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Optimization of the Light-On system in a lentiviral platform to a light-controlled expression of genes in neurons

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ABSTRACT

Background: Molecular brain therapies require the development of molecular switches to control gene expression in a limited and regulated manner in time and space. Light-switchable gene systems allow precise control of gene expression with an enhanced spatio-temporal resolution compared to chemical inducers. In this work, we adapted the existing light-switchable Light-On system into a lentiviral platform, which consists of two modules: (i) one for the expression of the blue light-switchable transactivator GAVPO and (ii) a second module containing an inducible-UAS promoter (UAS) modulated by a light-activated GAVPO.

Results: In the HEK293-T cell line transfected with this lentiviral plasmids system, the expression of the reporter mCherry increased between 4 to 5 fold after light induction. A time expression analysis after light induction during 24 h revealed that mRNA levels continuously increased up to 9 h, while protein levels increased throughout the experiment. Finally, transduction of cultured rat hippocampal neurons with this dual Light-On lentiviral system showed that CDNF, a potential therapeutic trophic factor, was induced only in cells exposed to blue light.

Conclusions: In conclusion, the optimized lentiviral platform of the Light-On system provides an efficient way to control gene expression in neurons, suggesting that this platform could potentially be used in biomedical and neuroscience research, and eventually in brain therapies for neurodegenerative diseases.

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1. Introduction

Molecular therapies for neurodegenerative diseases require the proper development of molecular switches to precisely control gene expression in a spatio-temporal manner [1,2]. Despite different approaches in accomplishing spatio-temporal control, current therapies are far from achieving a regulated gene expression in specific brain nuclei, when therapeutically desired. Theoretically, the spatial resolution could be accomplished with the precise delivery of the transducing vectors in specific brain areas, and the temporal activation could be achieved by timing the availability of the inducer.

Inducible gene switches have been developed based on nuclear receptors, which are ligand-controlled transcription factors regulated by estradiol or tamoxifen [3,4]. Other widely used inducible switches are the Tet-On/Off systems, whose transcription factors are controlled by tetracycline [5]. These chemical inducers, although useful in regulating gene expression, have the disadvantage of freely diffusing in the tissues of an organism losing spatial control. Also, these inducers present difficulties in crossing biological barriers, which are essentials for brain-oriented therapies [6,7]. An accurate temporal and spatial control of therapeutical gene expression is essential to avoid harmful effects produced by

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non-localized, untimely, or overexpressed protein, particularly in sensitive cells such as neurons. Part of this problem has been solved by replacing constitutive promoters with cell type specific promoters, restricting the expression of the trans-activator or trans-repressor to some specific cells [8,9]. However, this solution is complex and requires prior knowledge regarding cell type–specific promoters and is challenging to implement in different brain areas and does not offer control of gene expression.

Light-switchable systems to control gene expression have emerged as an alternative to chemically induced gene expression systems [10,11,12]. Easy control of spatio-temporal resolution, tunable intensity, and low toxicity for cells, make light an ideal inducer in complex cells such as neurons and could be easily adaptable to brain gene therapies.

The generation of a light-switchable gene expression system compatible with neurons requires a viral platform to deliver the genetic material efficiently [13,14]. Lentiviruses are suitable vectors for central nervous system (CNS) gene therapy since they efficiently transduce both mitotic and post-mitotic cells, integrating genetic information into the host genome resulting in a long term stable expression [15,16].

The *in vivo* application of these light-switchable systems could be easily implemented from the hardware use for opsin activation that have been used in culture cells or in *in vivo* experiments with mice or rats [18,19,20]. Different micro and nanometric systems have been created [17] and these developments have allowed a punctual and controlled illumination both in mice brains and out of this in the peripheral nervous system and the spinal cord [21,22,23,24]. Although light-controlled gene therapy in humans has yet to be accomplished, the use of a nano technology for light control along with a light-switchable gene expression system could be practically applied in the future to treat neurological disorders.

In this work, we optimized and adapted the Light-On light-switchable system, initially developed by Wang and colleagues [10], to a lentiviral platform compatible with the efficient transduction of neurons. The Light-On system is composed of a synthetic light-switchable trans-activator GAVPO, whose homodimerization is triggered by blue light. The activated GAVPO homodimer binds to the UAS regulatory sequence fused to a minimal promoter E1b (UAS inducible promoter), driving the expression of the gene of interest downstream [10]. The platform developed here consists of two lentiviruses, one that expresses GAVPO under the constitutive Ubiquitin promoter and a second lentivirus to induce the expression of a gene of interest driven by the UAS inducible promoter. As proof of concept, we demonstrated the expression of the cerebral dopamine neurotrophic factor (CDNF) induced by blue light. The developed light-switchable lentiviral system is suitable for the expression of genes in neurons in a regulated manner by blue light, which makes it amenable for use in biomedical and neuroscience research.

