Heliyon 9 (2023) e15656

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Neurite outgrowth induced by stimulation of angiotensin II AT_2 receptors in SH-SY5Y neuroblastoma cells involves c-Src activation

Helga M. Blanco [a], Celia N. Perez [a,b], Claudia Banchio [c], Sergio E. Alvarez [a,b,*], Gladys M. Ciuffo [a,b,**]

[a] Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis (UNSL), Ejército de los Andes 950, 5700 San Luis, Argentina
[b] Instituto Multidisciplinario de Investigaciones Biológicas (IMIBIO-SL), CONICET, Argentina
[c] Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET) Ocampo y Esmeralda, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (UNR), 2000 Rosario, Argentina

ARTICLE INFO

Keywords:
Ang II AT_2 receptors
Neurite outgrowth
c-Src phosphorylation/ dephosphorylation
Neural growth factor

ABSTRACT

Neuroblastoma, the most common extracranial solid tumor occurring in childhood, originates from the aberrant proliferation of neural crest cells. Accordingly, the mechanism underling neuronal differentiation could provide new strategies for neuroblastoma treatment. It is well known that neurite outgrowth could be induced by Angiotensin II (Ang II) AT_2 receptors; however, the signaling mechanism and its possible interaction with NGF (neural growth factor) receptors remain unclear. Here, we show that Ang II and CGP42112A (AT_2 receptor agonist) promote neuronal differentiation by inducing neurite outgrowth and βIII-tubulin expression in SH-SY5Y neuroblastoma cells. In addition, we demonstrate that treatment with PD123319 (AT_2 receptor antagonist) reverts Ang II or CGP42112A-induced differentiation. By using specific pharmacological inhibitors we established that neurite outgrowth induced by CGP42112A requires the activation of MEK (mitogen-activated protein kinase kinase), SphK (sphingosine kinase) and c-Src but not PI3K (phosphatidylinositol 3-kinase). Certainly, CGP42112A stimulated a rapid and transient (30 s, 1 min) phosphorylation of c-Src at residue Y^{416} (indicative of activation), following by a Src deactivation as indicated by phosphorylation of Y^{527}. Moreover, inhibition of the NGF receptor tyrosine kinase A (TrkA) reduced neurite outgrowth induced by Ang II and CGP42112A. In summary, we demonstrated that AT_2 receptor-stimulated neurite outgrowth in SH-SY5Y cells involves the induction of MEK, SphK and c-Src and suggests a possible transactivation of TrkA. In that regard, AT_2 signaling pathway is a key player in neuronal differentiation and might be a potential target for therapeutic treatments.

* Corresponding author. Instituto Multidisciplinario de Investigaciones Biológicas (IMIBIO-SL CONICET), Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Ejército de los Andes 950, 5700 San Luis, Argentina.
** Corresponding author. Instituto Multidisciplinario de Investigaciones Biológicas (IMIBIO-SL CONICET), Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Ejército de los Andes 950, 5700 San Luis, Argentina.
E-mail addresses: helgamyrna@gmail.com (H.M. Blanco), celiaperez.mixta@gmail.com (C.N. Perez), banchio@ibr-conicet.gov.ar (C. Banchio), sealvarez98@gmail.com, sealvar@email.unsl.edu.ar (S.E. Alvarez), gciuffo@gmail.com (G.M. Ciuffo).

https://doi.org/10.1016/j.heliyon.2023.e15656
Received 19 January 2022; Received in revised form 31 March 2023; Accepted 17 April 2023
Available online 21 April 2023

1. Introduction

The regulation of neuronal differentiation is a critical step in nervous system development and regeneration. The formation of new synapses and development of neural plasticity are modulated by neurotrophins, such as neural growth factor (NGF) and brain derived neurotrophic factor (BDNF), which act trough membrane receptors, including tyrosine kinase A (TrkA) receptor [1]. Although promotion of differentiation has been proposed as a potential therapy in the treatment of children suffering from neuroblastoma [2], a better understanding of the mechanisms that regulate neurite outgrowth and differentiation will provide further evidences to support new clinical trials.

Neuroblastoma cells are neural crest derived cells capable of unlimited proliferation *in vitro*, and retain the ability to differentiate into neuronal cell types [3]. In that regard, Neuro 2A and SH-SY5Y are mouse and human neuroblastoma cell lines widely used to explore the mechanisms involved in neuronal differentiation, neurite outgrowth and used as models of Parkinson and Alzheimer's disease [4–7].

Angiotensin II (Ang II), the major active peptide of the renin-angiotensin-system (RAS), acts via two membrane receptors classified as subtypes AT_1 and AT_2. Both belong to the seven transmembrane G protein-coupled receptor's family (GPCR). Interestingly, components of the RAS are present in SH-SY5Y neuroblastoma cells [8] and local production of Ang II might modify the responses to other stimuli, such as neurotrophins, estrogen or retinoic acid (RA) [9]. Indeed, increasing evidence suggests that Ang II plays an important role in mediating neurodegenerative diseases via a brain-specific RAS [10,11], but the mechanisms have not been fully elucidated.

We have previously demonstrated a prevalence of AT_2 receptors in brain developing tissues [12–14]. The timing of AT_2 receptor expression in coincidence with the physiological processes of differentiation and synaptogenesis suggests it may be involved in neural development. Certainly, neurite outgrowth induced by AT_2 receptors has been reported in different cell lines [15–18] and in primary cultured cells [19–21]. Although the signaling pathways that link AT_2 receptor activation with neurite outgrowth have been studied, the results are still controversial. While Chao et al. [22] described an important role of extracellular-regulated kinases (ERK) in AT_2 receptor-induction of cell proliferation in cultured neural stem cells from rat hippocampus; Guimond et al. showed that AT_2 receptor-induced neurite outgrowth of neuroblastoma cells involves the activation of Fyn, a member of the c-Src family, but not ERK pathway [17]. Remarkably, we have previously described the involvement of c-Src, Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP1) and Focal Adhesion Kinase (FAK) in the signaling pathway of Ang II AT_2 receptor in membrane preparations from different tissues [23,24]. On the contrary, it has also been suggested that NGF-mediated neurite outgrowth was suppressed by AT_2 receptors in SH-SY5Y cells [19]. The role of AT_2 receptors in differentiation has also been reported in NG108-15 cells [25].

Neural differentiation and protection induced by AT_2 receptors also involves the activation of NGF [26]. Certainly, Ang II stimulates TrkA phosphorylation [17,27]. Conversely, the effect of NGF on neurite outgrowth of cultured dorsal root ganglia (DRG) cells was inhibited by PD123319 [19]. Moreover, it has also been shown that NGF-induced neurite extension requires the activation of sphingosine kinase 1 (SphK1), the enzyme that produce the bioactive lipid sphingosine-1-phosphate (S1P) [28,29]. Concomitantly, down-regulation of S1P receptor 1 (S1PR1) also reduces neurite extension [29].

Here, we show that stimulation of Ang II AT_2 receptors induce neurite outgrowth in SH-SY5Y neuroblastoma cells through a signaling mechanisms that involves MEK/ERK, SphK and c-Src and provide evidence for a possible involvement of TrkA receptors in the pathway.

