously been used to differentiate cultured chick neuroretinal cells.[27] These cells had been transformed with a temperature-sensitive strain of the Rous sarcoma virus into mitotically active cells by the introduction of active $pp60^{v\text{-}src}$ tyrosine kinase; sConA interacted with the Golgi/lysosomal membrane in an unknown manner and prevented the $pp60^{v\text{-}src}$-induced phosphorylation of a 41-kDa phosphoprotein.[27] Because the RGC5 cells were originally transformed in a completely different manner, by way of the Ψ2 E1A adenovirus, it is unclear how sConA could induce the differentiation of RGC-5 cells at all. The process of transformation was not the same and most likely involved the induction of different cellular reactions. It is possible that the same tyrosine phosphorylation reactions had taken place to be inhibited by sConA. It is also conceivable that this compound can act on other tyrosine kinases present in RGC-5 cells. Nonetheless, though many published studies of RGC-5 cells have involved nondifferentiated cells, several authors have followed the original protocol and treated cells with sConA,[28–30] but no mention of the effect of this agent was made in any of these studies. In the present study, it is clear that sConA did not induce RGC-5 differentiation into a ganglion cell-like phenotype or even a more general neuron-like phenotype. It did, however, cause an alteration in cell morphology. The physical nature of sConA as a nonagglutinating lectin is likely to be responsible for this action: this compound is known to react with membrane glycoprotein mannose resi-