2. Materials and methods

2.1. Lentiviral plasmid construction

The PolyA-UASx5-E1b ($UAS_{(L)}$) (FP PAU5TA: 5′-GCTTTAAT TAACTTGGAGCGGCCGCAATA-3′ and RP PAU5TA: 5′-TTTACCGGTATGGTGGCCAAGCTTACTT-3′) and UASx5-E1b ($UAS_{(S)}$) (FP PAU5TA: 5′-GCGTTAATTAAAAGTGCAGGTGCCAGAAC-3′ and RP U5TA: 5′-TTTACCGGTATGGTGGCCAA GCTTACTT-3′) sequences were amplified by PCR from pU5-Gluc plasmid. The $UAS_{(L)}$ and $UAS_{(S)}$ sequences were sub-cloned into the pFU-mCherry-W plasmid, by replacing the Ubiquitin promoter between *PacI* and *AgeI* enzyme restriction sites to obtain the UAS-gene reporter lentiviral plasmids pF-$UAS_{(L)}$-mCherry-W and pF-$UAS_{(S)}$-mCherry-W, respectively. The bicistronic lentiviral plasmid pF-$UAS_{(S)}$-mCherry-1D/2A-HA-CDNF-W was obtained by sub-cloning the $UAS_{(S)}$ sequence, from pF-$UAS_{(S)}$-mCherry-W into pFU-mCherry-1D/2A-HA-CDNF-W, replacing the Ubiquitin promoter between *PacI* and *AgeI* enzyme restriction sites. The bicistronic light-switchable GAVPO lentiviral plasmid pFUG-1D/2A-HA-GAVPO-W was obtained by amplification of the GAVPO sequence (FP GAV: 5′-GG GTCTAGAGTCTATGAAGCTACTGTCTTCT-3′ and RP GAV: 5′-GGGTGA TCAATTACTTGTCATCATCGTCTTTG-3′) from pGAVPO plasmid, adding the *XbaI* and *BclI* enzyme restriction sites. The GAVPO amplified was sub-cloned into the pFUG-1D/2A-HA-W bicistronic plasmid between *NheI* and *BamHI* enzyme restriction sites, which are compatible for *XbaI* and *BclI* enzyme restriction sites, respectively. The pGAVPO plasmid was kindly provided by Dr. Yi Yang (East China University of Science and Technology). The pFUG-1D/2A-HA-CDNF-W plasmid described in [25] was used to replace GAVPO expression and evaluate whether blue light-induced expression strictly depends on the GAVPO presence.

2.2. Antibodies

Primary antibodies used: mouse monoclonal anti-GFP (1:1000, Santa Cruz), mouse monoclonal anti-mCherry (1:1000, Abexxa), goat polyclonal anti-CDNF (1:1000, RyD system), mouse monoclonal anti-β-Actin (1:10,000, Sigma-Aldrich), mouse monoclonal anti-β-Tubulin (1:5000, Abexxa), mouse monoclonal anti-Flag (1:1000, Millipore) and rabbit monoclonal anti-HA (1:1000, Cell Signaling). Secondary antibodies for immunofluorescence were: Alexa Fluor® 647 anti-goat (1:500, Invitrogen, USA). For immunoblotting, horseradish peroxidase-conjugated antibodies were used to detect primary antibodies (1:5000, Invitrogen, USA).

2.3. Cell culture and transfection

HEK293-T cells were cultured in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. PC-12 cells were cultured in DMEM with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum and penicillin and streptomycin antibiotics (all culture reagents were from Gibco). Both cell cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. Cells were transfected with a 1:1 ratio of UAS-gene reporter plasmid and light-switchable GAVPO plasmid, as previously reported in [26], with a Calfectin agent following the manufacturer's recommendations (Calbiotech). The percentage of transfection efficiency was 50% (HEK293-T) and 10% (PC-12).

2.4. Lentivirus production and transduction

HEK293-T cells were transfected with Lipofectamine 2000 (Invitrogen™) using the plasmids pCMVΔR8.91, pCMV-VSVg, and the corresponding transfer plasmids pFUG-1D/2A-HA-GAVPO-W, pF-$UAS_{(S)}$-mCherry-W and pF-$UAS_{(S)}$-mCherry-1D/2A-HA-CDNF-W to generate lentiviral particles [27]. A volume of 3 mL of supernatant was collected from a 60 mm culture plate at 48 and 72 h after transfection, centrifuged, passed through a 0.45 μm filter, and stored at −80°C.

2.5. Rat hippocampal neurons culture and lentiviral transduction

Primary cultures of hippocampal neurons were prepared, as previously reported [28]. Lentiviral transduction of hippocampal neuron cultures was performed at DIV 4 by adding 150 μl of the virus recollected from filtered supernatant. Neurons were incubated until DIV 7 at 37°C with 5% CO_2.

2.6. Induction assay with blue light (465 nm)

For induction experiments, a 16 × 20 cm acrylic box with blue LED tapes (each one with a maximum emission at 465 nm) attached to one of the inner faces of the box, was prepared (Supplementary Fig. 1). The acrylic box was completely sealed, leaving a small hole through which a connector passes to a charge. The box was sterilized with chlorine, ethanol and UV (30 min). After that, the acrylic box was placed inside the cell incubator. After transfection or transduction, plates were maintained in darkness at 37°C with 5% CO_2. Twenty-four hours after transfection or 76 h after transduction, a plate was exposed to intermittent illumination (15 min light/on, 15 min in dark/off) with blue light (acrylic box with blue LED tapes). Cells were 9.5 cm away from the acrylic box, and their light intensity was 116 lux measured with luminometer model UT383 (UNI-Trend Technology, Hong-Kong). The other culture plate was maintained in darkness in a different incubator until the end of the experiment. The period of light-induction was 24 h for experiment with cell lines, in neurons light-induction was carried out for 3 h at DIV7. Then, cell culture and neurons were fixed to Immunofluorescence or homogenized for further analysis.

2.7. Immunoblotting and densitometric analysis

For immunoblotting experiments, the samples were homogenized with a RIPA buffer (Millipore) containing protein inhibitors. Protein concentration was quantified by the BCA method (Thermo Scientific). Homogenized samples were fractionated and analyzed, as previously indicated [29].

Quantification of bands was performed using ImageJ software as shown in Janes [30]. Briefly, lane profiles were plotted with the gel analysis plug-in and the magic-wand tool was employed to calculate the integrated area within the band profile of interest, obtaining a final raw densitometry value. The intensity of bands of interest (mCherry and CDNF) was normalized with the intensity of band of a housekeeping gene (GAPDH or beta-Tubulin) obtaining a corrected intensity (CI).