2. Materials and methods

2.1. Materials

Reagents were obtained from commercial suppliers: Bovine serum albumin (BSA) fraction V, Triton X-100, sodium orthovanadate, Ang II, CGP42112A (Nα-Nicotinoyl-Tyr-(Nα-Cbz-Arg)-Lys-His-Pro-Ile), retinoic acid (RA), PD123319 (1-(4-dimethylamino)-3-methylphenyl)-methyl-5-diphenylacetyl-4,5,6,7-tetrahydro-1-himidazo [4,5c]pyridine-6-carboxylic acid) ditrifluoroacetate, UO126 (MEK inhibitor), Tyrphostin AG879 (TrkA inhibitor), LY294002 (PI3K inhibitor) were from Sigma Chemical Co. (St. Louis, MO). PP2 (c-Src inhibitor), PP3 (inactive analog) and NGF (7S NGF) were from Calbiochem (La Jolla, CA, USA). SKI-II (SphK inhibitor) was from Cayman Chemical (Ann Arbor, MI, USA). Nitrocellulose membranes were from BioRad (CA, USA). All other reagents were from the highest available quality.

The following antibodies were used: rabbit anti βIII tubulin (Cat. #AB15708, Millipore. MA, USA); rabbit β tubulin (H235) (Cat. #sc-9104, Santa Cruz Biotechnology, CA, USA); goat anti AT_2 (Cat. #sc7421, Santa Cruz Biotechnology CA, USA); mouse anti β actin (Cat #3700, Cell Signaling, Danvers, MA, USA); goat anti rabbit coupled to horseradish peroxidase (HRP) (Cat. #115035062, Jackson Inmuno Research, West Grobe, PA, USA) and Src antibody sampler kit (Cat. #9935, Cell Signaling Technology, Danvers, MA, USA).

MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma Aldrich) viability reagent was prepared freshly in PBS as a 5 mg/ml stock solution. Solution was subjected to five cycles of 15′ vortexing and 2′ sonication and centrifuged at 12.000×*g*, 4 °C for 10'. The supernatant was diluted 1:10 in DMEM serum free and stored at 4 °C as described previously [30].

2.2. Cell culture

Mouse neuroblastoma Neuro 2A (ATCC CCL131) and human neuroblastoma SH-SY5Y cells (kindly provided by Dr. S. Quiroga, University of Córdoba, Argentina) were cultured in Dulbecco's modified Eagle's essential medium (DMEM) (Gibco-BRL, San Francisco,

CA) containing high glucose, L-glutamine, and sodium pyruvate supplemented with 10% heat inactivated fetal bovine serum, (FBS, Natocor, Cordoba-Argentina), penicillin G (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Cell differentiation and morphometric analysis

To study cell differentiation, SH-SY5Y cells were cultured in 6 (4×10^4 cells) or 12 (2×10^4 cells) well plates for 24 h in DMEM 10% FBS (proliferation media). After that, the medium was changed to DMEM 2% FBS (differentiation media) and supplemented with the following agonists: Ang II (0.1 μM), retinoic acid (RA, 10 μM), NGF (100 ng/ml) or the specific AT_2 receptor agonist CGP42112A, (0.01 μM) for 3 days. Cells grown in differentiation media were used as a control of the effect induced by the agonists. As a basal control of differentiation, cells were cultured in proliferation media. The percentage of neurite outgrowth in proliferating cells was 2.5 ± 1.5 (n = 2, data not shown).

When indicated, the following inhibitors or competitors were added 40 min prior to agonist stimulation: PD123319 (AT_2 receptor antagonist, 10 μM), PP2 or its inactive analog PP3 (c-Src inhibitor, 2.5 μM), Tyrphostin AG879 (TrkA receptor inhibitor, 0.6 μM),

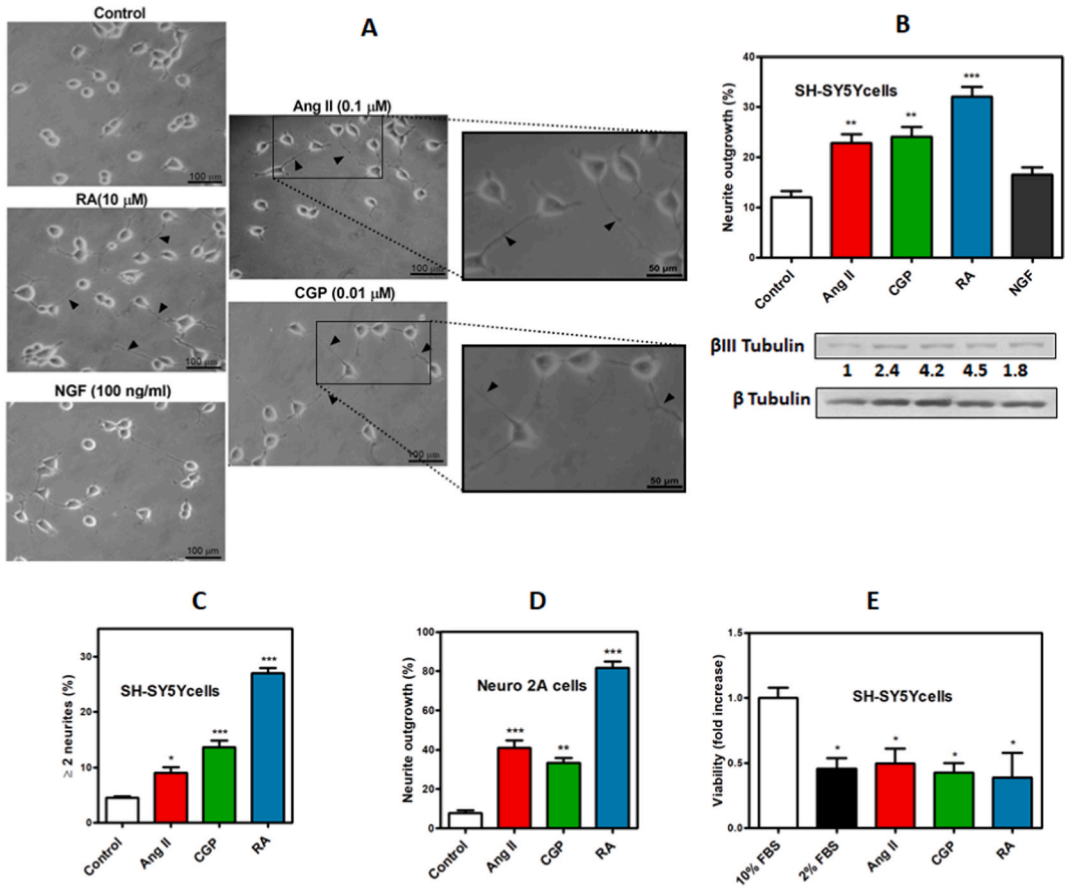

Fig. 1. Involvement of AT_2 receptor in induced neurite outgrowth in SH-SY5Y cells. (A) SH-SY5Y cells cultured in DMEM 2% FBS alone (Control) or supplemented with Ang II (0.1 μM), CGP42112A (0.01 μM, AT_2 receptors agonist), RA (10 μM) or NGF (100 ng/ml) and allowed to differentiate for 3 days. Representative images of photomicrographs are shown. Arrowheads point to differentiated cells. The insets in Ang II and CGP42112A panels show a magnification to better visualize the neurite enlargement. (B) Graph showing the percentage of cells exhibiting at least one neurite longer that a cell body. Values represent means ± S.E.M (n = 4). **p < 0.01, ***p < 0.001 vs. control. Lower panel: equal amounts of cell lysates from the same treatments were separated by SDS-PAGE and immunoblotted with anti-βIII tubulin, marker of differentiation. Blot was stripped and reprobed for β tubulin to ensure equal loading and transfer. Numbers indicate relative protein levels determined by densitometry. Blots are representative of results from at least three independent experiments. (C) Graph showing the percentage of cells exhibiting 2 or more neurites. Values represent means ± S.E.M (n = 4). *p < 0.05, ***p < 0.001 vs. control. (D) Neuro 2A cells cultured in DMEM 2% FBS alone (Control) or supplemented with Ang II (0.1 μM), CGP42112A (0.01 μM) or RA (10 μM)) and allowed to differentiate for 3 days. Graph represents the percentage of cells exhibiting at least one neurite longer that a cell body. Values represent means ± S.E.M (n = 3). **p < 0.01, ***p < 0.001 vs. control. (E) SH-SY5Y cells were grown as indicated by 72 h. MTT reagent was added and incubated 3 h additionally. Formazan crystals were dissolved in DMSO and absorbance was measured at 540 nm. Each treatment was normalized to the control in FBS 10% (proliferating conditions). The results are shown as the mean ± SEM of 3 independent experiments. *p < 0.05 vs. 10% FBS.

