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Differential responses of the mammalian retinal ganglion cell line RGC-5 to physiological stimuli and trophic factors

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ABSTRACT

The rat retinal ganglion cell (RGC) line RGC-5 constitutes a widely used model for studying physiological processes in retinal cells. In this paper we investigated the expression of clock and immediately early genes, and calcium mediated responses to physiological stimuli in differentiated and mitotically active RGC-5 cells. To this end, we attempted to differentiate the RGC-5 cells with a variety of effectors classically used to induce morphological differentiation. No sign of morphological differentiation was observed after 24 h of treatment with BDNF (80 ng/mL), NGF (100 ng/mL) and retinoic acid (20 ng/mL), among others. Only staurosporine (SSP) was able to promote neurite outgrowth at concentrations ranging from 53.5 to 214 nM. However, apoptotic nuclei were seen at 24 h of treatment using DNA staining, and a few cells remained at 72 h post-treatment. Concentrations of SSP lower than 214 nM were partially effective in inducing cell differentiation. Dividing RGC-5 cells express the RGC marker Thy-1 and different clock genes such as Per1, Clock and Bmal1. When characterizing the responsiveness of proliferative RGC-5 cells we found that in most of them, brief pulses of 50% FBS induced c-Fos and PER1 expression. Subsets of RGC-5 cells displayed significant changes in intracellular Ca^{2+} levels by ATP (100 μM) but not by glutamate (100–200 μM) stimulation. On the basis of cell morphology, size and complexity and effector responsiveness it was possible to distinguish different subpopulations within the cell line. The results demonstrate that only SSP is effective in promoting RGC-5 morphological differentiation, though the treatment provoked cell death. Proliferative cells expressing the RGC marker Thy-1 and a number of clock genes, responded differentially to diverse physiological stimuli showing a rapid c-Fos and PER1 induction by FBS stimulation, and an increase in intracellular Ca^{2+} by ATP.

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Short Retinal ganglion cells (RGCs) send visual and photic information to the brain through the axons forming the optic nerve and projecting to areas in the central nervous system involved in image- and non-image-forming tasks (Tessier-Lavigne, 1991; Valdez et al., 2009) RGCs may also be severely affected in optic neuropathies such as glaucoma, characterized by optic nerve degeneration resulting in a progressive loss of the visual field and eventual blindness (Ju et al., 2005; Osborne et al., 2001).

A clonal cell line originally established from postnatal rat retinal cells, named RGC-5, displays RGC characteristics based on expression of specific markers such as Thy-1, Brn-3c, Neuritin, NMDA and GABAb receptors, sensitivity to glutamate excitotoxicity and neurotrophin withdrawal (Agarwal et al., 2007; Krishnamoorthy et al., 2001). This cell line has been previously used as a cellular model to study diverse aspects related to glaucoma (i.e. glutamate excitotoxicity and cell death by serum deprivation) (Lipton, 2003). RGC-5 cells do not express the astrocyte marker glial fibrillary acidic protein (GFAP) or the amacrine or horizontal cell markers HPC-1 and 8A1 (Krishnamoorthy et al., 2001). However, the features of the RGC-5 line differ significantly from those of mature RGCs in that they are proliferative, morphologically undifferentiated and do not express the ion channels present in adult RGCs (Moorhouse et al., 2004). Recent works have shown that RGC-5 cells may have a mouse origin; clones used in these studies express neuronal markers and a cone opsin (Van Bergen et al., 2009; Wood et al., 2010). In order to induce differentiation of RGC-5 cells, different laboratories have used a variety of agents such as succinyl Concanavalin A (S-ConA), staurosporine (SSP), trichostatin (TSA) and human non-pigmented ciliary epithelium conditioned medium (HNPE) (Frassetto et al., 2006; Krishnamoorthy et al., 2001; Schwechter et al., 2007; Tchedre and Yorio, 2008). Results reported mainly with the S-ConA treatment have shown discrepancies in promoting visible cell differentiation (Krishnamoorthy et al., 2001; Van Bergen et al., 2009).

SSP is a protein kinase inhibitor acting as a strong inducer of apoptosis in different cell types when a concentration in the

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μmolar range is used. On the nmolar scale, however, SSP induces differentiation (Hashimoto and Hagino, 1989; Kabir et al., 2002; Raffioni and Bradshaw, 1995; Tafani et al., 2002; Yao et al., 1997; Zhang et al., 2003, 2005). SSP has been used to differentiate RGC-5 cells at concentrations ranging from 316 nM to 3.16 μM. Under these conditions RGC-5 cells do not divide and they present multiple branched neurites resembling a typical neuronal phenotype (Frassetto et al., 2006). Although SSP has been used by different labs to differentiate RGC-5 cells, little is known about apoptosis in these cells after such pharmacological treatment. Most studies have reported cell differentiation up to 24 h of treatment (Frassetto et al., 2006; Harper et al., 2009) and indirect information has been provided regarding a toxic effect of SSP on RGC-5 cells (Harvey and Chintala, 2007; Inokuchi et al., 2006; Schallenberg et al., 2009). In the present work, we investigated the time course of cell death after constant or transient SSP treatment at different concentrations. In addition, we examined the effect of different agents and trophic factors on RGC-5 cells, not studied before, in the attempt to achieve the cell differentiation.

Retinal illumination and other extracellular signals may induce significant changes in levels of intracellular Ca^{2+} (Tessier-Lavigne, 1991) and immediate-early gene (IEG) expression in RGCs of mammals and birds as reviewed in Caputto and Guido (2000). IEGs encode for, among others, the inducible transcription factors Fos and Jun; they are rapid and transiently expressed in response to diverse physiological stimuli and may act as cellular third messengers in the coupling of extracellular signals to long-term cellular changes. The inner retina and particularly RGCs of mammals have been shown to differentially express diverse clock genes such as *Clock*, *Bmal1* and *Per1* and *2* (Tosini et al., 2008) which are responsible for the molecular clockwork and generation of retinal circadian rhythms (Takahashi et al., 2008; Tosini et al., 2008). In this respect, the expression of selected clock genes was shown to be rapidly induced in response to physiological stimuli that synchronize intrinsic biological clocks resembling a typical IEG induction (Balsalobre et al., 2000, 1998). These and other cellular responses may be mediated by glutamate (Ebling, 1996) and other effectors such as ATP. RGCs exhibit heterogeneous responses to these effectors since they express different types of glutamatergic and purinergic receptors (Das et al., 2006; Ebling, 1996; Hartwick et al., 2007; Meyer-Franke et al., 1995; Morgan et al., 2008). Glutamate has been implicated in a number of functions related to retinal physiology and pathophysiology as well as in survival and growth of RGCs and RGC-5 cells (Dun et al., 2007; Fan et al., 2005; Meyer-Franke et al., 1995; Suemori et al., 2006).

In the present work we investigated: i) the morphological differentiation, neurite outgrowth and cell death of RGC-5 cell cultures in response to a variety of neurotrophic factors and other effectors (SSP, BDNF, NGF, vitamin A, etc.); and ii) whether RGC-5 cells under proliferation conditions maintain the typical intrinsic features of RGCs. To this end we examined the differential responses of these cells to serum, glutamate and ATP stimulation in order to stimulate the induction of IEG expression or changes in the mobilization of intracellular Ca^{2+}.

