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ROLE OF CEREBRAL DOPAMINE NEUROTROPHIC FACTOR IN ENDOPLASMIC RETICULUM STRESS

Thesis submitted to the Pontificia Universidad Católica de Chile in partial compliance with the requirements for the Ph.D. in Biological Sciences with a mention in Cellular and Molecular Biology

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DEDICATORY

This thesis is dedicated to my wonderful parents, Jorge Arancibia and Isabel Radich, my brother Jorge, my sister Isabel, my nephew Dante and my beloved Rafaella.

Whose selfless love, support, faith, and trust in me have brought me here. They have always allowed me to pursue my wildest dreams, still believing that I could achieve what I wanted.

Your encouragement and unwavering love have given me the greatest motivation to continue and achieve this degree. I hope you feel proud of me. For this, and for all the countless things they have given me, thank you, thank you very much.

EPIGRAPH

“Don't wait until everything is just right. It will never be perfect. There will always be challenges, obstacles, and less than perfect conditions. So what. Get started now. With each step you take, you will grow stronger and stronger, more and more skilled, more and more self-confident and more and more successful.”

-Mark Victor Hansen

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I must thank my beloved, Rafaella, for always putting a smile on my face and showing me the right side of things when I was discouraged. I cannot thank her enough for being the yin of my yang, often ungovernable, for the happiness, joy, and laughter that she brought to my life, completing it.

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ABBREVIATIONS

6-OHDA: 6-hydroxydopamine

ATF4: Activating transcription factor 4

ATF6: Activating transcription factor 6

BBB: Blood-Brain Barrier

CDNF: Cerebral Dopamine Neurotrophic Factor

DA: Dopamine

eIF2α: Initiating factor of eukaryotic translation 2α

ER: Endoplasmic reticulum

GRP78: Chaperone regulated by glucose-78

HEK293-T: Human Embryonic Kidney 293 T

IF: Immunofluorescence

IRE1: Enzyme that requires inositol-1

MANF: Mesencephalic astrocyte-derived Neurotrophic Factor

PERK: Pancreatic endoplasmic reticulum kinase

ROS: Reactive oxygen species

UPR: Unfolded Protein Response

WB: Western Blot

XBP1: Box binding protein X

RESUMEN

El factor neurotrófico cerebral dopaminérgico (CDNF) es un péptido de 187 aminoácidos localizado en el retículo endoplasmático de numerosos tipos celulares y conservado evolutivamente. CDNF ejerce un efecto protector sobre las neuronas en modelos celulares y animales de diversas enfermedades neurodegenerativas, pero se desconoce el mecanismo molecular de este efecto. Muchas enfermedades neurodegenerativas se han relacionado con una desregulación de la proteostasis en el retículo endoplásmico. La desregulación de la proteostasis produce estrés reticular lo que gatilla inicialmente la respuesta adaptativa a proteínas mal plegadas y, si el estrés persiste en el tiempo, se inducen vías de señalización relacionadas a la muerte celular o apoptosis. Varios estudios han revelado que una respuesta adaptativa resuelve el estrés de retículo endoplasmático atenuando la síntesis de proteínas, induciendo la expresión de chaperonas y metabolizando las proteínas mal plegadas. En esta tesis, investigamos si CDNF regula la proteostasis del retículo endoplásmico. Con este fin, se determinó la función de CDNF en un modelo de estrés de retículo endoplásmico inducido por Tapsigargina, en células HEK293-T y neuronas hipocampales en cultivo. Se observó que la expresión de CDNF aumentó significativamente la viabilidad de células HEK293-T expuestas a Tapsigargina. Este efecto, correlacionó con aumento de los niveles de proteínas protectoras de la respuesta a proteína mal plegada temprana tales como BiP, ATF4, ATF6 y XBP-1, tanto en las células HEK293-T como en neuronas. Además, la expresión de CDNF redujo los niveles de las proteínas pro-apoptóticas CHOP y caspasa-3 activa. Se determinó que CDNF protege a las células desde el retículo endoplásmico, dado que una versión mutante de CDNF carente de la secuencia de retención en retículo endoplásmico, y por lo tanto secretada, no protegió a las células frente al estrés inducido por Tapsigargina. En conclusión, CDNF regula la proteostasis en retículo endoplásmico induciendo la respuesta a proteína mal plegada adaptativa e inhibiendo las vías pro-apoptóticas activadas por el estrés de retículo endoplásmico. Finalmente, en la última parte de esta tesis se diseñó y construyó un dispositivo molecular de expresión regulada de CDNF, que combina métodos optogenéticos con una plataforma lentiviral.

ABSTRACT

The cerebral dopamine neurotrophic factor (CDNF) is a peptide of 187 amino acids located in the endoplasmic reticulum of numerous cell types and evolutionarily conserved. CDNF exerts a protective effect on neurons in cellular and animal models of various neurodegenerative diseases, but the molecular mechanism of this effect is unknown. Many neurodegenerative diseases are associated with the deregulation of proteostasis in the endoplasmic reticulum. Deregulation of proteostasis produces reticular stress, which initially triggers the response to misfolded proteins and, if stress persists over time, signaling pathways related to cell death or apoptosis are induced. Several studies have revealed that an adaptive response resolves the endoplasmic reticulum stress by attenuating protein synthesis, inducing the expression of chaperones, and metabolizing misfolded proteins. In this thesis, it was investigated whether CDNF regulates proteostasis of the endoplasmic reticulum. To this end, the function of CDNF was determined in an endoplasmic reticulum stress model induced by Thapsigargin, in HEK293-T cells and hippocampal neurons in culture. It was observed that the induced expression of CDNF significantly increased the viability of HEK293-T cells exposed to Thapsigargin. This effect correlated with the increase in protective protein levels of the early misfolded protein response such as BiP, ATF4, ATF6, and XBP-1, both in HEK293-T cells and in neurons. Also, the induced-expression of CDNF reduced the levels of pro-apoptotic proteins CHOP and active caspase-3. It was determined that CDNF protects cells from the endoplasmic reticulum, since a mutant version of CDNF, lacking the endoplasmic reticulum retention sequence, and therefore secreted, did not protect the cells against stress-induced by Thapsigargin. In conclusion, CDNF regulates proteostasis in the endoplasmic reticulum by inducing the response to adaptive misfolded protein and inhibiting pro-apoptotic pathways activated by endoplasmic reticulum stress. Finally, in the last part of this thesis, a molecular device for the regulated expression of CDNF that combines optogenetic methods with a lentiviral platform was designed and constructed.

1. INTRODUCTION

1.1 Neurotrophic factors

Neurotrophic factors are essential peptides for growth, maturation, and survival of neurons. To date, many neurotrophic factors have been discovered and it has been recognized that they have several biological effects, among them: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4; neurotrophic factor derived from the glial cell line (GDNF) and its family of ligands, such as neurturin, and neurotrophic cytokines. Along with these, cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) have been discovered and characterized as a new family of neurotrophic factors (Figure i) (Lindholm et al. 2007; Petrova et al. 2003).

Preclinical data using several models of neurodegenerative disorders show alterations in the levels, and even in the function of neurotrophic factors. Therefore, neurotrophic factors have received increasing attention for their therapeutic potential, especially for Alzheimer's and Parkinson's diseases (Rangasamy et al. 2010). Neurodegenerative diseases are complex, and their etiology is diverse. Current treatments aim to alleviate the symptoms, instead of preventing the loss of synapses and neuronal death. Therefore, neurogenic agents, including neurotrophic factors, represent a new therapeutic strategy to address the degenerative process itself.

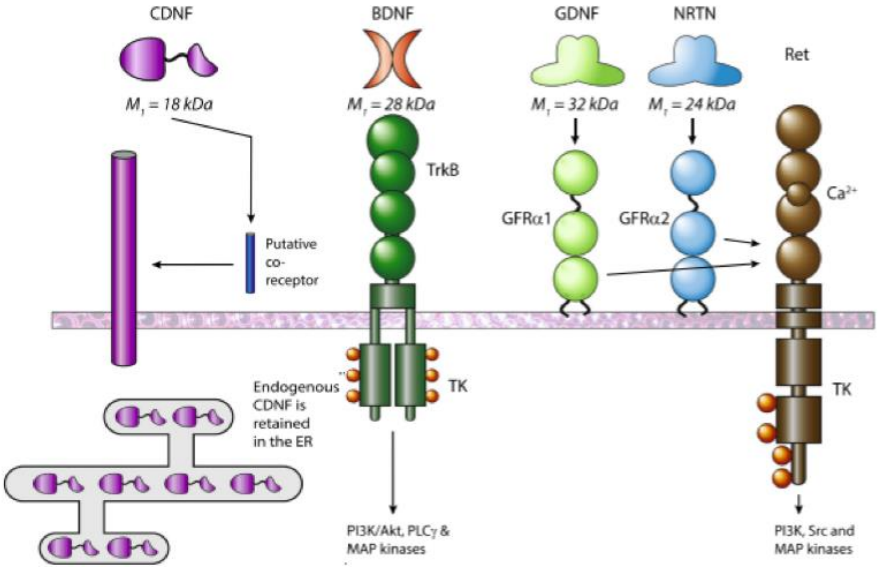

Figure i. The mode of action of CDNF is drastically different from other known neurotrophic factors such as BDNF, GDNF, or NRTN. Neuronal survival-promoting actions of BDNF, GDNF, and NRTN are mediated by transmembrane tyrosine kinase receptors leading to the activation of PI3K/Akt, PLCc, and MAP kinases pathways. On the other hand, different from other neurotrophic factors, CDNF is retained in the endoplasmic reticulum (Voutilainen, Arumäe, Airavaara, & Saarma, 2015).

1.2 Cerebral Dopamine Neurotrophic Factor (CDNF)

A few years ago, a new class of neurotrophic factors was discovered, mesencephalic astrocyte-derived neurotrophic factor (MANF) (Petrova et al. 2003) and cerebral dopamine neurotrophic factor (CDNF) (Lindholm et al. 2007). They form a distinct family that does not share structural similarities with other families of neurotrophic factors (Figure 1). MANF was initially purified from conditioned media from an immortalized ventral mesencephalic astrocytic cell line (Petrova et al. 2003) and its sequence determined by a combination of proteomics and bioinformatics technologies. CDNF was discovered by Lindholm and colleagues in 2007 using a bioinformatics approach and confirmed by biochemical and molecular studies (Lindholm et al., 2007). MANF and CDNF proteins function as secreted neurotrophic factors, but some of their activities might occur while they are resident in the endoplasmic reticulum (ER) lumen due to a non-canonical ER retention signal.

CDNF is an 18 kDa peptide of 187 aminoacids, of which the N-terminal 26 amino-acids are a signal peptide that is cleaved at the ER. This neurotrophic factor is expressed in both neuronal and non-neuronal tissues, and although databases report a splicing variant for CDNF, no experimental confirmation is available. The structural analysis of the mature peptide shows two featured domains, a saposin-like domain localized in the N-terminal and a disulfide bridge formed by two cysteine residues localized in the C-terminal (Apostolou et al., 2008; Parkash et al., 2009). Besides, the C-terminal domain has a non-canonical KDEL retention sequence (QTEL in rat, KTEL in human), suggesting that CDNF could be an ER-resident protein (Lindahl and Lindholm, 2017). However, many studies in cell lines have shown that CDNF could be secreted to the extracellular medium (Latge et al. 2015; Mei and

Niu 2014; Norisada et al., 2016; Sun et al. 2011). This dual localization of CDNF has led to propose that the neuroprotector effect could be exerted either by secreted CDNF or by direct effect in the ER.

Regarding the mode of action of CDNF, as shown in Figure ii, the N-term saposin motif of this protein could confer the capacity to interact with lipids in the plasma membrane, but this idea has not been demonstrated to date. On the other hand, the C-terminal contains a disulfide bond between conserved cysteines in a CXXC motif. The CXXC motif is a consensus sequence of proteins of the thiol-protein oxidoreductase superfamily, other members of which include thioredoxins, glutaredoxins, and peroxiredoxins. Common to this enzyme superfamily is that all members are involved in disulfide-mediated redox reactions and glutathione metabolism in which the CXXC domain takes center stage. However, no enzymatic oxidoreductase activity has been detected for CDNF (Figure ii).

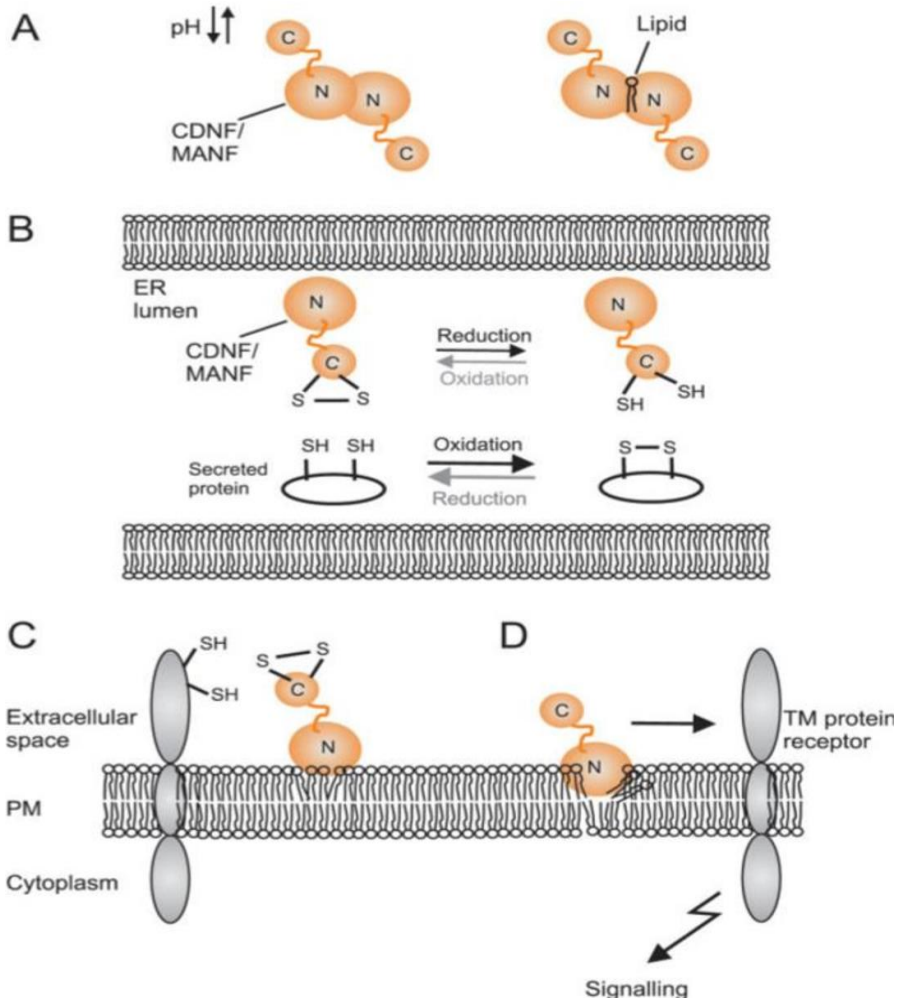

Figure ii. Proposed modes of action of CDNF. (A) Changes in pH or lipid binding may induce the dimerization of CDNF. (B) The C-terminal domain of CDNF may facilitate the formation of disulfide bridges on secretory proteins in the ER. (C) CDNF may reduce disulfide bonds on a transmembrane receptor protein on the cell surface. (D) CDNF may interact with the cell surface membrane and subsequently activate a transmembrane receptor protein. (Adapted from Lindholm & Saarma, 2010).

1.3 Role of CDNF in neurodegenerative diseases

CDNF is significantly expressed in neurons of the cortex, the hippocampus, and the striatum (Lindholm et al., 2007). However, CDNF is not detectable in positive tyrosine hydroxylase cells, a protein present in dopamine neurons. Despite not being expressed in dopamine neurons, the infusion of CDNF in the striatum and substantia nigra has neuroprotective and, more importantly, neurorestorative effects in animals treated with 6-OHDA and MPTP toxins (Airavaara et al., 2012; Lindholm et al., 2007; Voutilainen et al., 2011). Intriguingly, the neuroprotective effect was also observed when neurons of the substantia nigra were transduced with an adeno-associated virus or lentivirus encoding CDNF, supporting the idea that it could protect the cells from intracellular compartments (Bäck et al. 2013; Cordero-Llana et al. 2015; Wang et al. 2017). Another study showed that CDNF and MANF together produced synergistic neuroprotection and neurorestoration effects on the nigrostriatal system (Cordero-Llana et al., 2015). It should be noted that the neuroprotective effect of CDNF is not only circumscribed to dopamine neurons, but also hippocampal neurons were protected in an animal model of Alzheimer's disease (Kemppainen et al., 2015). Finally and, in accordance with the above, other study showed that CDNF alleviates synaptotoxicity induced by β-amyloid (Zhou et al., 2016), suggesting that CDNF could be of importance in others neurodegenerative diseases.

In this context, several studies have suggested that a common component among neurodegenerative disorders such as Parkinson's and Alzheimer's diseases is the aggregation of misfolded proteins. Regardless of the cause, the aggregation of proteins would cause the

death of specific populations of neurons due to an alteration of the proteostasis of the ER. Supporting this idea, it was reported that the treatment with 6-OHDA causes ER stress in dopamine neurons (Holtz et al., 2006; Ryu et al., 2002; Ryu et al., 2005), and in PC-12 cells (Elmazoglu et al., 2017). Similarly, Alzheimer's disease has been related to a disturbance in the homeostasis of the ER (Hoozemans et al., 2005; Hoozemans et al., 2012). Taken together, the data discussed above suggest that the protection and restorative effect exerted by CDNF could be due to its role in relieving ER stress.

1.4 Endoplasmic Reticulum and the Unfolded Protein Response

The ER is an essential organelle for the functioning of the cell since it participates in the folding and processing of secretory and membrane proteins. Therefore, ER is pivotal for cellular proteostasis, which refers to the balance between synthesis, folding, and protein degradation. Alterations of ER homeostasis results in the accumulation of misfolded proteins, a condition called ER stress. In turn, ER stress activates several signaling pathways known as "unfolded protein response" (UPR) (Ron & Walter, 2007).

The UPR is mediated by three receptors present in the ER membrane that act as ER stress sensors: Enzyme requiring inositol-1 (IRE1), pancreatic ER kinase (PERK), and activator transcription factor-6 (ATF6) (Walter and Ron 2011). It has been proposed that ER stress triggers the dissociation of these three receptors from the chaperone protein regulated by glucose-78 (GRP78/BiP), which binds to hydrophobic residues of misfolded proteins (Figure 3) (Reviewed in Ron & Walter, 2007). However, it has been reported recently that UPR can

be activated of a BiP-independent manner, which misfolded proteins during ER stress can bind to PERK and IRE1, promoting their oligomerization and activation (Adams et al. 2019; Wang et al. 2018).

Essentially, the progression of the UPR comprises two main phases: the adaptive UPR and the terminal UPR that play a restorative and apoptotic role, respectively (Rutkowski & Kaufman, 2007; Tabas & Ron, 2011; Erguler, Pieri, & Deltas, 2013). In the adaptive phase, UPR signaling pathways are vital to restoring the ER proteostasis. During this phase, both PERK and IRE1 are activated by oligomerization and phosphorylation (Hamanaka et al., 2005; Ron & Hubbard, 2008). At the same time, ATF6 is cleaved in the Golgi apparatus, and its N-terminal domain translocates to the nucleus as well as the spliced form of the X-box binding protein (XBP1), which is subjected to an IRE1 ribonuclease activity. In the nucleus, both ATF6 and XBP1, which act as transcription factors, increase the expression of chaperones and genes related to degradation mediated by the ER (ERAD) (Chen et al., 2002).

In parallel, global protein translation is attenuated by the PERK-eIF2a-ATF4 pathway, thus reducing the overload of the ER, while the rate of degradation of accumulated proteins is markedly increased. Finally, if the ER stress persists, the UPR activates the signaling pathways PERK-eIF2a-ATF4-CHOP and IRE1-TRAF2-ASK1-JNK, which lead to mechanisms of programmed cell death or apoptosis (Reviewed in Chan et al. 2015).