2.8. Immunofluorescence

Indirect immunofluorescence (IF) was performed using 4% paraformaldehyde for 10 min at room temperature. After fixation, coverslips were permeabilized in PBS 1× containing 0.05% Triton X-100. Then, coverslips were washed with PBS 1× and were incubated with a blocking solution (PBS 1×, 2% glycine, 2% BSA, 5% FBS, 50 mM NH_4Cl, pH 7.4) for 1 h. The incubations with primary antibodies were made for 2 h at room temperature or overnight at 4°C. Then, three washes with PBS 1× solution were performed, and incubation with secondary antibodies was carried out for 1 h at room temperature. Three washes were performed, and a final wash in distilled water was made to remove excess of salt. Finally, the coverslips were mounted with a Vectashield/DAPI solution (Vector).

2.9. Imaging and quantification of fluorescence intensity

Images were acquired in an Olympus DS-Fi2 epifluorescence microscope equipped with 40× and 100× Olympus UplanFI oil immersion objectives and a Nikon DS-Fi2 camera operated with QC software (Q-Imaging). For quantification, images were processed employing ImageJ software (NIH, USA), obtaining the corrected total cell fluorescence (CTCF) of mCherry, CDNF, and EGFP per cell given by the software. Datasets are presented as fluorescence intensity of mCherry/EGFP or CDNF/EGFP, normalized to dark situations.

2.10. RNA extraction and RT-qPCR

Total RNA was extracted using Trizol reagent (Ambion) according to the manufacturer's instructions. cDNA was synthesized by RT-PCR using 1 μg of total RNA employing ImProm-II RT (Promega). Quantitative real-time PCR was performed using EvaGreen qPCR Mix plus (Solis Byodine) and a LightCycler thermocycler (Roche). For the quantification of mCherry mRNA levels, the following primers were used: Forward: 5′- GATAACATGGCCATCATCAAGGA -3′ and Reverse: 5′-CGTGGCCGTTCACGGAG -3′ and for measure the CDNF mRNA levels the following primers were employed: Forward: 5′-AAAGAAAACCGCCTGTGCTA-3 and Reverse: 5′-TCATTTTCCACAGGTCCACA-3. The values were normalized to GAPDH mRNA levels. To detect GAPDH the following primers were used: Forward: 5′-AACGGGAAGCTTGTCATCAATGGAAA-3′ and Reverse: 5′-GCATCAGCAGAGGGGG CAGAG-3′.

2.11. Statistical analysis

The data represent the mean ± SEM (standard error of the mean) of results obtained from three independent experiments. For immunoblot and immunofluorescence analysis, data were normalized using the mean CI and CTCF, respectively. The dark condition was set as 100% for each dataset. The light induction was presented as the fold induction of the fraction obtained in normalized dark condition. Each dataset in the blue light condition was compared with its own normalized dark condition. To compare two groups (Dark vs Light), a "Student *t*-test" was performed using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA). Differences between experimental groups were considered statistically significant at a confidence level greater than 95% ($p < 0.05$). In figures $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

3. Results

3.1. Construction of the light-switchable GAVPO trans-activator and UAS-gene reporter lentiviral plasmids

This work proposed to generate a light-switchable lentiviral platform to control the expression of genes of interest in the CNS. For this purpose, we adapted the Light-On system into a lentiviral platform that could allow us efficient transduction of neurons. To this end, we first constructed two lentiviral plasmids and tested their expression in heterologous cells. This set of plasmids consisted of a plasmid that encodes the light-switchable GAVPO trans-activator protein and a second plasmid containing the UAS inducible promoter (UASx5-E1b) upstream of mCherry as a reporter gene. In their original description, the UAS inducible promoter contains a PolyA sequence upstream of the UASx5 element, which is not compatible with the generation of functional lentiviral particles. Therefore, two versions of the UAS-gene reporter lentiviral plasmid were constructed to test the functionality of the system by transfection in heterologous cells. A large version of the UAS inducible promoter containing the PolyA sequence (pF-$UAS_{(L)}$-mCherry-W) and a short version lacking the PolyA sequence (pF-$UAS_{(s)}$-mCherry-W) were constructed, both driving the expression of the reporter gene mCherry (Fig. 1A and Fig. 1B). To easily monitor GAVPO expression by EGFP fluorescence, we generated a bicistronic light-switchable GAVPO lentiviral plasmid (pFUG-1D/2A-HA-GAVPO-W), which encodes EGFP and GAVPO separated by 1D/2A bicistronic element (Fig. 1C). Also, to demonstrate the functionality of this lentiviral platform, we constructed a bicistronic UAS-gene reporter plasmid (pF-$UAS_{(s)}$-mCherry-1D/2A-HA-