1. Experimental procedures

1.1. Materials

All reagents were analytical grade. The specific antibody against rat c-Fos was raised in rabbit (Sigma Aldrich) and used in a dilution of 1:500 for Inmunocytochemistry (ICC) and 1:10,000 for Western Blot (WB). The α-tubulin (α-Tub) protein was detected by the mouse monoclonal DM1A antibody (Sigma–Aldrich) dilution 1:1000 for ICC and WB. The PERIOD 1 (PER1) antibody (dilution 1:500) was raised in rabbit (Alpha Diagnostics) and the BMAL1 antibody (dilution 1:200) was raised in goat (Santa Cruz Biotechnology). The specific antibody against Thy-1 was raised in rabbit (Biosynthesis) from a synthetic peptide with a chicken sequence KNITVIKDKLEKC (Contin et al., 2006) and used in a dilution of 1:2000 for ICC. This sequence has about 70% and 62% of homology with the rat and mouse Thy-1 protein sequence respectively. The specificity of this antibody has been validated in our laboratory by immunochemistry using rat retinal sections (Contin MA, unpublished results). According to the sequence alignment (Blast NCBI), this antibody may recognize the two allelic forms seen in the mouse (Thy1.1 and Thy1.2) and the only one variant existing in the rat (Thy1.1) (Zwerner et al., 1977; Williams and Gagnon, 1982; Bradley et al., 2009). The mouse monoclonal anti-GFAP antibody (1:1000) was from Chemicon, Temecula CA. The secondary antibodies used for ICC were: Alexa Fluor 546 goat anti-mouse IgG; Alexa Fluor 488 goat anti-rabbit; and Alexa Fluor 488 goat anti-mouse IgG (dilution 1:1000) from Invitrogen. The secondary antibodies used for WB were anti-rabbit IgG peroxidase conjugate and anti-goat IgG peroxidase conjugate from SIGMA (dilution 1:1000); anti-rabbit IgG IRDye® 800CW conjugated goat polyclonal and anti-mouse IgG IRDye® 680CW conjugated goat polyclonal from Li-COR® IRDye® Infra Red Imaging Reagents (dilution 1:25,000). The FluorSave was from Calbiochem®. Staurosporine, DAPI, propidium iodide (PI), NGF, BDNF, Retinoic Acid, retinaldehyde, protease inhibitor, and other biochemical reagents were purchased from Sigma–Aldrich (USA). Vitamin A (Tanvimil) was from Raymos SAIC; Pluronic acid F-127 and Fura-2 AM were from Invitrogen and B-27 Supplement 50× was from GIBCO.

1.2. RGC-5 cell cultures

The RGC-5 cell line was provided by Dr. N. Agarwal (North Texas Health Science Center, USA) at passage 13 on June 2005. RGC-5 cells were grown in 100-mm tissue culture plates in Dulbecco's modified Eagle medium (DMEM, Sigma–Aldrich), 10% FBS (Gibco), 200 U/mL of penicillin and 100 μg/mL of streptomycin according to Krishnamoorthy et al. (2001), and were incubated at 37 °C with 5% CO_2. Successive passages were made after 2–3 days in culture when cells achieved 80% confluence. For selected experiments, cells were serum-deprived during 36 h, stimulated by 50% FBS (time 0) as reported (Balsalobre et al., 2000, 1998) and harvested every 15–30 min during 2 h after the serum shock.

Our RGC-5 clones express the RGC marker Thy-1 but not astrocyte markers such as GFAP (Suppl. Fig. 1); these observations are in agreement with their retinal origin as RGCs and previously published data (Agarwal et al., 2007; Krishnamoorthy et al., 2001; McKernan et al., 2007).

1.3. Treatment of RGC-5 cell cultures with neurotrophic factors and effectors

RGC-5 cells were grown at 50–60% confluence and the culture medium was replaced by DMEM supplemented with BDNF (80 ng/mL), NGF (100 ng/mL), vitamin A (20 μg/mL), retinoic acid (20 μM) retinaldehyde (2 μM), staurosporine (53.5–214 nM), B-27 Supplement (10 μL/mL), or vehicle (controls), during 24, 48 and 72 h. The vehicles utilized for the different effectors were DMSO, ethanol or distilled water at a final concentration $< 0.1\%$. After 24 h of treatment, cells were fixed for immunocytochemistry (ICC) and DAPI staining. For studies involving SSP, two series of experiments were performed: in the first, a dose-time response curve was carried out by assessing SSP concentrations from 53.5 to 214 nM and incubation times from 24 to 72 h; and in the second, pulses of 214 nM SSP were given to the cell cultures for 1, 2, 6 or 12 h, after which the effector was removed and replaced by fresh culture medium (DMEM + B27).

1.4. Immunocytochemistry and DAPI staining

RGC-5 cells were grown to 60–70% confluence on 10 mm cover-slips, serum-deprived during 36 h, stimulated by 50% FBS for 15, 30, 60 and 120 min, and then fixed with 3% paraformaldehyde–4% sucrose in PBS for 20 min at room temperature (Bussolino et al., 2001). Cells were permeabilized with PBS–0.2% Triton X 100 for 10 min, blocked 2 h with 1% BSA-PBS at room temperature and incubated overnight at 4 °C with primary antibodies in PBS. They were then incubated with the corresponding secondary antibody for 1 h at 37 °C, washed and mounted with FluorSave. The c-Fos ICC was visualized using an Olympus FV 1000 spectral confocal microscope equipped with a 63× and 100× Uplan SApo oil-immersion objective and fluorescence was acquired using a 488 laser line (excitation) and emission collected between 500 and 530 nm. Quantification of fluorescence associated to c-Fos was carried out by the Image J software (National Institute of Health Bethesda, Maryland, USA).

DAPI (1 μg/mL) staining was carried out during 5 min at room temperature after secondary antibody incubation. The tubulin ICC and DAPI staining were visualized with a Zeiss Axiovert-200 (Carl Zeiss, Oberkochen, Germany) epifluorescence microscope using a 60× or 100× objective equipped with a Micromax camera (Princeton Instruments, Trenton, NJ) controlled with the Metamorph Imaging 4.5 Software (Universal Imaging Corporation, Downingtown, PA).

1.5. Western blotting (WB)

RGC-5 cells harvested in PBS buffer containing protease inhibitors were lysed by repeated cycles of sonication and freezing (3 times at −20 °C). Total protein content in the homogenates was determined by Bradford's methods. Homogenates were resuspended in sample buffer (62.5 mM Tris HCl pH 6.8; 2% SDS; 10% glycerol; 50 mM DTT; 0.1% bromophenol blue) and heated at 90 °C for 5 min. 50 μg of protein

were separated by SDS-gel electrophoresis on 12–15% polyacrylamide gels, transferred onto nitrocellulose membranes, blocked for 1 h at room temperature with 5% skimmed milk in PBS and then incubated overnight at 4 °C with specific antibodies. Membranes were incubated with the corresponding secondary antibody in PBS during 1 h at room temperature followed by three washes with PBS for 15 min each. For Li-COR® IRDye® antibodies, membranes were scanned using an Odyssey IR Imager (LI-COR Biosciences). For peroxidase conjugate antibodies, membranes were developed with 4-Cl-Naftol/H_2O_2 as substrate and scanned with an HP Photosmart C3180 scanner. Densitometric quantification of specific PER1 and Tubulin immunolabeling was carried out with Image J software.

1.6. RT-PCR assays

Total RNA was extracted from RGC-5 cells and rat retina (positive control) using TRIzol® reagent following manufacturer's specifications (Invitrogen). The yield and purity of RNA were estimated by optical density at 260/280 nm. Following DNAse treatment (Promega), c-DNAs were synthesized from RNAs using MMLV reverse transcriptase polymerase (Promega) with oligo dT as primers according to the manufacturer's specifications. The polymerase chain reaction was performed in a Labnet Multigen Thermal cycler using the GoTaq® DNA Polymerase (Promega). The following primers were employed: Clock Forward: 5′TCTCTTCCAAACCAGACGCC3′; Clock Reverse: 5′TGCGGCATACTGGATGGAAT3′. GAPDH Forward: 5′AGACAGCCGCATCTTCTTGT3′; GAPDH Reverse: 5′TGATGGCAACAATGTCCACT3′ according to (Kamphuis et al., 2005). These primers display a complete alignment with the corresponding rat mRNA sequence and they also amplify the respective mouse mRNAs as shown in Fig. 6A. The PCR products were resolved in 1–2% agarose gel.

1.7. Propidium iodide staining and flow cytometry

The DNA content was determined as described (Sampedro et al., 2001). Briefly, RGC-5 cell cultures grown to 70% confluence were treated with 53.5 nM SSP or vehicle for 0, 24, 48 and 72 h. The cells were then washed twice and collected in cold PBS and permeabilized with cold 70% ethanol at 4 °C overnight in the dark. A solution containing propidium iodide (PI, 50 μg/mL) and ribonuclease-A (500 U/mL) was subsequently added to stain DNA since PI is the typical dye for these experiments (Nicoletti et al., 1991). The permeabilization step was included to allow the dye to reach the intracellular compartment and stain DNA; this approach allows DNA assessment in all cells and the identification of subpopulations with a sub-diploid DNA content revealing both apoptotic and necrotic cells. After two washes with PBS, cells were resuspended on Isotón-II. Data was collected from 10,000 cells in each sample using a flow cytometer (ORTHO Inmunocount Flow Cytometry Systems). The analysis program used was WinMDI.