LY294002 (PI3K inhibitor, 5 μM), SKI-II (SphK inhibitor, 5 μM) or UO126 (MEK inhibitor, 10 μM). After 72 h, cells were observed by phase contrast microscopy (Zeiss, Axiovert 25C) and 15–20 random fields were counted. Cells with at least one neurite longer than the cell body were considered positive for neurite outgrowth as previously described [31]. The percent of neurite outgrowth and morphometric analysis were performed by analyzing at least 20 fields for each condition. The percent of cells exhibiting two or more neurites, the number of neurites per cell and the length of the principal neurite were also measured. All these analyses were done with the ImageJ (NIH) software after calibration. Similar protocols were used to evaluate differentiation in different cell lines [7,18,27,31,32].

2.4. Cell viability assay (MTT)

All the viability experiments were performed in sextuplicates as reported [30]. Briefly, 5×10^3 SH-SY5Y cells were seeded in each well of 96-well plates and allowed to adhere overnight at 37 °C. Then, the medium was replaced by DMEM 10% FBS or DMEM 2% FBS with or without CGP42112A, Ang II or RA for 72 h as indicated in figure legends. Cells were then incubated for 3 additional hours in 110 μl of 0.5 mg/ml solution of MTT. Formazan crystals were dissolved in DMSO and absorbance determined at 540 nm using a microplate reader (Epoch, Biotek, USA).

2.5. Western blot analysis

Cells were treated as indicated, washed with ice-cold PBS and scraped into 1 × lysis buffer (50 mM Tris-HCl pH 8.0 containing 50 mM KCl, 10 mM EDTA, 20 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1:1000 protease cocktail inhibitor and Triton X-100 1%). Protein concentration was determined by Bradford assay, using BSA as standard. Equal amount of proteins (40 μg) were resolved on 7 or 10% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane (GE Healthcare, England, UK) and analyzed by Western blotting with the indicated primary antibodies (1:1000 for 3 h) followed by incubation with the appropriate HRP-coupled anti-rabbit IgG (1:20,000) or anti-mouse (1:10,000) secondary antibodies (Jackson Immuno Research). Blots were developed with ECL detection kit from GE Healthcare (England UK) and the results analyzed with Scion Image J software. Membranes were stripped by incubation on 62.5 mM Tris buffer containing 2% SDS and 100 mM β-mercaptoethanol for 10 min at 50 °C and loading control performed with anti-βI-tubulin (1:1000), Sigma, St. Louis, MO) antibody.

For AT_2 receptor western blotting, immunoreactive signals were detected with fluorescent secondary antibodies (IRD, Li-COR, 1/30,000). After scanning in OddysseyClx device, optical densities of AT_2 bands were quantified and normalized to β actin with Image Studio 5.2 Software.

2.6. Statistical analysis

Results are expressed as the mean ± SEM of at least three independent experiments. Statistical analysis was performed using one-way ANOVA, followed by the Tukey post-analysis (GraphPad PRISM Software package 8.0.1). Western blots were quantified with Scion Image J software. Differences were considered to be statistically significant when p values were <0.05.

3. Results

3.1. Ang II and CGP42112A induce neurite outgrowth in neuroblastoma cells

To establish the role of Ang II in neuronal differentiation, we used the human neuroblastoma cell line SH-SY5Y as a model. Cells were cultured in DMEM 2% FBS (Control) and RA was used as positive control of differentiation as previously reported [7,9,31,33]. Fig. 1A shows a representative image of photomicrographs of SH-SY5Y cells treated as indicated (arrowheads point to differentiated cells). Remarkably, Ang II and CGP42112A (AT_2 receptor agonist) significantly increased the percentage of neurite bearing cells to a similar level than RA treatment (Fig. 1B). Due to its higher affinity, the agonist CGP42112A displayed a similar effect to Ang II even at lower concentration (0.01 μM versus 0.1 μM) [24]. The expression of βIII tubulin, a marker of cell differentiation in SH-SY5Y cells [34,35], also increases in the presence of Ang II and CGP42112A (Fig. 1B, lower panel) and further supports a positive role of AT_2 receptor in SH-SY5Y cell differentiation. NGF did not induce neurite outgrowth and, as expected, also failed to induce βIII-tubulin expression (Fig. 1B). The uncropped images of the western blots are shown in Fig. S1 (MW βIII tubulin 50 kDa and MW β tubulin 55 kDa according to the datasheets).

We next decided to analyze if these factors modulate the number of neurites and the length of the principal neurite. Both Ang II and CGP42112A augment the percentage of cells that have two or more neurites (Fig. 1C). In a similar fashion, they also increase the average number of neurites per cell (data not shown). Conversely, none of the stimuli that we used modulate the average length of the principal neurite (Fig. S2).

Similarly, we further confirmed the role of AT_2 receptor in neurite outgrowth in Neuro2A mouse neuroblastoma cells, suggesting that the effect is not only restricted to one cell line (Fig. 1D).

As previously indicated [33], differentiation of SH-SY5Y cells also induces a reduction in proliferation. In agreement, our results show that a decrease in the concentration of FBS from 10 to 2%, which is required for differentiation, reduces cell viability (Fig. 1D). Moreover, treatment with Ang II, CGP42112 and RA in 2% FBS did not further affect cell viability (Fig. 1E).

Since our results and previous evidences suggest that Ang II-induced neurite outgrowth depends on AT_2 receptor [17,18], we also

evaluated the expression of these receptors in SH-SY5Y cells by Western blot. Stimulation with CGP42112, Ang II, RA (which induce neurite outgrowth) or NGF (which does not affect the same process), did not modulate AT_2 protein levels (Fig. 2). Of note, we have previously established the specificity of the anti-AT_2 antibody used in this study by Western blot/immunoprecipitation [23,24,36] and immunohistochemistry [12]. Fig. S3 shows the full image of the Western blot.

3.2. Blocking of AT_2 receptor reverts neurite outgrowth induced by CGP42112A

To further confirm the participation of AT_2 receptors in the induction of neurite outgrowth, we performed a differentiation assay in the presence of PD123319, an AT_2 antagonist. Fig. 3A shows representative images of SH-SY5Y cells treated as indicated. PD123319 (10 μM) completely reverted Ang II and CGP42112A-induced differentiation as well as the increase in the number of cells that exhibit two or more neurites (Fig. 3B and C). Overall, these results indicate that Ang II promotes neurite outgrowth and differentiation in neuroblastoma cells through the engagement of AT_2 receptor.

3.3. c-Src is important in AT_2 receptor-induced neurite outgrowth in SH-SY5Y cells

Activation of different signaling mechanisms have been linked to neurite outgrowth induced by Ang II via AT_2 receptors, including Ras, ERK, NO and GMPc [17,25,27,37]. Thus, to identify the possible signaling pathway leading to differentiation of SH-SY5Y cells, we first used the following inhibitors: UO126 (MEK inhibitor), LY294002 (PI3K inhibitor), PP2 (c-Src inhibitor) and its inactive analog PP3.