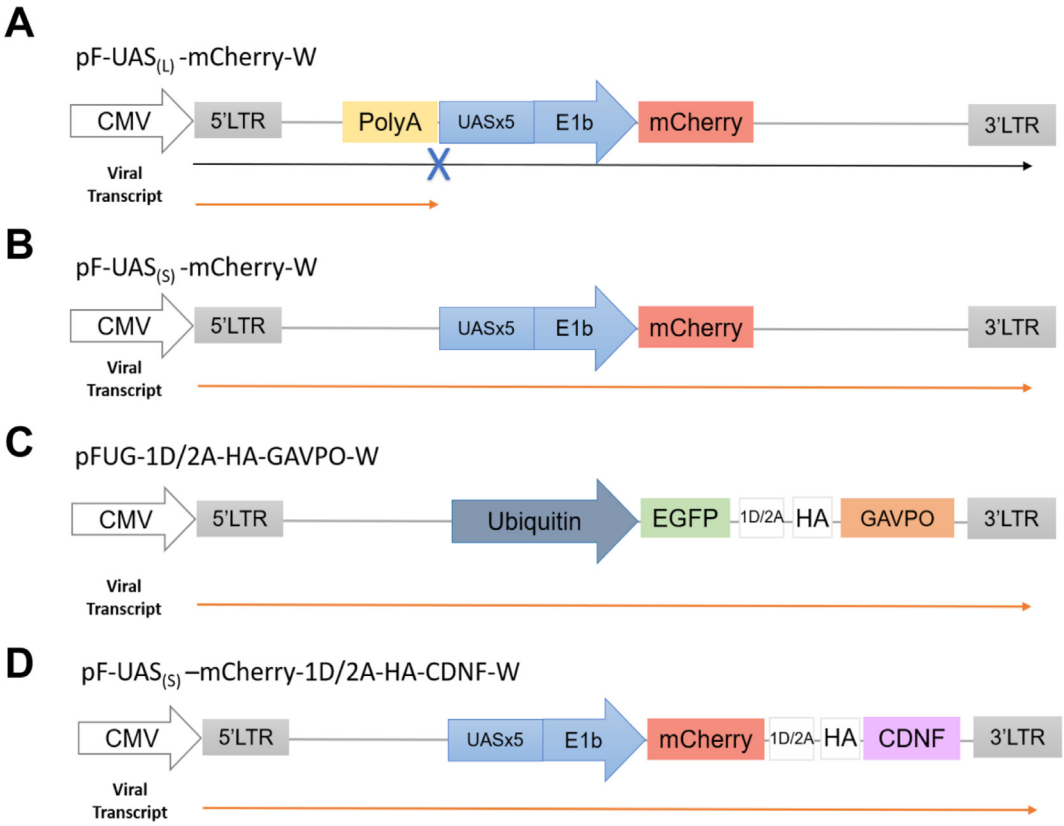

Fig. 1. Schematic illustration of the Light-On lentiviral plasmids. (Aand B) Schematic representation of both large and short versions of the UAS-gene reporter plasmids: pF-$UAS_{(L)}$-mCherry-W and pF-$UAS_{(S)}$-mCherry-W (without PolyA region, yellow). In these plasmids, mCherry (pink) expression is driven by the UASx5-E1b inducible promoter (light blue). (C) Schematic representation of pFUG-1D/2A-HA-GAVPO-W light-switchable GAVPO lentiviral plasmid, expressing EGFP (green) and the light-switchable transactivator GAVPO (orange) in a bicistronic manner, using 1D/2A bicistronic element from Foot-and-Mouth disease virus. (D) Schematic representation of the bicistronic UAS-gene reporter plasmid pF-$UAS_{(s)}$-mCherry-1D/2A-HA-CDNF-W, which encodes mCherry and CDNF (purple) as blue-light reporter genes in a bicistronic manner using the same bicistronic element (1D/2A) as in (C). In orange is shown the expected viral transcript for viral particle production step.

CDNF-W) that encodes mCherry and CDNF, a protein with significant therapeutic potential (Fig. 1D).

3.2. Expression and induction of Light-On lentiviral plasmids by blue light

A functional light-switchable expression system must have a low leakage expression in the absence of light; meanwhile, the induction of gene expression should be induced and tunable over a wide range of light intensities. To characterize the Light-On lentiviral platform, we transfected HEK293-T and PC-12 cell lines with the lentiviral plasmid system. The expression of EGFP and mCherry reporter proteins was determined by immunoblot and immunofluorescence. As shown in Fig. 2A, HEK293-T cells transfected with the bicistronic plasmid pFUG-1D/2A-HA-GAVPO-W expressed the GAVPO protein at the expected size as a result of the cleavage of the 1D/2A sequence. However, a portion close to 5% of the recombinant peptide remains non-cleaved (Supplementary Fig. 2), as previously reported [31]. To activate GAVPO and induce the expression of the reporter mCherry, the cell cultures were intermittently exposed to blue light for 24 h (15 min on/15 min off). As shown in Fig. 2A, the expression of GAVPO is constitutive and, as expected, not affected by blue light exposure. Interestingly, a significant 4–5 fold increase in mCherry protein levels is observed in HEK293-T exposed to blue light compared to cells maintained in darkness (Fig. 2A–C). Similar results were found by fluorescence analysis in HEK293-T (~4 fold) (Fig. 2D and Fig. 2E) but not in PC-12 cells, which exhibited a higher fold increase (20–30 fold) in mCherry fluorescence intensity (Fig. 2F and Fig. 2G). The difference in fold increase exhibited by these two cell lines is due to the different background signal detected for each cell line. PC-12 cells presented a 2–5 times lower background signal compared to HEK293-T cells (Supplementary Fig. 3). Despite these differences in fold induction, the lentiviral Light-On plasmid platform behaves as expected, allowing the light-controlled expression of a reporter mCherry in the tested cell-lines.

3.3. Inducing the expression of cerebral dopamine neurotrophic factor by blue light

As proof of concept, we tested in the optimized Light-On lentiviral platform the expression of Cerebral Dopamine Neurotrophic Factor (CDNF), a neurotrophic factor that shows promising therapeutic potential in neurodegenerative diseases [32]. We co-transfected HEK293-T cells with the light-switchable transactivator plasmid pFUG-1D/2A-HA-GAVPO-W and the bicistronic UAS-gene reporter plasmid pF-$UAS_{(s)}$-mCherry-1D/2A-HA-CDNF-W, which encodes mCherry and CDNF. Only cells exposed to blue light showed mCherry and CDNF expression (Fig. 3A). In the immunoblot analysis, while a low expression of mCherry was observed in dark conditions, (Fig. 3B, DARK), a significant 3–4 fold increase was detected for mCherry and CDNF in light conditions (Fig. 3B, LIGHT and Fig. 3D–E), compared to the dark situation. In order to establish that GAVPO was crucial to gene activation, we replaced the pFUG-1D2A-HA-GAVPO-W plasmid that allows GAVPO expression, with the pFUG-1D/2A-HA-CDNF-W plasmid that permits CDNF expression from a constitutive promoter. As expected, the expression of mCherry was not detected in the absence of GAVPO