1.8. Calcium imaging by FURA-2 AM fluorescence microscopy

RGC-5 cells were grown in an 8-well Lab-Tek recording chamber (Nunc™, NY-USA) until 40–50% confluence and then incubated in a colorless DMEM (GIBCO) containing 0.1% of Pluronic acid F-127 and 5 μM fura-2 AM (Invitrogen) Ca^{2+} indicator dye for 30–40 min at room temperature. The fluorescent imaging technique was performed as described (Sekaran et al., 2003) with modifications. Briefly, the dual-wavelength Ca^{2+}-sensitive indicator fura-2 was excited alternately at 340 and 380 nm with a 175 W Xenon lamp LX175F and the appropriate filters (XF04-2 Ratio Imaging Set). A filter shutter (model Lambda DG4, Shutter Instrument Company-USA) alternated the 340/380 nm excitation filters during 400 ms for each wavelength. The emitted fluorescence at 510 nm was captured every 2 s with a Micromax camera (Princeton Instruments, Trenton, NJ) fitted to a Zeiss Axiovert-200 microscope, using a 100× Uplan SApo oil-immersion objective (NA: 1.4; Olympus). The shutter and the camera were controlled with the MetaFluor software generating 12 bits—4 × 4 binned fluorescence images for each wavelength. The quantification of fluorescence levels in the cells was carried out using the MetaMorph_ 4.5 software; the measurements of mean fluorescence intensity in each cell were background-corrected by subtracting the mean fluorescence of an area with no cells. The Fura-2 ratios (R) were calculated as the ratio of the corrected mean fluorescence obtained for excitation at 340 nm to the corrected mean fluorescence obtained for excitation at 380 nm. The normalization of the ratios (relative 340/380 ratio) was done as $R_t - R_0/R_0$, where R_t is the ratio at a given time "t" and R_0 is the mean of 40 pre-stimulus ratios. ATP (100 μM) or glutamate (100 μM) stimulations were carried out by carefully pipetting a 3× solution of ATP or glutamate into the Lab-Tek chamber. Vehicle controls were added 5 min beforehand. No significant vehicle effects or changes in focus were detected. Effector responses were considered significant when a rise in the Fura-2AM ratio was greater than three times the change in the baseline obtained by vehicle treatment (≤0.007). A similar approach has been previously used (Hartwick et al., 2007).

1.9. Statistics

Statistical analyses involved one-way analysis of variance (ANOVA) with both Newman–Keuls and Tukey post hoc tests when appropriate.

2. Results

2.1. Effect of neurotrophic factors and other effectors on differentiation of RGC-5 cultures

We examined the effect of a variety of neurotrophic factors and other effectors such as BDNF, NGF, retinoic acid, enriched medium for neuronal growth (B27) and SSP, on the morphological differentiation of RGC-5 cell cultures (Figs. 1 and 2). After 24 h of treatment, no differentiation effect was observed with BDNF (80 ng/mL) (Fig. 1A), NGF (100 ng/mL) (Fig. 1B), retinoic acid (20 ng/mL) (Fig. 1C) and enriched medium for neuronal growth B27 (10 μL/mL) (Fig. 1D) as compared with controls treated with the vehicle only (Fig. 1F). We also treated RGC-5 cultures with vitamin A, retinaldehyde, differentiation medium (DM) (Rotstein et al., 2003), and co-cultures with disaggregated embryonic retinal cells; none of the treatments had a significant effect on cellular morphology as compared to vehicle-treated controls (data not shown). Moreover, no sign of neurite outgrowth was observed after 3 days of treatment with most neurotrophic effectors tested or DM, and even after a week of NGF treatment (data not shown). However, when we assessed the response of the RGC-5 cells to SSP at concentrations ranging from 53.5 to 214 nM (Figs. 1E to 4), we found that this effector induced cell differentiation at even lower concentrations than those reported previously (Frassetto et al., 2006). It is noteworthy that the morphological changes were observed as early as 1 h after SSP administration at the maximal concentration used (Fig. 4A). However, when SSP remained in the medium during 24, 48 or 72 h, cell death was clearly observed, with the typical cell shrinkage and apoptotic bodies (Fig. 2A; Suppl. Figs. 2 and 3). Nuclei evaluation and quantification revealed that 42% of cells died by 24 h of treatment with SSP, the number increasing to 71% and 86% at 48 and 72 h of treatment, respectively. Cells treated with vehicle (controls) and subjected to FBS deprivation did not show any sign of differentiation or indication of cell death (Fig. 2B and C). Furthermore, when we tested the effect of SSP without removal of 10% FBS at 24 h post stimulation, we observed morphological differentiation of RGC-5 cultures but cells eventually died (data not shown).

Lower concentrations of SSP (53.5 nM) were also found to induce neurite outgrowth in RGC-5 cells (Fig. 3C). However, the effect was not uniform and was not observed in all cells examined, even at 48 h of treatment (Fig. 3A). In addition, the DNA histogram of RGC-5 cells (Fig. 3E) clearly denotes the presence of a sub-G_1 peak after 48 h-SSP treatment, becoming apparent at 24 h of treatment (Fig. 3F). The appearance of this sub-diploid DNA peak is a marker of cell death and confirms the toxic effect of SSP on RGC-5 cells.

To avoid cell death after differentiation by prolonged SSP exposure, pulses of 214 nM of this compound were given to the cultures for 1, 2, 6 or 12 h, and the effector was then removed and replaced by fresh culture medium (Fig. 4). After 24 h, cells displayed a clear differentiation showing neurite outgrowth irrespective of the SSP pulse duration. However, nuclear DNA staining by DAPI showed the presence of apoptotic nuclei for all time intervals assessed during the SSP pulses (Fig. 4A). When pulses of SSP in a lower range of concentrations (53.5–107 nM) were utilized, the cell morphology was similar to that of the controls (vehicle) (data not shown). RGC-5 cells treated with the vehicle exhibited no sign of differentiation or cell death at any time during the course of the experiments (Fig. 4B). Taken together, our results strongly suggest that only SSP (214 nM) was effective in causing RGC-5 differentiation (Fig. 1). However, though morphologically differentiated, the cells had an unhealthy appearance and apoptotic bodies were observed even at 24 h of treatment (Fig. 2). At a lower concentration (53.5 nM), SSP was partially effective in inducing morphological differentiation but still had a toxic effect