As was previously reported in NG108-15 [27,37] and Neuro2A cells [31], MEK/ERK activity is required for neurite extension induced by engagement of AT_2 receptors in SH-SY5Y cells (Fig. 4A). Likewise, PP2 was also able to reduce neurite outgrowth induced by CGP42112A (Fig. 4C), thus supporting the involvement of c-Src in the signaling pathway downstream of AT_2 receptors. On the other hand, PI3K is not required for neurite outgrowth in SH-SY5Y cells treated with CGP42112A (Fig. 4B).

Bearing in mind that S1P has also been pointed as an inductor of neurite extension and differentiation [28,29]; we decided to explore its role in our model. Interestingly, pre-treatment with the dual SphK inhibitor SKI-II, that prevents S1P production [38], reverts the effect of CGP4212A on neurite elongation (Fig. 4D). Comparable results were obtained when we measured the percentage of cells showing two or more neurites; UO126, PP2 and SKI-II revert the effect of CGP42112A (Fig. S4).

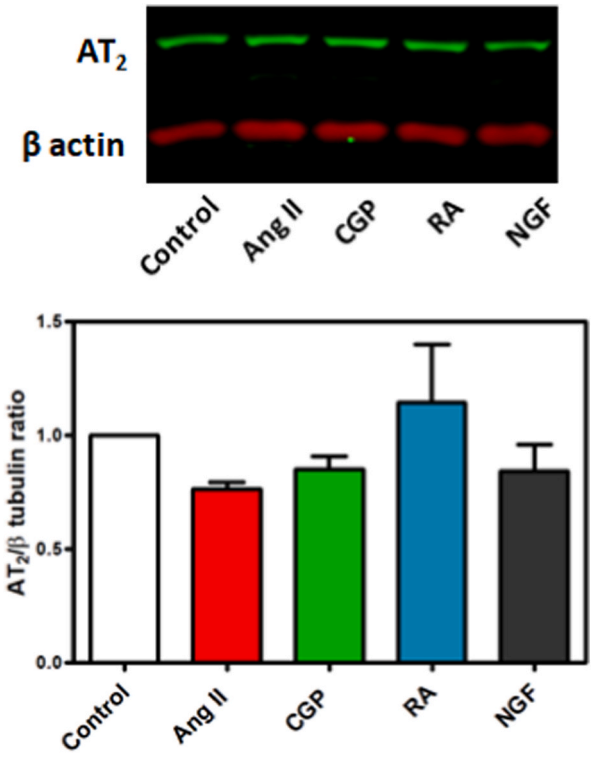

Fig. 2. Differentiation does not modulate protein levels of AT_2 receptor. SH-5Y5Y cells were stimulated with Ang II, CGP42112A, RA or NGF and allowed to differentiate for 3 days. Cell lysates were prepared, equal amount of proteins were separated by SDS-PAGE and western blots were developed with anti-AT_2 and anti-β-actin antibodies. A representative blot and densitometry data are shown. Values represent means ± S.E.M (n = 3).

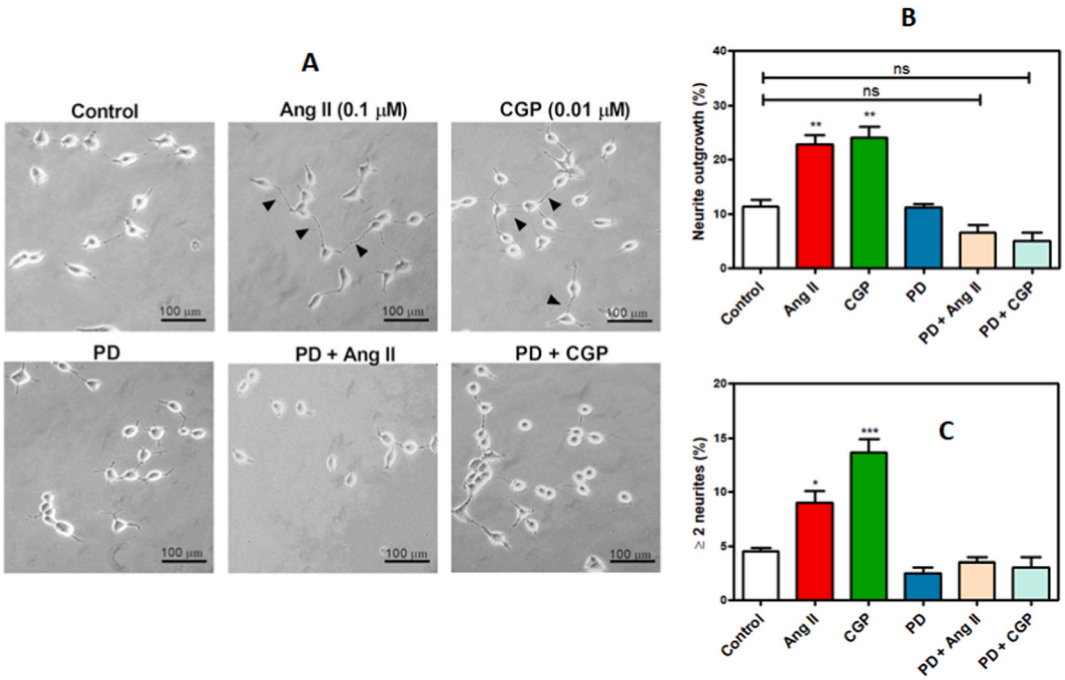

Fig. 3. PD123319 reverts neurite induction by Ang II or CGP42112A. (A) SH-SY5Y cells were treated with Ang II (0.1 μM) or CGP42112A (0.01 μM) in the absence or the presence of PD123319 (10 μM) and allowed to differentiate for 3 days. Representative images of photomicrographs for control and different treatments are shown. Arrowheads point to differentiated cells. (B) Graph showing the percentage of cells exhibiting at least one neurite longer that a cell body. Values represent means ± S.E.M (n = 4). **p < 0.01 vs. control. (C) Graph showing the percentage of cells exhibiting 2 or more neurites. Values represent means ± S.E.M (n = 4). *p < 0.05, ***p < 0.001 vs. control.

3.4. CGP42112A induces c-Src activation

Considering the effect of the c-Src inhibitor PP2 (Fig. 4C) and our previous reports demonstrating that AT_2 receptors activation induce recruitment of c-Src in rat fetal and brainstem PND15 membranes [23,24], we decided to evaluate whether or not CGP42112A activate c-Src phosphorylation in SH-SY5Y cells. Since phosphorylation happens at the initial stages of receptor activation, SH-SY5Y cells were stimulated during short times (30 s, 1, 3, 5, 10 min) with CGP42112A. Cell lysates were analyzed by Western blot using anti-Y^{416}pSrc and Y^{527}pSrc specific antibodies. Interestingly, while rapid and transient (30 s, 1 min) phosphorylation was detected at residue Y^{416}, phosphorylation at residue Y^{527} was induced later (1 min) but maintained during at least 10 min (Fig. 5A and B). Considering that phosphorylation of residue Y^{416} is associated to c-Src activation, while Y^{527} phosphorylation is indicative of c-Src inactivation [39], it is tempting to speculate CGP42112A induces both activation and deactivation of c-Src in a timely fashion that is characteristic of signal transduction pathways. The uncropped Western blot images are displayed in Fig. S5.

3.5. Inhibition of TrKA receptor reduced neurite outgrowth induced by CGP42112A

Previous data suggest a possible crosstalk between NGF and AT_2 receptors [17,19,26,27]. In order to explore this possibility, we assayed the effect of pre-treatment with tyrphostin AG879 (inhibitor of the NGF receptor TrkA) [27] in AT_2 receptor-induced neuronal differentiation. Remarkably, AG879 reverts neurite outgrowth induced by Ang II and CGP42112A in SH-SY5Y cells (Fig. 6A). Correspondingly, AG879 also reduces the number of cells that display two or more neurites (Fig. 6B).