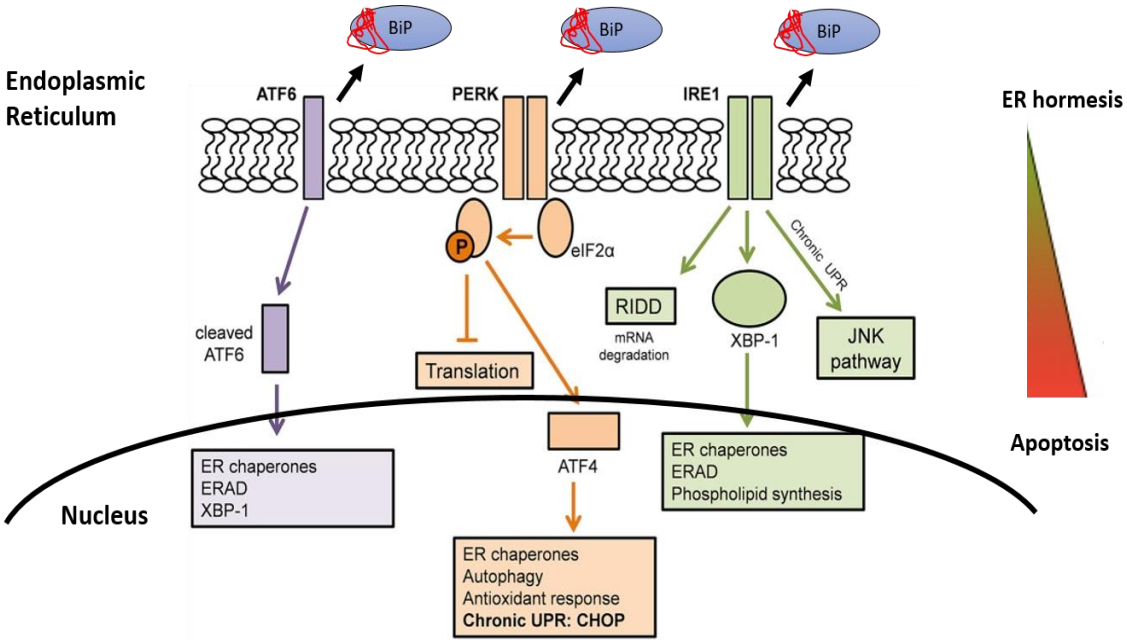

Figure iii. The Unfolded Protein Response is mediated by three receptors present in the ER. Three membrane proteins are the ER stress sensors: Enzyme requiring inositol-1 (IRE1), pancreatic ER kinase (PERK), and activating transcription factor-6 (ATF6). During ER stress, these receptors dissociate from the protein chaperone regulated by glucose-78 (BiP/GRP78), which joins to a hydrophobic place of misfolded proteins (Adapted from Hetz and Mollereau, 2014).

1.5 Unfolded Protein Response and Neurodegenerative Diseases

Activation of UPR signaling pathways has been observed in various diseases, such as diabetes, inflammation, and neurodegenerative diseases (Lin, Walter, & Yen, 2008; Scheper & Hoozemans, 2015; Wang & Kaufman, 2012). One of the characteristics observed in these diseases is the presence of aggregated or misfolded proteins. Among them are the aggregates of α-synuclein in Parkinson´s disease, pTau, and beta-amyloid in Alzheimer´s disease, and Huntingtin in Huntington's disease. In recent years, there has been growing evidence that alterations in proteostasis in the ER contribute to the pathological process (Brown & Naidoo, 2012; Matus, Glimcher, & Hetz, 2011). Interestingly, post-mortem brain samples of patients suffering Alzheimer's and Parkinson's diseases show strongly increased UPR markers such as phospho-PERK and phospho-eIF2α, a feature also observed in animal models of these neurodegenerative diseases (Hartmann, 2004; Hoozemans et al., 2005; Hoozemans et al., 2007).

1.6 CDNF: Modulator of the Unfolded Protein Response?

As discussed in previous paragraphs, CDNF has a protective and restorative effect on DA neurons that degenerate due to the application of 6-OHDA and MPP in preclinical models of Parkinson's disease or neuronal cultures. Both 6-OHDA and MPP have been strongly associated with ER stress (Ryu et al. 2002), and the inhibition of ER stress could exert neuroprotection in these models (Cai et al., 2016). The non-conventional neurotrophic factor CDNF is an ER-resident protein, and it is unclear whether it can be secreted during ER stress. Thus, as an ER-resident protein, it could have a role inside the ER, possibly regulating the unfolded protein response pathways.

2. HYPOTHESIS AND OBJECTIVES

The molecular mechanism of action of CDNF remains unresolved. Our preliminary data show that overexpressing CDNF increases the expression of BiP (Figure 1), a protein that has been reported that is involved in the modulation of the UPR. Thus, the neuroprotection observed by CDNF in different cellular and animal models could be mediated by inducing a preemptive activation of the UPR.

The maintenance of proteostasis in the ER is fundamental for the proper functioning of the secretory capacities of cells that produce hormones and neurotransmitters, and like-wise essential in cells with high structural complexity as observed in neurons (Walter & Ron 2011). Aberrant activation of the UPR signaling pathways has been found in various diseases such as diabetes, inflammation, and neurodegenerative diseases. As a consequence, a disturbance in the ER proteostasis could lead to cell death. It is the mechanism that has been proposed as the main pathway leading to the loss of neurons in neurodegenerative diseases (Hoozemans et al., 2005; Hoozemans et al., 2007).

Neurons as secretory cells of high structural complexity are characterized by a constant vesicle production and protein demand to maintain its function and structure. Therefore, the overload in the ER is critical and makes the neurons more susceptible to a disturbed UPR. The protective and restorative effect of CDNF on dopaminergic neurons in animal models of Parkinson’s disease locates this neurotrophic factor among those with high therapeutic potential (Lindholm et al., 2007; Airavaara et al. 2012). However, the cellular and molecular

mechanisms of neuroprotection and restoration exerted by CDNF have not been elucidated, waiting to be uncovered to assess its full therapeutic potential. In summary, given the protective effect of CDNF against neuronal degeneration and the emerging evidence pointing to presumed participation in the UPR during ER stress, the following hypothesis is presented:

"CDNF exerts a cytoprotective effect by restoring ER proteostasis through the activation of the misfolded protein response (UPR) pathways."

Main Objective

To determine whether CDNF exerts a cytoprotective effect by activation of the misfolded protein response pathways (UPR) during the induction of a pharmacological ER stress by either an extracellular or intracellular action.

Specific objectives

1. To determine if CDNF exerts a cytoprotective effect by modulating the UPR

1.1 To evaluate whether the gain of function of CDNF exerts a cytoprotective effect in HEK293-T cells and neurons through the induction of UPR pathways, during a pharmacological ER stress condition.

1.2 To evaluate whether the gain of function of CDNF exerts a cytoprotective effect in HEK293-T cells and neurons, through the attenuation of the apoptosis pathways induced by ER stress.

2. To determine whether the cytoprotective effect of CDNF is intra or extracellular.

2.1 To evaluate the cytoprotective effect of a CDNF mutant lacking the non-canonical ER retention sequence QTEL (CDNF-ΔQTEL).

2.2 To evaluate whether the gain of function of CDNF-ΔQTEL exerts a cytoprotective effect in HEK293T cells through the induction of UPR pathways, during a pharmacological ER stress condition.

2.3 To evaluate if the gain of function of CDNF-ΔQTEL exerts a cytoprotective effect in HEK293T cells, through the attenuation of apoptosis pathways induced by ER stress.

3. To generate a lentiviral platform to assess the delivery of CDNF in a spatially and temporally dosed manner with potential therapeutic application.

3.1 To construct a lentiviral vector that encodes CDNF and whose expression is regulated through an optogenetic system.

3.2 To determine the light conditions that allow controlled expression of CDNF in neurons.

3.3 To assess the activation of the UPR pathways in cells expressing CDNF regulated by blue-light.

FIRST PART DOCTORAL THESIS

CDNF is part of a group of unconventional neurotrophic factors with cytoprotective and regenerative effects in neurons of animal models of different diseases (Bäck et al. 2013; Cordero-Llana et al. 2015; Glembotski et al. 2012; Lindahl, Saarma, and Lindholm 2017; Lindholm et al. 2007; Mätlik et al. 2017; Voutilainen et al. 2009). The exact function of MANF and CDNF in the nervous system is not yet understood, but their mechanism of action differs from traditional neurotrophic factors, which exert their actions through binding and activation of cell trans-membrane receptors (Airaksinen and Saarma 2002). Thus, the molecular mechanism behind the cellular protective effects of CDNF in animal models of different diseases is still not understood, but it has been suggested to be related to modulation of ER stress.

In the first part of this thesis, we explored the possibility of whether CDNF, given its intracellular location, could be regulating the survival pathways that modulate ER homeostasis. Among the many survival pathways that have been reported so far, we decided to evaluate the UPR pathway signaling, due to its location in the ER and its association with many neurodegenerative diseases. In this context, this first part of our study focuses on 1) To evaluate whether CDNF protects against ER stress in a thapsigargin-induced cellular model of ER stress. 2) To determine if CDNF modulates the adaptive response of the three main UPR pathways, and finally 3) To evaluate whether CDNF modulates cell death pathways associated with ER stress.

Establishing a cellular model for the study of ER stress

Although most studies with CDNF show a protective role in dopaminergic neurons, CDNF is expressed in non-neural tissues, and its cytoprotective activity extends beyond the dopaminergic system (Latge et al. 2015; Liu et al. 2018; Mei and Niu 2014). To characterize the expression of CDNF, we performed western blot analysis in different cell lines. The results show that CDNF protein levels vary among the different cell lines, observing a considerable endogenous expression in rat PC-12, human HEK293-T, and mouse HT22 cell lines (Figure 1). Thus, CDNF is expressed not only in neurons but also in a wide range of cell lines. In the present study, we choose HEK293-T due to this cell line is efficiently transfected.

To study the potential protective effect of CDNF against ER stress, we established in HEK293-T cells an ER stress model induced by Thapsigargin (Tg), an inhibitor of an ER calcium transporter (Chen et al. 2000). Tg causes ER stress and in the long-term, triggers cell death by apoptotic mechanisms (Fribley et al. 2009). The treatment of HEK293-T cells with Tg increased the expression of BiP and XBP1 and CHOP (Figure 2), demonstrating that the Tg treatment induces ER stress.

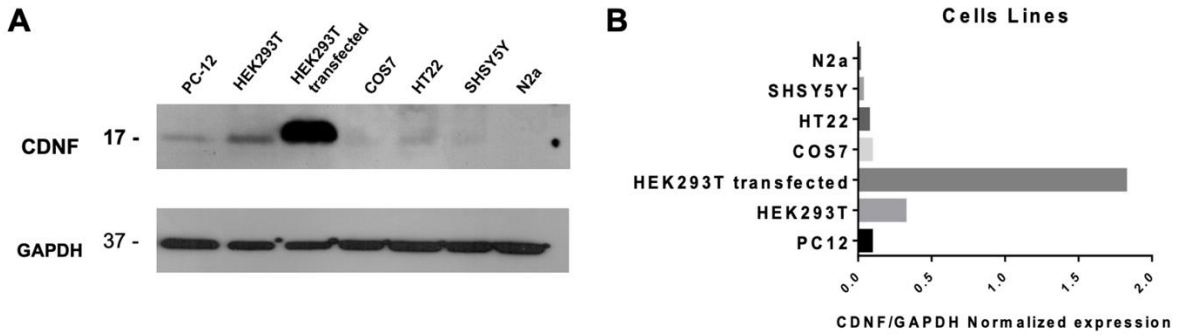

Figure 1. CDNF is expressed in different cell lines. A) Whole-cell extracts of rat PC-12, human HEK293-T, human SHSY5Y, monkey COS7, mouse HT22, and N2a were fractionated on SDS-PAGE and analyzed by Western blot assay to detect the CDNF peptide. The whole extract of HEK293-T cells transfected with FU-CDNF-W plasmid was used as a positive control, and GAPDH was used as load control. B) Protein levels of CDNF were quantified by densitometry using the ImageJ program.

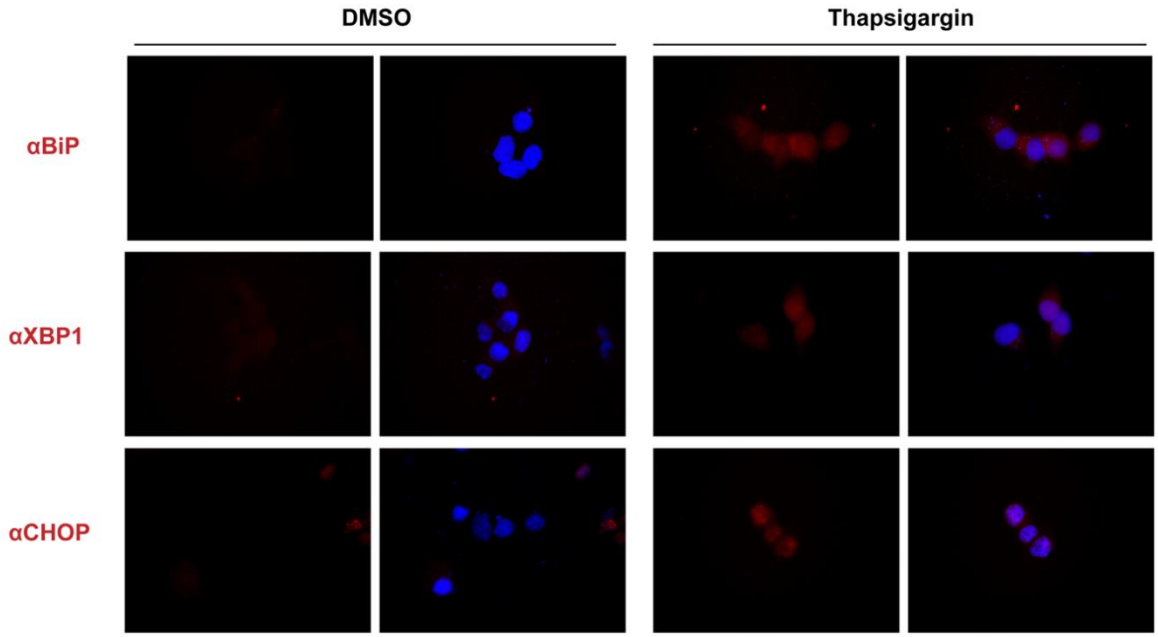

Figure 2. Establishing a model of ER stress using Thapsigargin (Tg). HEK293-T cells were treated with DMSO (A) or 1 uM of Tg (B) for 24h. Then, cells were fixed, and indirect immunofluorescence was performed to detect the UPR markers BiP, XBP1, and CHOP.

Establishing a CDNF gain-of-function model in HEK293-T cells

Considering the distinctive localization of CDNF in the ER, we hypothesized that its protective effect emerges from its subcellular location as a key regulator of the UPR. To test this hypothesis, first, we expressed rat CDNF (87% homology to human CDNF) using a bicistronic plasmid that encodes GFP as a reporter of transfection and the complete rat CDNF cDNA sequence, including its signal peptide (Figure 3A). Transfection of FUG-CDNF-W plasmid in HEK293-T cells resulted in a 5- fold increase of CDNF protein levels, and a similar increase of its mRNA (Figure 3B, C) compared to control transfected cells (mock).

Overexpressed CDNF showed a cytoplasmic reticular and perinuclear localization (Figure 3D), indicative of ER localization. To demonstrate that expressed CDNF localizes at the ER, the plasmid FU-CDNF-W was co-transfected with the FU-mRFP-KDEL-W plasmid, an ER marker. With the same purpose, we performed immunofluorescence for PDI, an ER endogenous marker, in cells transfected with FU-CDNF-W. As shown in Figure 4, the staining of CDNF distinctly overlaps with the fluorescence of mRFP-KDEL and PDI, demonstrating that the expressed CDNF specifically localizes in the ER compartment.

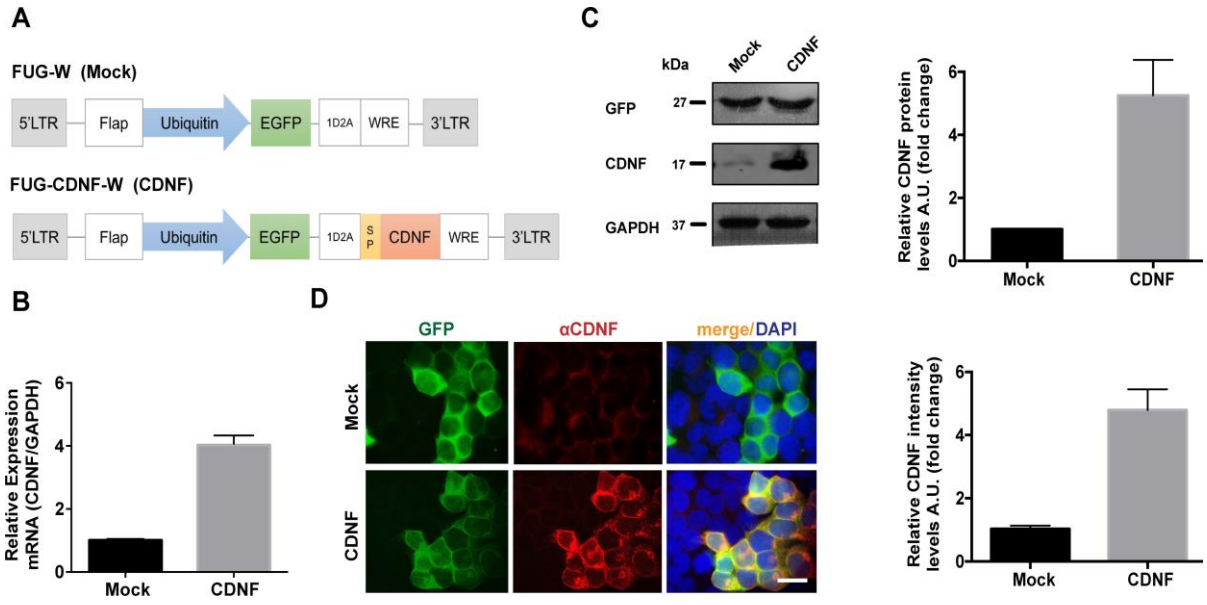

Figure 3. Transfecting FUG-CDNF-W plasmid in HEK293-T cells induces a five-fold increase of CDNF (A) Scheme of parental bicistronic FUG-W (mock) and derived FUG-CDNF-W (CDNF) plasmids. (B-D) HEK293-T cells were transiently transfected with Mock or CDNF plasmids. Twenty-four hours later, cells were harvested to quantify the expression of CDNF by RT-qPCR (B), Western blots (C), and immunofluorescence (D). GAPDH was used as a reference gene in Western blots and RT-qPCR assays to normalize CDNF expression. Data are expressed as the mean of two independent experiments, performed each by duplicate. A.U.= arbitrary units. Scale bar: 20 µm.