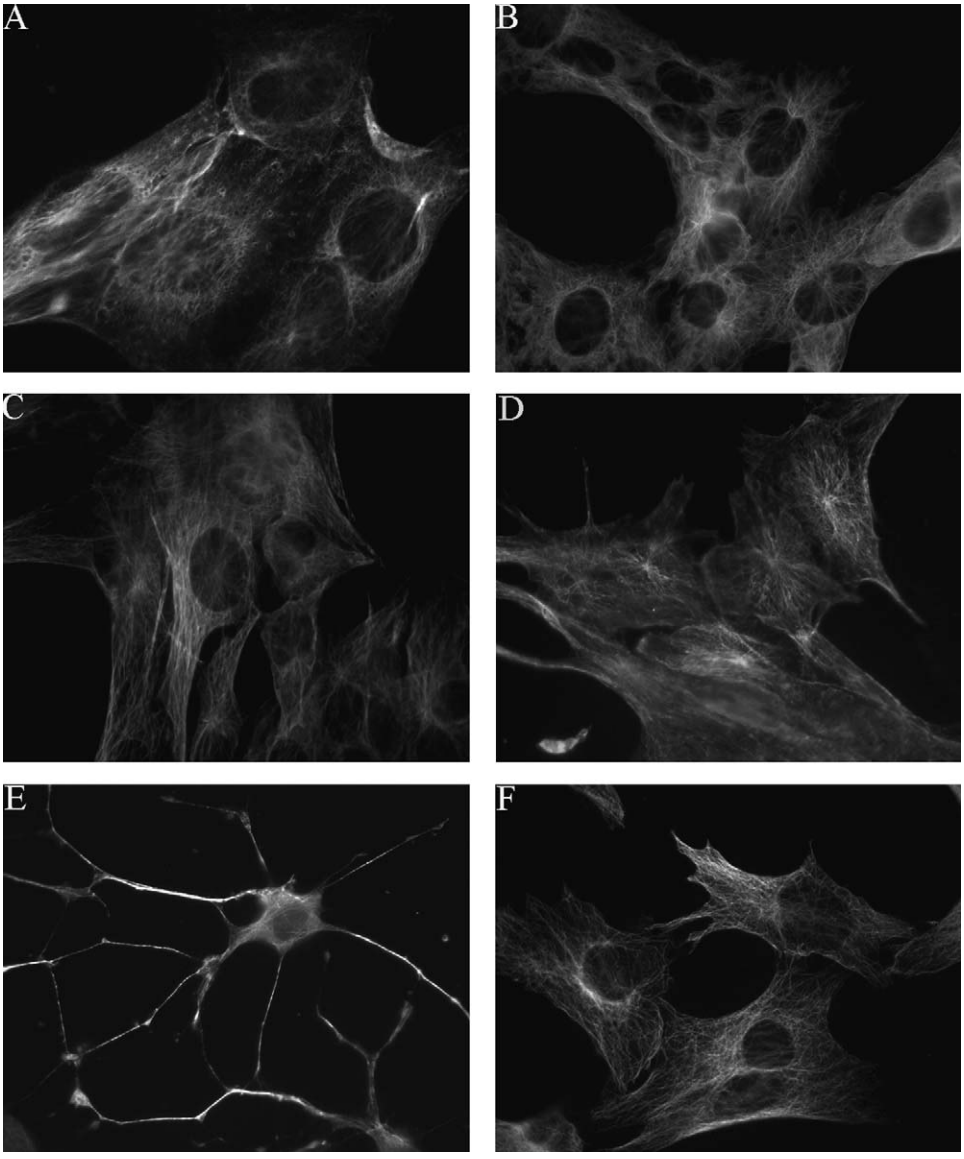

Fig. 1. Morphological differentiation of RGC-5 cells in culture: The effect of neurotrophic factors and other effectors. Effectors such as BDNF (A), NGF (B), retinoic acid (C), enriched medium for neuronal growth (B27) (D), Staurosporin (SSP) (E) and vehicle (F) were tested on RGC-5 cultures after 24 h of treatment. No morphological changes were observed with BDNF, NGF, retinoic acid, and enriched medium for neuronal growth (B27) as compared with controls treated with vehicle only, even after 3 days in culture. However, SSP (214 nM) was able to cause morphological differentiation of RGC-5 cells with several well-defined neurites in each cell. Photomicrographs show the α-Tubulin immunofluorescence highlighting the whole cell bodies and processes. See Section 1 for further details.

on RGC-5 cell viability (Fig. 3) which could not be prevented by the administration of SSP during a brief pulse (Fig. 4).

In view of the unsatisfactory morphological differentiation of RGC-5 by SSP and the other effectors utilized, we carried out the following series of studies with proliferating RGC-5 cells in order to evaluate similarities between this cell line and RGCs in terms of their intrinsic functioning and cell responsiveness to different physiological effectors.

2.2. Induction of c-Fos protein in RGC-5 cells by serum stimulation

RGCs of different vertebrate species have been shown to induce the expression of several IEG proteins in response to physiological stimuli (Caputto and Guido, 2002; Semo et al., 2003). Here we investigated the induction of c-Fos protein in RGC-5 cells after serum shock with 50% FBS for 30 and 60 min. The results presented in Fig. 5 strongly indicate that c-Fos protein was rapidly induced in the serum-stimulated RGC-5 cell cultures within 30 min as observed either by WB or ICC. NIH-3T3 cells were used as positive controls of c-Fos induction in response to serum stimulation (Bussolino et al., 2001). The quantification of relative fluorescence levels associated to c-Fos immunoreactivity in RGC-5 cells upon serum stimulation (Fig. 5, bottom panel) showed a 30% and 50% increase at 30 min ($n = 68$) and 60 min ($n = 124$) post-stimulation, respectively, as compared with background levels at time 0 ($n = 65$). The ANOVA revealed a significant time and treatment effect ($p < 0.001$). Strikingly, the immunofluorescence observed after 30 min of serum treatment was mainly localized in the cytoplasm whereas at 60 min, immunoreactivity was also visualized in the nuclei (see Fig. 5 middle panel).

2.3. Expression of clock genes in RGC-5 cells

The mammalian retina contains an endogenous clock that regulates a number of physiological processes in a rhythmic manner (Bai et al., 2008; Dorenbos et al., 2007; Guido et al., 2002; Kamphuis et al., 2005; Tosini et al., 2007, 2008) through an intrinsic molecular mechanism that involves, at least nine clock

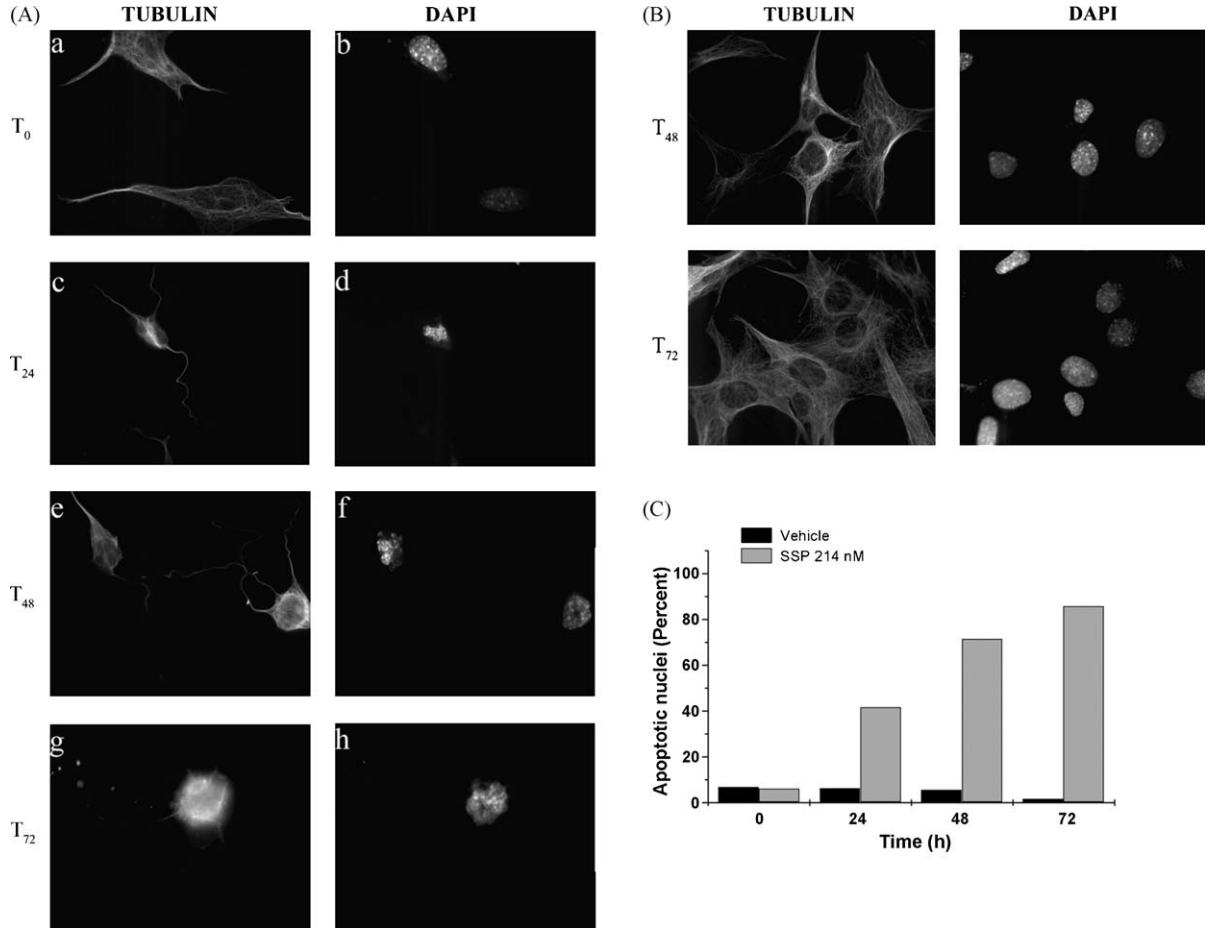

Fig. 2. Time course of Staurosporine (SSP) effect on the RGC-5 cell morphology. (A) SSP (214 nM) largely induced cell differentiation after 24 h of treatment as observed by ICC with the α-Tubulin antibody (a, c, e) (left panel). Although cells looked morphologically differentiated (c and e), cell death was clearly observed by nuclear staining with DAPI at 24, 48 and 72 h post-treatment (d, f, h). (B) Vehicle-treated cells (controls) do not show morphological differentiation at 48 and 72 h (left panel) and no apoptotic nuclei were seen at any time examined (right panel). Apoptotic bodies were clearly distinguished from normal cell nuclei (A vs. B). (C) Quantification of apoptotic nuclei in each condition revealed that levels of cell death at time 0 were similar between SSP-treated and vehicles controls (6%). An increase in cell death was observed at 24, 48 and 72 h of SSP treatment (42%, 71% and 86% respectively) whereas controls presented basal levels as observed at time 0 (6.2%, 5.43%, and 1.5% respectively). Number of cells assessed in each group was between 30–130/condition.