4. Discussion

Neuronal differentiation is a crucial process during embryogenesis, but has also been suggested to be important in different neuropathological conditions, including neuroblastoma. Indeed, the strategy of inducing neuronal differentiation to inhibit tumor growth has been proposed as a new therapeutic approach to improve the prognosis of children suffering from neuroblastoma [2].

Neuroblastoma cell lines are useful models for the investigation of neuronal differentiation and could be suitable to study neurological diseases such as Parkinson and Alzheimer's disease [4,6]. In particular, SH-SY5Y human neuroblastoma cells derive from the SKN-SH human neuroblastoma cell line that was subcloned three times and are used as a model for studying the molecular mechanisms involved in neuronal differentiation [33].

Here, we evaluated the level of differentiation of human SH-SY5Y cells towards neuronal type by measurement of neurite's length as described by the group of a coauthor of this study [7,31]. Although many stimuli induce differentiation of SH-SY5Y cells, we used RA

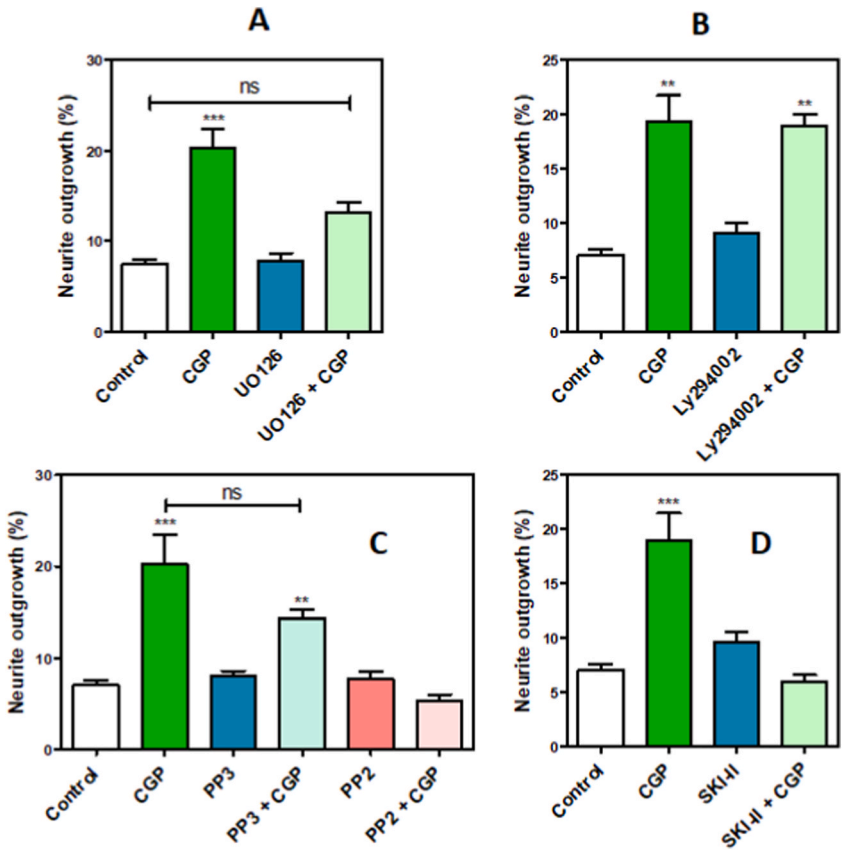

Fig. 4. Differentiation induced by AT_2 receptor stimulation is independent of PI3K, but requires ERK, SphK and c-Src. SH-SY5Y cells were pretreated for 40 min with the indicated inhibitors, stimulated with CGP42112A (0.01 μM) and allowed to differentiate during 3 days. The graphs show the percentage of cells exhibiting at least one neurite longer that a cell body. (A) Neurite outgrowth induced by CGP42112A in the absence or the presence of MEK inhibitor UO126 (10 μM). Values represent means ± S.E.M (n = 4). ***$p < 0.001$ vs. control. (B) Neurite outgrowth induced by CGP42112A in the absence or the presence the PI3K inhibitor LY294002 (0.5 μM). Values represent means ± S.E.M (n = 2). **$p < 0.01$ vs. control. (C) Neurite outgrowth induced by CGP42112A in the absence or the presence the c-Src inhibitor PP2 (2.5 μM) or its inactive analog, PP3 (2.5 μM). Values represent mean ± S.E.M (n = 5). ***$p < 0.001$ and **$p < 0.01$ vs. control. (D) Neurite outgrowth induced by CGP42112A in the absence or the presence of SphK inhibitor SKI-II (5 μM). Values represent means ± S.E.M (n = 3). ***$p < 0.001$ vs. control.

treatment as positive control since it has been the most widely used [33]. Our results demonstrate that both Ang II and CGP42112A (selective AT_2 receptor agonist) induced a 2 fold increase in neurite outgrowth in SH-SY5Y cells, comparable to the 3 fold induction observed after RA treatment. The observed increase in neurite length is similar to data obtained by other authors in different cell lines [7,9,17,18,31]. Furthermore, Ang II and CGP42112A not only enhance neurite outgrowth but also increase the number of neurites per cell without affecting the average length of the principal neurite. On the other hand, tough previous studies indicate that 7 days of RA treatment were necessary to induce differentiation [40], in our conditions Ang II, CGP42112A and RA stimulate neurite outgrowth in SH-SY5Y cells after only 3 days. Certainly, recent studies demonstrated that SH-SY5Y cells display neurite extension when treated with Ang II for a period of only 24 h [41]. Moreover, neurite elongation occurs in parallel with reduction in cell soma size. Consistent with the literature [33], we show here that differentiation in FBS 2% is accompanied by a decrease in proliferation. On the other hand, it is important to remark that neither Ang II nor CGP42212 further reduce viability, suggesting that AT_2 engagement may be a useful strategy to induce differentiation without affecting cell viability.

To further confirm the previous results, we show that expression level of βIII tubulin, marker of neuronal differentiation [40], was also increased by Ang II and CGP42112. In that regard, it is important to mention that dissimilar results have been reported regarding the expression of neuronal markers in differentiated SH-SY5Y cells. While RA increased the levels of the neuronal marker βIII tubulin, it does not modulate tyrosine hydroxylase (TH) levels [42]. On the contrary, neurite outgrowth induced by RA in SH-SY5Y cells is accompanied by increased TH levels [43], βIII tubulin and enolase 2 (Eno2) [44]. Previous reports from a coauthor from this study have shown that RA and phosphatidylcholine (PtdCho) induce differentiation and increased expression of βIII tubulin in Neuro 2A cells [31]. Moreover, phosphatidylethanolamine (PtdEtn) failed to induce βIII tubulin expression and differentiation, demonstrating the specificity of the neural marker. Thus, our results suggest that neurite outgrowth induced by Ang II and CGP42112A are accompanied by increased expression of βIII tubulin, a hallmark of differentiation toward a neuronal phenotype.