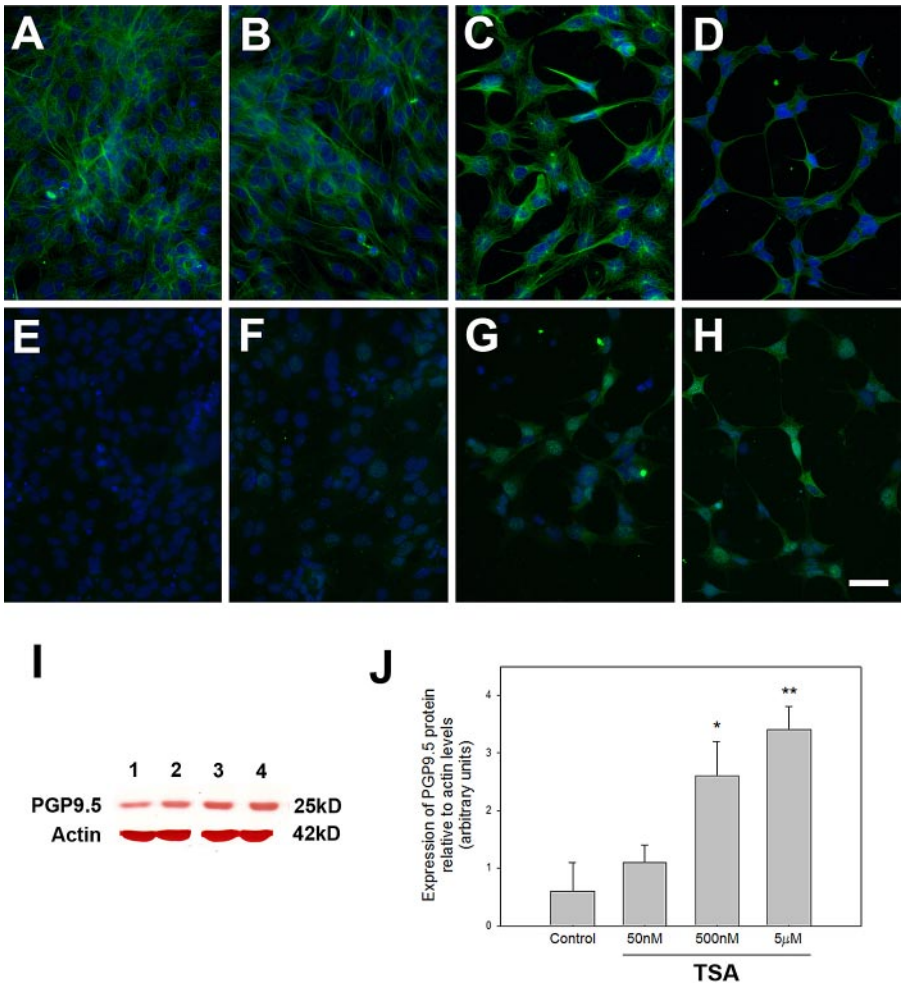

FIGURE 5. Effect of increasing concentrations of TSA on RGC-5 cells and their expression of neuron-specific PGP9.5. (**A–D**) α-Tubulin immunolabeling, identifying that general RGC-5 cell morphology (**A**) altered slightly after treatment with 50 nM TSA (**B**) but more markedly after treatment with 500 nM (**C**) and 5 μM (**D**) TSA. (**E–H**) Similarly, expression of PGP9.5 was TSA dose dependent, with only traces of this protein present in control cells (**E**) or cells treated with 50 nM TSA (**F**) but much more marked levels after treatment with 500 nM (**G**) or 5 μM (**H**) TSA. (**I**) Western immunoblot analysis confirmed this effect on PGP9.5 protein (*lane 1*, control; *lane 2*, 50 nM TSA; *lane 3*, 500 nM TSA; *lane 4*, 5 μM TSA), which was quantified in **J**. $^{*}P < 0.05$, $^{**}P < 0.01$, one-way ANOVA followed by post hoc Bonferroni test ($n = 4$). (**A–H**) Magnification, 200×. Scale bar, 10 μm. Nuclei were labeled with DAPI for visualization.

dues and has been shown to cause an inhibition of growth and a change in cell morphology.[31,32]

Studies by Levin's group[21,22] have clearly shown that the nonselective protein kinase inhibitor STSN can induce rapid alteration of RGC-5 cells to a neuronal-like morphology, provoke increased expression of MAP-2 and Thy-1, and establish an outward rectifying channel current. Importantly, it was conclusively proven that the action of STSN is not related to its known capacity[33] to induce apoptosis in (RGC-5) cells.[21] The present studies confirmed both the rapid alteration in morphology after STSN treatment and the increased expression of MAP-2 (mRNA). There was, indeed, a marked induction of neuritogenesis, consistent with the report from Frassetto et al.[21] In the present study, however, no Thy-1 expression was observed in STSN-treated RGC-5 cells, even by RT-PCR, using either mouse-specific (Fig. 2) or rat-specific Thy-1 primers (data not shown). The Thy-1 antibody used was a mouse-specific Thy-1.2 monoclonal antibody, which showed routine positive labeling in ganglion cells in mouse retinal sections (data not shown), but this was not the same one used by Frassetto et al.[21] STSN has been previously shown to induce differentiation of SH-SY5Y neuroblastoma and prostatic cancer TSU-Pr1 cells into those with neuron-like phenotypes.[34–36] The action of this compound has been stated to involve changes in expression and phosphorylation status of epidermal growth factor (EGF) receptors in neuroblastoma preparations, but this was not found to be the case in RGC-5 cells.[21] Recently, because of the success in this process described for RGC-5 cells by Levin's group,[21,22] the use of STSN has become the technique of choice for differentiating these cells.[37–41] The present data confirm previous results and show that STSN can indeed induce the differentiation of cultured RGC-5 cells to a more neuron-like phenotype but not specifically to that of a retinal ganglion cell.

To control gene expression, cells must first manage the coiling and uncoiling of DNA around histones in the nucleosomes. This is primarily accomplished by histone acetylases, which acetylate lysine residues in core histones and thus decrease their binding to the DNA phosphate backbone.[42] This leads to a less compact and transcriptionally active chromatin. HDACs catalyze the removal of acetyl groups from histones, which restores positive charges and causes condensation of the nucleosome.[42] This represses transcription because of the increased stearic interference of histones preventing access to transcription factors, regulatory complexes, and RNA polymerases.[42] Because HDAC inhibitors prevent these deacetylase reactions from occurring, they are well established as epigenetic modulators and have been shown to be potent inducers of the growth arrest, differentiation, and apoptosis of transformed cells.[42] Results shown in the present study demonstrated that of the tested differentiating agents, the HDAC inhibitor TSA induced the greatest change of gene expression in RGC-5 cells toward a neuron-like phenotype, as defined by the parameters examined. The effects of TSA were also concentration dependent. The results were exciting, because of all the neuron-specific markers analyzed, only synaptophysin, which was not induced in these cells under any tested circumstances, was not upregulated by TSA. The results were also in agreement with those of Schwechter et al.,[23] who demonstrated that the HDAC inhibitors TSA and sodium butyrate