genes (*Clock, Bmal1, Per1, Per2, Cry1, Cry2*, etc.). Different laboratories have shown the expression of clock genes restricted to the inner retina and RGCs of mammals (Bai et al., 2008; Dorenbos et al., 2007; Guido et al., 2002; Kamphuis et al., 2005; Tosini et al., 2007, 2008). Here we investigated the expression of the clock genes *Clock* (at the mRNA level) and *Bmal1* and *Per1* (at the protein level) in RGC-5 cells after serum stimulation with 50% FBS. The results presented in Fig. 6A clearly indicate that RGC-5 cells expressed the three clock genes tested as observed in positive controls (total retina and NIH 3T3 cells). Moreover, PER1 protein was rapidly induced in the serum-stimulated RGC-5 cell cultures within 15 min as observed by WB (Fig. 6B). The relative levels associated to PER1 immunoreactivity in RGC-5 cells upon serum stimulation (Fig. 6B) showed a significant increase at 15 min and elevated levels at 30 and 60 min post-stimulation as compared with background levels at time 0 ($p < 0.001$). Similarly, Balsalobre et al. (1998, 2000) and Mateju et al. (2009) reported an immediate early expression of PER1 protein in Rat-1 cells and in the rat suprachiasmatic nuclei, respectively.

2.4. Differential Ca^{2+} responses to ATP and glutamate stimulation in RGC-5 cells

We assessed changes in intracellular Ca^{2+} levels in individual RGC-5 cells after stimulation by ATP (100 μM) or glutamate (100–200 μM) using the fluorescent indicator fura-2 AM and image analysis as described previously (Sekaran et al., 2003; Uchida and Iuvone, 1999). Fig. 7A, shows the differential response of RGC-5 cells to ATP stimulation in three different individual cells representative of all cells tested ($n = 63$). We found that around half the cell population (~54%) responded to ATP by increasing their intracellular calcium levels. The other half (~46%) showed no significant responses. In the former case, two different types of responsiveness to ATP stimulation were observed: one subset of RGC-5 cells responded strongly to ATP treatment, sometimes generating several bursts of intracellular Ca^{2+} mobilization (29%) (Fig. 7A, left panel), whereas the second subset of cells exhibited intermediate responses (25%) (Fig. 7A, middle panel). In contrast, RGC-5 cells did not respond to glutamate stimulation in concentrations ranging from 100 μM (Fig. 7B, left panel) to 200 μM (data not shown); however, positive controls of disaggregated chicken embryonic retinal cells kept in culture strongly responded to the same stimuli of 100 μM glutamate (Fig. 7B, middle panel). In addition, a series of observations obtained by flow cytometry of proliferative RGC-5 cultures (controls, Fig. 7C), together with the cytoskeleton immunocytochemistry of these cells denoting the whole cell bodies (Fig. 7D), show diverse cellular morphologies, sizes and complexities (see also Suppl. Fig. 1). These findings together with the differential ATP responses described above strongly suggest that there are different subsets of cell

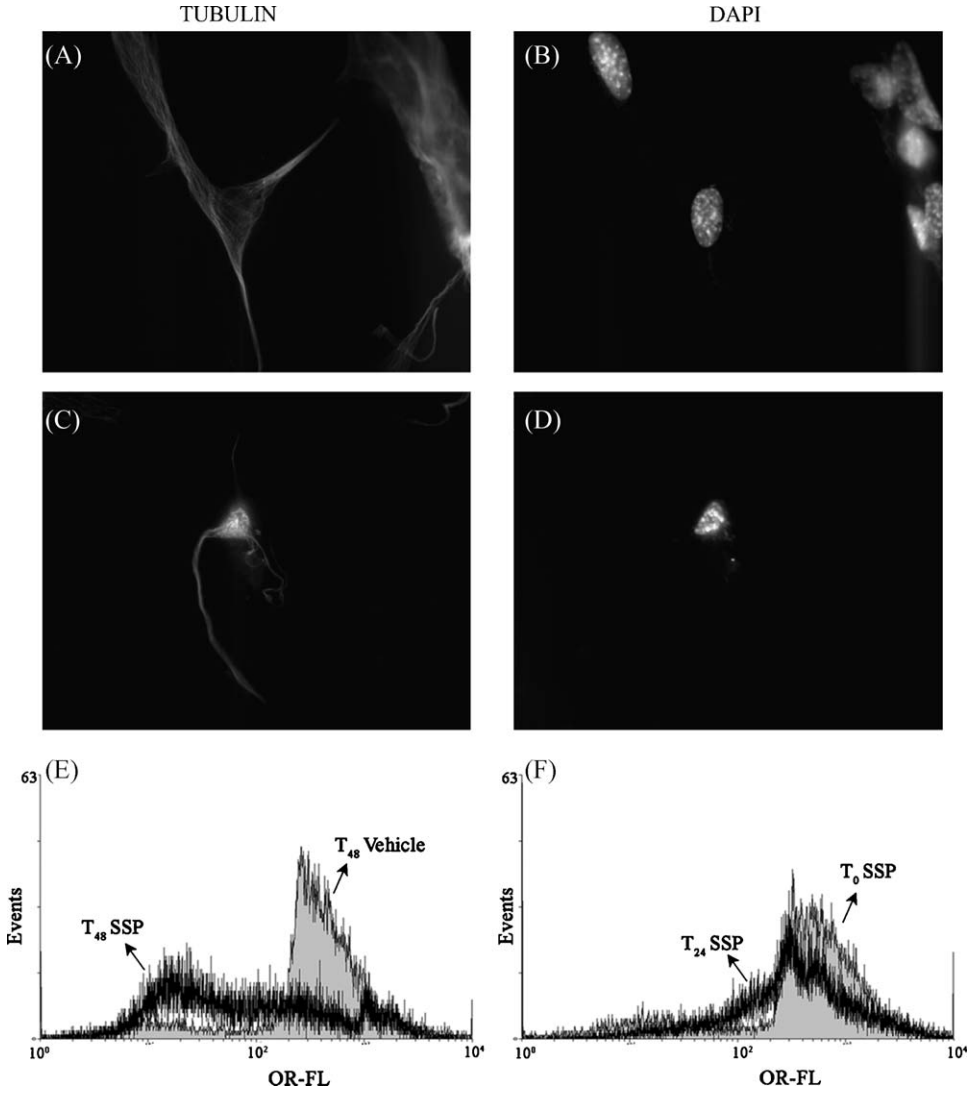

Fig. 3. Effect of a low-dose SSP treatment (53.5 nM) on RGC-5 cell morphology. (A–D) RGC-5 cultures were exposed to 53.5 nM for 48 h. Each pair of photomicrographs shows the α-Tubulin (left panel) and DAPI immunofluorescence (right panel) in RGC-5 cultures. A and B show representative RGC-5 cells with no morphological changes; C and D show a RGC-5 cell exhibiting a neuronal-like morphology after SSP treatment. (E and F) DNA histograms with PI staining for RGC-5 cells treated with SSP or vehicle at time 0 and after 24 h (F) or 48 h of treatment (E). A significant subpopulation of RGC-5 cells showed a sub-G1 peak when exposed to SSP for 24 and 48 h, suggesting cell apoptosis.

populations present in the RGC-5 cell line we used. However, there was no evident correlation between morphology and ATP responses.

3. Discussion

The adequate functioning of RGCs is essential for optimal retinal physiology, visual processes, light detection and the setting of biological clocks (Contin et al., 2006; Foster et al., 2003; Garbarino-Pico et al., 2004; Guido et al., 2002). When these cells are severely affected in optic neuropathies such as glaucoma, the progressive loss of the visual field and other functions may occur, leading eventually to blindness.