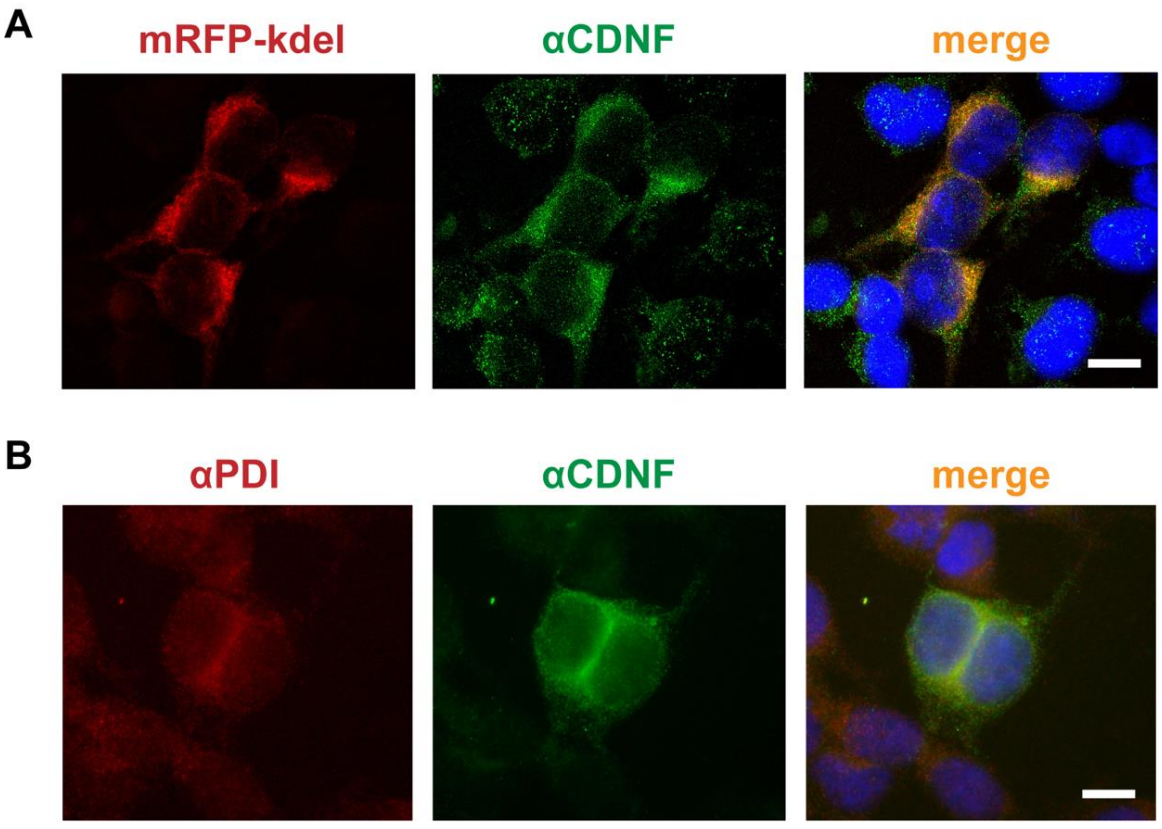

Figure 4. CDNF localizes in the ER. HEK293-T cells were co-transfected with the FU-CDNF-W plasmid, which encodes only full-length rat CDNF, and FU-mRFP- KDEL-W (A), that encodes the monomeric red fluorescent protein fused to a signal peptide and containing the ER retention signal KDEL to mark the ER. CDNF immunofluorescence shows a distribution pattern that coincides with the mRFP-KDEL signal (red). Also, PDI immunofluorescence was performed, as an endogenous ER marker, indicating that CDNF (green) localizes in the ER (B). Scale bar: 10 µm.

First Scientific Article: Published

CDNF induces the adaptive Unfolded Protein Response and Attenuates Endoplasmic Reticulum Stress-Induced Cell Death

This article encompasses the execution of the following objectives:

1. To determine if CDNF exerts a cytoprotective effect by modulating the UPR

1.1. To evaluate whether the gain of function of CDNF exerts a cytoprotective effect in HEK293-T cells and neurons through the induction of UPR pathways, during a pharmacological ER stress condition.

1.2. To evaluate whether the gain of function of CDNF exerts a cytoprotective effect in HEK293-T cells and neurons, through the attenuation of the apoptosis pathways induced by ER stress.

2. To determine whether the cytoprotective effect of CDNF is intra or extracellular.

2.1. To evaluate the cytoprotective effect of a CDNF mutant lacking the non-canonical ER retention sequence QTEL (CDNF-ΔQTEL).

2.2. To evaluate whether the gain of function of CDNF-ΔQTEL exerts a cytoprotective effect in HEK293T cells through the induction of UPR pathways, during a pharmacological ER stress condition.

2.3. To evaluate if the gain of function of CDNF-ΔQTEL exerts a cytoprotective effect in HEK293T cells, through the attenuation of apoptosis pathways induced by ER stress.

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CDNF induces the adaptive unfolded protein response and attenuates endoplasmic reticulum stress-induced cell death

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ABSTRACT

The Cerebral Dopamine Neurotrophic Factor (CDNF) is a neurotrophic factor that has a protective effect in cell and animal models of several neurodegenerative diseases. The molecular mechanism of the protective effect of CDNF is unclear. Many neurodegenerative diseases have been related to a proteostasis dysregulation in the endoplasmic reticulum (ER). A failure of proteostasis produces ER stress, triggering the unfolded protein re- sponse (UPR) and, in the long-term, induces cell death. An adaptive UPR solves ER stress by attenuating protein synthesis, inducing chaperones expression, and degradation of misfolded proteins. Since CDNF is an ER resident protein, we investigated whether the role of CDNF is to regulate ER proteostasis. To this end, we determined the effect of CDNF in thapsigargin-induced ER stress in HEK293-T cells and cultured hippocampal neurons. Our results show that CDNF improved the viability of HEK293-T cells exposed to thapsigargin. CDNF increased levels of protective proteins of the early UPR, such as BiP, ATF4, ATF6, and XBP-1 in both HEK293-T cells and neurons. Conversely, expression of CDNF attenuated ER stress-induced apoptotic proteins, CHOP and cleaved caspase-3 in HEK293-T cells and neurons. A mutant CDNF lacking the ER retention sequence failed to protect against ER stress. In conclusion, CDNF regulates proteostasis in the ER by inducing the adaptive UPR response and in- hibiting apoptotic pathways triggered by ER stress. We propose that neuroprotection induced by CDNF is mediated by regulating ER proteostasis.

1. Introduction

The endoplasmic reticulum (ER) is an organelle that participates in the synthesis, folding and sorting of many proteins [1]. The equilibrium among these processes is called proteostasis of the ER and its disturbance is produced by an accumulation of misfolded proteins. A disruptive proteostasis triggers the unfolded protein response (UPR) [2,3], an evolutionary conserved mechanism in which the Binding Immunoglobulin Protein (BiP, also called GRP78) acts as a master regulator binding to the misfolded proteins in the ER. During this process, BiP detaches from the UPR sensors localized in the ER membrane resulting in the activation of three associated signaling pathways: protein kinase R-like endoplasmic reticulum kinase (PERK), activated transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). Autophosphorylation of PERK leads to the eIF2a phosphorylation [4] that attenuate protein translation, but selectively increases the expression of proteins involved in oxidative stress, protein folding, and apoptosis. Thus, PERK mediates pro-survival and pro-death signaling [5]. ATF6 is cleaved at Golgi and the N-terminal domain translocate to the nucleus to induce expression/transcription of chaperones and ER-associated degradation proteins [6,7]. The activation of IRE1 induces the cytoplasmic splicing of XBP1 transcript and the protein translocate to the nucleus to induce ER stress-related gene expression [8].

A dysregulated UPR has been associated with many pathologic states such as cancer, diabetes and neurodegenerative diseases [9,10]. Many studies have found a causal link between the progress of neurodegenerative diseases and an alteration in the proteostasis in the ER, characterized by elevated UPR markers associated with cell death [11–13]. Particularly, in cellular and animal models of Parkinson dis- ease, the treatment with neurotoxic agents such as 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), or α-synuclein induces ER stress and pro-apoptotic related proteins

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[14–16] supporting the idea that this neurodegenerative disease is related to a disturbed ER proteostasis.

The Cerebral Dopamine Neurotrophic Factor (CDNF) has emerged as a potential treatment against Parkinson's disease and other neurodegenerative disorders due to its neuroprotective and restorative effects of neurons in *in vitro* and *in vivo* studies [17,18]. The application of CDNF to the extracellular milieu protected cultured mesencephalic neurons against α-synuclein-induced toxicity [19]. Likewise, overexpression of CDNF protected PC-12 cells against 6-OHDA-induced cell death [20], and alleviated Aβ-induced synaptotoxicity of hippocampal neurons [21]. *In vivo* studies have shown that a direct injection of CDNF into the striatum [22] or transduction with adeno-associated viruses encoding CDNF protected midbrain dopamine neurons against 6-OHDA neurotoxic effects [23–25].

CDNF is a non-conventional neurotrophic factor, which along with its homologue, the mesencephalic astrocyte-derived neurotrophic factor, MANF [26–28], reside in the ER, but can also be secreted to the extracellular medium [22,29,30]. Molecular studies showed that CDNF is retained in the ER through a non-canonical QTEL retention signal [28,30]. In spite that the three-dimensional structure of CDNF has been revealed [19], the molecular mechanism and the cell compartment from which CDNF exerts a cytoprotective effect are unknown. Con- sidering that CDNF is an ER resident protein that protects against da- mages associated to ER stress, we tested the hypothesis that CDNF protects cells and neurons against ER stress by modulating the UPR. We determined the role of CDNF in the UPR using HEK293-T cells treated with thapsigargin (TG), a well-established ER stressor and in cultured hippocampal neurons. Our data show that increasing CDNF levels in the ER of HEK293-T cells and hippocampal neurons triggers an early adaptive UPR and blocks the increase of pro-apoptotic proteins, indicating that the protective effect of CDNF is due to a key regulatory role in ER proteostasis. A mutant CDNF lacking the ER retention sequence failed to protect against ER stress and inducing BiP, indicating that CDNF must be localized in the ER to induce UPR and protect cells against TG-induced ER stress.

2. Material and methods

2.1. Plasmids

FUG-W is a lentiviral plasmid that encodes green fluorescent protein (GFP, G in the plasmids) under the human ubiquitin promoter [31]. The bicistronic lentiviral plasmid FUG-CDNF-W [32] encodes separately GFP and rat full-length CDNF, including its signal peptide. CDNF was cloned downstream from the 1D2A sequence that allow cutting CDNF protein [33]. A mutated version of CDNF lacking QTEL sequence in the C-terminal was cloned in FUG-W plasmid. To analyze the localization of overexpressed CDNF, we co-transfected FU-CDNF-W (without EGFP) along with pFUmRFP-KDEL-W plasmid, which encodes the monomeric red fluorescent protein (mRFP) fused to an ER peptide signal and to the canonical ER retention sequence KDEL. All plasmids were verified by sequencing.

2.2. Antibodies

The following primary antibodies were used: goat polyclonal anti-CDNF (1:1000, R&D Systems), rabbit polyclonal anti-BiP (1:1000, Santa Cruz Biotechnology), rabbit polyclonal anti-XBP1 (1:1000, Santa Cruz Biotechnology), rabbit polyclonal anti-PDI (1:1000, Santa Cruz Biotechnology), rabbit polyclonal anti-ATF6 (1:500, Abexxa Biologics), rabbit polyclonal anti-ATF4 (1:500, Abexxa Biologics), rabbit polyclonal anti-CHOP (1:500, Abexxa Biologics), mouse monoclonal anti-active caspase-3 (1:100, Abexxa Biologics), mouse monoclonal anti-GAPDH (1:10,000, Millipore) and Alexa Fluor 568 Phalloidin (Thermo Fisher Scientific). The secondary antibodies for immunofluorescence were Alexa Fluor® 488, Alexa Fluor® 568 anti-mouse, anti-rabbit and anti-goat (1:500, Invitrogen, USA). For immunoblotting, horseradish peroxidase-conjugated antibodies were used to detect goat, mouse, and rabbit primary antibodies (1:5000, Invitrogen, USA).

2.3. Cell culture, transfection and thapsigargin treatment

HEK293-T cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Gibco), supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 units/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco), and maintained at 37 °C in an atmosphere of 95% air and 5% CO_2. Cells were transfected with Calfectin agent following manufacturer's recommendations (Calbiotech). Twenty-four hours after transfection the cells were treated in free-serum medium with thapsigargin (Sigma-Aldrich) or dimethyl sulfoxide DMSO (Sigma-Aldrich) during times and at concentrations indicated in the figures.

2.4. Primary culture of rat hippocampal neurons

Pregnant Sprague-Dawley rats were obtained from Pontificia Universidad Católica de Chile. Hippocampi were dissected from E18 embryos and neurons were prepared using a modified Banker's culture protocol [34]. Cells were dissociated with trypsin and plated at 2.5×10^5 cells/cm^2 on poly-D-lysine (Sigma-Aldrich) coated coverslips in 24-well cell-culture dishes. Neuronal cultures were maintained in Neurobasal media supplemented with B27 (Gibco, USA), GlutaMAX-I (Gibco), 100 U/ml Ampicillin and 100 μg/ml Streptomycin (Gibco, USA) at 5% CO_2 and at 37 °C. Hippocampal neurons were transfected with Lipofectamine 2000 following manufacturer's recommendations (Invitrogen). The cultures were stained with MAP2, showing over 90% of MAP2 positive cells.

2.5. MTS assay

Cells were seeded in 96-well plates at a density of 2×10^4 cells/ml and incubated overnight. Then, cells were transfected with FUG-W and FUG-CDNF-W plasmids. Twenty-four hours after transfection, the media was changed for fresh media containing different amounts of TG, and cells were incubated for different times. At the end of the incubation period, 20 μl of MTS was added to each well and the plates were incubated for 3 h at 37 °C. Cell viability was evaluated by measuring the mitochondrial-dependent conversion of the yellow tetrazolium salt of MTS to purple formazan crystals by metabolic active cells. The optical density at 570 nm (proportional to the number of live cells) was assessed with an Epoch (Biotek).

2.6. RNA extraction and RT-qPCR

Total RNA was extracted using Trizol reagent (Ambion) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA by RT-PCR using ImProm-II RT (Promega). Quantitative real-time PCR was performed using Evagreen qPCR Mix plus (Solis Byodine) in a LightCycler thermocycler (Roche), using specific primers for UPR markers described in [35]. Primers efficiency was calculated from a standard curve of increasing concentrations of cDNA targets (Supplementary Table 1).

2.7. Immunoblotting

Whole cell extracts were obtained by homogenization in RIPA buffer (Millipore) containing inhibitors (1 mM PMSF, 7 μg/ml Pepstatin, 5–10 μg/ml Leupeptin and 10 μg/ml Aprotinin) and sonication. Protein concentrations were quantified by the BCA method (Thermo scientific). Samples were heated in Laemmli's loading buffer at 95 °C for 5 min, fractionated by SDS-PAGE, and transferred to a nitrocellulose membrane (Hybond, Amersham Biosciences). Membranes were blocked with Tris-buffered-saline (TBS) pH 7.4, containing 5% non-fat milk, and

0.1% Tween-20. After incubation with the appropriate primary and secondary antibodies western blots were revealed by chemiluminescence (ECL, Amersham, USA).

2.8. Immunofluorescence imaging and quantification of fluorescence intensity

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Then, cells were permeabilized in 0.05% Triton X-100 in PBS and incubated with blocking solution (2% glycine, 2% BSA, 5% FBS, 50 mM NH_4Cl in PBS pH 7.4) for 1 h. After incubation with ap- propriate primary and secondary antibodies, coverslips were mounted with Vectashield/DAPI solution (Vector). Images were acquired with an Olympus DS-Fi2 epifluorescence microscope furnished with 40× and 100× Olympus UplanFI oil immersion objective and equipped with a Nikon DS-fi2 camera operated with the standard QC capture software (Q-Imaging). Quantification was performed with ImageJ software (NIH, Baltimore, MD) using the corrected total cell fluorescence (CTCF) method.

2.9. Statistical analysis

Statistical analyses were performed by One-way or two-way ANOVA followed by Bonferroni post-test, using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA). Data are reported as mean ± SEM. Values of $P < 0.05$ were considered significant.

2.10. Ethical statement

All experimental procedures (protocol number 161213007) were approved by the Ethic Committee of Pontificia Universidad Católica de Chile (CA 1492008598726). Efforts were made to minimize the number of animals used and their suffering.

3. Results

3.1. CDNF protects HEK293-T cells against TG-induced cell death

Considering the distinctive localization of CDNF in the ER, we hypothesized that its protective effect emerges from its subcellular location as a key regulator of the UPR. To test this hypothesis, we expressed rat CDNF (87% homology to human CDNF) using a bicistronic plasmid that encodes GFP as a reporter of transfection and the complete rat CDNF cDNA sequence including its signal peptide (Suppl. Fig. 1A). Transfection of FUG-CDNF-W plasmid in HEK293-T cells resulted in a 5-fold increase of CDNF protein levels and a similar increase in mRNA (Suppl. Fig. 1B, C) compared to control transfected cells (mock). Overexpressed CDNF showed a cytoplasmic reticular and perinuclear localization (Suppl. Fig. 1D), indicative of ER localization. To further demonstrate that expressed CDNF is localized at the ER, the plasmid FU-CDNF-W was co-transfected in COS-7 cells with FU-mRFP-KDEL-W, an ER-directed marker. As shown in Suppl. Fig. 2, staining of CDNF distinctly overlaps with the fluorescence of mRFP-KDEL, demonstrating that expressed CDNF specifically localizes in the ER.

To determine the mechanism by which CDNF protects cells, we modeled an altered ER proteostasis using HEK293-T cells exposed to TG, a potent inhibitor of the ER calcium transporter SERCA [36], which causes ER stress and in the long-term triggers cell death by apoptotic mechanisms [37]. Cell viability of HEK293-T cells cultured with increasing concentrations of TG (0, 0.25, 0.5, 1, 5, 10 μM) was assessed at 24 h by the MTS assay. A significant decrease in cell viability was evident at 1 μM TG and nearly two-thirds of cells died after 24 h of culture with 10 μM TG compared to untreated cells (Mock, Fig. 1A). These results confirm that HEK293-T cells are sensitive to TG that induced a dose-dependent cell death. As expected, CDNF induced a significant increase in HEK293-T viability (Fig. 1A). Interestingly, the protection induced by CDNF was lost when the cells were exposed to 10 μM TG during 24 h, indicative of a limit to the protective role of CDNF when ER-stress is excessive. To further characterize CDNF protective effect against ER-stress, a time-course study was carried out by treating HEK293-T cells with 2 μM TG at different times (0, 3, 6, 9, 12, 24 h). Cell viability decreased after 9 h of treatment, reaching sig- nificance at 12 and 24 h (Fig. 1B). Expression of CDNF induced a sig- nificant protection against TG-induced HEK293-T cell death at 12 and 24 h (Fig. 1B). Altogether, these results show that expression of CDNF in the ER exerts a cytoprotective effect against TG-induced cell death.

3.2. CDNF promotes an adaptive UPR

Considering that CDNF protected the cells from TG-induced cell death and UPR is a conserved protective mechanism that emerges during ER stress, we tested the hypothesis that the protective effect of CDNF is due to a modulation of UPR. For this purpose, we assessed the expression of BiP, XBP1 and ATF6. Similar to a previous report [38], we observed a significant increase of BiP protein levels in HEK293-T cells treated with 1 μM TG at 24 h (Fig. 2A and B), confirming that TG induces ER stress and triggers the UPR. Expression of CDNF *per se* also increased BiP levels in HEK293-T cells (Fig. 2A and B), as compared with cells transfected with the control plasmid FUG-W (Mock). The time-course analysis of BiP mRNA expression and protein levels showed that TG induced a progressive increase in BiP that reach 4-fold increment at 12 h of exposure compared to untreated cells (Fig. 2C and D). Interestingly, expression of CDNF induced significantly higher levels of BiP mRNA and protein above TG-induced levels, until reaching a plateau at 12 h of treatment. These data indicate that CDNF regulates the expression of BiP, suggesting that CDNF is an inductor of an early UPR.

The fact that CDNF increased BiP prompted us to identify the UPR signaling pathway stimulated by CDNF. BiP activates three pathways: IRE1, ATF6, and PERK [39–41]. Therefore, we studied XBP1, activated ATF6, and ATF4, downstream signals of IRE1, ATF6 and PERK pathways, respectively. Immunofluorescent assays show a significant increase in XBP1 (Fig. 3A, B) and ATF6 (Fig. 3C, D) in HEK293-T cells treated with 2 μM TG. Interestingly, CDNF induced a significant increase in basal levels of XBP1 (Fig. 3A, B) and ATF6 (Fig. 3C, D) proteins. In addition, at all times tested, CDNF expression induced a further increase in XPB1 and ATF6 protein levels compared to cells exposed to TG 2 μM alone (Fig. 3E, F and G).