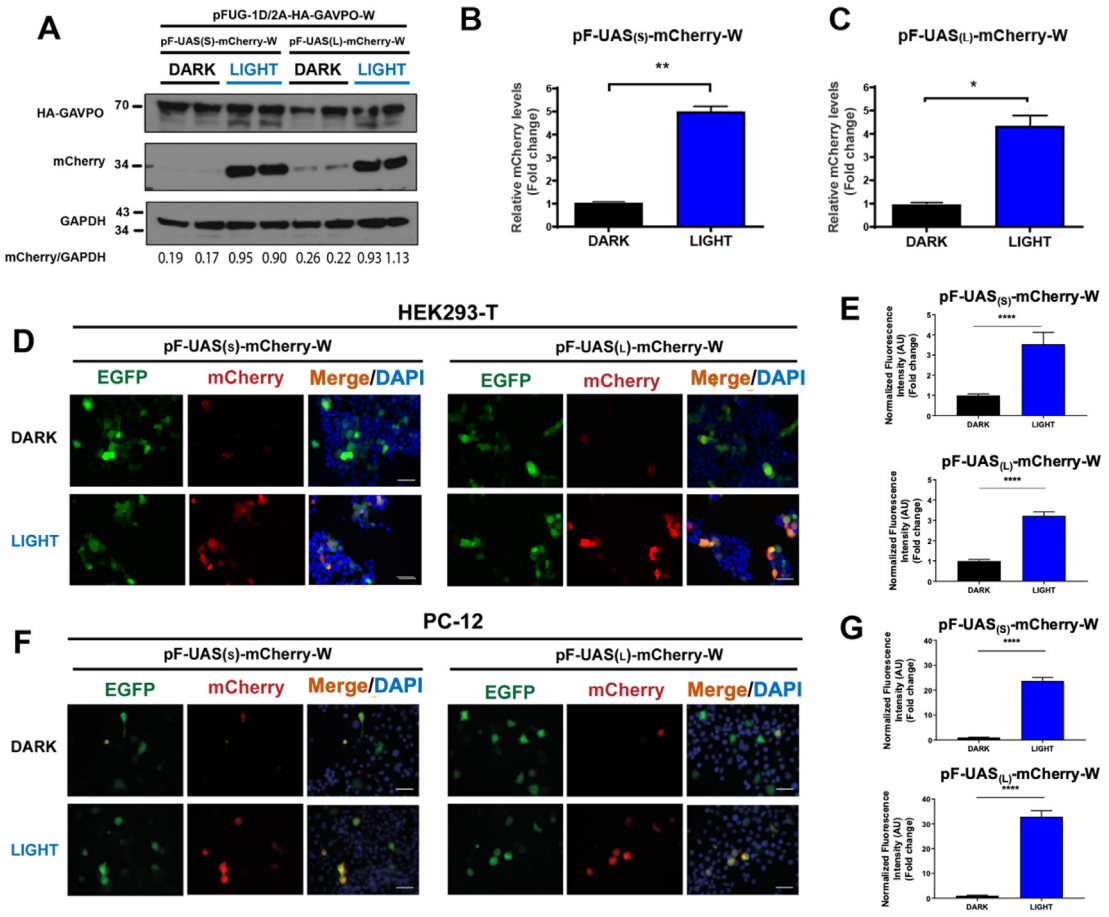

Fig. 2. Expression of lentiviral plasmids modulated by blue-light. (A) HEK293-T were co-transfected with pFUG-1D/2A-HA-GAVPO-W and pF-UAS$_{(L)}$-mCherry-W or pF-UAS$_{(S)}$-mCherry-W. Then, cells were exposed to darkness (DARK) or blue illumination (LIGHT) during 24 h, and western blots were performed to detect mCherry protein levels. HA epitope was used to reveal GAVPO expression. GAPDH was used as the loading control. (B and C) mCherry protein levels were quantified by densitometry as indicated in material and methods. The intensity of mCherry bands was normalized with the intensity of GAPDH bands. The normalized value is indicated in the figure. The dark condition was normalized using the CI mean as the 100% of all the dataset and results are expressed as fractions of the dark condition (fold change). (D and F) HEK293-T and PC-12 cells were visualized by fluorescence microscopy to observe EGFP and mCherry expression from UAS$_{(L)}$ and UAS$_{(S)}$ versions of UAS-gene reporter plasmids in darkness or blue illumination conditions. DAPI staining (blue) was used to visualize the nuclei of cells. (E and G) Quantification of fluorescence intensity of mCherry was performed in EGFP positive cells. Scale Bar: 50 μm. Data are presented as mean ± SEM. Student's *t*-test. *n* = 100 cells per condition.

(Fig. 3C), confirming that background expression observed in dark conditions is due to a background activation of GAVPO (Fig. 3B, DARK). It is important to note that the protein levels of CDNF obtained through induction by a constitutive promoter were similar to the protein levels of CDNF induced by blue light (Fig. 3B, LIGHT and Fig. 3C).

On the other hand, we evaluated a time-course expression of mCherry and CDNF induced by blue light during different time-windows (0.5, 1, 3, 6, 9, 12, and 24 h) in HEK293-T cells. Immunoblot and real-time PCR data showed a gradual increase in mCherry and CDNF protein levels (Fig. 3G and Fig. 3H) and the expression of their transcripts (Fig. 3I) during intermittently light-exposure (15 min on/15 min off) over 24 h. The profile of mCherry and CDNF protein levels is similar, with a constant increase until 24 h. In contrast, mCherry and CDNF mRNA levels were up-regulated until nine hours of light exposure and plateauing after this time point. Taken together, these results show that the two-component bicistronic lentiviral platform induces the expression of a gene of interest by blue light.