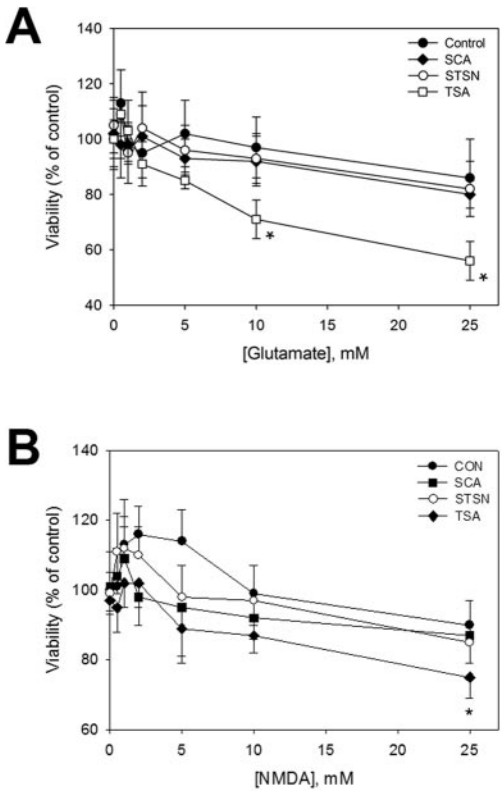

FIGURE 6. Glutamate (**A**) and NMDA (**B**) sensitivity of RGC-5 cells treated with vehicle control, 50 μg/mL sConA, 316 nM STSN, or 500 nM TSA, as measured by MTT reduction cell viability assay. Only TSA rendered the cells sensitive to glutamate and NMDA, and then only at significant levels at 10 mM (glutamate) or 25 mM (NMDA). $^*P < 0.05$, one-way ANOVA followed by post hoc Bonferroni test ($n = 6$).

initiated differentiating changes in RGC-5 cells. TSA was also able to induce a level of glutamate/NMDA sensitivity that was not present in control cells and that was not replicated by any other differentiating agent. Undifferentiated RGC-5 cells have been described to express NMDA receptor subunits,[10,41] which are evidently inactive because these cells are known to be unresponsive to glutamate/NMDA. Although data from the present study implies that TSA may induce an active state for these receptors, perhaps by stimulating the expression of regulatory NMDA receptor subunits, this seems unlikely given that the concentrations of excitotoxins used were extremely high. One possibility is that these compounds were toxic to RGC-5 cells through an oxidative or a pH-based mechanism and that, in altering genetic expression, TSA had altered cellular biochemistry to the extent that the cells could no longer tolerate these compounds at the tested concentrations. In terms of RGC-5 cells, however, TSA certainly appeared to induce the most neuron-like phenotype of all the tested agents.