XBP1 mRNA is spliced during the UPR in the cytoplasm of stressed cells by IRE1, and this spliced XBP1 (sXBP1) mRNA isoform is the one that is translated into the XBP1 protein [42]. Therefore, we studied the effect of expressing CDNF in XBP1 splicing, by RT-qPCR using primers specific to quantify the sXBP1 isoform and total mRNAs. Expression of CDNF did not change total levels of XBP1 mRNA, but significantly increased sXBP1 mRNA isoform (Fig. 3H and I). Conversely, TG treatment induced a big increment of total XBP1 mRNA expression along with an increase of sXBP1 mRNA and protein. These data suggest that the mechanism by which CDNF induces an increase of the XBP1 protein is by increasing the IRE1 signaling. Finally, we measured the levels of a chaperone that belongs to the Glucose-Regulated Proteins (GRP), GRP94, which increases during ER stress [43]. We observed that TG 2 μM induced a significant increase of GRP94 mRNA levels. However, expression of CDNF did not change GRP94 mRNA levels (Fig. 3J), indicating that CDNF effect is specific over some UPR signaling pathways.

To rule out the possibility of an induction of UPR is due to increasing the load in protein levels in the ER, we carried out two experiments. First, we analyzed the expression of the Protein Disulfide Isomerase (PDI) an ER-resident chaperone, whose expression is induced by protein accumulation [44]. Immunofluorescence and Western blot assays show that PDI levels were not modified by CDNF expression (Suppl Fig. 3). Second, we studied the effect of expressing mRFP-KDEL using the same promoter used to induce CDNF expression. The expression of mRFP-KDEL in the ER did not change BiP and XBP1 levels in

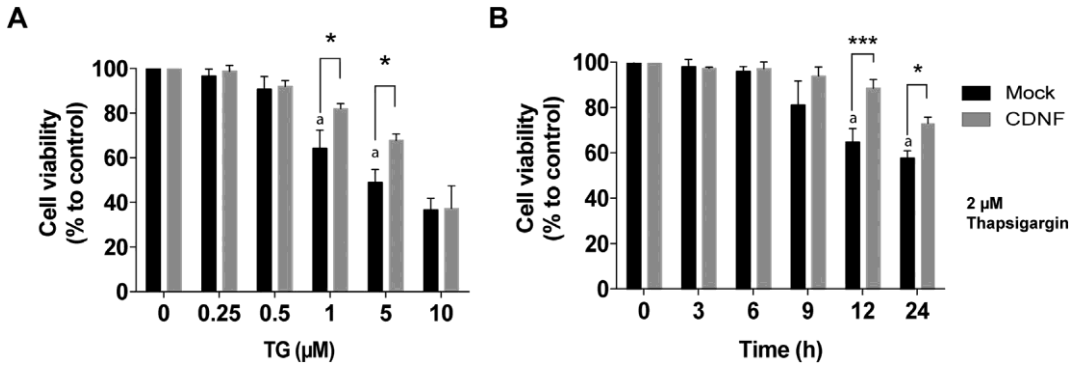

Fig. 1. CDNF protects HEK293-T cells against TG-induced cell death. HEK293-T cells were transfected with FUG-W (black bars) and FUG-CDNF-W (gray bars) plasmids. Twenty-four hours later, cells were treated with different concentrations of TG (A) or with 2 μM TG during different time-length (B). Cell viability was quantified by MTS assays. The data are presented as % of control (mock). Data correspond to the mean ± SEM of 3 independent experiments, each performed in duplicates. The statistical analysis was performed with two-way ANOVA and Bonferroni post hoc test, *$p < 0.05$ and ***$p < 0.001$. a, ***$p < 0.01$ compared to control without TG treatment.

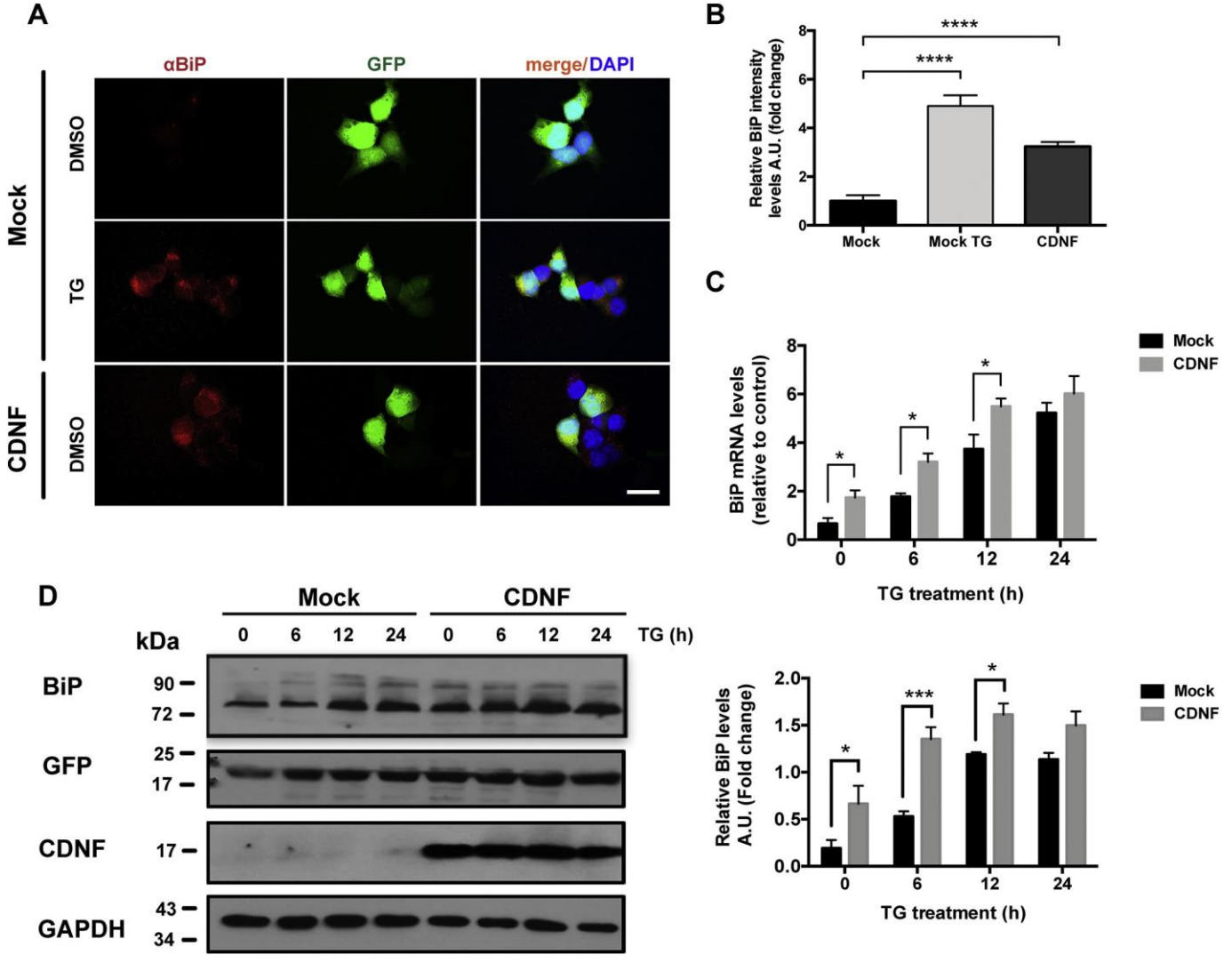

Fig. 2. CDNF increases BiP protein and mRNA levels. HEK293-T cells were transfected with FUG-W (Mock) or FUG-CDNF-W (CDNF). Twenty-four hours after transfection, cells were treated with TG (1 μM) for additional 24 h for immunofluorescent assays to detect BiP (A, B), or the indicated times for RT-qPCR (C) and western-blots (D) to quantify BiP mRNA expression and protein levels. GAPDH was used to normalize western blot and RT-qPCR data. The bars represent the mean ± SEM of 3 independent experiments, each performed in duplicates. The statistical analysis was carried out with ANOVA and a post hoc Bonferroni test, *$p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ Scale bar: 20 μm.

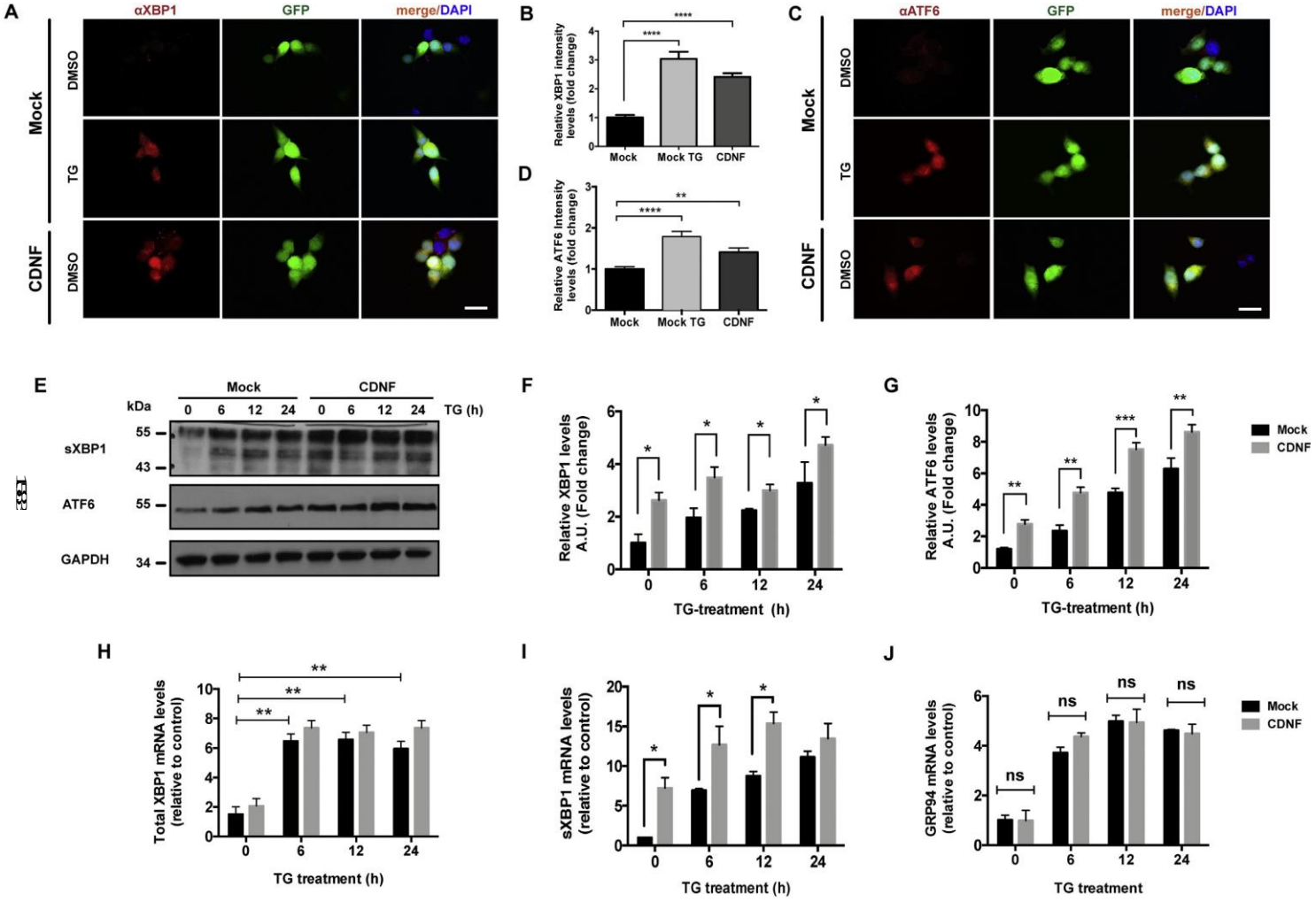

Fig. 3. CDNF increases splicing of XBP1 and ATF6 protein levels. HEK293-T cells were transfected with FUG-W (Mock) or FUG-CDNF-W (CDNF). Twenty-four hours after transfection, cells were treated with TG (1 μM) for 24 h to perform immunofluorescent assays to detect XBP1 (A, B) and ATF6 (C, D). Western-blots were carried out at indicated times after TG treatment to quantify XBP1 (E, F) and ATF6 (G) protein levels. Expression of unspliced (H) and spliced (I) XBP1mRNA, and GRP94 mRNA (J) were quantified by RT-qPCR at indicated times after TG treatment. GAPDH was used to normalize RT-qPCR and western blot data. Data correspond to the mean ± SEM of 3 independent experiments performed as duplicates. Statistical analysis was performed with ANOVA and a *post hoc* Bonferroni test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Scale bar is 20 μm.

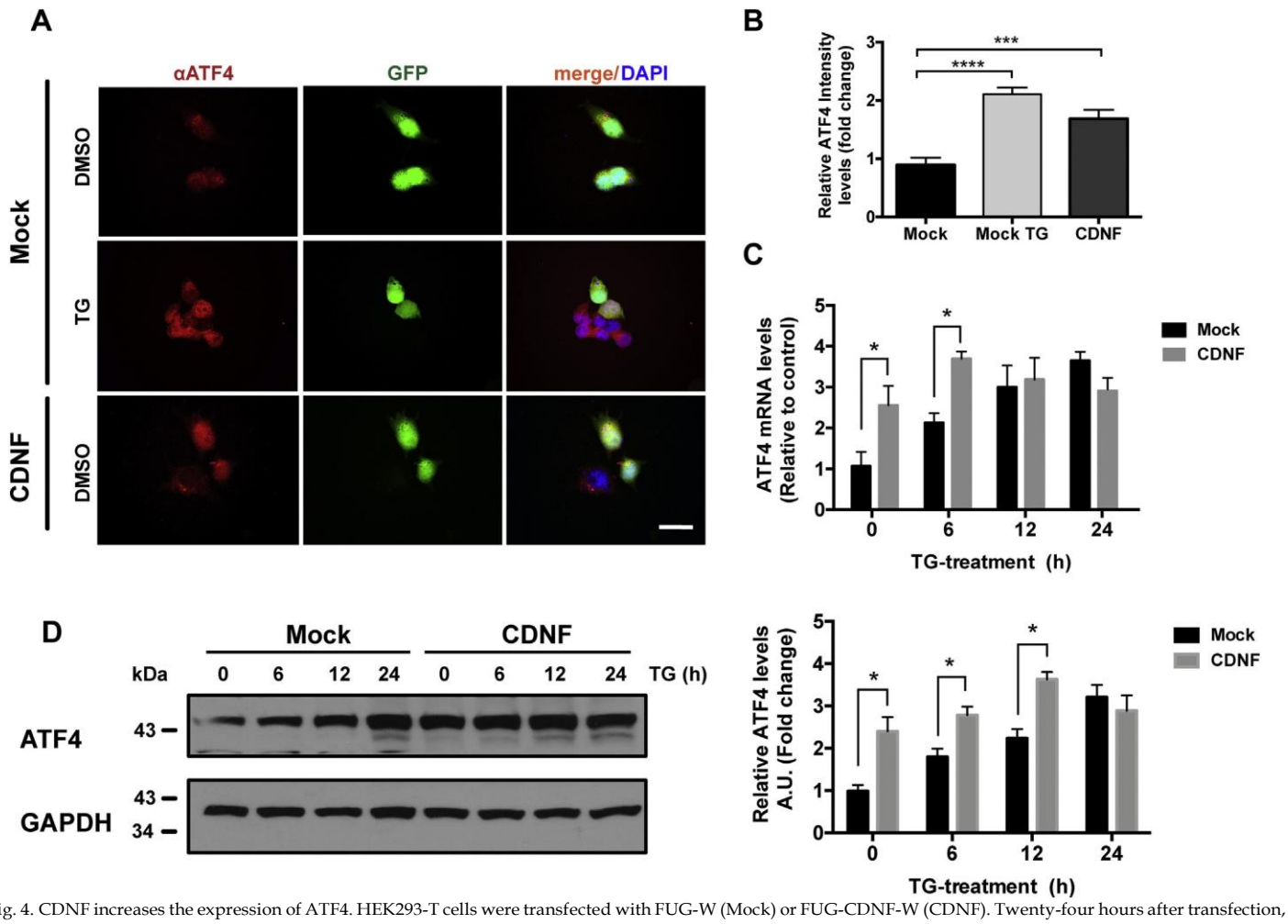

Fig. 4. CDNF increases the expression of ATF4. HEK293-T cells were transfected with FUG-W (Mock) or FUG-CDNF-W (CDNF). Twenty-four hours after transfection, cells were treated with TG (1 μM) for 24 h to perform immunofluorescent assays to detect ATF4 (A, B). Expression of ATF4 mRNA (C) and protein levels (D) were assessed respectively by RT-qPCR and western blots at indicated times after TG treatment. GAPDH was used to normalize RT-qPCR and western blot data. Data correspond to the mean ± SEM of 3 independent experiments performed as duplicates. Statistical analysis was performed with ANOVA and a post hoc Bonferroni test, *$p < 0.05$, ***$p < 0.001$ and **** $p < 0.0001$. Scale bar: 20 μm.

HEK293-T cells (Suppl Fig. 4A and B). Altogether, the data indicate that CDNF triggers an early UPR response.

3.3. CDNF attenuates ER stress-induced apoptosis

Cell fate during an ER stress is determined by the balance between the adaptive UPR and the terminal UPR [45]. Therefore, we evaluated whether CDNF prevents the induction of the terminal UPR during an ER stress. To this end, we choose ATF4 protein to evaluate the PERK/ATF4/CHOP signaling pathway, which is activated by prolonged ER stress to induce apoptosis [46]. Since expression of CDNF reduced cell death induced by TG, we expected a decrease in the expression of ATF4. Surprisingly, HEK293-T cells expressing CDNF showed a significant increase of ATF4 (Fig. 4). RT-qPCR assays showed that CDNF induced the expression of ATF4 mRNA in the basal condition, and produced a significantly greater increase at 6 h after treatment with TG, but not at later tested times (Fig. 4C). This increase in ATF4 mRNA levels was reflected in increased protein levels of ATF4 up to 12 h of TG treatment (Fig. 4D).

The intriguing previous result suggested that CDNF could counteract TG-induced cell death by blocking pro-apoptotic pathways. Thus, we evaluated the pro-apoptotic protein CCAAT-enhancer-binding protein homologous protein (CHOP) induced in terminal UPR and cleaved caspase-3, a recognized marker of apoptosis. As expected, TG increased the expression of CHOP and cleaved caspase-3 in HEK293-T cells (Fig. 5). A significant increase in CHOP mRNA levels (Fig. 5C) and a concomitant increase in protein levels were observed in cells treated with TG (Fig. 5A, B, D). Similarly, increased levels of active caspase-3 were observed at 12 and 24 h (Fig. 5E). Consistent with a protective effect, the expression of CDNF counteracted the increase in CHOP and caspase-3 in HEK293-T cells induced by TG (Fig. 5). Interestingly, CDNF *per se* induced a low but significant increase of CHOP basal levels, but arrested further increases induced by TG. Taken together, the data suggest that CDNF protects the cells by inhibiting the pro-apoptotic mechanisms activated by the PERK pathway.