3.4. Expression of Light-On components by lentiviral particles in neurons

Finally, we evaluated the functionality of this light-switchable lentiviral platform in cultured rat hippocampal neurons. Lentiviral particles were produced in HEK293-T cells (see material and methods for details) and neurons at DIV 4 were co-transduced with FUG-1D/2A-HA-GAVPO-W and F-UAS$_{(S)}$-mCherry-W viruses with a MOI < 1 to avoid hyperinfection, this result with a low transduction efficiency, allowing to evaluate gene induction at a single cell level. At DIV 7, a group of neurons were exposed to blue light for three hours of intermittent light exposure (15 min on/15 min off). After this illumination protocol, we detected a significant higher mCherry expression in neurons exposed to blue light (6–7 fold induction) compared to neurons maintained in dark conditions (Fig. 4A and Fig. 4B). As expected, neurons expressing mCherry were also positive for EGFP expression (the reporter gene for GAVPO expression) and not mCherry expressing neurons were observed in the dark condition. We also performed the same experiment in neurons co-transduced with FUG-1D/2A-HA-GAVPO-W and F-UAS$_{(S)}$-mCherry-1D/2A-HA-CDNF-W viruses. Some neurons showed a distinctive mCherry and CDNF protein expression, as observed by immunofluorescence (Fig. 4C). Data analysis showed a 6-fold increase in mCherry and CDNF expression after blue light activation (Fig. 4D). Similar to the experiments using F-UAS$_{(S)}$-mCherry-W virus (Fig. 4A), cells expressing mCherry and CDNF were also positive for EGFP expression, indicating that expression was GAVPO-dependent. Moreover, we did not detect any cells expressing mCherry in dark conditions, further suggesting that a blue light-activated GAVPO mediates the expression. These results

