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Staurosporine induces ganglion cell differentiation in part by stimulating urokinase-type plasminogen activator expression and activation in the developing chick retina

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ABSTRACT

Here, we investigated whether staurosporine-mediated urokinase-type plasminogen activator (uPA) activation is involved in retinal ganglion cell (RGC) differentiation. Retinal cells were isolated from developing chick retinas at embryonic day 6 (E6). Relatively few control cells grown in serum-free medium started to form processes by 12 h. In contrast, staurosporine-treated cells had processes within 3 h, and processes were evident at 8 h. Immunofluorescence staining showed that Tuj-1-positive cells with shorter neurites could be detected in control cultures at 18 h, whereas numerous Tuj-1 positive ganglion cells with longer neuritic extensions were seen in staurosporine-treated cultures. BrdU-positive proliferating cells were more numerous in control cultures than in staurosporine-treated cultures, and the BrdU staining was not detected in post-mitotic Tuj-1 positive ganglion cells. Western blotting of cell lysates showed that staurosporine induced high levels of the active form of uPA. The staurosporine-induced uPA signal was localized predominantly in the soma, neurites and axons of Tuj-1-positive ganglion cells. Amiloride, an inhibitor of uPA, markedly reduced staurosporine-induced Tuj-1 staining, neurite length, neurite number, and uPA staining versus controls. In developing retinas in ovo, amiloride administration remarkably reduced the staurosporine-induced uPA staining and RGC differentiation. Taken together, our in vitro and in vivo data collectively indicate that uPA plays a role in the staurosporine-mediated stimulation of RGC differentiation.

1. Introduction

Retinal development is a complex process wherein a single type of neuroblast migrates and differentiates into all seven retinal cell types, which are then organized into three morphologically distinct layers in the mature retina [1]. Early in retinal development, the neuroblasts of the inner layer of the optic cup initially proliferate and expand in number, but then later limit their proliferation. The distinct processes of cell division and differentiation must be coordinated so differentiation is initiated when the cells stop dividing [2]. The signaling that induces cells to exit the cell cycle and differentiate in the vertebrate neural retina involves extensive cross-talk among numerous regulators, including neurotrophins, transcription factors, growth factors, hormones, and signaling molecules [3]. In retinas from chick embryos between E3 and E8 Tuj-1 positive cells were located exclusively in the post-mitotic ganglion cells, the major neuronal cell type generating at these ages [4–6].

Plasminogen activators (PAs) are serine proteases that are synthesized and secreted by many different cell types to convert inactive plasminogen to active plasmin. PAs activate a cascade of proteolytic activities that allow the PA-producing cells to degrade specific components of the extracellular matrix (ECM) [7]. uPA is involved in controlling cell migration, tissue remodeling, and the movement of cell processes [8]. The binding of uPA to its cell surface receptor (uPAR) activates a variety of other proteases. This may initiate the downstream activation of several matrix metalloproteinases [9–11] leading to the digestion of various components of the ECM and the activation of certain growth factors [12]. In addition to its pericellular proteolytic role, uPAR interacts with integrins and vitronectin to regulate cell adhesion and migration [13–15].

Staurosporine is an alkaloid isolated from the culture broth of *Streptomyces staurosporesa*. One of its major effects is to inhibit protein kinase C (PKC) activity, but it can also affect other kinases involved in intracellular signaling [16]. It can mimic the effects of nerve growth factor (NGF) in promoting neurite outgrowth [17],

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and induce the development of axon-like neuritis [18]. In addition, staurosporine reportedly has a variety of effects on neuronal cell lines [17,19,20]. Although the RGC-5 cell line is widely used in RGC research, recent data have showed that RGC-5 cells express any specific RGC marker mRNA and proteins in control and staurosporine treated cells [21], but RGC-5 cells express photoreceptor-specific markers. Staurosporine increases the number of photoreceptor cells in primary cultured early developing chick retinal cells [22], but there are no reports indicating the involvement of staurosporine on RGC differentiation. We herein decided to focus the in vitro analysis on the mechanisms of RGC differentiation in staurosporine-treated cultures. Furthermore, we examined the effects of staurosporine on the early developing retina in ovo.