3.4. CDNF induces an early adaptive UPR and blocks the terminal UPR in hippocampal neurons

CDNF has emerged as a potential therapeutic tool for clinical use given its protective and restorative effects of neurons in models of neurodegenerative diseases. Our previous results prompted us to study whether CDNF protection of neurons is due also to its capacity of inducing the adaptive UPR. To answer this question, we transfected hippocampal neurons with FUG-CDNF-W to express CDNF (Suppl Fig. 5) and tested the expression of UPR marker proteins by quantitative immunofluorescence. As shown in Fig. 8, the expression of CDNF triggered an increase in BiP, XBP1, and ATF6 protein levels, suggesting that

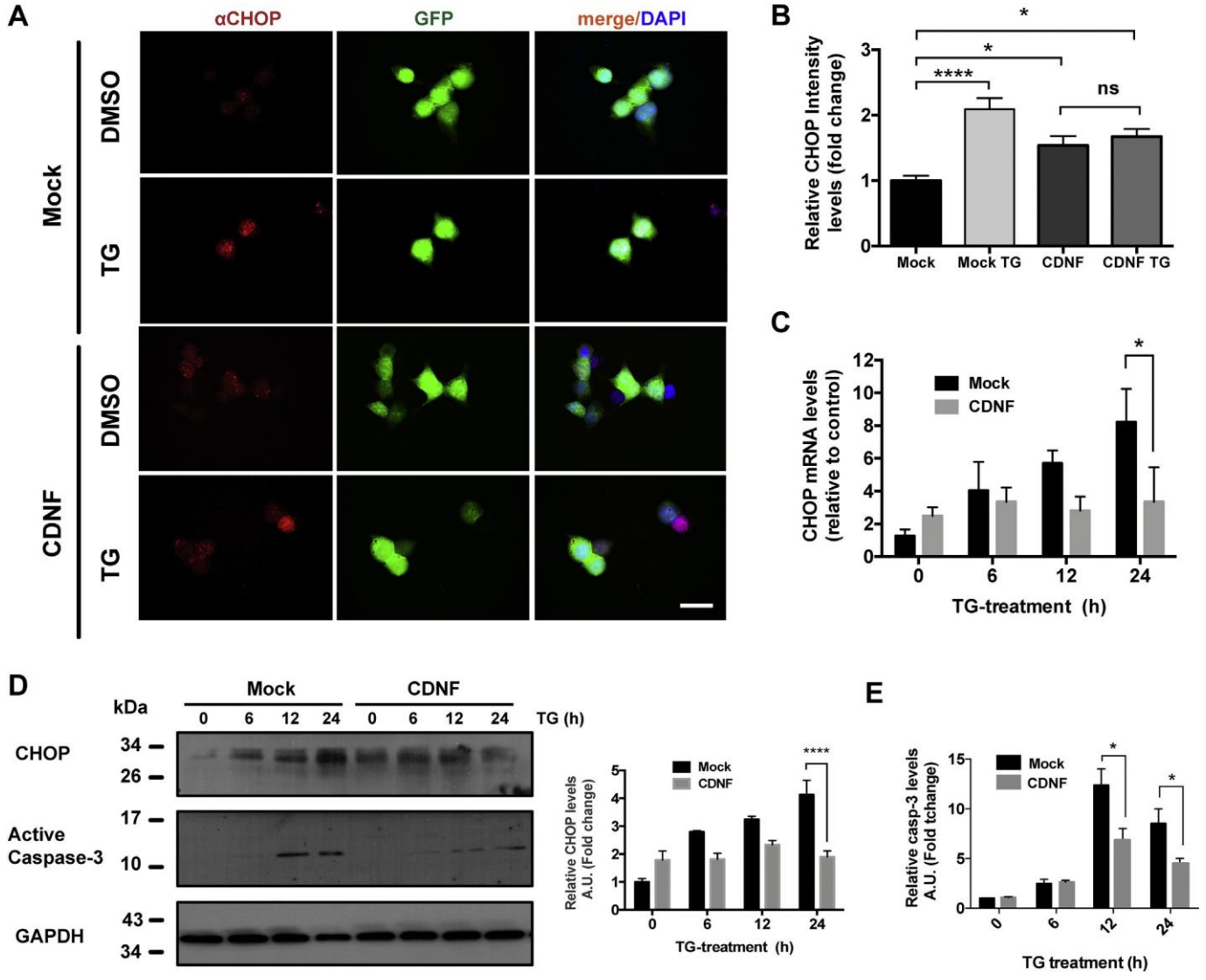

Fig. 5. CDNF attenuates TG-induced expression of CHOP and active caspase-3 in HEK293-T cells. HEK293-T cells were transfected with FUG-W (Mock) or FUG- CDNF-W (CDNF). Twenty-four hours after transfection, cells were treated with TG (1 μM) for 24 h to perform immunofluorescent assays to detect CHOP (A, B). Expression of CHOP mRNA (C) and protein levels (D) were assessed respectively by RT-qPCR and western blots at indicated times after TG treatment. Caspase-3 (casp-3) protein levels were quantified by western blots (D, E). GAPDH was used to normalize RT-qPCR and western blot data. Data correspond to the mean ± SEM of 3 independent experiments performed as duplicates. Statistical analysis was performed with ANOVA and a post hoc Bonferroni test, *$p < 0.05$ and ****$p < 0.0001$. Scale bar: 20 μm.

CDNF can protect hippocampal neurons against ER stress by inducing an adaptive UPR. As with HEK293-T cells, expressing CDNF in hippocampal neurons induced ATF4 levels (Fig. 6D), and no changes were observed in PDI expression levels (Suppl Fig. 6) discarding an alteration of ER by CDNF in neurons.

Next, we evaluated the protective effect of CDNF on hippocampal neurons against the induction of CHOP produced by the stressor TG. Hippocampal neurons treated with TG (1 μM) showed levels of CHOP 5 times higher than control cells. Remarkably, hippocampal neurons expressing CDNF showed significant lower levels of CHOP compared to neurons transfected with mock control, when treated with TG. (Fig. 7). The results in both HEK293-T cells and hippocampal neurons are consistent, suggesting that CDNF protects neurons from ER stress by promoting an adaptive UPR and inhibiting cell death-inducing mechanisms.

3.5. *Secreted CDNF FAILS to protect cells AGAINST TG-induced ER stress*

The neuroprotective effect of CDNF has been attributed to both intracellular and extracellular CDNF. Given that CDNF can be secreted, we wanted to answer the question of whether the protective effect and the induction of UPR are due to intracellular CDNF. To answer this question, we generated a mutated version of CDNF lacking its ER retention sequence, QTEL (Fig. 8A). As expected, CDNF-Δ-QTEL is not efficiently retained as shown by decreased CDNF-Δ-QTEL protein levels in whole cell extracts while increased levels of the mutant were observed in the extracellular medium, suggesting a continuous secretion of CDNF-Δ-QTEL (Fig. 8B). Besides, mutant CDNF-Δ-QTEL failed to increase BiP levels compared to wild-type CDNF (Fig. 8B). Finally, cell viability assays showed that HEK293-T cells expressing the mutant CDNF-Δ-QTEL displayed a similar death rate compared to controls when treated with TG, while cells expressing wild-type CDNF displayed significantly higher cell viability (Fig. 8C). Altogether, these results show that the protective effect of CDNF requires that CDNF localize in

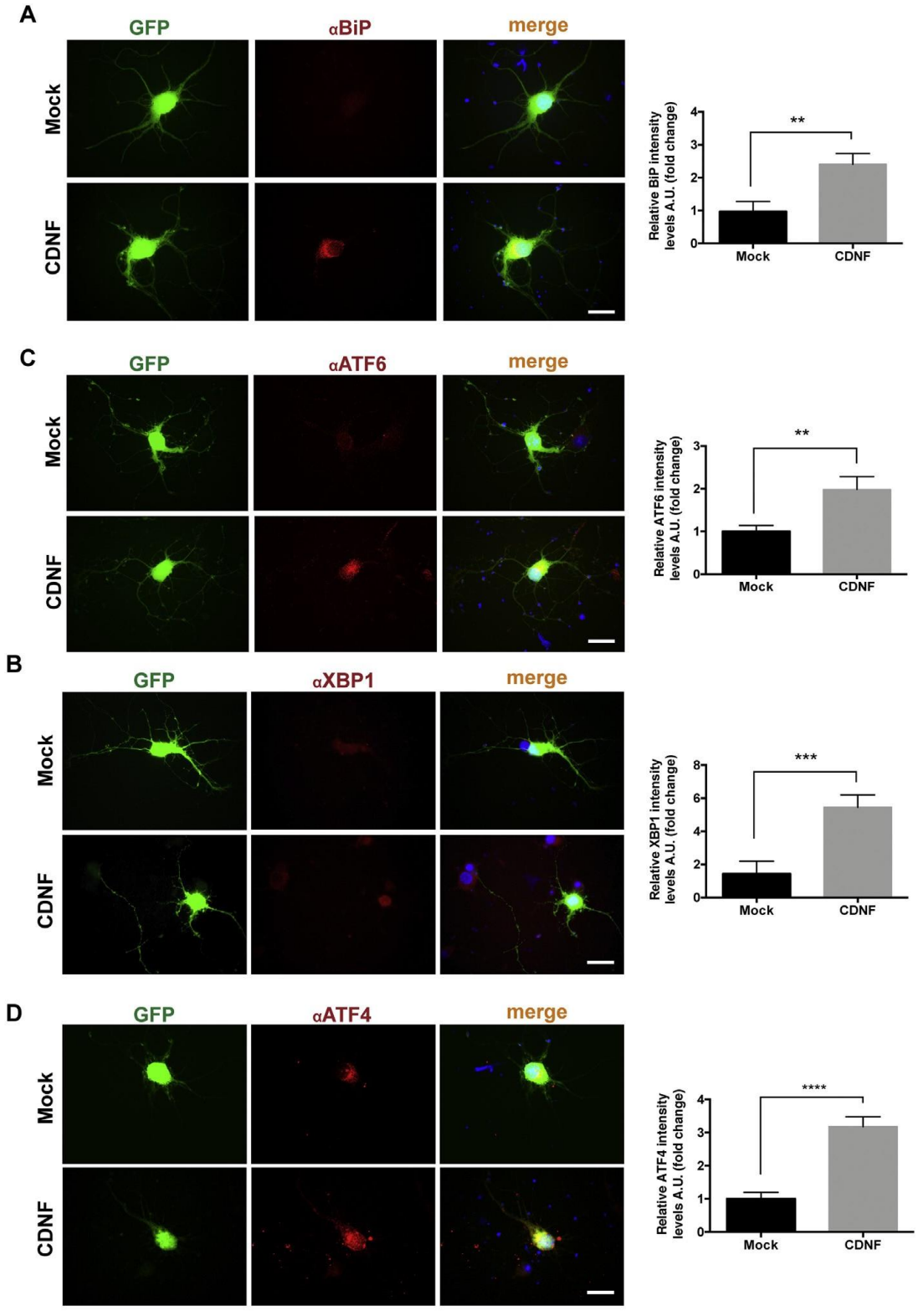

Fig. 6. CDNF triggers adaptive UPR in hippocampal neurons. Hippocampal neurons at 4DIV were transfected with FUG-W (Mock) and FUG-CDNF-W plasmids. Neurons were paraformaldehyde-fixed at 7DIV to perform immunofluorescence assays to quantify BiP (A), XBP1 (B), ATF6 (C) and ATF4 (D). Data are expressed as the mean ± S.E.M. of two independent experiments. At least 10 neurons were quantified in each experiment. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Bar represents 10 μm.

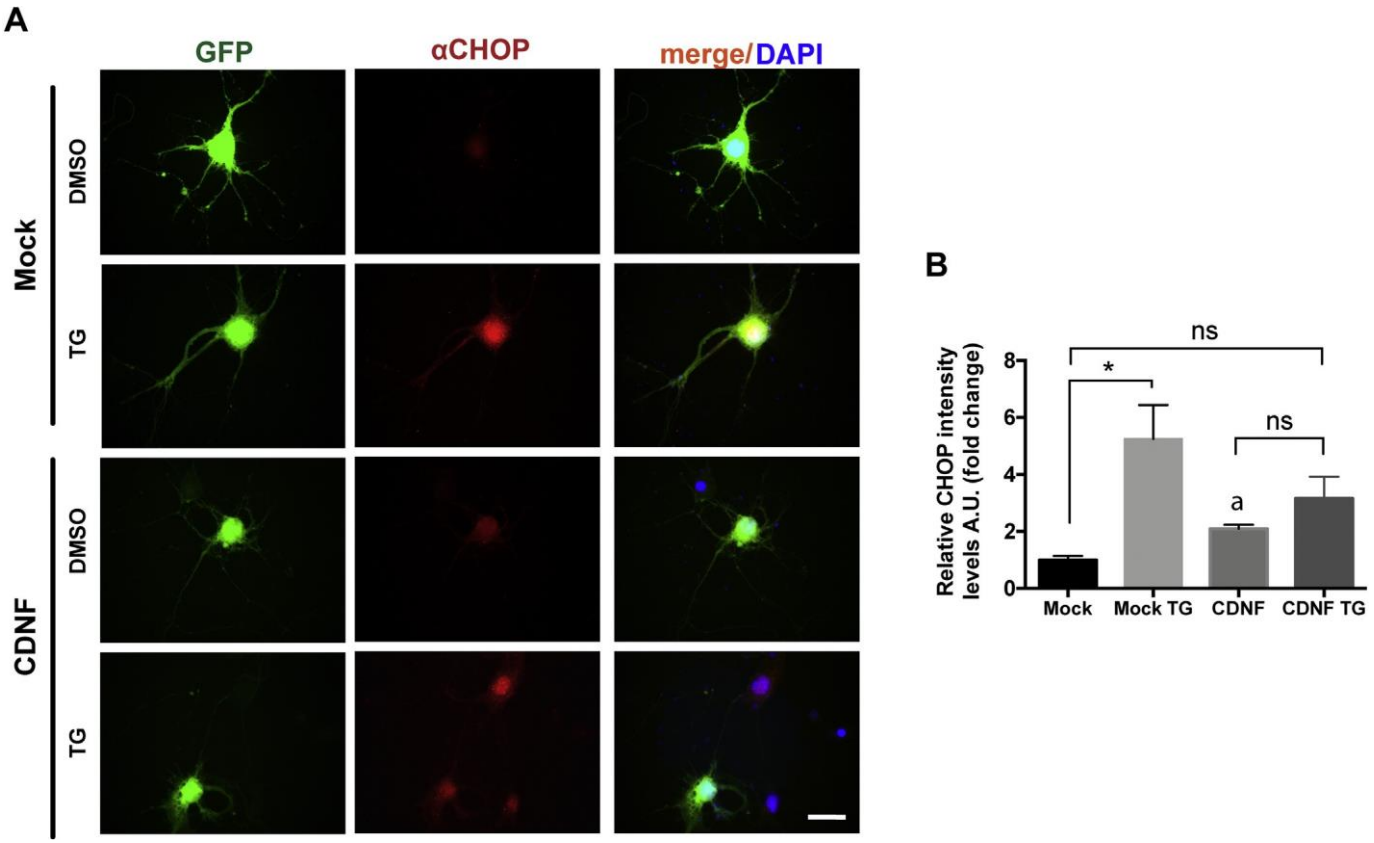

Fig. 7. CDNF attenuates TG-induced expression of CHOP in hippocampal neurons. Hippocampal neurons at 4DIV were transfected with FUG-W (Mock) and FUG-CDNF-W plasmids and were treated with TG (1 μM) or vehicle (DMSO) during 24 h. At 7DIV, neurons were paraformaldehyde-fixed and immunofluorescence assays carried out to quantify CHOP protein levels. Data are expressed as the mean ± S.E.M. of two independent experiments. CHOP levels were quantified in at least 10 neurons by condition. Statistical analysis was performed by One-way ANOVA followed by Bonferroni post-test. *$p < 0.05$, Δ $p < 0.05$ compared to Mock. Bar represents 10 μm.

the ER, where it can promote an early UPR.

4. Discussion

Growing evidence shows that the neurotrophic factor CDNF exerts potent protective and restorative functions in cellular and animal models of several neurodegenerative diseases such as Parkinson and Alzheimer. However, we lack information regarding the mechanism by which CDNF protects neurons. Here, we provide evidence that CDNF induces an early adaptive UPR and inhibits pro-apoptotic pathways in HEK293-T cells and hippocampal neurons.

An adaptive UPR increases cell capacity to deal with stressors by inhibiting translation of unnecessary proteins while inducing chaperones expression that augments protein-folding activity. Our data show that expressing CDNF in HEK293-T cells and neurons triggers an increase of BiP, the master regulator of UPR. CDNF *per se* induced significant increases of XBP1 splicing and protein, and mRNA expression and protein levels of activated ATF6 and ATF4, suggesting that it turns on the three UPR pathways. These data allow proposing that CDNF elicits signals of a mild ER stress, increasing ER hormesis, which has been propose to limit the effects of a posterior insult [47,48]. The fact that CDNF itself induces a mild early UPR explains why it is an effective protective agent in numerous types of damage in different cell types. For instance, brain transduction with lentivirus or AVV2 encoding CDNF protected dopamine neurons from damage caused by the neu- rotoxins MPP+ and 6-OHDA, and the abused drug methamphetamine [18,23,25]. In addition, expression of CDNF reduced synaptotoxicity induced by β-amyloid in cultured hippocampal neurons [21]. Recently, it was shown that the expression of CDNF protected cardiomyocytes from apoptosis induced by tunicamycin [49]. Taking together the evidence indicate that CDNF protect cells and neurons against several mechanisms related to ER-stress induced cell death.

The increase in adaptive UPR players induced by CDNF was accompanied by a blockage of pro-apoptotic signals, indicating a dual protector function for CDNF. Our data show that expression of CDNF itself induced a little but significant CHOP expression, as previously reported by Zhou and colleagues [21]. In addition, CDNF limited the increase of CHOP and active caspase-3 in cells and neurons treated with TG. Supporting our data, other study showed that CDNF improves PC-12 cells viability treated with 6-OHDA, effect that was associated to a down-regulating of active caspase-3 [20]. These evidences support arole for CDNF blocking apoptosis induced by ER stress.

The compartment from which CDNF exerts cellular protection is a matter of active study. Our data indicate that the protective effect requires that a certain amount of CDNF be present in the ER. Others have shown that CDNF can also protect from outside cells [19–22]. In 2015, it was proposed that CDNF could interact with lipids or proteins in the plasma membrane that would allow its endocytosis [30]. However, a recent study showed that although part of the CDNF applied in the extracellular is internalized, it was only found in endosomes and multivesicular bodies, but not in the ER [50], ruling out the possibility that by endocytosis CDNF can accumulate in the ER. On the other hand, our data do not support that a receptor in the plasma membrane would mediate the protective effect of CDNF. Since significant amounts of CDNF-Δ-QTEL were detected in the extracellular medium, but the protective effect and the induction of BiP were lost. Whether CDNF has a dual protective role exerted by intra and extracellular mechanism requires further analysis.