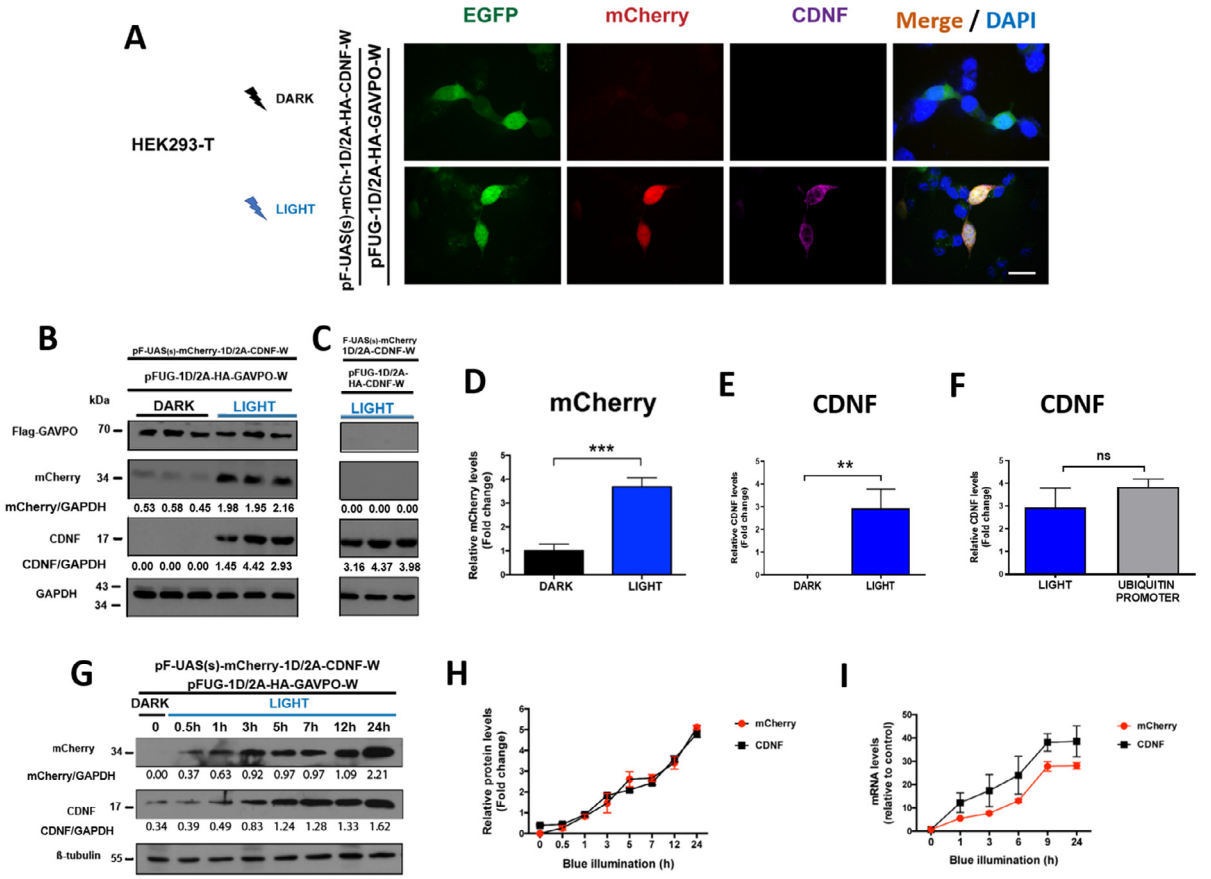

Fig. 3. Blue-light inducible expression of CDNF in HEK293-T cells. Plasmids pF-UAS$_{(s)}$-mCherry-1D/2A-HA-CDNF-W and pFUG-1D/2A-HA-GAVPO-W were co-transfected in HEK293-T cells. Then, cells were exposed to darkness or blue illumination conditions for 24 h. (A) Immunofluorescence against CDNF (purple) was performed, and cells expressing EGFP and mCherry were visualized by fluorescence microscopy. DAPI staining (blue) was used to visualize the nuclei of cells. Scale Bar: 20 µm. (B and C) Immunoblot in HEK293-T cells co-transfected with pF-UAS$_{(s)}$-mCherry-1D/2A-HA-CDNF-W and pFUG-1D/2A-HA-GAVPO-W or pFUG-1D/2A-HA-CDNF-W (as a control to substitute GAVPO expression). After 24 h with blue illumination or darkness condition, cells were homogenized, and western blot was performed to detect Flag-GAVPO, mCherry, CDNF, and GAPDH proteins. GAPDH was used as loading control. mCherry and CDNF bands were normalized with GAPDH bands. The normalized values are indicated in the figure. Bar graphs show the quantification of relative mCherry and CDNF protein levels. (F) Temporal characterization of the inducible expression of mCherry and CDNF under blue-light illumination. HEK293-T cells were co-transfected with pFUG-1D/2A-HA-GAVPO-W and pF-UAS$_{(s)}$-mCherry-1D/2A-HA-CDNF-W for 24 h and then exposed to darkness (0 h) or blue illumination conditions by times indicated in the figure. Cells were homogenized, and western blot was performed to detect mCherry and CDNF. GAPDH was used as loading control. (G) Quantification of mCherry and CDNF protein levels in (F). (H) Quantification of mCherry and CDNF mRNA levels quantified by real-time PCR using specific primers.

show the functionality of the Light-On lentiviral platform to induce light-dependently the expression of a gene of interest in neurons.

4. Discussion

In this first adaptation of the Light-On system to a lentiviral platform, we constructed two lentiviral plasmids that express separately the previously reported Light-On system components [10]; a transactivator lentiviral plasmid that expresses GAVPO and a UAS-gene reporter plasmid, in order to facilitate viral particle production, and their subsequent use. The original version of the Light-On plasmid described by Wang and co-workers has a PolyA region upstream of the UASx5 sequence [10], presumably to be used as an insulator element to stop any upstream promoter activity. As was explained before, this PolyA region is incompatible with the lentiviral platform because it interferes with the synthesis of the complete viral transcript to be pack during viral assembly. Therefore, we constructed a bicistronic UAS-gene reporter plasmid that expresses mCherry and CDNF without the PolyA sequence (UAS$_{(S)}$). The results show that this lentiviral plasmid containing the UAS$_{(S)}$ promoter without the PolyA sequence allows the proper synthesis of viral transcripts and packaging of a functional lentivirus. Neurons transduced with these lentiviral particles expressed mCherry and CDNF in the presence of GAVPO under light conditions, while a low background expression was observed in darkness. Thus, these results indicate that the Light-On system is fully compatible in the presented configuration of the lentiviral platform.

When testing the Light-On lentiviral system in blue-light induction experiments, we observed a significant increase in mCherry protein levels (Fig. 2), in a manner that is strictly dependent on the presence of GAVPO, confirming that the UAS promoter can be used as an orthogonal system in the tested cell-lines. Indeed, although some mCherry background expression is observed in cells maintained in the dark, this is caused by background activation of GAVPO, which completely disappears when GAVPO is not transfected into cells (Fig. 3C).

The induction levels reached in our experiments are lower than the values reported by Wang et al. [10], which could be attributed to differences in plasmid size or a difference in the quantification of the reporter gene. Wang and co-workers evaluated transcript levels of a stabilized reporter gene by RT-qPCR and obtained 200–300-fold induction levels. Here, we quantified the transcript levels of reporter genes obtaining an induction of ~40-fold (Fig. 3H). This discrepancy could be explained due to different mRNA degradation rates since we used non-stabilized transcripts.