Although our previous studies established a potential role of AT_2 receptors in neuronal differentiation and development [12,13],

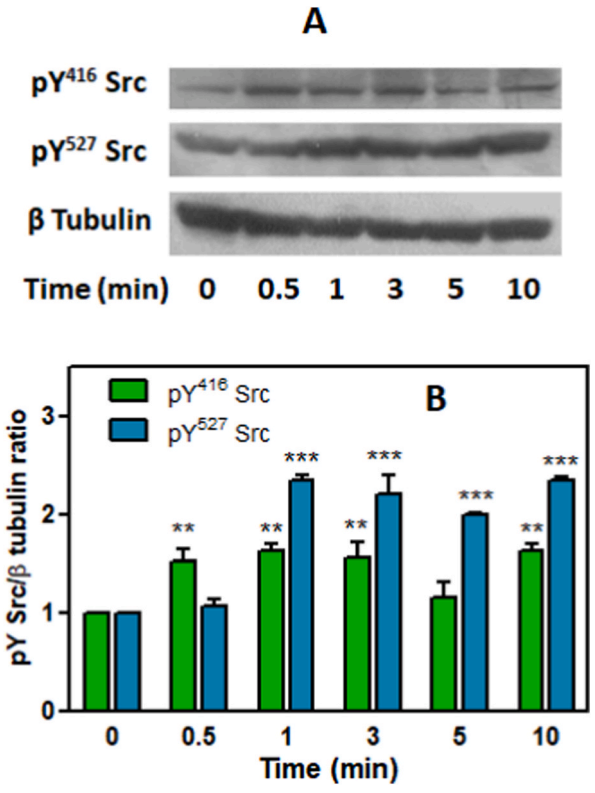

Fig. 5. CGP42112A stimulates c-Src phosphorylation in SH-SY5Y dells. (A) SH-SY5Y cells were stimulated with CGP42112A (0.01 μM) for the indicated times. Cell lysates were prepared, proteins separated by SDS-PAGE and immunoblotted with anti-pY^{416}Src or anti-pY^{527}Src antibodies. Membranes were stripped and reprobed with anti-β tubulin for loading control. (B) Densitometry of western blots for pY^{416}Src (green bars) and pSrcY^{527} (blue bars) normalized to β tubulin. The data are shown as the mean ± S.E.M (n = 3), $^{**}p < 0.01$, $^{***}p < 0.001$ vs. controls (time = 0).

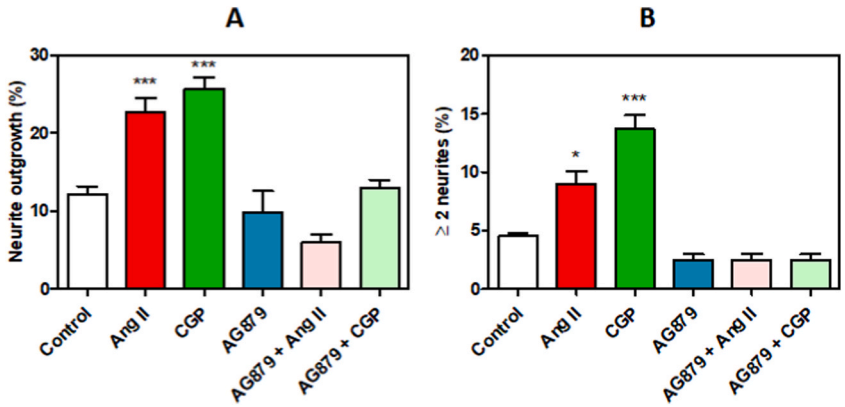

Fig. 6. Tyrphostin AG879, a TrkA inhibitor, prevents neurite outgrowth induced by Ang II and CGP42112A. (A) SH-SY5Y cells were pre-treated with AG879 (0.6 μM, 40 min), stimulated with CGP42112A, allowed to differentiate for 3 days and neurite outgrowth was quantified. Values represent mean ± S.E.M (n = 3), $^{***}p < 0.001$ vs. control. (B) Graph showing the percentage of cells exhibiting 2 or more neurites. Values represent means ± S.E.M (n = 3). $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control.

SH-SY5Y cells contain both AT_1 and AT_2 Ang II receptor subtypes [45]. Here, by using CGP42112A and PD123319 (the high selectivity AT_2 receptor agonist and antagonist respectively) we confirmed the specific participation of AT_2 receptors in neurite outgrowth. As mentioned, these results are consistent with the literature in different models, including SH-SY5Y cells [41], NG108-15 cells [17,27], PC12W cells [46], rat DRG neurons [19,47] and primary rat neural cells [21] among others. On the other hand, it has also been reported that both AT_1 and AT_2 receptors are important for *in vivo* and *in vitro* neurite outgrowth [48].

It was recently reported that mRNA expression of AT_2 increased *in vivo* after induction of cutaneous inflammation that in turn leads

to neurite outgrowth [48]. On the contrary, our results show that protein levels of Ang II AT_2 receptors remain unaltered under differentiation conditions. Whether this discrepancy may be related to differences in mRNA versus protein levels or the distinct models that were used is currently not known.

Although a number of studies have been performed in order to evaluate the pathways and the possible roles of Ang II AT_2 receptors in the differentiation process, the activation of dissimilar signaling mechanism have been described (ERK, NO, GMPc and Fyn among others) in different models [11,15–17,20,21,27,37]. Indeed, opposite results have been revealed in NG108-15 mouse neuroblastoma cells: while in some reports ERK was important for AT_2 receptor induction of neurite outgrowth [27,37], in other it was not involved [17]. Consequently, the signaling pathways conducting neurite outgrowth mediated by Ang II AT_2 receptors remain unclear and deserve further attention.

Here, we used specific inhibitors to enlighten the role of different signaling in neurite outgrowth. Although previous reports proposed a role of PI3K in RA-mediated differentiation of SH-SY5Y cells [49], our results suggest that PI3K is not important in the process induced by Ang II AT_2 receptor engagement. On the contrary, inhibition of MEK/ERK, SphK1 and c-Src pathways prevented neurite outgrowth induced by CGP41112A (Fig. 7). As mentioned above, the importance of ERK activation in neurite outgrowth has been highlighted in different models [27,37,50].

Furthermore, our results are in agreement with previous report suggesting a role of SphK and S1P signaling in neurite elongation. Certainly, it was shown that NGF activated SphK1 increasing the levels of S1P that in turn stimulates S1PR1 enhancing neurite outgrowth [29]. Moreover, S1P increased neurite elongation provoked by suboptimal doses of NGF [28]. However, since this is the first report of a possible link between activation of AT_2 receptor and induction of SphK and S1P signaling in neurite outgrowth, the mechanism needs to be elucidated (Fig. 7).

Interestingly, the role of Fyn, a member of the Src-family of kinases (SFK) in neurite outgrowth induction has been established [17]. Moreover, we were among the first group to describe the participation of c-Src and demonstrate the physical association between AT_2 receptors, c-Src, SHP-1 and FAK, following stimulation in *ex vivo* models [23,24]. Thus, we decided to further study the signaling mechanism.

Now, we confirm that CGP42112A induces the rapid and transient phosphorylation of c-Src at residue Y^{416}, which has been described as crucial to increase the enzyme activity [39]. Remarkably, the phosphorylation of c-SrcY^{416} was followed by phosphorylation of residue Y^{527}, indicative of a later inactivation [39]. Our previous studies established that activation and phosphorylation of SHP-1 by c-Src are common factors in the signaling mechanism induced by Ang II AT_2 receptors in *ex vivo* models [23,24]. Interestingly, a role of SHP-1 in AT_2-induced neural differentiation has also been described in rat fetal brain cultured cells [21]. Thus, the rapid phosphorylation and subsequent inactivation of c-Src observed in the present study suggest a similar process to the one described earlier [21,24].