The RGC-5 cell line displays features of RGCs based on the expression of specific markers (Agarwal et al., 2007; Krishnamoorthy et al., 2001), but has been reported to have characteristics significantly different from mature RGCs (Moorhouse et al., 2004). Supporting this former evidence our RGC-5 cell clones (R13, see Section 1) maintain the expression of Thy-1 and do not express the glial cell marker GFAP (Suppl. Fig. 1). Our results are in agreement with most publications up to date in which Thy-1 expression was observed in the RGC-5 cells (Agarwal et al., 2007; Frassetto et al., 2006; Ganapathy et al., 2010; Ju et al., 2009; Krishnamoorthy et al., 2001; Maher and Hanneken, 2008; McKernan et al., 2007). Nevertheless, absence of Thy-1 expression in RGC-5 clones was recently demonstrated (Van Bergen et al., 2009; Wood et al., 2010). In this respect, Van Bergen and colleagues have used RGC-5 clones at a passage number 19 while our clones were from an earlier passage (R13). It is possible that the differences observed in Thy-1 expression could be related to the use of RGC-5 cells from different passages. Nevertheless, further studies will be necessary to clarify this point.

3.1. Morphological differentiation of RGC-5 cultures

Cell differentiation can be described in terms of functional criteria as indexed by variations in the responsiveness to a given extracellular stimulus such as glutamate; or in terms of morphological changes visualized mainly by neuritogenesis (Krishnamoorthy et al., 2001; Schwechter et al., 2007; Van Bergen et al., 2009). In the present paper, we examined the acquisition of mature neuronal features and neurite formation of cultured RGC-5

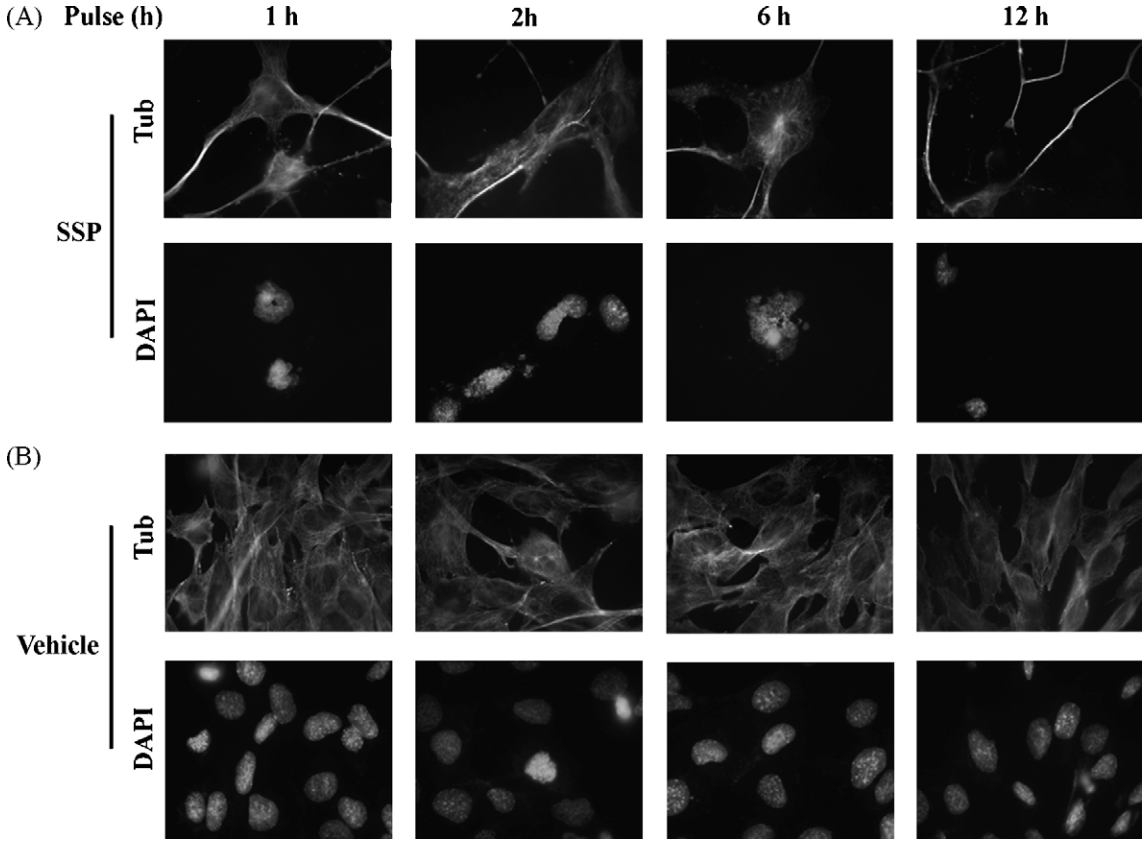

Fig. 4. The effect of pulses of SSP (214 nM) on RGC-5 cells. RGC-5 cultures exposed to SSP pulses of 1, 2, 6 and 12 h at 214 nM (A) or vehicle only (controls) (B). Top panel: photomicrographs of RGC-5 cultures at each time showing immunofluorescence associated to α-Tubulin in whole cell. Bottom panel: photomicrographs illustrating the cell nuclei stained with DAPI.

cells in response to treatment with a variety of neurotrophic factors and other effectors such as BDNF, NGF, vitamin A, retinoic acid, retinaldehyde, SSP and the enriched medium for neuronal growth (B27) (Figs. 1–3). Of all these effectors, only SSP was able to morphologically differentiate RGC-5 cultures even at lower concentrations than those previously reported by other laboratories, in such studies SSP was shown to induce neurite outgrowth and significantly reduces BrdU incorporation (Frassetto et al., 2006; Harvey and Chintala, 2007).

3.2. SSP-induced differentiation and cell death

RGC-5 cells treated with SSP clearly showed neuritogenesis (Figs. 1 and 2); however, cells exhibiting signs of morphological differentiation with a typical neuronal phenotype died after 24 h of treatment as seen by staining with DAPI or PI. The few cells remaining at 72 h post-treatment showed characteristic cell shrinkage (Figs. 2 and 3).

When cell death owing to prolonged SSP treatment was prevented in RGC-5 cultures by exposing cells to an SSP pulse of different durations, cells became apoptotic at the highest effector concentration used (Fig. 4) or did not differ from the vehicle-treated controls at the lowest concentration (53.5 nM) (data not shown). SSP has been characterized as a strong inducer of apoptosis in many different cell types; however, the mechanism(s) by which it induces apoptosis remain(s) controversial. Although most studies report caspase activation under SSP treatment, caspase-independent mechanisms (Belmokhtar et al., 2001; Xue et al., 2003) or both caspase-dependent and -independent apoptotic pathways in selected cell populations (Zhang et al., 2005, 2004) have been proposed. Apoptotic cells were originally identified by the characteristic changes in their nuclei and this is still one of the best methods for confirming cell death. Since they have reduced DNA stainability following staining with different fluorochromes, the presence along the cell cycle of cells with DNA stainability lower than that of G_1-Go cells (hypodiploid or sub-G_1 peaks, A_0 cells), has been considered a marker of cell death by apoptosis (Darzynkiewicz et al., 1992; Nicoletti et al., 1991). Remarkably, the DNA histogram for RGC-5 cells presented in this work clearly indicates the presence of a sub-G_1 peak after SSP treatment (Fig. 3E and F); this sub-diploid DNA peak highlights the toxic effect of SSP on RGC-5 cells. Strikingly, both DAPI and PI staining experiments indicate that SSP has a lethal effect on RGC-5 cells leading to cell death after 24 h in culture. The elucidation of the exact death mechanism (apoptosis or necrosis) triggered by SSP is beyond the scope of this work, however, evidences reported above strongly suggest that SSP treatment causes apoptosis.