2. Materials and methods

2.1. Retinal cell preparation and cell culture

At E6, developing chick retinas were carefully dissected, and dissociated retinal cell cultures were prepared as previously described [23]. Briefly, neural retinas were incubated in 0.1% trypsin in Ca^{2+}- and Mg^{2+}-free HBSS for 15 min at 37 °C, and then gently triturated for 5 min to yield a single-cell suspension. The retinal cells (3.5×10^6 cells per plate) were plated on 60-mm culture dishes coated with 1 mg/ml of poly-DL -ornithine (Sigma). The cells were cultured in F-12 medium supplemented with 10% FBS, 1% penicillin and streptomycin, 1% chick serum (Sigma), and ITS (5 mg/ml insulin, 30 nM selenium, 25 mg/ml human transferrin; Sigma). After 3 h of culture, the culture dishes were washed twice with serum-free F-12 medium. Thereafter, the cells were cultured either in the presence or absence of 50 nM staurosporine (Calbiochem, San Diego, CA) and 200 μM amiloride (Sigma, St. Louis, MO). To exclude the possibility that staurosporine inducing cell death, retinal cells were cultured in serum-free F-12 medium for 18 h, but staurosporine does not affect cell viability up to cultures for 36 h.

2.2. Preparation of conditioned media

Secreted proteins were precipitated from equal-volume aliquots of supernatant, using 10% ice-cold trichloroacetic acid. The precipitates were washed twice with 100% acetone, air dried, dissolved in RIPA buffer, and stored at −20 °C until use.

2.3. Western blot analysis

Equal volumes of conditioned media or equal amounts of protein lysate (30 μg) in RIPA buffer were separated by 8% SDS–PAGE under reducing conditions. After transfer, the nitrocellulose membranes were incubated for 1 h with primary antibodies against Tuj-1 (R&D systems, Minneapolis, MN), uPA (Technoclone, Vienna, Austria), or β-actin (Santa Cruz, CA). The results were visualized using horseradish peroxidase (HRP)-conjugated second antibodies, along with an enhanced chemiluminescence kit.

2.4. Administration of drugs onto developing embryos in ovo

The egg shell was cracked at the blunt pole, the air sac was punctured with a yellow pipette tip, and drugs were administered onto the chorioallantoic membrane around the developing embryo at E6. Embryos received 100 μl of 500 nM staurosporine (0.1% DMSO/PBS), 100 μl of 2 mM amiloride, or both. Control embryos received the equivalent volume of diluted 0.1% DMSO in PBS. The puncture holes were sealed with tape, and the treated eggs were returned to the incubator. After two days, the embryos were dissected and paraffin embedded.

2.5. Immunohistochemistry

Immunohistology was performed as described before [24]. Paraffin sections (3-μm) were incubated with primary antibodies against Tuj-1, and BrdU (Cell signaling Technology, Danvers, MA) at RT for 1 h. The corresponding IgGs coupled to Alexa-Fluor 488 or Alexa-Fluor 555 was used as secondary antibodies. Nuclei were counterstained with DAPI (Invitrogen, Carlsbad, CA). Digital images were captured under a Zeiss fluorescence microscope (Carl Zeiss, Germany).

2.6. Immunofluorescence staining

Briefly, equal numbers of retinal cells were cultured for 18 h in the presence or absence of 50 nM staurosporine and/or 200 μM amiloride in serum-free F-12 medium. Staining was performed as described before [24]. Cells were incubated with primary antibodies against Tuj-1, uPA, and BrdU at RT for 1 h. The corresponding IgGs coupled to Alexa-Fluor 488 or Alexa-Fluor 555 was used as secondary antibodies. Nuclei were counterstained with DAPI, and digital images were captured by confocal microscopy.