Several lines of evidence support the existence of a possible modulation between Ang II AT_2 and TrkA receptors in different models [17,19,27]. Thus, we evaluated the participation of TrkA receptor in SH-SY5Y cells neurite outgrowth by using the inhibitor tyrphostin AG879 as previously reported [27]. We observed a clear inhibition of neurite outgrowth following stimulation with Ang II or CGP42112A in the presence of AG879, suggesting a modulation between both receptors. Remarkably, it was reported that EMA1087 (compound 21, C21), a non-peptide AT_2-agonist, increased expression of TrkA and TrkB neurotrophin receptors and promoted neurite outgrowth of primary neurons [51]. Moreover, the latter effect was reduced by inhibition of Trk. The possible intermodulation between AT_2 and TrkA has also been proposed to induce neurite enlargement of cultured DRG cells [50].

Although these findings strongly suggest that signaling of AT_2 and TrkA receptors regulate each other and may have a synergistic effect in neuronal differentiation, the underlying mechanisms have not been elucidated. However, based on several evidences [17,24,27], it is tempting to speculate that transactivation of TrkA by AT_2 receptors involve the phosphorylation and activation of c-Src (Fig. 7).

5. Conclusions

Taken together, our results demonstrate that stimulation of Ang II AT_2 receptors with Ang II or the selective agonist CGP42112A induce neurite outgrowth in SH-SY5Y neuroblastoma cells. Remarkably, the process implicates the phosphorylation of c-Src at specific residues suggesting early activation followed by inactivation of the kinase. Moreover, neurite outgrowth induced by CGP42112A was prevented by the use of specific inhibitors of c-Src, MEK, ShpK and TrkA receptors. Fig. 7 summarizes the experimental data obtained in the present study.

Author contribution statement

Helga M. Blanco, Celia N. Perez: Performed the experiments; Analyzed and interpreted the data.

Dr. Claudia Banchio: Contributed reagents, materials, analysis tools or data.

Dr. Sergio E Alvarez, Dr. Gladys M. Ciuffo: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

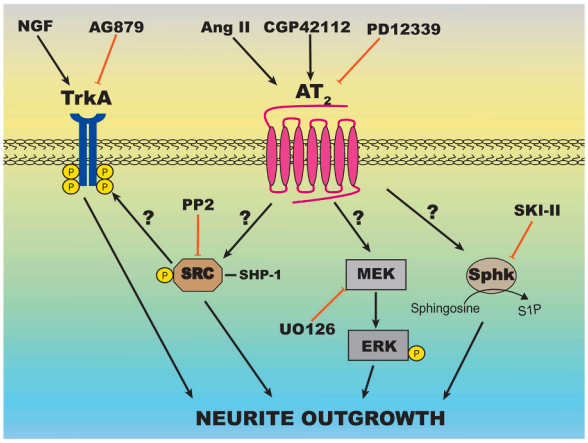

Fig. 7. Proposed model of AT_2 receptor mediated neurite outgrowth. The figure summarizes the present and some past findings related to the role of Ang II AT_2 receptors in the mechanism of neurite elongation. We explored the participation of three different pathways in the neurite outgrowth induced by Ang II or CGP42112A by using specific inhibitors of c-Src (PP2), MEK (UO126), TrkA (AG879) and SphK (SKI-II). Besides, we evaluated the phosphorylation and thus activation of c-Src. Once activated, c-Src may enhance neurite outgrowth either directly or through the transactivation of TrkA receptor (this work and [17,27]). Moreover, activation of SphK that increases S1P levels (this work and [28]) and induction of the MEK/ERK pathway are also important for neurite elongation (this work and [27,37]). The role of SHP-1 in the AT_2 receptor signaling pathway is also displayed since it has been demonstrated by our group and others [21,23,24].

Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

G.M. Ciuffo, S.E. Alvarez and C. Banchio are members of the researcher's career of CONICET (Consejo Nacional de Investigaciones Científicas y Tecnológicas, Argentina). H.M. Blanco and C.N. Perez have a fellowship from CONICET, Argentina. This work was supported by grants from CONICET (PIP 656 to GMC and SEA), ANPCYT (PICT 15-32350 to GMC and PICT 2378-2014 to SEA) and Universidad Nacional de San Luis (PROICO 02-0616 to GMC and 02-0318 to SEA).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15656.

References

[1] M. Mitre, A. Mariga, M.V. Chao, Neurotrophin signalling: novel insights into mechanisms and pathophysiology, Clin. Sci. 131 (1) (2017) 13–23.
[2] Z. Jin, et al., Development of differentiation modulators and targeted agents for treating neuroblastoma, Eur. J. Med. Chem. 207 (2020), 112818.
[3] M. Gautier, et al., Plasticity in neuroblastoma cell identity defines a noradrenergic-to-mesenchymal transition (NMT), Cancers 13 (12) (2021).
[4] B. Huo, et al., Pax3 inhibits Neuro-2a cells proliferation and neurite outgrowth, J. Cell Mol. Med. 25 (2) (2021) 1252–1262.
[5] H. Xicoy, B. Wieringa, G.J. Martens, The SH-SY5Y cell line in Parkinson's disease research: a systematic review, Mol. Neurodegener. 12 (1) (2017) 10.
[6] B.P. Coelho, et al., Dual effect of doxazosin: anticancer activity on SH-SY5Y neuroblastoma cells and neuroprotection on an in vitro model of alzheimer's disease, Neuroscience 404 (2019) 314–325.
[7] P. Domizi, et al., KDM2B regulates choline kinase expression and neuronal differentiation of neuroblastoma cells, PLoS One 14 (1) (2019) e0210207.
[8] L. Chen, et al., Angiotensin-converting enzyme inhibition reduces neuroblastoma cell growth rate, Proc. Soc. Exp. Biol. Med. 196 (3) (1991) 280–283.
[9] H. Teppola, et al., Morphological differentiation towards neuronal phenotype of SH-SY5Y neuroblastoma cells by estradiol, retinoic acid and cholesterol, Neurochem. Res. 41 (4) (2016) 731–747.
[10] T. Kobiec, et al., The renin-angiotensin system modulates dopaminergic neurotransmission: a new player on the scene, Front. Synaptic Neurosci. 13 (2021), 638519.
[11] M.O. Guimond, N. Gallo-Payet, How does angiotensin AT(2) receptor activation help neuronal differentiation and improve neuronal pathological situations? Front. Endocrinol. 3 (2012) 164.
[12] M.E. Arce, et al., Purkinje cells express Angiotensin II AT(2) receptors at different developmental stages, Neuropeptides 45 (1) (2011) 69–76.
[13] M.E. Arce, et al., Age-related changes in Ang II receptor localization and expression in the developing auditory pathway, Neurochem. Res. 44 (2) (2019) 412–420.
[14] S.I. Sanchez, et al., Prenatal blockade of Ang II receptors affects neonatal rat hindbrain structure and receptor localization, Exp. Neurol. 220 (2) (2009) 246–254.
[15] L. Laflamme, et al., Angiotensin II induction of neurite outgrowth by AT2 receptors in NG108-15 cells. Effect counteracted by the AT1 receptors, J. Biol. Chem. 271 (37) (1996) 22729–22735.