In most of the literature citing the use of SSP, morphological differentiation was studied up to 24 h (Frassetto et al., 2006; Harper et al., 2009). However, our observations lead us to infer that the cell-death program has already been initiated by this time, since apoptosis is clearly visualized at 24 h of SSP treatment (Figs. 2–4). It is also very important to take into account the concentration of SSP used to provoke differentiation, since it is known that SSP concentrations in the nmolar range cause neurite outgrowth whereas on the μmolar scale they can cause apoptosis. Our experiments were performed at concentrations on the nmolar scale and at similar SSP concentrations to those used by other laboratories (Frassetto et al., 2006; Harper et al., 2009). Nevertheless, even when we used low SSP concentrations, cells died after 24 h of treatment. In agreement with our observations, other reports have suggested RGC-5 cell death after 24 h of SSP

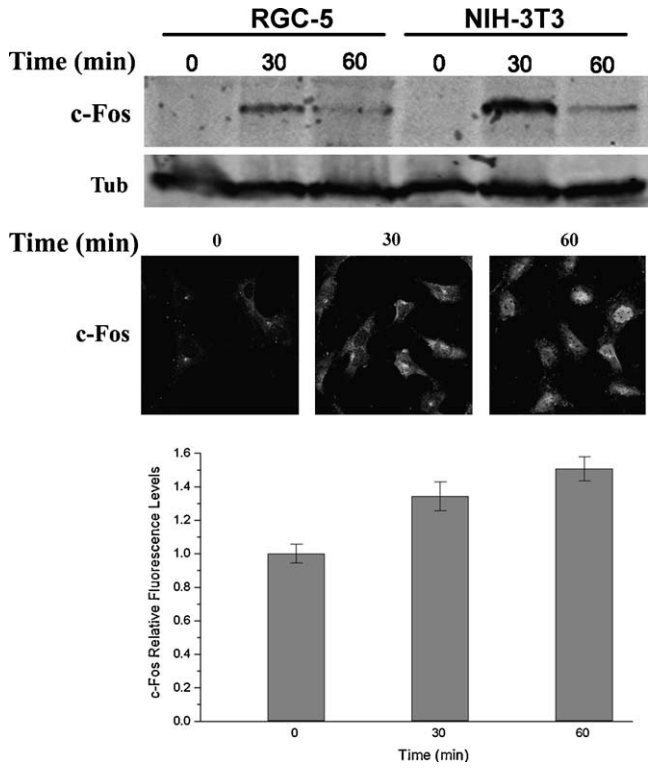

Fig. 5. Induction of c-Fos protein in RGC-5 cells by serum-stimulation. Top panel: c-Fos induction in RGC-5 and NIH 3T3 cells at 0, 30 and 60 min by WB. α-Tubulin (Tub) was used as a housekeeping protein. Center and bottom panel: Immunocytochemistry showing the c-Fos protein positive immunostaining in RGC-5 cells and quantification of relative fluorescence levels at 0, 30 and 60 min after serum stimulation. Data are mean ± SEM (n = 65–125/group). The ANOVA revealed a significant effect of time ($p < 0.001$, F = 17,2865). The pairwise comparisons indicate no differences between levels of serum stimulation at 30 and 60 min, though both were significantly higher than at time 0.

treatment at similar or higher concentrations than those we used (Harvey and Chintala, 2007; Inokuchi et al., 2006; Schallenberg et al., 2009) Our work is the first report to directly demonstrate the lethal effect of SSP on RGC-5 cells regardless the induction of morphological differentiation. Based on our observations, the use of SSP to obtain differentiated healthy RGC-5 cells should be reconsidered.

3.3. Other differentiation effectors

A number of laboratories have attempted to morphologically differentiate RGC-5 cells using effectors such as S-ConA, with varied results (Krishnamoorthy et al., 2001; Van Bergen et al., 2009). We also tested the effect of this compound (25–50 μg/mL) on RGC-5 cells but saw no sign of morphological differentiation (data not shown). Prior work from other laboratories attempted to achieve morphological differentiation of this cell line using neurotrophic factors and other substances such as CTNF, insulin or forskolin, but these compounds alone or in combination did not differentiate RGC-5 cells (Schwechter et al., 2007). In the present work we tested a variety of agents (B27, vitamin A, retinoic acid), neurotrophic factors (NGF, EGF and BDNF), enriched medium (DM, Rotstein et al., 2003), conditioned medium from primary retinal cell cultures and co-cultures of RGC-5 with embryonic retinal cells. In Fig. 1, we showed representative results for these treatments; unfortunately, neither of them exhibited a detectable effect on RGC-5 morphology. Further studies will be required to investigate the effect of other neurotrophic factors or chemical signals (i.e. SSP) on the morphological differentiation of RGC-5 cells in order to better understand the mechanisms operating in these cells; such mechanisms may regulate the balance between the proliferation events, cell differentiation and cell-death programs. Even effectors reported to cause neuritogenesis in RGC-5 cells, such as TSA, a histone deacetylase inhibitor, appear to act through different mechanisms that also cause cell death (Schwechter et al., 2007).

3.4. Differential Ca^{2+} responses to glutamate and ATP stimulation in RGC-5 cells

Harvey and Chintala (2007) recently reported that the synthesis and secretion of the tissue plasminogen activator (tPA) and the urokinase plasminogen activator (uPA) into the SSP-differentiated RGC-5 cell-conditioned medium is concentration- and time-dependent. These proteases, in turn, directly cause the death of

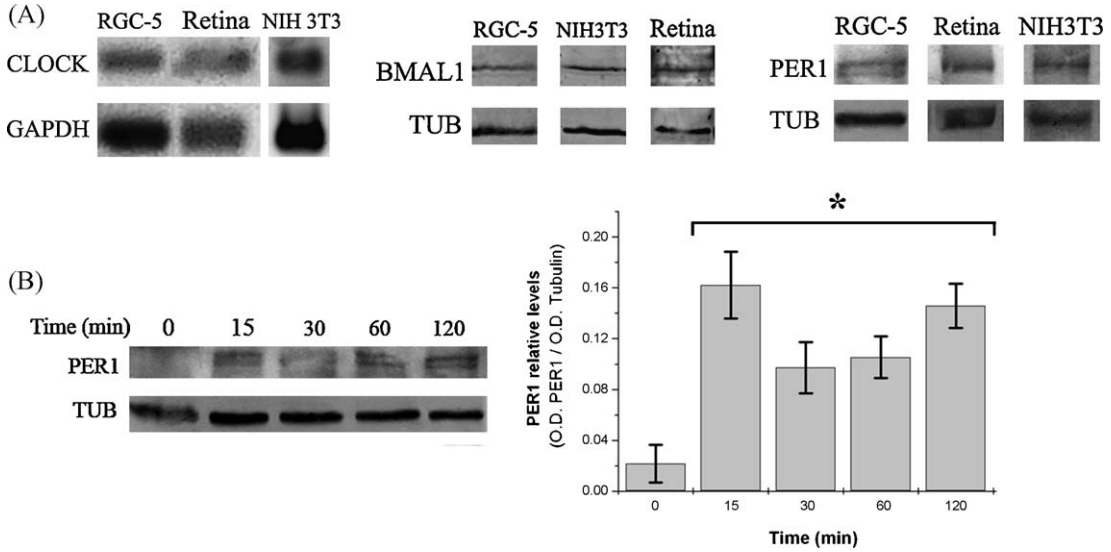

Fig. 6. Expression of Clock Genes in RGC-5 cells. (A) Assessment of *Clock* mRNA by RT-PCR and of BMAL1 and PER1 proteins by WB in positive controls (the whole rat retina and NIH 3T3 cells) and RGC-5 cells after a 2 h-serum shock with 50% FBS. GAPDH mRNA and α-Tubulin (Tub) protein were used as housekeeping genes in the RT-PCR and WB respectively. (B) PER1 induction in RGC-5 cells at 0, 15, 30, 60 and 120 min after serum stimulation and quantification of relative optical density levels at the same times post stimulation. Data are mean ± SEM from 5 independent experiments. The ANOVA revealed a significant effect of time ($p < 0.001$, F = 7.9073); levels at 15 min were significantly higher than those at time 0, $^{*}p < 0.01$.