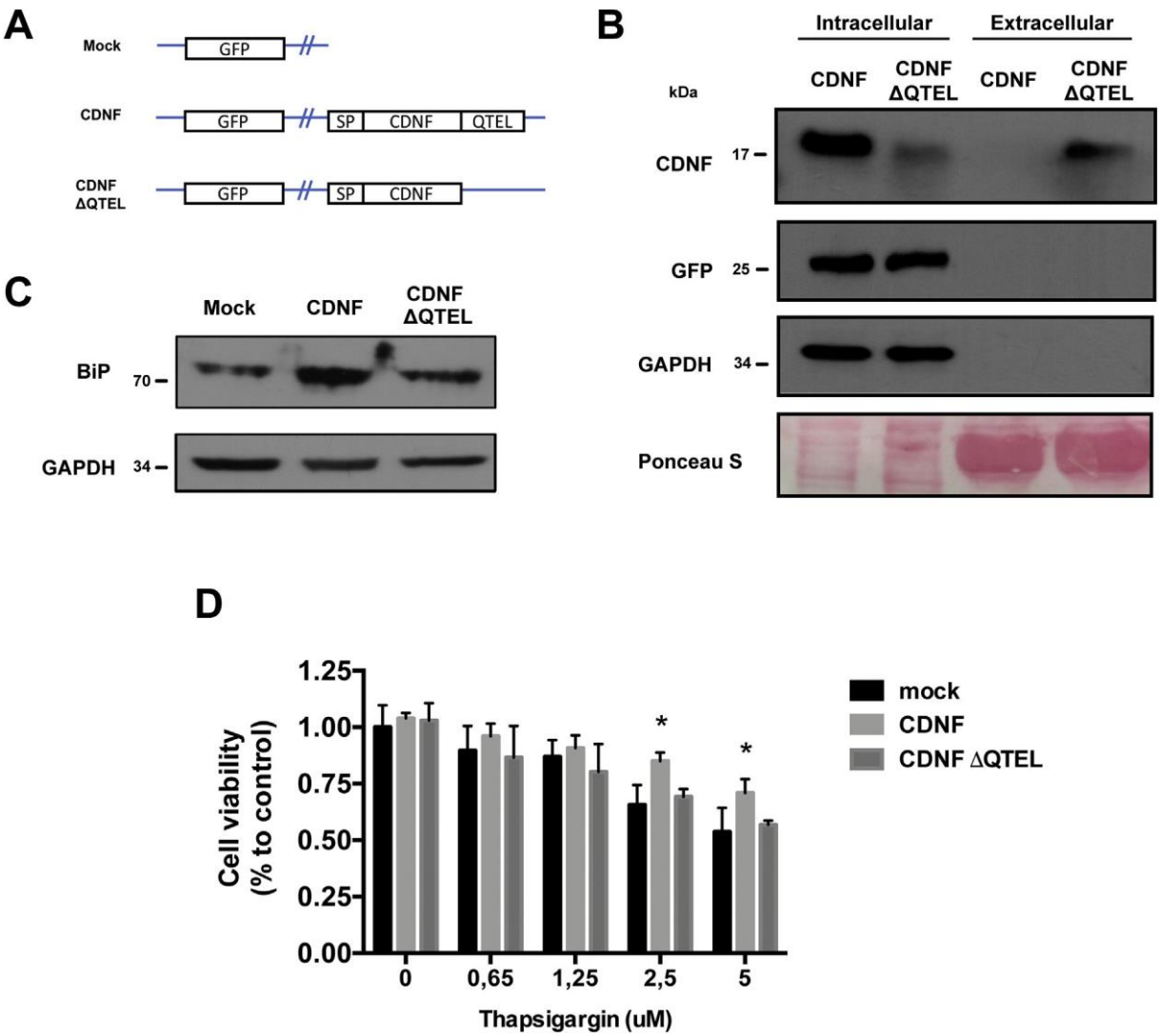

Fig. 8. Secreted CDNF fails to protect cells against TG-induced ER stress and induce BiP expression. (A) Scheme of plasmids enconding wild-type CDNF and mutant lacking QTEL sequence. (B) HEK293-T cells were transfected with FUG-W (mock) and FUG-CDNF-W and FUG-CDNF-ΔQTEL-W and the amount of CDNF in whole cell extracts (intracellular) and in the medium (extracellular) was analyzed by western blot. GFP was used as transfection control. Red Ponceau membrane staining shows that the amount of protein loaded in each well are equivalent. (C) BiP signaling in western blots of whole cell extracts indicate that mutant CDNF-Δ-QTEL is unable to induce BiP. (D) MTS cell viability was performed in HEK293-T cells transfected with FUG-W (mock) and FUG-CDNF-W and FUG-CDNF-Δ-QTEL-W. Statistical analysis was performed by One-way ANOVA followed by Bonferroni post-test. *$p < 0.05$, compared to mock.

The protection mechanism induced by CDNF seems to be different compared to the analog MANF. In our experimental system, CDNF levels were not induced in HEK293-T cells by TG treatment (Suppl. Fig. 7). Unlike CDNF, it has been shown that MANF is induced by ER- stress. For instance, Apostolou and colleagues showed that treating osteosarcoma U2OS cells with tunicamycin and TG induced a significant increase of MANF, but not CDNF [27]. Similar results were reported by Glembotski and colleagues [51]. It has been proposed that MANF interacts with BiP allowing its binding to ATF6, XBP1 and PERK sensors [52], thus limiting the ER stress response. Therefore, even though the high similarity between MANF and CDNF, the mechanisms by which these neurotrophic factors protect neurons is different and could be complementary. Several questions remain unanswered regarding CDNF regulation. Among them, what are the signals that trigger CDNF expression and secretion are unknown. Revealing these signals will open new therapeutic opportunities for treating neurodegenerative diseases.

In conclusion, CDNF reduces ER stress-induced cell death by inducing an adaptive UPR and by limiting the terminal UPR.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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Competing interests

The authors declare that they have no competing interests.

Author contributions

DA, acquisition and analysis of the data, drafting the manuscript; MEA, analysis and interpretation of the data, drafting and revising the

manuscript; and PZ, conception and design, analysis, drafting and revising the manuscript. All the authors have read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2018.08.012.

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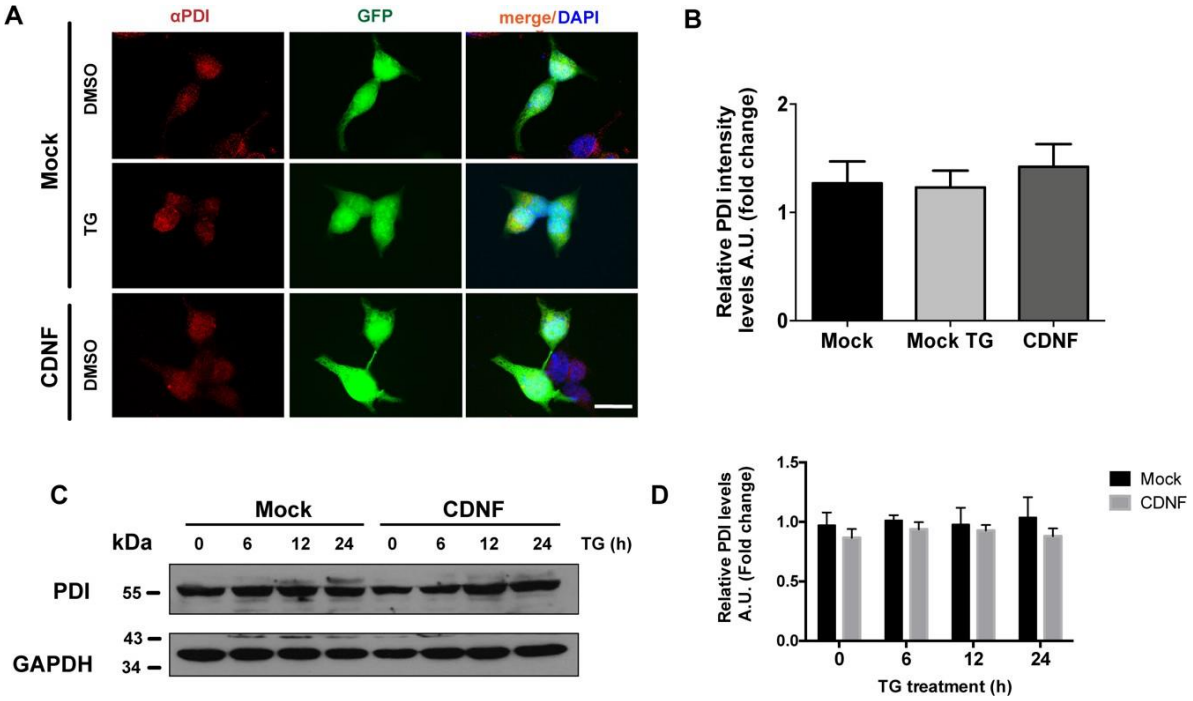
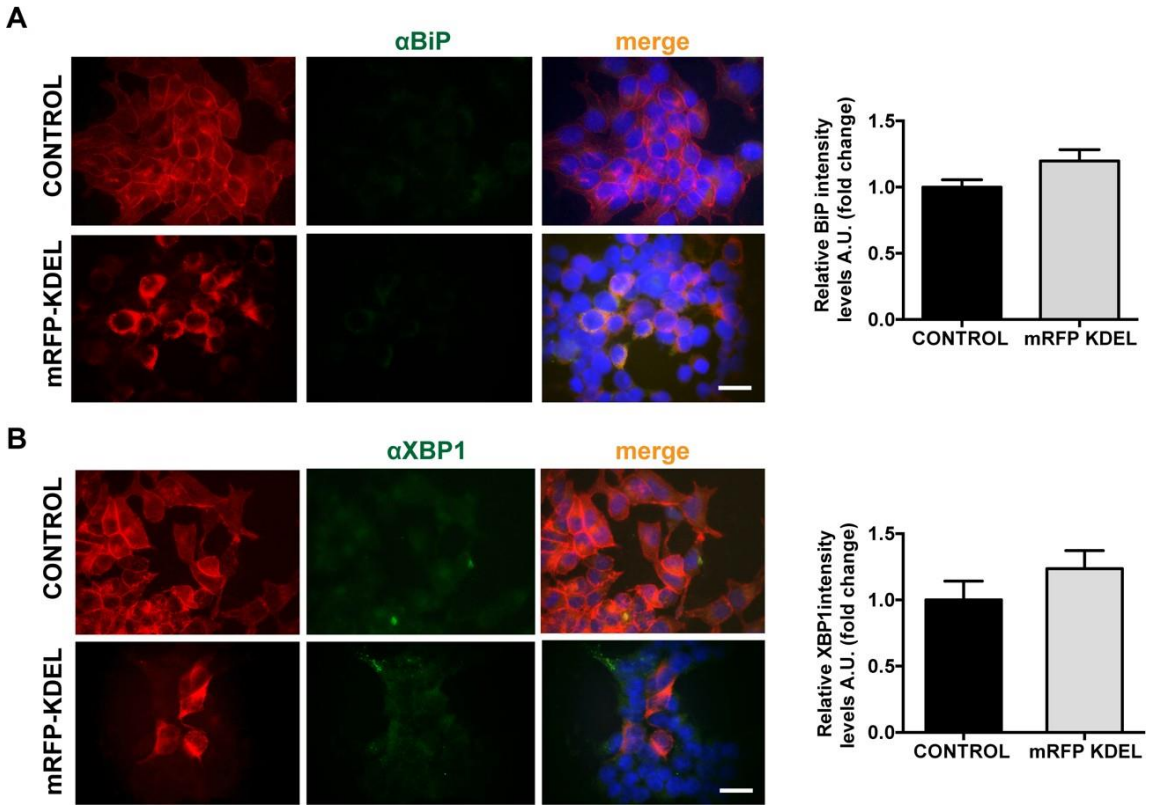
SUPPLEMENTARY FIGURES

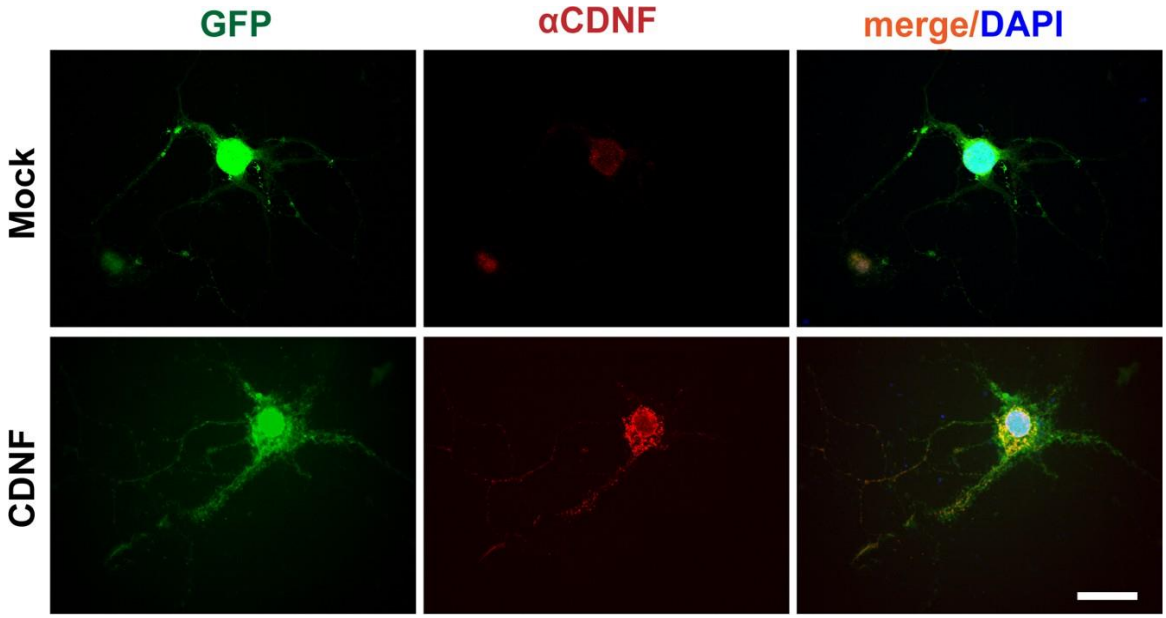
(Of the previous scientific article: CDNF induces the adaptive Unfolded Protein Response and Attenuates Endoplasmic Reticulum Stress-Induced Cell Death)



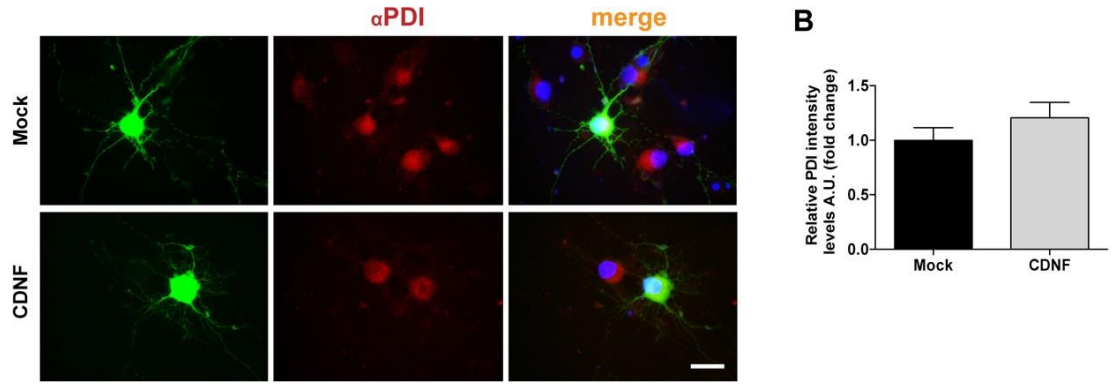
Supplementary Figure 1. CDNF does not change PDI in HEK293-T cells. HEK293-T cells were transfected with FUG-W (Mock) and FUG-CDNF-W plasmids and were treated with TG (1 uM) or vehicle (DMSO) for 24 hours or the indicated times. Immunofluorescence and western blot were performed to quantify PDI protein levels. Data correspond to the mean ± SEM of two independent experiments performed by duplicate. Scale bar: 20 μm.



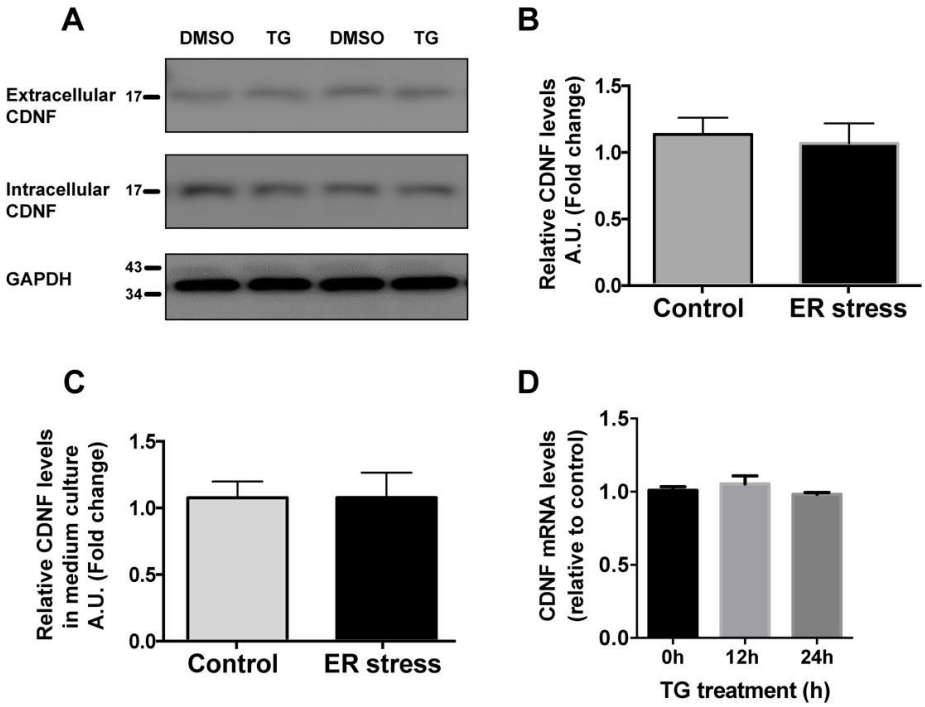
Supplementary Figure 2. The expression of mRFP-KDEL in the ER of HEK293-T cells does not induce UPR markers. HEK293-T cells were transfected with the FU-mRFP-KDEL plasmid. Phalloidin 568 was used to visualize the cell bodies in non-transfected cells (red, CONTROL). Immunofluorescence was performed to detect and quantify BiP (A, upper panel green) and XBP1 (B, lower panel green). Nuclei were labeled with DAPI. Data correspond to the mean ± SEM of two independent experiments, performed in duplicates. Scale bar: 20 μm.



Supplementary Figure 3. Transfection of FUG-CDNF-W increases CDNF in cultured hippocampal neurons. Hippocampal neurons were transfected with FUG-W (Mock) and FUG-CDNF-W (CDNF) plasmids at 4DIV and fixed at 7 DIV. Immunofluorescence against CDNF (red) was performed to test the efficacy of the plasmid to inducing CDNF expression. Scale bar: 10 μm.



Supplementary Figure 4. CDNF does not change PDI in hippocampal neurons. Hippocampal neurons were transfected with FUG-W (Mock) and FUG-CDNF-W plasmids at 4DIV and fixed at 7 DIV. A) Immunofluorescence assays were performed to detect PDI (red) and quantified (B). Scale bar 10 µm.



Supplementary Figure 5. The expression and secretion of CDNF do not change during a TG-induced ER stress. HEK293-T cells were treated with TG 1µM or DMSO (vehicle) for 24 hours. (A, B) Intracellular CDNF was quantified in total cell extracts obtained in the RIPA buffer. Extracellular CDNF levels were assessed in equivalent supernatant volumes from each culture (A, C). Quantification was performed using ImageJ software using GAPDH as load control. D) RT-qPCR was performed to quantify CDNF mRNA levels during TG-treatment, and GAPDH was used to normalize RT-qPCR data. The bars represent the mean ± SEM for two independent experiments.

Supplementary Table 1. The efficiency of primers used for RT-qPCR

Gene	Slope	Efficiency
GAPDH	-3,140	2,081
GRP78	-3,116	2,093
GRP94	-3,058	2,114
Total XBP1	-3,2588	2,027
sXBP1	-3,1361	2,057
CHOP	-3,4833	1,937
ATF4	-3,3361	1,994

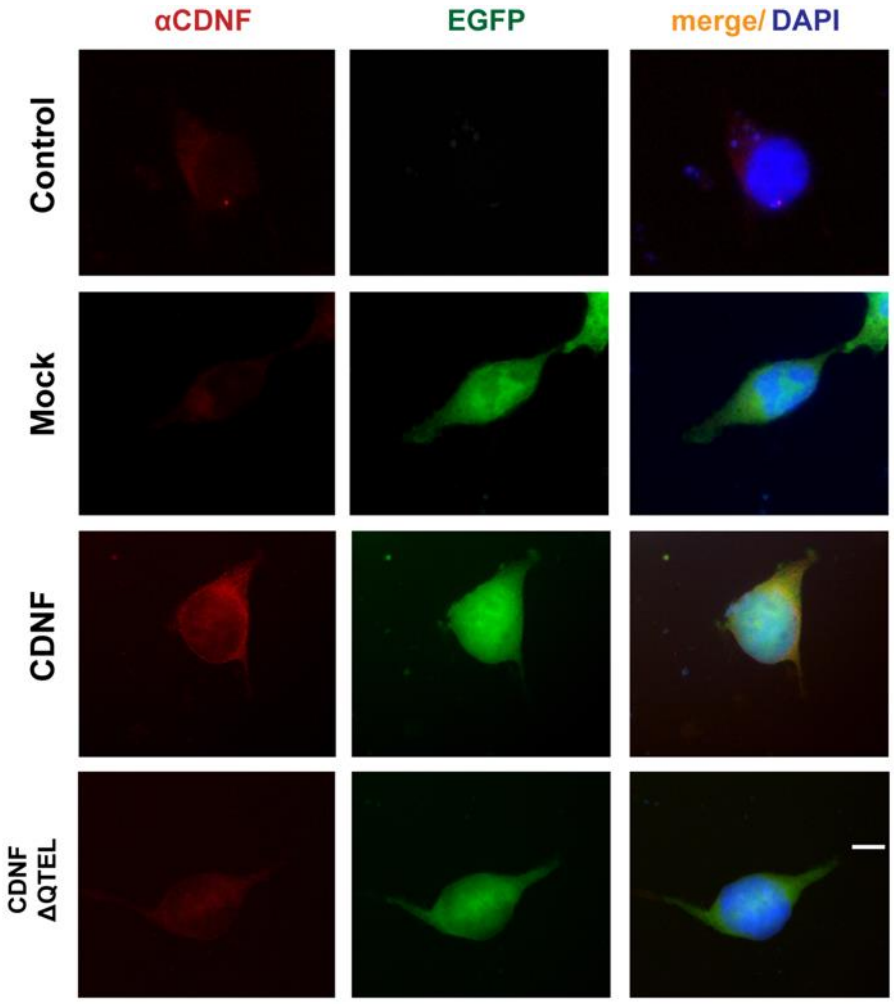


Figure Supplementary 6. Characterization of the CDNF mutant lacking the QTEL sequence. (CDNFΔQTEL). HEK293-T cells were transfected with the plasmids used, FUGW (vacuum control), FUG-1D2A-CDNF (CDNF), and the vector expressing CDNF without its non-canonical ER retention sequence QTEL, FUG-1D2A-CDNFΔQTEL-W (CDNF ΔQTEL) and the mutant were characterized by immunofluorescence against CDNF.