2.7. BrdU staining

Equal numbers of cells were exposed to the indicated drugs for 12 h, treated with BrdU (25 μg/ml) for 6 h, and harvested. The cells were then washed in PBS, fixed in 4% PFA in PBS for 10 min, washed in PBS, incubated in 2 N HCl in PBS for 30 min. and then subjected to immunostaining.

2.8. Statistical analysis

Data are expressed as the mean ± S.E.M from three independent experiments. The statistical analyses were performed using one-way ANOVA, followed by the student's *t*-test. $P < 0.05$ was taken as indicating statistical significance.

3. Results

3.1. Effects of staurosporine on morphological changes, cell cycle parameters, and RGC differentiation

The morphologies of cultured chick retinal cells were observed at 3, 8, and 12 h under phase-contrast microscopy (Fig. 1A). Control retinal cells were rounded at 3 and 8 h, and relatively few cells started to form processes by 12 h. In contrast, staurosporine (50 nM) treatment induced the cells to extend processes within 3 h, and many of these cells had evident processes at 8 and 12 h. We then examined whether RGC differentiation was influenced by staurosporine treatment by immunofluorescence analysis. Although some Tuj-1-positive cells (20%) with shorter neurites were detected in control cultures for 18 h, Tuj-1-positive ganglion cells (55%) with longer neuritic extensions were seen in the staurosporine-treated cultures (Fig. 1B). We next examined proliferating cells by BrdU staining. As expected, the post-mitotic Tuj-1 positive ganglion cells at 18 h of culture did not label with BrdU (purple color). Furthermore, there were more BrdU-positive cells in control cultures than in the staurosporine-treated cultures (Fig. 1C).