The finding that RGC-5 cells express nestin is an interesting but not altogether surprising one. Undifferentiated RGC-5 cells behave as neuronal precursor cells. They undergo rapid proliferation, they are not sensitive to glutamate or growth factors, and they can be induced into maturing neuron-like cells by agents such as STSN or TSA.[21,23] Furthermore, the intermediate filament nestin represents a characteristic marker of multilineage progenitor cells and, in particular, of neural progenitor cells.[43] What was surprising was that no other neural precursor or stem cell-type markers could be determined in RGC-5 cells. No mRNA was expressed for the neuronal precursor MAP, doublecortin, or the developmental RNA-binding protein Musashi-1, each of which is associated with retinal neuron development.[44–46] Furthermore, the homeobox-containing transcription factor protein CHX10, which is critical for progenitor cell proliferation and bipolar/ganglion cell determination in the developing retina,[47] could also not be detected in RGC-5 cells. It is possible that the RGC-5 cells represent a cell line at a defined and specific stage of cell fate committal, one at which the other markers are not expressed.[43] Another possibility derives from the fact that injured nervous tissue is known to upregulate nestin,[48] which may indicate the initiation of remodeling or the reversion to a more immature phenotype for the cells in which this filament protein is expressed,[43] usually astrocytes or, in the case of the retina, Müller cells.[49,50] In breaking up the retina in the process of initial primary culture, proteins are known to be induced in glial cells in response to the enormous stress to which cells are subjected by this method; for example, GFAP is expressed by Müller cells and astrocytes when producing mixed retinal cultures.[26] If, then, the cells in culture are transformed at this stage, which may be the case for the RGC-5 cells, they may be effectively "fixed" as cells expressing such stress marker proteins. Thus, these proteins would be expressed under ordinary culturing

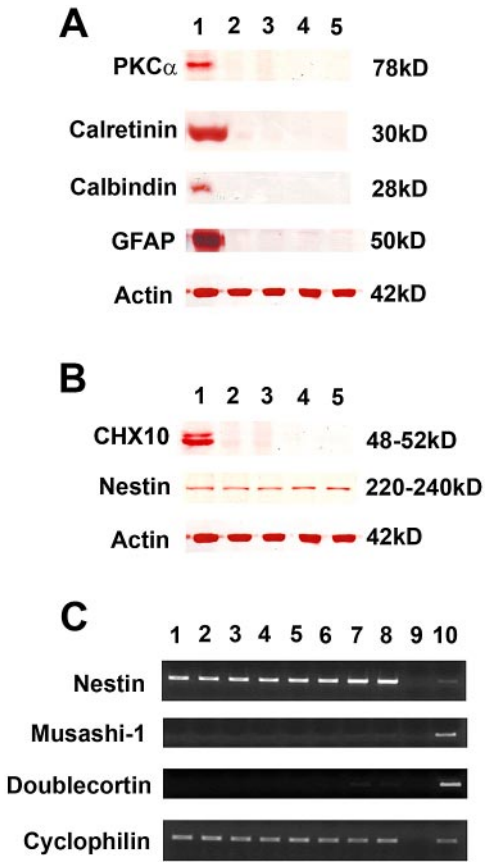

FIGURE 7. Expression of other retinal proteins and mRNAs in RGC-5 cells. *Lane 1*, mouse retina positive control extract; *lane 2*, control cells; *lane 3*, cells treated with 50 μg/mL sConA; *lane 4*, cells treated with 316 nM STSN; *lane 5*, cells treated with 500 nM TSA. (**A**) PKCα, calretinin, calbindin, and GFAP were not expressed by the cells after any treatment. (**B**) Retinal development marker CHX10 was not expressed by treated or untreated RGC-5 cells, but, interestingly, the retinal progenitor cell intermediate filament nestin was expressed in all extracts at equal levels. (**B**) *Lane 1* represents an extract prepared from a "stressed" mouse brain, as a positive expression control. (**C**) Musashi-1 and doublecortin mRNAs were not expressed by RGC-5 cells, but nestin mRNA was present. *Lanes 1, 2*: control; *lanes 3, 4*: sConA; *lanes 5, 6*: STSN; *lanes 7, 8*: TSA; *lane 9*, mouse retina negative RT; *lane 10*, mouse retina positive control sample.