Non-canonical retention sequence QTEL is necessary to induce the expression of proteins related to the UPR and decreases protein related to apoptosis.

In order to assess the importance of the QTEL sequence in the regulation of the UPR response by CDNF, we evaluated whether the overexpression of the mutant CDNF ΔQTEL on the UPR response. First, the levels of the PDI protein were assessed and no differences were found. Interestingly, the CDNF version lacking QTEL is not present in the whole homogenate and does not increase the levels of BiP, ATF4, and ATF6 compared to CDNF wild type version (Figure 9). Finally, the CHOP protein levels were evaluated in cells treated with two μM of TG for 24 h, where a lower increase is observed in cells expressing native CDNF, but not in the version lacking QTEL where CHOP increases the protein levels in comparison to control (Figure 9). These results together confirm that the protection exerted by the CDNF is due to its retention in the RE, where it can promote an early UPR and inhibit apoptosis induced by ER stress.

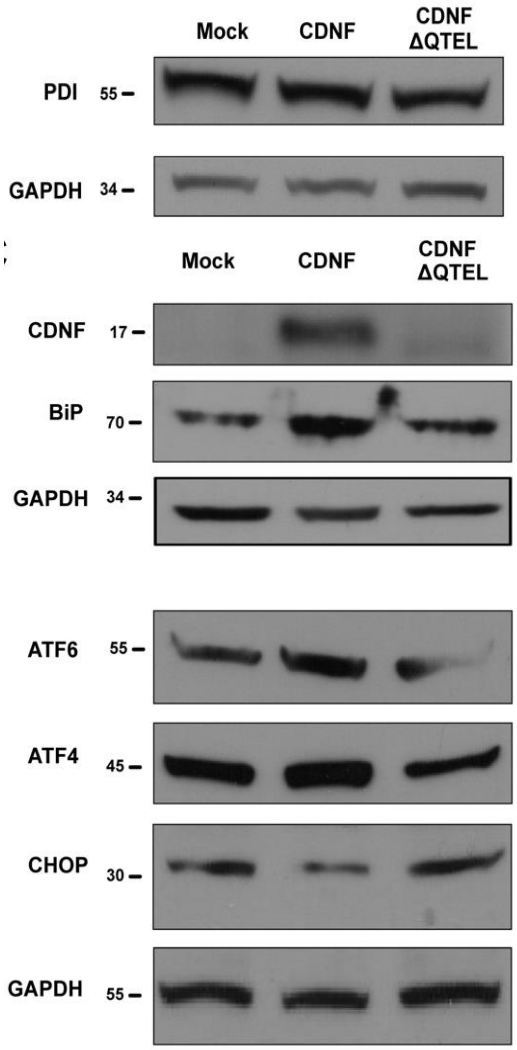


Figure 5. Non-canonical retention sequence QTEL is necessary to induce the expression of proteins related to the UPR and decreases protein related to apoptosis. HEK293-T cells were transfected with FUG-W (Mock), and FUG-CDNF-W and FUG-CDNFΔQTEL-W and western blot assays were performed to detect PDI, BiP, ATF4, ATF6 and CHOP, proteins related to UPR.

SECOND PART DOCTORAL THESIS

Growing evidence shows a protective role of CDNF against various types of alterations related to ER stress that culminates with neuronal death (Arancibia et al, 2018, Bäck et al., 2013; Cheng et al. al., 2013; Liu et al., 2017, Mei & Niu, 2014, Voutilainen et al., 2011, Zhou et al., 2016). Given the therapeutic potential of CDNF, there is a need to generate a new methodology for the efficient and regulated delivery of CDNF in the central nervous system, a feature mostly lacking on the current vector used for brain gene therapy (Voutilainen et al., 2015, Lindahl and Lindholm, 2017, Houtarinen et al., 2018). Optogenetics emerges as a proper tool to control the delivery of proteins of interest within cellular systems, such as neuronal tissues.

In this work, we have optimized and adapted an optogenetic system LightOn, originally developed by Wang and collaborators (Wang et al., 2012), to a lentiviral platform to obtain an adjustable and inducible switch system of gene expression compatible with neurons. The LightOn system is composed of: (i) a light-regulated transcription factor, GAVPO, which homo-dimerizes with blue light and recognizes a specific activation sequence; and (ii) a GAVPO-sensitive promoter composed of the UASG sequence linked to a minimal adenovirus promoter E1b (see diagram in Figure 1 of the second article). In this way, we constructed a bicistronic lentiviral vector that expresses the reporter gene EGFP and GAVPO separately. On the other hand, we modified the inducible promoter to adapt it to the lentiviral platform. To achieve this, we generated a reporter vector containing the modified promoter

that manages the expression of mCherry and the neurotrophic factor CDNF, both by blue light.

The functionality of the LightOn lentiviral system was evaluated by transfection in HEK293-T and transduction in cultured neurons. The results show that cells exposed to blue light display a significant increase in mCherry and CDNF, compared to cells kept in darkness. Finally, we generated an all-in-one plasmid that combines all elements to regulate the expression of the protein of interest by blue-light, called the OPTO vector. CDNF was cloned into this vector, and the effect on UPR markers was evaluated. In summary, the expression of CDNF regulated by blue-light was able to induce UPR markers.

In conclusion, the modified optogenetic lentiviral system can be used to finely tune the expression of genes in cells and neurons, thus providing a tool for biomedical research for controlled delivery of genes in neurons. Future studies using this tool will allow the development of a potential therapy against Parkinson's disease by controlling the dose of CDNF delivered to the neurons.

Second Scientific Article: Manuscript in Preparation

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

This article encompasses the execution of the following objectives:

3. To generate a lentiviral platform to assess the delivery of CDNF in a spatially and temporally dosed manner with potential therapeutic application.

3.1 To construct a lentiviral vector that encodes CDNF and whose expression is regulated through an optogenetic system.

3.2 To determine the light conditions that allow controlled expression of CDNF in neurons.

3.3 To assess the activation of the UPR pathways in cells expressing CDNF regulated by blue-light.

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

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ABSTRACT

The development of precision therapies requires molecular switches to control the expression of genes in a limited space and time. Optogenetic tools allow the control of gene expression with a better spatiotemporal resolution compared with chemical inducers, standing up a promise for gene therapy in complex tissues such as the central nervous system. In this work, we adapted the Light-On optogenetic system, composed of a GAVPO (gal4-vivid-p65 optimized) photosensitive transcriptional activator and a reporter controlled by Upstream Activation Sequence of Gal4 (UASG), in a lentiviral genome that permits efficient transduction of neurons. This Light-On lentiviral system was tested first in cell lines, which showed the expression of the mCherry reporter and the cerebral dopamine neural factor (CDNF) in response to blue light. Both mRNA expression and protein levels of mCherry and CDNF resulted in a function of the intensity and time of exposure to blue light and depended on GAVPO presence. Cyclic blue light exposure with a pattern of 15 minutes on and 15 minutes off for 12 or 24 h, allowed reaching stable levels of CDNF mRNA and increasing amounts of the CDNF protein. Hippocampal neurons in culture were transduced with this Light-On lentiviral system and, the expression of CDNF and mCherry was observed only under light conditions. In conclusion, we optimize the Light-On system in a lentiviral platform that allows the regulation of gene expression in neurons.

INTRODUCTION

Gene switches are a valuable tool in cell and molecular biology. They were developed to study gene function, especially of those involved in diseases [1,2]. Gene switches became an alternative approach to overexpression experiments since the former can regulate the dose, space, and time of gene expression. A wide range of gene expression dose may avoid some cellular defects produced by an overexpressed protein [3-5]. In addition, gene switches may be useful in cell types sensitive to overexpression, such as neurons, which in the face of any disturbance, activate cell death mechanisms.

Chemical inducers regulate most of the gene switches; however, they can diffuse freely, being difficult to remove quickly, not allowing a fine regulation [6-8]. Optogenetic systems to control gene expression have emerged as an alternative to chemical-induced gene switches [9-11]. The high spatiotemporal resolution, the easily tunable light intensity and low toxicity for cell, make light an ideal inducer. An optogenetic gene switch system could be useful in neurons improving the studies of genes involved in neurodegenerative diseases, many of which its role is not completely elucidated. Also, it could contribute to a novel strategy with therapeutic importance, evaluating the effects of neuroprotective molecules in a wide range of expression.

The generation of an optogenetic gene switch system compatible with neurons needs the lentiviral platform to deliver genetic material [12,13]. Lentiviruses are used to infect both mitotic and post-mitotic cells, integrating genetic information into the host genome and maintaining a long term stable expression [14,15]. Most of the optogenetic systems incorporated into neural cells have used photosensitive channels to manipulate neuronal

excitability [16,17]. So far, one study has reported an optogenetic system in neurons for controlling endogenous transcription. This system is based on an optogenetic two-hybrid approach [18]. Although this gene switch offers control of endogenous expression, it is based on the formation of a heterodimer that requires the expression of two modules for reconstituting a functional transcription factor. Also, this approach requires the design and construction of DNA binding domains for each endogenous gene that needs to be manipulated.

In this work, we optimized and adapted the optogenetic LightOn system, originally developed by Wang and co-workers [9], to a lentiviral platform to obtain an inducible and adjustable neuronal compatible gene switch system. The LightOn system is composed of (i) a light-switchable transcription factor GAVPO, which homodimerizes upon blue light and recognizes a specific activating sequence; and (ii) a GAVPO responsive promoter composed by the UASG sequence linked to a minimal adenovirus E1b promoter. Thus, we constructed a bicistronic lentiviral vector expressing the reporter gene EGFP and GAVPO. On the other hand, we modified the GAVPO responsive promoter to adapt it to the lentiviral platform. For this, we generated a light reporter vector containing the modified promoter and driving the expression of mCherry and other proteins of interest by blue light. Finally, the functionality of the LightOn lentiviral system was evaluated by transfection and transduction in HEK293-T, PC-12, and neurons. All cells exposed to blue light showed a significant increase in mCherry and CDNF expression in comparison to cells maintained in darkness. Taken together, the developed optogenetic lentiviral system can be used to control gene expression in neural cells, providing an important tool for biomedical research.

MATERIALS AND METHODS

DNA manipulation and lentiviral plasmid construction.

The reporter plasmids F-UAS$_{(L)}$-mCherry-W and F-UAS$_{(s)}$-mCherry-W were sub-cloned in the FU-mCherry-W plasmid replacing the ubiquitin promoter, between *PacI* and *AgeI* restriction sites. PolyA-UASGx5-TATA (FP PAU5TA: 5'-GCTTTAATTAACTTGG AGCGGCCGCAATA-3' and RP PAU5TA: 5'- TTTACCGGTATGGTGGCCAAGCTT ACTT-3') and UASGx5-TATA (FP PAU5TA: 5'-GCGTTAATTAAAA GTGCAGGTGCCAGAAC-3' and RP U5TA: 5'-TTTACCGGTATGGTGGCCAAGCTTA CTT-3') amplified by PCR were obtained from pU5-Gluc plasmid. The bicistronic lentiviral plasmid F-UAS$_{(s)}$-mCherry-1D2A-HA-CDNF-W was obtained by sub-cloning UASGx5-TATA sequence, flanked between *PacI* and *AgeI*, from F-UAS$_{(s)}$-mCherry-W into FU-mCherry-1D2A-HA-CDNF-W, by replacing ubiquitin promoter. On the other hand, the bicistronic lentiviral plasmid FUG-1D2A-HA-GAVPO-W was obtained by sub-cloning GAVPO by PCR from a pGAVPO plasmid (FP GAV: 5'-GGGTCTAGAGTCTATGAA GCTACTGTCTTCT-3' and RP GAV: 5'-GGGTGATCAATTACTTGTCATCATCGTCTT TG-3'), which was inserted between *NheI* and *BamHI* restriction sites in FUG-1D2A-HA-W vector [19]. All plasmids were verified by sequencing.

Antibodies

The following primary antibodies were 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 (1:10000, 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). The secondary

antibodies for immunofluorescence were Alexa Fluor® 647 anti-goat (1:500, Invitrogen, USA). For immunoblotting, horseradish peroxidase-conjugated antibodies were used to detect mouse, goat, and rabbit primary antibodies (1:5000, Invitrogen, USA).

Cell culture and transfection

HEK293-T cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Gibco), supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 units/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). PC-12 cells were cultured in DMEM supplemented with 10% (v/v) horse serum (Gibco), 5% (v/v) fetal bovine serum (Gibco), 100 units/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). Both cell cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. Cells were transfected with Calfectin agent following the manufacturer's recommendations (Calbiotech). For fluorescence microscopy experiments, 8 x 10^4 cells were plated in a 24-well culture plate with coverslips poly-L-lysine-pretreated. For western blot, 2 x 10^5 cells were plated in a 6-well culture plate. All transfections were performed for 24 hours.

Lentivirus production and transduction

HEK293-T Cells were transfected with Lipofectamine 2000 following the manufacturer's recommendations (Thermo Fisher). Transfection of cells was carried out with a three-plasmid lentiviral system containing pCMVΔR8.91, pCMV-VSVg, and the corresponding transfer plasmids pFUG-1D2A-HA-GAVPO-W and pF-$UAS_{(s)}$-mCherry-1D2A-HA-CDNF-W to produce a functional lentivirus as described previously [21]. Twenty-four hours later, the culture medium was replaced, and lentiviral particles were collected from the supernatants at 48 and 72 h after transfection, centrifuged, and stored at -80ºC.

Rat hippocampal neuron culture and lentiviral transduction

Primary cultures of hippocampal neurons were prepared from fetal rat brains (embryonic day 19; E19) according to the method of Kaech and Banker [20]. The brains were collected in precooled Hank's balanced salt solution (HBSS), and after that, the hippocampi were carefully dissected out. Cell suspensions were prepared by treating the tissue with 0,25% trypsin for 15 minutes at 37°C, followed by homogenization. After dilution with neurobasal medium containing 0,5 mM GlutaMAX, B-27 supplement (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin, neurons were plated on culture dishes coated with 0,1 mg/ml poly-L-lysine. Lentiviral transduction of the hippocampal neuron cultures was performed at 4 DIV by adding 150 µl of the virus recollected from filtered supernatant. The neurons were incubated until 7 DIV at 37°C with 5% CO_2.

Induction assay with blue light (465 nm)

Immediately after transfection and transduction, both plates were maintained in darkness at 37ºC with 5% CO_2. Twenty-four hours after transfection, a culture plate was exposed to illumination cycles with blue light (465 nm) with a sterile house-made blue light system consisting of a blue LED tape with 294 blue led. Cells were 9.5 cm away from blue led, and their intensity was 116 lux. The other culture plate was maintained in darkness until the end of the experiment. The illumination cycles consisted of 15 minutes with blue light and 15 minutes in darkness, to avoid overheating, for 24 hours or periods indicated in the figures.

Immunoblotting

The samples were homogenized with RIPA buffer (Millipore) containing inhibitors (1mM PMSF, 7 µg/ml Pepstatin, 5-10 µg/ml Leupeptin and ten µg/ml Aprotinin) and sonication. Protein concentration was quantified by the BCA method (Thermo Scientific). Homogenized

samples were heated in Laemmli´s loading buffer at 95°C for 5 minutes and then separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Hybond, Amersham Biosciences) and blocked with a solution containing Tris-buffered saline at pH 7.4 (TBS), 5% non-fat milk, and 0.1%. After incubation with primary antibody for two hours at room temperature or overnight at 4ºC, the membranes were washed three times with 1x TBS-T and subsequently incubated with secondary antibody conjugated with horseradish peroxidase for 45 minutes at room temperature. Three final washes with 1x TBS-T for 10 minutes were carried out. Finally, proteins were visualized by chemiluminescence (ECL, Amersham, USA).

Immunofluorescence

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

Imaging and quantification of fluorescence intensity

Images were acquired with an Olympus DS-Fi2 epifluorescence microscope obtained with 40X and 100X Olympus UplanFI oil immersion objective and equipped with a Nikon DS-

Fi2 camera operated with the standard QC capture software (Q-Imaging). For quantification, images were processed employing ImageJ software (NIH, Baltimore, MD) using the corrected total cell fluorescence (CTCF) method.

Statistical analysis

The data in figures represent the mean ± SEM (standard error of the mean) of results obtained from at least three independent experiments. To compare two groups (darkness and light conditions), a "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% ($p < 0.05$).

RESULTS

3.1. Construction of GAVPO and reporter lentiviral vectors

Vector gene expression with controllable promoters is a useful tool for studying gene function. As the LightOn system has proven its suitability with tight expression control [9], we addressed the question of whether this system can also be used for expression in neurons with lentiviral vectors. For this purpose, we constructed two types of lentiviral vectors: 1) a light-reporter vector that contains the GAVPO-responsive promoter (PolyA-UASGx5-TATA) upstream of the transgene of interest, and 2) a transactivator vector that expresses GAVPO (schematized in Fig. 1). For doing this, we optimized the LightOn system to make it compatible with lentiviral vectors. The original GAVPO-responsive promoter contains a PolyA sequence upstream of the UASGx5 element, which is not compatible with the lentiviral particle production. Lentiviruses used in this work have a CMV promoter upstream

of the 5'LTR region that drives the expression of the viral transcript located between LTRs sequences (gray boxes, Fig. 1). Thus, the PolyA sequence could interfere with the production of the viral transcript producing an incomplete viral RNA. For this reason, we generated both large ($UAS_{(L)}$, with PolyA sequence) and short ($UAS_{(S)}$, without PolyA sequence) versions of the light-reporter vector (Fig. 1A and B). We also constructed a bicistronic light-reporter vector with $UAS_{(S)}$ promoter to express a protein of our interest, to test the functionality of the optimized system by transfection and transduction (Fig. 1C). Furthermore, we generated a bicistronic transactivator lentiviral vector that expresses the reporter EGFP and GAVPO by using a 1D/2A bicistronic sequence (Fig. 1D). The constructions are detailed in the materials and methods section.