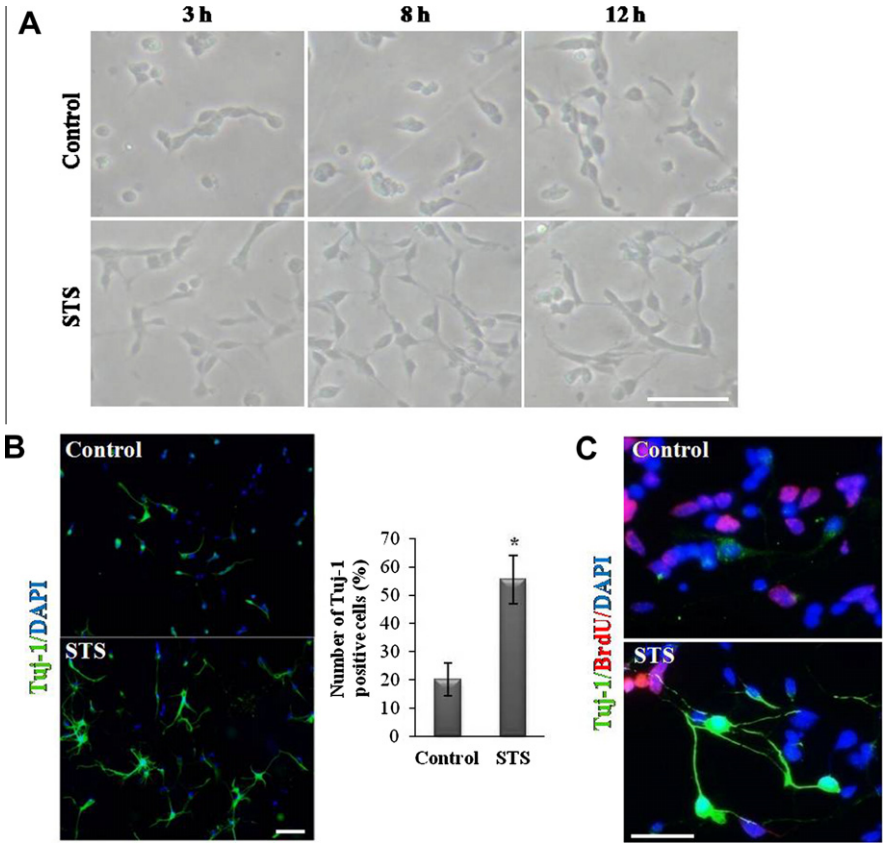

Fig. 1. Staurosporine affects cell cycle arrest, stops cell proliferation, and induces neurite extension and RGC differentiation. (A) The morphological features of cultured cells were observed by phase-contrast microscopy. Scale bar: 10 μm. (B) Immunofluorescence staining for Tuj-1-positive ganglion cells after culture for 18 h. Quantification of Tuj-1 positive cell number was performed using the Image-j program. $^{*}P < 0.01$ versus corresponding value for control. Scale bar: 50 μm. (C) Immunofluorescence staining of BrdU-positive proliferating cells (purple) after culture for 18 h. Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Expression patterns of uPA and the inhibitory effect of amiloride on staurosporine induced RGC differentiation in vitro

No prior study has assessed uPA in relation to retinal development. In this study, we examined the expression patterns and roles of uPA in RGC differentiation during retinal development. Western blot analysis (Fig. 2A) showed that high levels of the secreted form of uPA (50-kDa) could be detected in control and staurosporine-treated cultures throughout the culture period, but co-treatment of amiloride (200 μM) plus staurosporine (50 nM) decreased the levels of secreted uPA seen at 12 and 18 h. Once secreted, uPA remains in a biologically inactive until it binds to uPAR at the cell surface [25]. Therefore, we examined the level of active uPA during the differentiation of cultured cells. At 12 and 18 h post-plating, we detected higher level of active uPA (100-kDa; presumably bound to uPAR) in staurosporine-treated cultures versus controls. We further found that this level was markedly reduced by amiloride co-treatment. We then used immunofluorescence staining to determine whether staurosporine-induced uPA activation was linked to RGC differentiation. As expected, the number of strongly Tuj-1-positive cells with longer neuritic extensions increased upon RGC differentiation in staurosporine-treated cultures for 18 h (Fig. 2B). In contrast, co-treatment with amiloride plus staurosporine remarkably reduced the number of Tuj-1-positive cells. Furthermore, uPA (red) proteins were localized predominantly in the soma of Tuj-1-positive ganglion cells (green) in cultures treated with staurosporine. The uPA signal was also detected on the neurites and axons (white arrows) of the Tuj-1-positive cells. Although some individual axons expressed uPA alone, the majority of the large-diameter axons were double-positive. We also examined the expression levels of Tuj-1 by Western blot analysis, and found that staurosporine-induced Tuj-1 expression was dramatically decreased by co-treatment with amiloride for 18 h (Fig. 2C). These findings collectively suggest that staurosporine-induced uPA activation may play a critical role in RGC differentiation in vitro.

3.3. Staurosporine-induced RGC differentiation in the developing retina is blocked by inhibition of uPA in ovo

To examine the effect of uPA inhibition on RGC differentiation in the developing retina in ovo, E6 embryos were treated with amiloride (2 mM) with or without staurosporine (500 nM). After an additional two days of incubation, immunofluorescence was used to analyze Tuj-1 (green) and uPA (red) staining in the developing retina (Fig. 3). In control retinas, both uPA and Tuj-1 were mainly detected in the GCL. In retinas treated with amiloride alone, these signals were diminished, while Tuj-1 and uPA were induced and co-localized (yellow arrows) in the GCL of retinas treated with staurosporine alone. Notably the staurosporine-induced enhancement of uPA and Tuj-1 staining was dramatically diminished in their definitive layer by amiloride co-treatment. Taken together, the results of our in vitro and in vivo studies suggest that staurosporine-induced uPA activation may stimulate RGC differentiation.