[16] F. Cote, et al., Nitric oxide, a new second messenger involved in the action of angiotensin II on neuronal differentiation of NG108-15 cells, Endocr. Res. 24 (3–4) (1998) 403–407.
[17] M.O. Guimond, C. Roberge, N. Gallo-Payet, Fyn is involved in angiotensin II type 2 receptor-induced neurite outgrowth, but not in p42/p44mapk in NG108-15 cells, Mol. Cell. Neurosci. 45 (3) (2010) 201–212.
[18] M.O. Guimond, et al., Comparative functional properties of two structurally similar selective nonpeptide drug-like ligands for the angiotensin II type-2 (AT(2)) receptor. Effects on neurite outgrowth in NG108-15 cells, Eur. J. Pharmacol. 699 (1–3) (2013) 160–171.
[19] N. Hashikawa-Hobara, N. Hashikawa, Angiotensin II AT2 receptors regulate NGF-mediated neurite outgrowth via the NO-cGMP pathway, Biochem. Biophys. Res. Commun. 478 (2) (2016) 970–975.
[20] F. Cote, et al., Activation of the AT(2) receptor of angiotensin II induces neurite outgrowth and cell migration in microexplant cultures of the cerebellum, J. Biol. Chem. 274 (44) (1999) 31686–31692.
[21] J.M. Li, et al., Angiotensin II-induced neural differentiation via angiotensin II type 2 (AT2) receptor-MMS2 cascade involving interaction between AT2 receptor-interacting protein and Src homology 2 domain-containing protein-tyrosine phosphatase 1, Mol. Endocrinol. 21 (2) (2007) 499–511.
[22] J. Chao, et al., Angiotensin II increased neuronal stem cell proliferation: role of AT2R, PLoS One 8 (5) (2013), e63488.
[23] S.E. Alvarez, et al., Involvement of c-Src tyrosine kinase in SHP-1 phosphatase activation by Ang II AT2 receptors in rat fetal tissues, J. Cell. Biochem. 105 (3) (2008) 703–711.
[24] L.R. Seguin, R.S. Villarreal, G.M. Ciuffo, AT(2)receptors recruit c-Src, SHP-1 and FAK upon activation by Ang II in PND15 rat hindbrain, Neurochem. Int. 60 (2) (2012) 199–207.
[25] L. Gendron, et al., Nitric oxide and cyclic GMP are involved in angiotensin II AT(2) receptor effects on neurite outgrowth in NG108-15 cells, Neuroendocrinology 75 (1) (2002) 70–81.
[26] G. Umschweif, et al., Angiotensin receptor type 2 activation induces neuroprotection and neurogenesis after traumatic brain injury, Neurotherapeutics 11 (3) (2014) 665–678.
[27] B. Plouffe, et al., Role of tyrosine kinase receptors in angiotensin II AT2 receptor signaling: involvement in neurite outgrowth and in p42/p44mapk activation in NG108-15 cells, Endocrinology 147 (10) (2006) 4646–4654.
[28] L.C. Edsall, G.G. Pirianov, S. Spiegel, Involvement of sphingosine 1-phosphate in nerve growth factor-mediated neuronal survival and differentiation, J. Neurosci. 17 (18) (1997) 6952–6960.
[29] R.E. Toman, et al., Differential transactivation of sphingosine-1-phosphate receptors modulates nerve growth factor-induced neurite extension, J. Cell Biol. 166 (2004) 381–392.
[30] L.E. Campos, et al., Hydroxynaphthalenecarboxamides and substituted piperazinylpropandiols, two new series of BRAF inhibitors. A theoretical and experimental study, Bioorg. Chem. 103 (2020), 104145.
[31] L. Paoletti, et al., Lysophosphatidylcholine drives neuroblast cell fate, Mol. Neurobiol. 53 (9) (2016) 6316–6331.
[32] H. Marcucci, et al., Phosphatidylcholine biosynthesis during neuronal differentiation and its role in cell fate determination, J. Biol. Chem. 285 (33) (2010) 25382–25393.
[33] J. Kovalevich, M. Santerre, D. Langford, Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology, Methods Mol. Biol. 2311 (2021) 9–23.
[34] S. Paik, R.K. Somvanshi, U. Kumar, Somatostatin-mediated changes in microtubule-associated proteins and retinoic acid-induced neurite outgrowth in SH-SY5Y cells, J. Mol. Neurosci. 68 (1) (2019) 120–134.
[35] M.A. Lauzon, O. Drevelle, N. Faucheux, Peptides derived from the knuckle epitope of BMP-9 induce the cholinergic differentiation and inactivate GSk3beta in human SH-SY5Y neuroblastoma cells, Sci. Rep. 7 (1) (2017) 4695.
[36] M.J. Manzur, et al., Focal adhesion kinase, RhoA, and p38 mitogen-activated protein kinase modulates apoptosis mediated by angiotensin II AT2 receptors, J. Cell. Biochem. 120 (2) (2018) 1835–1849.
[37] L. Gendron, et al., Signals from the AT2 (angiotensin type 2) receptor of angiotensin II inhibit p21ras and activate MAPK (mitogen-activated protein kinase) to induce morphological neuronal differentiation in NG108-15 cells, Mol. Endocrinol. 13 (9) (1999) 1615–1626.
[38] P. Gao, et al., Characterization of isoenzyme-selective inhibitors of human sphingosine kinases, PLoS One 7 (9) (2012), e44543.
[39] R. Roskoski Jr., Src protein-tyrosine kinase structure, mechanism, and small molecule inhibitors, Pharmacol. Res. 94 (2015) 9–25.
[40] J.M. Hernandez-Martinez, et al., Quinolinic acid induces neuritogenesis in SH-SY5Y neuroblastoma cells independently of NMDA receptor activation, Eur. J. Neurosci. 45 (5) (2017) 700–711.
[41] B.J. Collazo, et al., Angiotensin II induces differentiation of human neuroblastoma cells by increasing MAP2 and ROS levels, J. Renin-Angiotensin-Aldosterone Syst. JRAAS 2021 (2021), 6191417.
[42] A.D. Ducray, et al., Quantitative characterization of phenotypical markers after differentiation of SH-SY5Y cells, CNS Neurol. Disord.: Drug Targets 19 (8) (2020) 618–629.
[43] K. Khwanraj, et al., Differential expression of tyrosine hydroxylase protein and apoptosis-related genes in differentiated and undifferentiated SH-SY5Y neuroblastoma cells treated with MPP, Neurol. Res. Int. 2015 (2015), 734703.
[44] A. Kunzler, et al., Changes in cell cycle and up-regulation of neuronal markers during SH-SY5Y neurodifferentiation by retinoic acid are mediated by reactive species production and oxidative stress, Mol. Neurobiol. 54 (9) (2017) 6903–6916.
[45] L.I. Chen, O.M. Prakash, R.N. Re, The interaction of insulin and angiotensin II on the regulation of human neuroblastoma cell growth, Mol. Chem. Neuropathol. 18 (1–2) (1993) 189–196.
[46] U. Stroth, et al., Angiotensin II and NGF differentially influence microtubule proteins in PC12W cells: role of the AT2 receptor, Brain Res. Mol. Brain Res. 53 (1–2) (1998) 187–195.
[47] U. Anand, et al., Angiotensin II type 2 receptor (AT2 R) localization and antagonist-mediated inhibition of capsaicin responses and neurite outgrowth in human and rat sensory neurons, Eur. J. Pain 17 (7) (2013) 1012–1026.
[48] S.G. Benitez, et al., Cutaneous inflammation differentially regulates the expression and function of Angiotensin-II types 1 and 2 receptors in rat primary sensory neurons, J. Neurochem. 152 (6) (2020) 675–696.
[49] Y. Nishida, et al., Identification and classification of genes regulated by phosphatidylinositol 3-kinase- and TRKB-mediated signalling pathways during neuronal differentiation in two subtypes of the human neuroblastoma cell line SH-SY5Y, BMC Res. Notes 1 (2008) 95.
[50] U. Anand, et al., Mechanisms underlying clinical efficacy of Angiotensin II type 2 receptor (AT2R) antagonist EMA401 in neuropathic pain: clinical tissue and in vitro studies, Mol. Pain 11 (2015) 38.
[51] P. Namsolleck, et al., AT2-receptor stimulation enhances axonal plasticity after spinal cord injury by upregulating BDNF expression, Neurobiol. Dis. 51 (2013) 177–191.