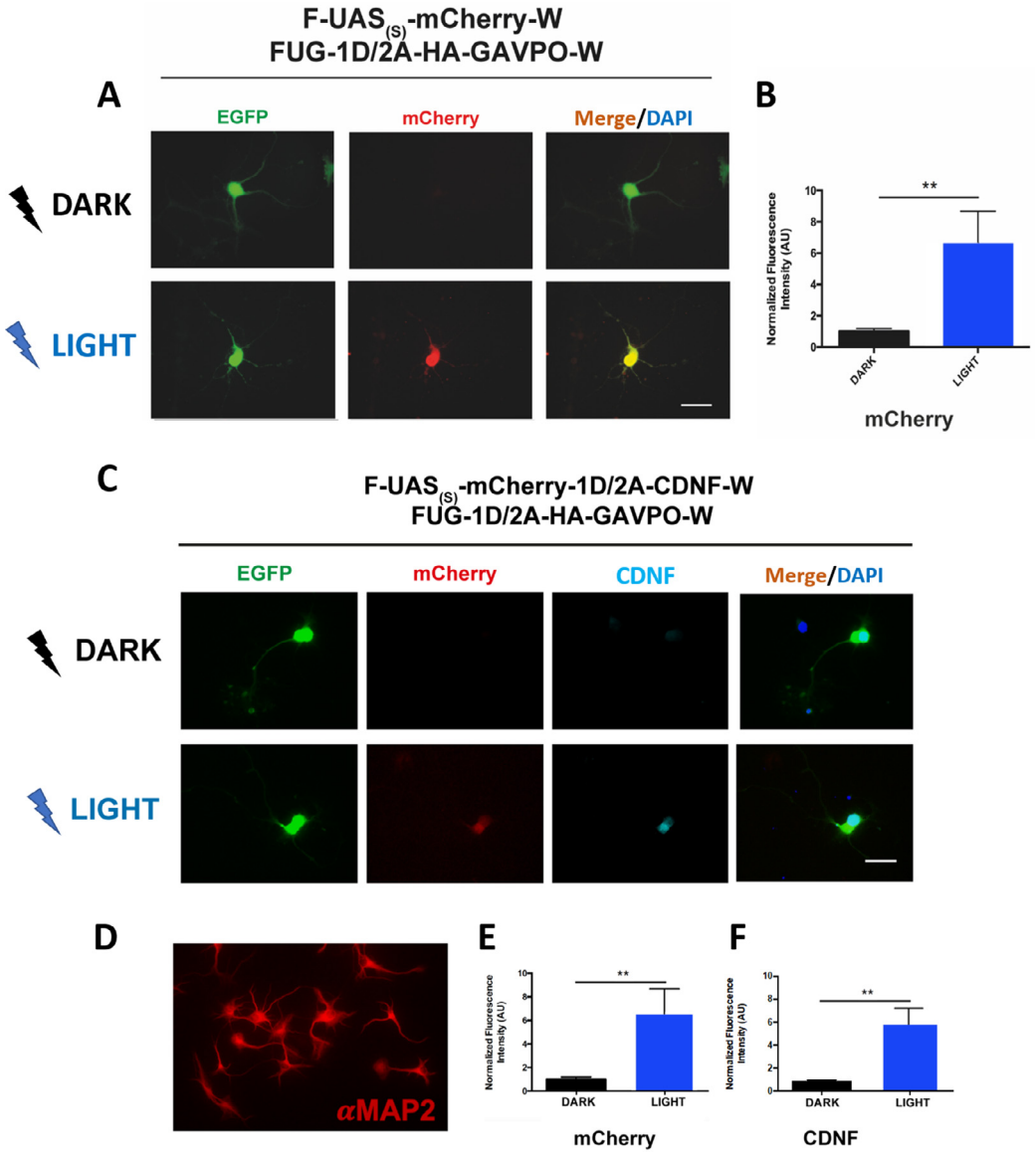

Fig. 4. Blue-light inducible expression of CDNF in rat cultured hippocampal neurons. (A and B) Rat hippocampal neurons were co-transduced with F-UAS$_{(S)}$-mCherry-W or F-UAS$_{(S)}$-mCherry-1D/2A-CDNF-W and FUG-1D2A-HA-GAVPO-W lentiviruses at DIV 4. Then, neurons at DIV 7 were illuminated with blue light for three hours and fixed. (A) Neurons were visualized by fluorescence microscopy to observe mCherry expression (red) in dark or light conditions. Also, EGFP was visualized as reporter of GAVPO expression. Scale Bar: 20 μm. (B) Quantification of fluorescence intensity of mCherry. (C) Immunofluorescence against CDNF was performed, and fluorescence microscopy was used to observe neurons expressing EGFP (green), mCherry (red), and CDNF (purple). DAPI staining (blue) was used to visualize the nuclei of neurons. Scale Bar: 20 μm. (D) MAP2 was used to visualize neurons. (E and F) Quantification of fluorescence intensity of mCherry and CDNF. *n* = 10 neurons per condition.

Despite these differences in transcript induction, a five-fold change in protein levels was obtained after 24 hours illumination for the mCherry reporter and the CDNF factor. These levels are similar when using a constitutive promoter, suggesting that this system could achieve a significant physiological relevant level of protein expression when activated (Fig. 3G). Furthermore, light-induction in HEK293-T cells reach CDNF protein expression levels similar to an average constitutive promoter (Ubiquitine C, Fig. 3C), which has been shown to modulate the unfolded protein response (UPR) in a previous work [29].

It is important to note that we detected differences in the induction of mCherry between HEK293-T and PC-12 at individual cells (Fig. 2D–G). The difference is produced by the different background signals between both cell lines analyzed. This variability in background signal in different cell lines is a phenomenon that was also reported by Wang and colleagues [10]. In our studies, the raw data in PC-12 cells showed 2–5 times lower background level for mCherry compared to HEK293-T cells, but after light-induction both cells achieved similar levels of mCherry expression (Supplementary Fig. 3). The normalized data result in a net 20–30 fold increased expression in PC-12 cells (Fig. 2G). Considering that PC-12 is neuronal in its origin, this result suggests that the platform could be of use in neurons.

The transduction experiments in hippocampal neuronal cultures revealed an inducible expression of mCherry and CDNF as a result of GAVPO blue-light activation. Interestingly, we did not detect the background expression of mCherry in EGFP positive neurons in dark conditions (Fig. 4A and Fig. 4C). Although it is possible to assume that some of the EGFP positive neurons were not co-transduced with the mCherry reporter virus, we did not observe neurons expressing mCherry in darkness conditions, suggesting that the activation of mCherry expression is due to the light activation of GAVPO. In dark conditions, low immunostaining of CDNF was observed in EGFP transduced neurons, that may well

correspond to the endogenous levels of CDNF in neurons as previously reported [33]. Interestingly, with three hours of light-induction the levels of CDNF immunostaining significantly increase 6-fold, suggesting that this platform could achieve therapeutic levels of trophic factors in neurons after short light exposure.

In the present study, we demonstrated that the Light-On system adapted to the lentiviral platform is inducible by blue light. Using light as an inducer, adjustments in expression levels can be easily made, and a high spatial and temporal resolution can be achieved. With the four-fold induction level observed by immunoblots, we suggest that this optogenetic tool could be applied in neuronal systems to regulate the expression levels of target genes finely, providing another suitable option for the existing gene therapy approaches, that in combination with new technologies of illumination through nanoparticles [34] could bring new developments for therapies oriented to CNS.

Conflict of interest

The authors declare that they have no competing interests.

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Supplementary material

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