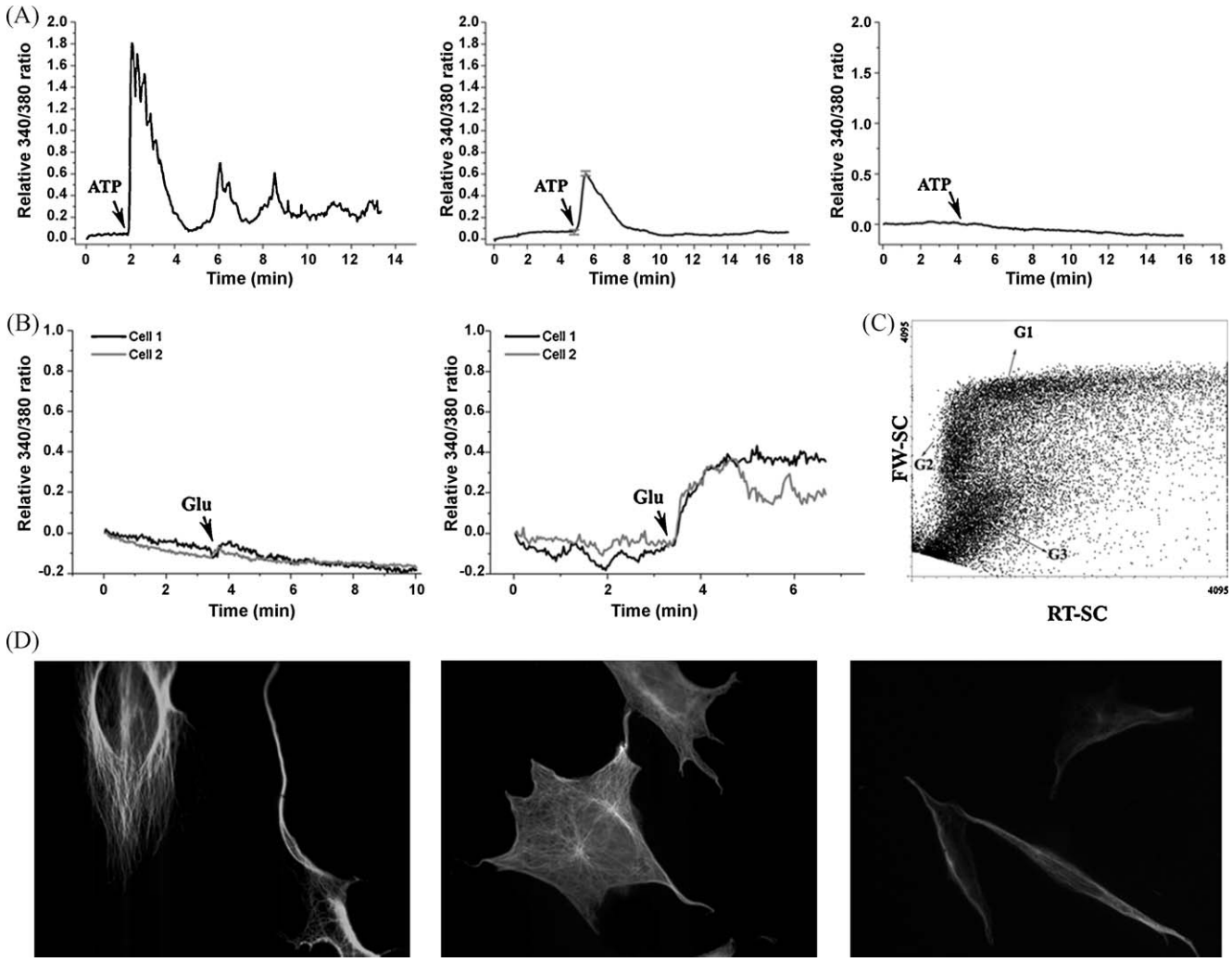

Fig. 7. Responses of RGC-5 cells to ATP (A) or Glutamate (B) by FURA-2AM Ca^{2+} Imaging. (A) Relative 340/380 FURA-2AM ratio for individual cells showing differential Ca^{2+} responses to 100 μM ATP added at the point marked with the arrow. (B) Relative 340/380 FURA-2AM ratio for individual cells showing differential Ca^{2+} responses over time to 100 μM glutamate added at the point marked with the arrow in RGC-5 (left plot) and disaggregated embryonic retinal cells (right plot). (C) Dot plots of RGC-5 cells after 48 h of vehicle treatment (control) illustrating differential cell complexities and showing at least three differential cell group subpopulations (G1, G2 and G3). (D) α-Tubulin immunocytochemistry of RGC-5 cells showing differences in cellular morphology in cell cultures.

RGC-5 cells. Remarkably, the inhibition of plasminogen activators attenuates the death of these differentiated RGC-5 cells and stabilizes their neurite network in vitro. Excess of tPA can provoke cell death by excessive processing of glutamate receptors, by increasing calcium influx or by other effects. Interestingly, undifferentiated RGC-5 cells may not express sufficient functional glutamate receptors for uPA to be able to act and induce Ca^{2+} influx. In this connection, we detected no glutamate responses in Ca^{2+} influx by fura-2 fluorescence in proliferating RGC-5 cells, whereas they did respond to ATP stimulation (Fig. 7A and B).

ATP has been involved in differentiation, cell proliferation, and survival; acting via P2 receptors, it is also a toxic agent implicated in cellular degeneration and death (Fries et al., 2004; Meyer-Franke et al., 1995; Wheeler-Schilling et al., 2001). Activation of the P2X(7) receptors present in most RGCs elevates Ca^{2+} influx and kills rat RGCs, thus having physiological and pathophysiological implications for ganglion cell function (Mitchell et al., 2008). The energy reduction-induced cell death of RGCs has been related to many ophthalmic diseases; glucose deprivation causes an acute decline in the intracellular ATP level, concomitantly decreasing cell viability (Li et al., 2009). Recent reports suggest that extracellular ATP modulates retinal processing and could play a role in the regulation of glial cells during retinal pathophysiology and retinal signaling within subsets of retinal neurons (Das et al., 2006).

3.5. Induction of IEG and clock gene expression in RGC-5 cells by serum stimulation

Another interesting characteristic of RGCs in the retina of different vertebrate species is their ability to respond to light stimulation by inducing the expression of IEG proteins such as c-Fos, the most studied member of the AP1 family of transcription factors (Caputto and Guido, 2000). Light induction of c-Fos in RGCs has been reported even in the absence of functional classical retinal photoreceptors (cones and rods), indicating that the responses observed were directly associated to an endogenous capacity of these neurons (Semo et al., 2003). RGC-5 cells in culture were also able to induce the expression of c-Fos in response to an extracellular stimulus as shown in Fig. 5. Serum stimulation rapidly promoted the expression of c-Fos in RGC-5 cells. In addition, *c-fos* has been involved in both apoptotic cell death and regeneration of damaged RGCs (Oshitari et al., 2002). The results nevertheless suggest that RGC-5 cells retain at least some characteristics of mature RGCs in terms of their intrinsic response

to extracellular and physiological signals that promote significant changes in IEG protein levels.

In the mammalian retina, an autonomous circadian clock regulates the temporal organization of physiology through a mechanism involving the expression of a number of clock genes that generates molecular loops of transcription and translation of clock proteins to determine daily rhythms (Dunlap et al., 2004). Molecular loops are conformed by positive and negative elements that regulate the expression of clock genes and clock-controlled genes by activating and repressing transcription along the 24 h period (Dunlap et al., 2004; Hastings et al., 2008; Takahashi et al., 2008). CLOCK and BMAL1 have been shown to act as positive elements while PER and CRY act as a negative elements inhibiting its own synthesis once they are translated. PER1 has also been involved in the clock entrainment mechanisms, thus it could mediate the shifting effects of external signals that synchronize the activity rhythms (Balsalobre et al., 1998; Dunlap et al., 2004; Mateju et al., 2009).

Expression of complete sets of key clock proteins is mainly restricted to individual inner retinal cells including RGCs (Tosini et al., 2007, 2008). In addition, our laboratory have previously demonstrated that RGCs cells contain autonomous circadian oscillators synthesising phospholipids and melatonin in a rhythmic manner (Garbarino-Pico et al., 2004; Contin et al., 2006). Based on these observations, we investigated the expression of clock genes in RGC-5 cells. As shown in Fig. 6, RGC-5 cells displayed the expression of Clock, BMAL1 and PER1 which are the most commonly studied key elements in the core of the molecular circadian clock, likely acting as regulatory factors responsible for the 24 h oscillation of diverse cellular functions. In addition, PER1 expression was rapidly induced in these cells in response to serum stimulation. The presence of these clock genes in RGC-5 cells resembles observations reported in the rodent retinas which exhibit clock gene expression mainly restricted to RGCs and inner retinal cells (Tosini et al., 2007, 2008).

We were able to identify different cell subpopulations in our RGC-5 cultures based on differential ATP responses, morphological immunostaining by ICC and diverse cell morphology, size and cell complexity by flow cytometry.

To summarize, the present work is the first report to directly demonstrate the toxic effect of SSP on RGC-5 cells despite the morphological changes observed and previously reported by other groups. Our overall findings lead us to infer that RGC-5 cells display distinct differentiation abilities and/or effector sensitivities for triggering morphological changes, neuritogenesis and/or of cell-death programs. Nevertheless, proliferating RGC-5 cells exhibit differential responses to physiological stimuli (ATP, glutamate and serum) in the mobilization of intracellular Ca^{2+} as well as in the induction of the IEG protein c-Fos and the clock genes (*Clock*, *Bmal1* and *Per1*) which resembles RGC features.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuint.2010.05.013.

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