4. Discussion

uPA converts inactive plasminogen to its active serine protease, plasmin, which has a broad range of activity. The plasminogen

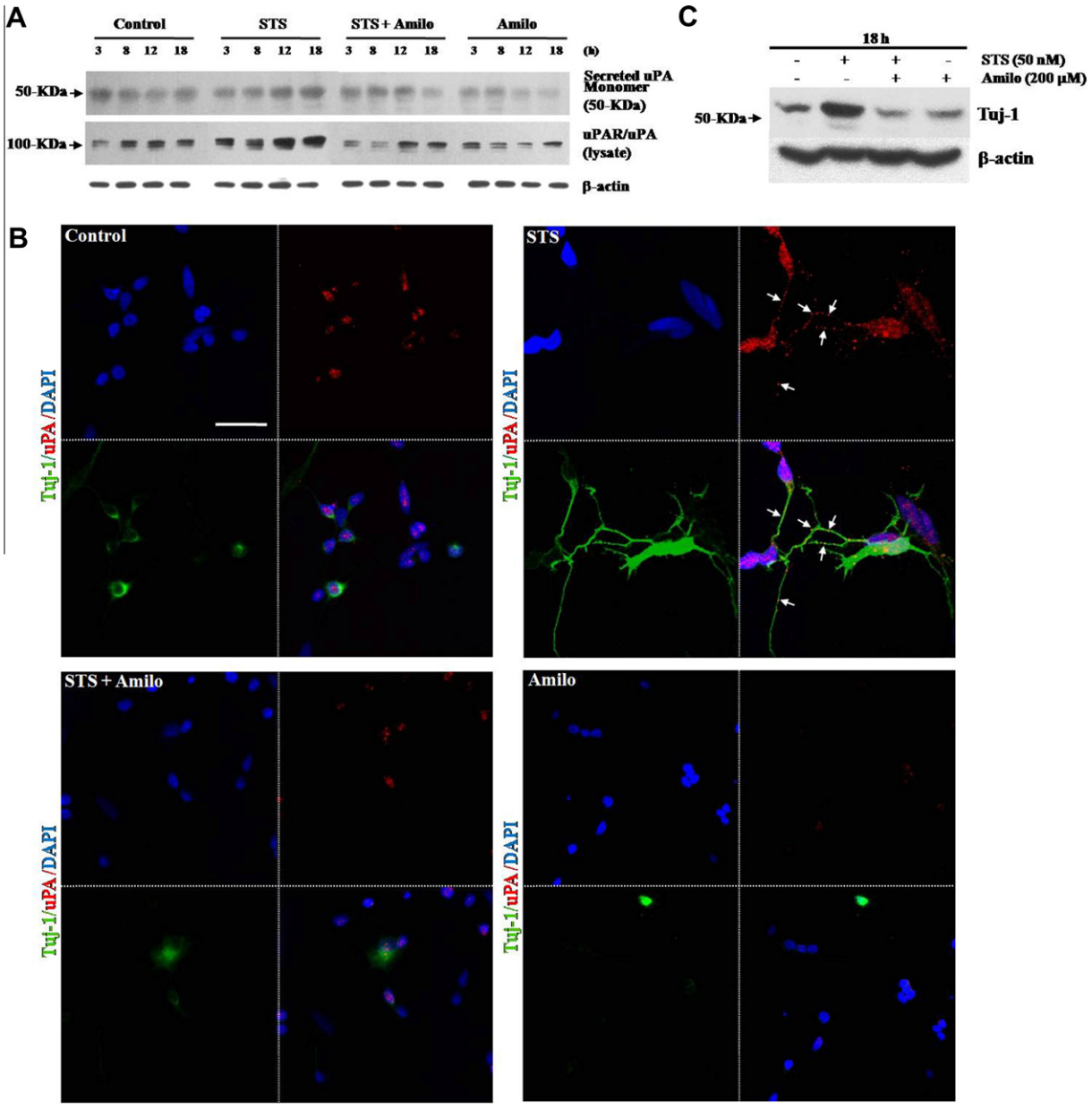

Fig. 2. Staurosporine-induced RGC differentiation is blocked by uPA inhibition. (A) Cells were cultured in serum-free media with or without 50 nM of STS and/or 200 μM of amiloride (Amilo). Equal amounts of cell lysates and conditioned media were assessed for uPA expression using Western blot analysis. (B) Immunofluorescence staining of uPA proteins (red) and Tuj-1-positive ganglion cells (green) in cultured cells for 18 h. Note that uPA signal was present on the axons (white arrows) of the Tuj-1-positive cells in staurosporine treated cultures. Inhibition of both uPA- and Tuj-1-positive signals by the inhibition of uPA. Images are representative of at least three different experiments. Scale bar: 20 μm. (C) Effect of staurosporine and Amilo on the expression of Tuj-1 by Western blot analysis. β-actin was used as the loading control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activators are secreted proteases that bind the cell surface and localize proteolytic activity to cell–cell and cell–substratum sites [26,27]. The binding of uPA to uPAR enhances uPA activity [27]. PA-mediated plasminogen activation induces RGC loss in adult mouse retina [28–32]. In the developing mouse retina, tissue-type PA (tPA) is localized in the neural retina, and it is down-regulated once the photoreceptors have differentiated [33]. uPA has been suggested to be involved in axonal growth and the tissue remodeling associated with neural development [8,26,34]. However, no previous study has examined the involvement of uPA in retinal development. Considering the multifunctional nature of uPA/uPAR and their possible role in retinal development, our findings (Figs. 2 and 3) indicate that staurosporine-induced uPA activity plays a significant role in retinal development and affects RGC differentiation both in vitro and in ovo.

Active uPA cleaves cell-surface plasmin, which can activate downstream extracellular proteases and latent growth factors to support directional remodeling of the local extracellular environment [35]. A large amount of the secreted, biologically inactive form of uPA was detected in the conditioned media of control and staurosporine-treated cultures throughout culture periods. However, co-treatment of amiloride with staurosporine decreased the level of uPA secretion by 12 and 18 h. (Fig. 2A). Neurons release both serine proteases and metalloproteinases from distal processes and growth cones [26]. Released uPA is bound to cell-surface receptors at focal adhesion sites [26], where it is thought to degrade adhesive contacts to promote movement of the leading edge of the cell [26]. The results presented in Fig. 1 are consistent with the notion that uPA plays a role in changing the cell shape during