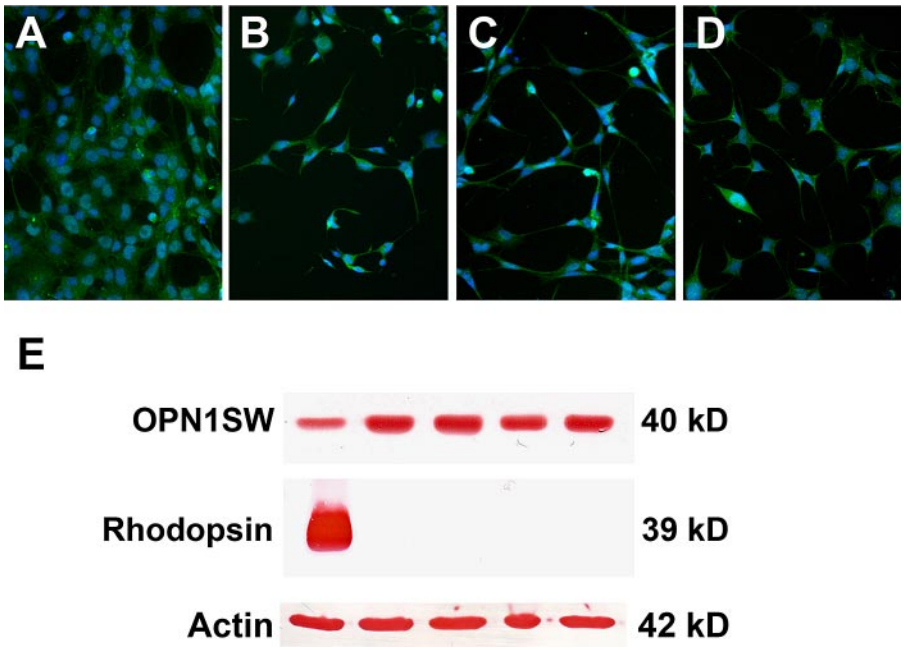

FIGURE 8. Expression of blue cone opsin in cultured RGC-5 cells. (**A**) Control cells. (**B**) Cells treated with 50 μg/mL sConA. (**C**) Cells treated with 316 nM STSN. (**D**) Cells treated with 500 nM TSA. (**A–D**) Magnification, 200×. (**E**) Western immunoblot labeling of photoreceptor cell protein markers in extracts from mouse retina (*lane 1*, positive control) and RGC-5 cells (*lane 2*, control cells; *lane 3*, 316 nM STSN; *lane 4*, 50 μg/mL sConA; *lane 5*, 500 nM TSA). Clearly, RGC-5 cells did not express rhodopsin but did express blue cone opsin; none of the treatments altered the levels of cone opsin expression.

thereafter. This, however, would imply a glial-type lineage for the RGC-5 cell line, and, as shown, these cells do not express glial markers such as GFAP. It has been shown that cells from the adult striatum harvested for primary culture do express nestin and can differentiate into either neurons or glia.[51] All data together imply that cells originally derived from a nestin-expressing population can resume or continue expressing this protein when subjected to different stimuli.[48] These data also imply that RGC-5 cells may not be derived from ganglion cells but from progenitor-like cells. In the present study, there was no downregulation in nestin expression after either TSA or STSN, which would be expected if terminal differentiation had been stimulated.[52]

One clear finding in the present study was that the RGC-5 cells expressed cone, but not rod, photoreceptor-specific opsin. All cells showed positive labeling for this protein (blue wavelength cone opsin), regardless of whether they were treated with a differentiating agent, and no agents altered its relative expression level. This striking result implies a cone photoreceptor lineage for the cells. This is the case with another ocular cell-line, the 661W cone photoreceptor cell line.[53] Analysis of images of 661W cells as originally characterized[53] indicated that these cells were morphologically similar to RGC-5 cells and had a similar expression profile. One might conjecture, perhaps, that RGC-5 cells have been mistakenly derived from the same stocks or have been mixed with the 661W cells at some point.

In conclusion, RGC-5 cells can be differentiated into neuron-like cells with either TSA or, to a lesser extent, STSN. TSA acts on RGC-5 cells in a concentration-dependent manner to induce neuron-like morphologic changes, neuritogenesis, expression of numerous neuron-specific marker proteins/mRNAs, and sensitivity to glutamate toxicity. In addition, RGC-5 cells expressed cone-specific opsin. This finding may help to identify the origins of this cell line.

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