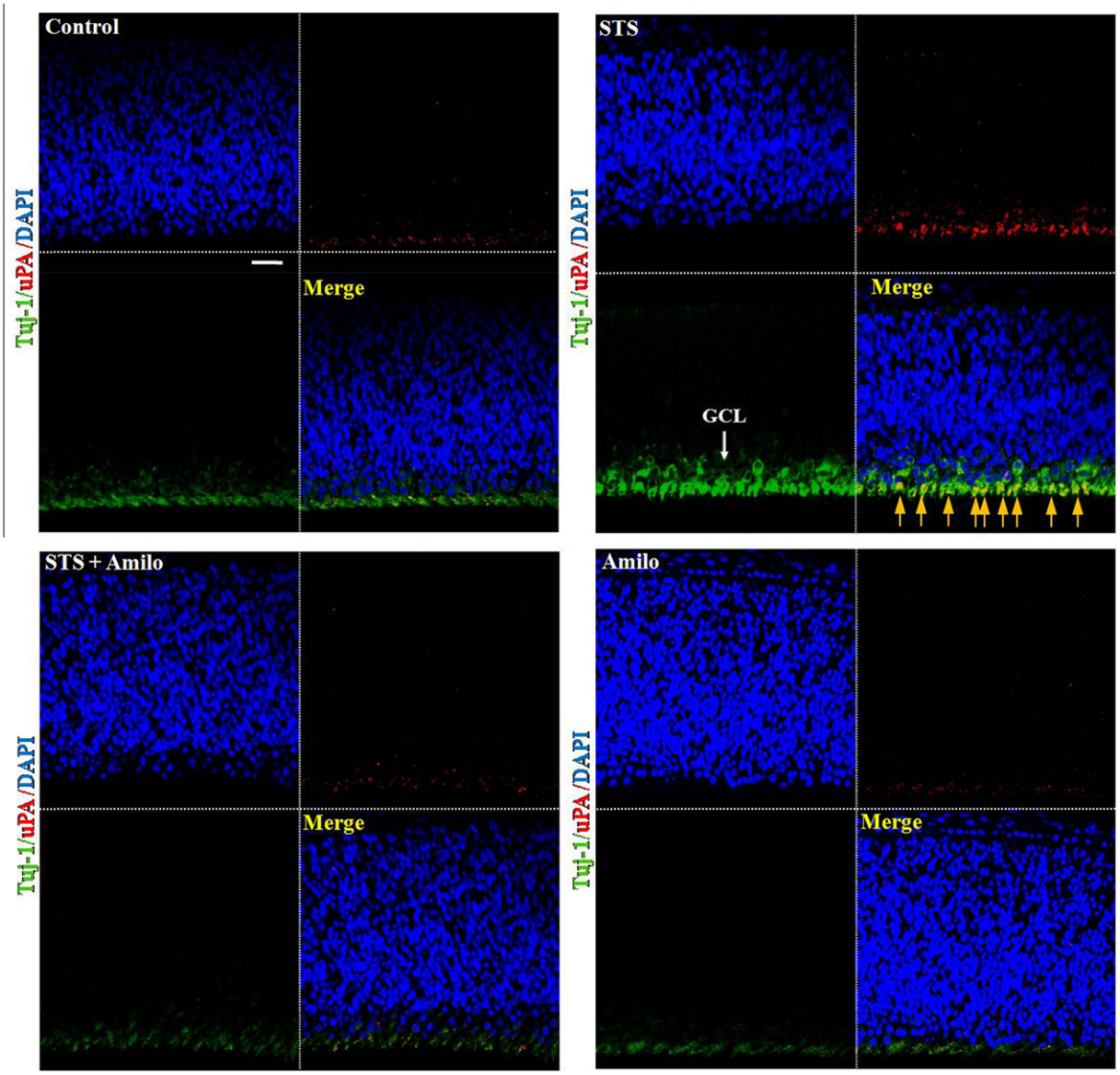

Fig. 3. Staurosporine-induced enhancement of ganglion cell numbers is blocked by uPA inhibition in the developing retina. E6 embryos were treated with or without STS (500 nM) and/or Amilo (2 mM) and incubated for two more days, and retinal sections were subjected to immunofluorescence staining. The uPA (red) signal was weaker than the Tuj-1 (green) signal in the GCL of control retinas. In contrast, strong both Tuj-1 (green) and uPA (red) signals were clearly co-localized (yellow arrows) in the expanded GCL of STS-treated retinas. Note that STS-induced both uPA and Tuj-1 signals were dramatically reduced by Amilo treatment. Images are representative of at least three different experiments. Scale bar: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mitosis and motility [7,36]. Meanwhile, uPAR plays key roles in cell adhesion, proliferation, differentiation, migration, invasion, tissue repair, and tissue remodeling [37]. Importantly, a significant amount of a high molecular weight form of uPA (presumably the active form of uPA bound to uPAR at the cell surface) was detected in staurosporine-treated cell lysates (Fig. 2A). Most neuronal uPA appears to be released as soon as it is synthesized, and very little is associated with neurons either intracellularly or on the cell surface [26]. Here, although weak cytoplasmic uPA signals were seen in control cultured cells, staurosporine treatment was associated with significant uPA immunoreactivity in the cytoplasm of large ganglion cells (Fig. 2B). Furthermore, the presumably active form of uPA was highly detected on neurites and the large-diameter axons of ganglion cells (Fig. 2B). Amiloride, which is a competitive inhibitor of uPA [28–32] strongly inhibited staurosporine-induced uPA expression and activation (Fig. 2), as well as staurosporine-induced RGC differentiation (Figs. 2 and 3). These observations are consistent with the notion that uPAR and uPA contribute to neurite outgrowth, neuronal migration, and the growth of axons and dendrites [8,26,34]. Here, we show that staurosporine activates uPA and significantly promotes RGC differentiation both in vitro and in ovo.

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