3.2 Expression and induction of LightOn lentiviral vectors by blue light

First, we characterized the expression of the LightOn system components. Lentiviral vectors were transfected in HEK293-T cells and were analyzed by immunoblot (Fig. 1A, Supplementary information). The bicistronic vector FUG-1D/2A-HA-GAVPO-W expresses both EGFP and GAVPO constitutively as a result of cleavage of the 1D/2A bicistronic sequence. However, a portion close to 5% of peptides remains non-cleaved, as previously reported (Torres et al. 2010). To analyze the functionality of the LightOn lentiviral system, FUG-1D/2A-HA-GAVPO-W and F-$UAS_{(L)}$-mCherry-W or F-$UAS_{(S)}$-mCherry-W vectors were co-transfected in HEK293-T cells for 24 h. Then, an induction protocol was carried out with blue light (see Material and methods). As shown in Fig. 2A, HA-GAVPO protein was observed in all conditions. Cell cultures under blue light exposure showed a significant increase in mCherry expression compared to cells maintained in darkness, as it was detected

by immunoblot (Fig. 2A-C) and immunofluorescence (Fig. 2D and E). Also, the same results were observed by immunofluorescence using PC-12 cells (Fig. 2F and G). In darkness condition, we detected a slight expression of a mCherry protein with both large and short versions of light-reporter. To clarify this point, we co-transfected both light-reporter versions with FUGW without induction. By immunoblot, the mCherry protein levels were just detected in $UAS_{(S)}$ version (Fig. 1B, Supplementary information). We also observed this background in HEK293-T cells transfected with each light-reporter version, in darkness and light conditions (Fig. 2A and B Supplementary information).

We thought that the background observed by transfection was due to the lentiviral CMV promoter. Thus, to determine this possibility, we generated a new light-reporter version without CMV promoter ($UAS_{(L)}$ ΔCMV). By immunofluorescence, we noticed that $UAS_{(L)}$ ΔCMV light-reporter version presented less background (Fig. 2C, Supplementary information). However, induced-cells (HEK293-T and PC-12) co-transfected with FUG-1D/2A-HA-GAVPO-W and F-$UAS_{(L)}$-mCherry-W ΔCMV, presented background levels similar to those presented by the other light-reporter versions (Fig. 3A and B, Supplementary information). Thus, we hypothesized this was due to the GAVPO availability, which is clarified below.

Taken together, the lentiviral vectors express correctly GAVPO, EGFP, and mCherry proteins. Also, the optimized LightOn lentiviral system can be activated by blue light in HEK293-T and PC-12 cells with different background levels.

3.3 Inducing the expression of Cerebral Dopamine Neurotrophic Factor (CDNF) by blue light

The background expression of mCherry observed in darkness led us to clarify whether this was because of the wide availability of GAVPO. Also, as proof of concept, we expressed a neurotrophic factor (cerebral dopamine neurotrophic factor, CDNF) regulated by our optimized blue light optogenetic lentiviral system. For this purpose, we co-transfected HEK293-T cells with F-$UAS_{(s)}$-mCherry-1D/2A-HA-CDNF-W, which expresses mCherry and CDNF under $UAS_{(S)}$ promoter sequence, and FUG-1D/2A-HA-GAVPO-W. Cells exposed to blue light showed a significant increase of mCherry and CDNF expression levels (Fig. 3A). We checked this expression was dependent on GAVPO availability because no background expression of mCherry was detected when we co-transfected F-$UAS_{(s)}$-mCherry-1D/2A-HA-CDNF-W and FUG-1D/2A-HA-CDNF-W, used as control (Fig. 4, Supplementary information).

Finally, we evaluated the temporal expression of blue light-modulated genes by co-transfecting HEK293-T with FUG-1D/2A-HA-GAVPO-W and F-$UAS_{(s)}$-mCherry-1D/2A-HA-CDNF-W. Then, cells were exposed under blue light for different periods (0.5, 1, 3, 6, 9, 12, and 24 h). By immunoblot, levels of Flag-GAVPO, mCherry, and CDNF were quantified (Fig. 3B-D). Also, we determined the transcripts of modulated genes (Fig. 3E-F). These experiments showed a gradual increase in mCherry and CDNF expression (protein and transcripts) during the time course of induction. Altogether, these results demonstrate that a neurotrophic factor-like CDNF can be modulated by blue light and that this expression is

dependent on GAVPO availability. Also, the expression level can be modulated according to blue light exposure times.

3.4 Expression of LightOn components and CDNF by lentiviral particles in neurons

Finally, we evaluated the functionality of this system in primary cultures of hippocampal rat neurons. Lentiviral particles were produced in HEK293-T cells (see Material and Methods for details). Neurons of 4 DIV were co-transduced with FUG-1D/2A-HA-GAVPO-W and F-$UAS_{(s)}$-mCherry-1D/the 2A-HA-CDNF-W virus. After seven days, neurons were induced with blue light for 12 hours. Co-transduced neurons exposed to blue light showed expression of mCherry and CDNF. This expression was GAVPO-dependent, given that cells expressing mCherry and CDNF were positive for EGFP expression, the transduction reporter gene of GAVPO expression. These results confirm the functionality of the LightOn lentiviral system in neurons by transduction.

DISCUSSION

In this work, we optimized the optogenetic system LightOn to adapt and combine it with a lentiviral platform. We constructed lentiviral vectors that express the LightOn system components previously reported, one vector that expresses GAVPO and light-reporter vectors that contains the original version of the GAVPO-responsive promoter and our new version of this inducible promoter. These lentiviral vectors demonstrated a specific expression induced by blue light.

The original version of Wang and co-workers has a PolyA region upstream of the UASGx5 sequence [9], presumably to stop any upstream expression. As it was explained before, this PolyA region is incompatible with the lentiviral platform because it could interfere with the complete synthesis of viral transcript during the viral particle production. For this reason, we constructed a bicistronic light-reported plasmid that expresses mCherry and CDNF considering the short version of UASGx5 inducible promoter ($UAS_{(S)}$), since our interest was to evaluate this system by transduction. The results show that the $UAS_{(S)}$ promoter is functional as the original version. Also, the production of lentiviral particles with the $UAS_{(S)}$ promoter was successful, showing that this version is compatible with the lentiviral platform.

In our first blue-light induction experiments, we observed a significant increase in mCherry protein levels. However, in darkness condition, we found a slight expression of mCherry (background) with both $UAS_{(L)}$ and $UAS_{(S)}$ versions of light reporter plasmids (Fig. 2D-G). Initially, we think that our background may have been a non-light induced expression, due to the CMV promoter localized upstream the 5'LTR sequence in lentiviral plasmids. For this reason, F-$UAS_{(L)}$-mCherry-W ΔCMV vector was constructed, which lacks an upstream CMV promoter. We almost did not observe the background expression of mCherry when F-$UAS_{(L)}$-mCherry-W ΔCMV plasmid was transfected in HEK293-T cells (Fig. 2, Supplementary information). Nevertheless, when F-$UAS_{(L)}$-mCherry-W ΔCMV vector was co-transfected with FUG-1D/2A-HA-GAVPO-W, we continued observing background expression (Fig. 3, Supplementary information). Thus, we thought that in co-transfection experiments, the background could be due to GAVPO availability. To clarify this point, we performed induction experiments by replacing the FUG-1D/2A-HA-GAVPO vector by the

other one without GAVPO sequence expression. Here, we observed mCherry and CDNF expression only under blue light illumination in the presence of GAVPO (Fig. 4, Supplementary information), which means that the expression is GAVPO-dependent. This also suggests that background in co-transfections could be because of a wide GAVPO availability. Concerning mCherry and CDNF induction experiments (Fig. 3), we did not observe expression of mCherry in darkness condition, but a slight expression of CDNF protein was detected, which is because CDNF is expressed endogenously (Arancibia, Zamorano, and Andrés, 2018).

We determine by quantification that blue light illumination increases protein levels of mCherry and CDNF significantly. Our induction levels are lower than Wang and co-workers reported. Probably, this is due to technical issues and the method employed to quantify. 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, getting an induction of ∼40-fold. The above could be explained due to the different mRNA degradation rates. We used non-stabilized reporter genes. In this work, we also measured protein levels induced by blue-light by western blot and obtained about 5-fold induction levels. Finally, the results by transduction in hippocampal neurons cultures showed similar results obtained by transfection in HEK293T; we visualized by epifluorescence microscopy mCherry and CDNF expression as a result of activation of GAVPO through blue light illumination.

Thus, we demonstrated that the LightOn system adapted to the lentiviral platform is inducible by blue light. With the induction levels obtained in this work, we suggest that this tool could be applied in neuronal systems to regulate the expression levels of target genes finely. This molecular tool could be another option for existing gene therapy, but, for this purpose, this system has to be evaluated in more detail. Particularly, gene switch systems have not been completely compatible with CNS, mainly because of the blood-brain barrier presence. This structure selectively filters the passage of molecules, which includes some molecular inducers used so far

.

Moreover, some gene switch systems use very small chemical inducers, which can spread freely and, therefore, can be hard to remove. In contrast, with light as inducer, adjustments can be made easily, and high spatial and temporal resolution can be achieved. Finally, we hope that combining this tool with new technologies of illumination through nanoparticles; it could be possible to develop new therapies oriented to CNS.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

RZ and DA, acquisition and analysis of the data, drafting the manuscript; MEA, analysis, and interpretation of the data drafting and revising the manuscript; and LR and PZ, conception and design, analysis, and interpretation of the data, drafting and revising the manuscript. All the authors have read and approved the final manuscript.

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FIGURES

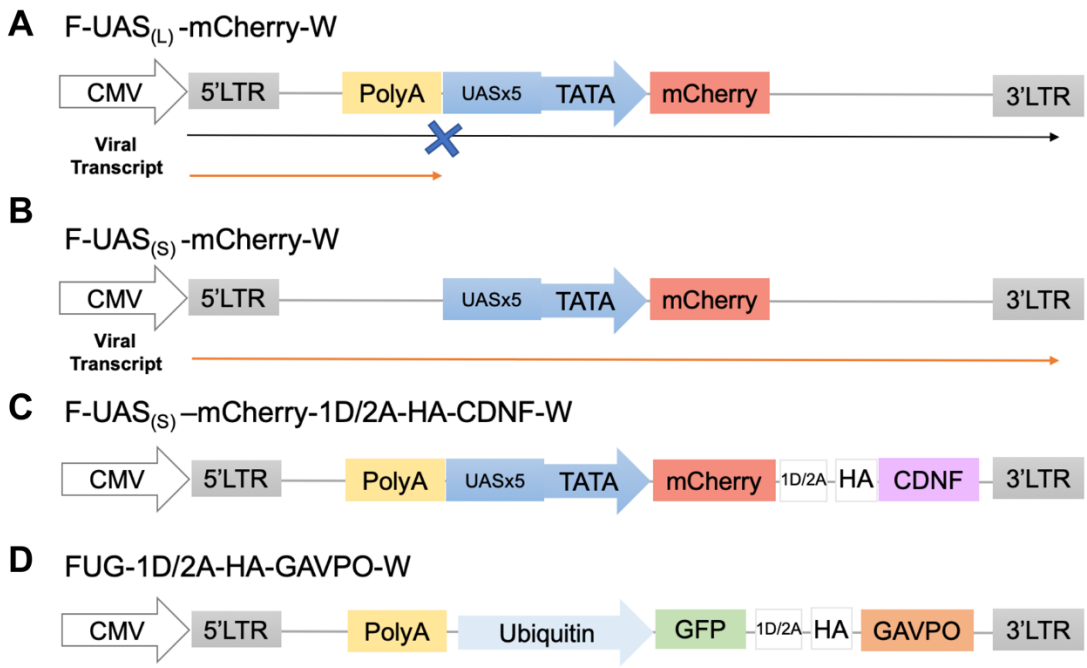


Figure 1. Schematic drawing of the blue-light inducible lentiviral expression vectors. Schematic representation of (A) F-UAS$_{(L)}$-mCherry-W and (B) F-UAS$_{(S)}$-mCherry-W (without PolyA region, yellow) light-reporter lentiviral plasmids. These plasmids were constructed from the pU5-Gluc plasmid, where UASGx5 inducible promoter (blue) was amplified by PCR and inserted into FU-mCherry-W, replacing the ubiquitin promoter. In orange is shown the viral transcript expected for viral particle production step. (C) Schematic representation of F-UAS$_{(s)}$-mCherry-1D/2A-HA-CDNF-W, where the UASGx5-TATA inducible promoter was sub-cloned into FU-mCherry-1D/2A-HA-CDNF-W plasmid. This plasmid express mCherry (red) and a neurotrophic factor (purple) in a bicistronic manner, given the presence of a 1D/2A bicistronic element from Foot-and-Mouth disease virus. (D) Schematic representation of FUG-1D/2A-HA-GAVPO-W transactivator lentiviral plasmid that allows the expression of EGFP (green) and photosensitive transactivator GAVPO (orange) in a bicistronic manner.

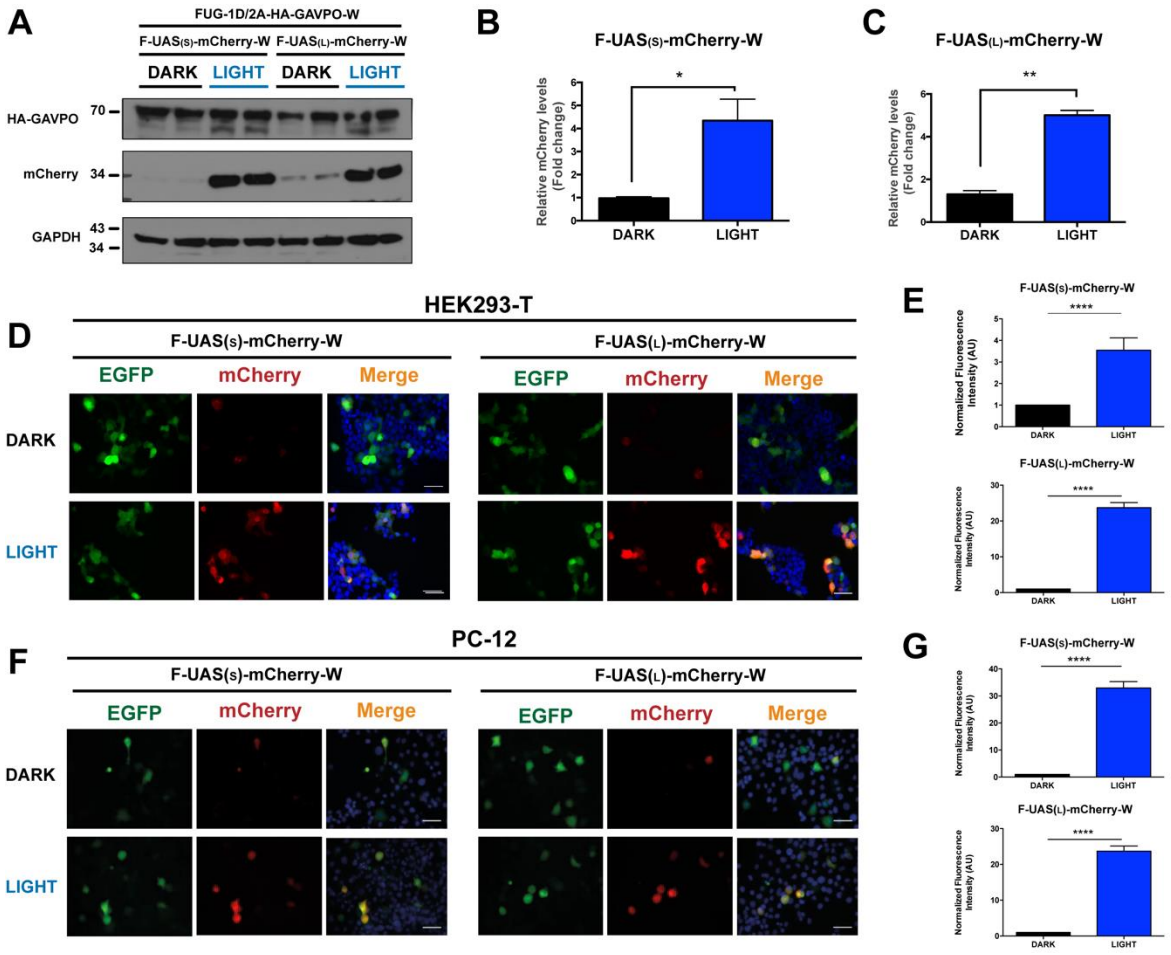


Figure 2. Expression of lentiviral plasmids modulated by blue light. A) HEK293-T were co-transfected with FUG-1D/2A-HA-GAVPO-W and F-UAS$_{(L)}$-mCherry-W or F-UAS$_{(s)}$-mCherry-W. Then, cells were exposed to darkness (DARK) or blue illumination (LIGHT) during 24 h, and western blots were performed to detect mCherry protein levels in both conditions. HA epitope was used to reveal GAVPO expression, and GAPDH was used as load control. B, C) mCherry protein levels were quantified by densitometry. D) HEK293-T and (F) PC-12 cells were visualized by fluorescence microscopy to observe EGFP and mCherry expression from UAS$_{(L)}$ and UAS$_{(S)}$ versions of the inducible promoter in darkness or blue illumination conditions. E, F) Fluorescence intensity quantification of mCherry was performed in EGFP positive cells. Scale Bar: 50 μm. Data are presented as mean ± SEM.

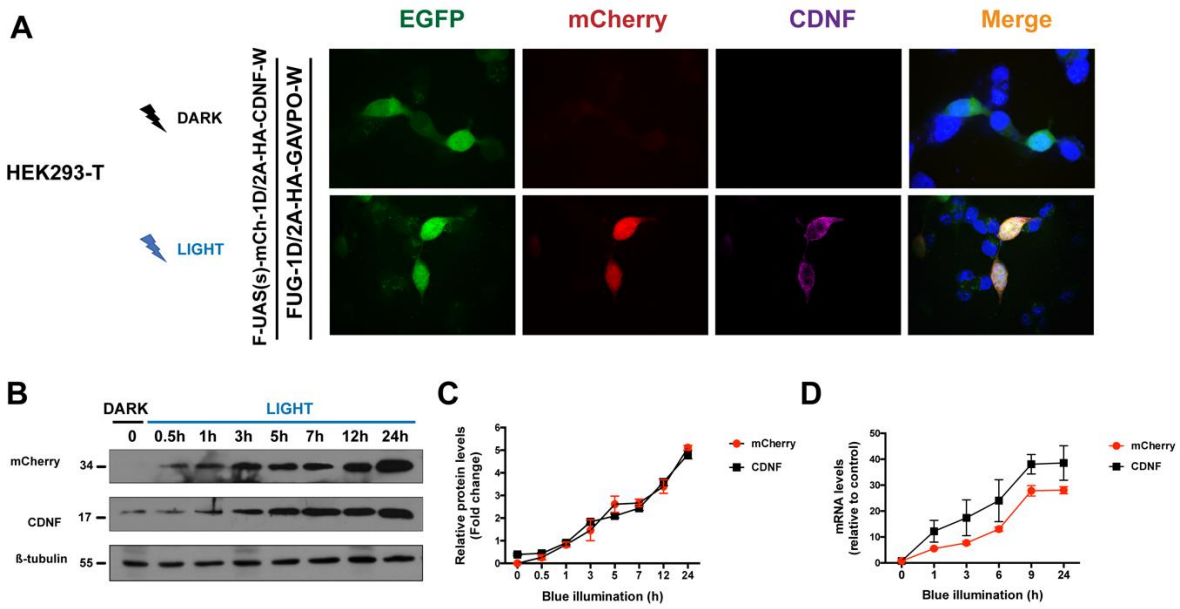


Figure 3. Blue light-induced CDNF expression as proof of concept. A) Plasmids F-UAS$_{(s)}$-mCherry-1D/2A-HA-CDNF-W and FUG-1D/2A-HA-GAVPO-W were co-transfected in HEK293-T cells. Then, cells were exposed to darkness or blue illumination conditions for 24 h. Immunofluorescence against CDNF (purple) was performed, and cells expressing EGFP and mCherry were visualized by fluorescence microscopy. Scale Bar: 20 μm. B) Temporal characterization of the expression induction of mCherry under blue light illumination. HEK293-T cells were co-transfected with FUG-1D/2A-HA-GAVPO-W and F-UAS$_{(s)}$-mCherry-1D/2A-HA-CDNF-W for 24 h and then exposed to darkness (0h) 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 load control. C) Graphs present the quantification of mCherry and CDNF protein levels. D) mRNA levels of mCherry and CDNF was quantified by real-time PCR using specific primers.

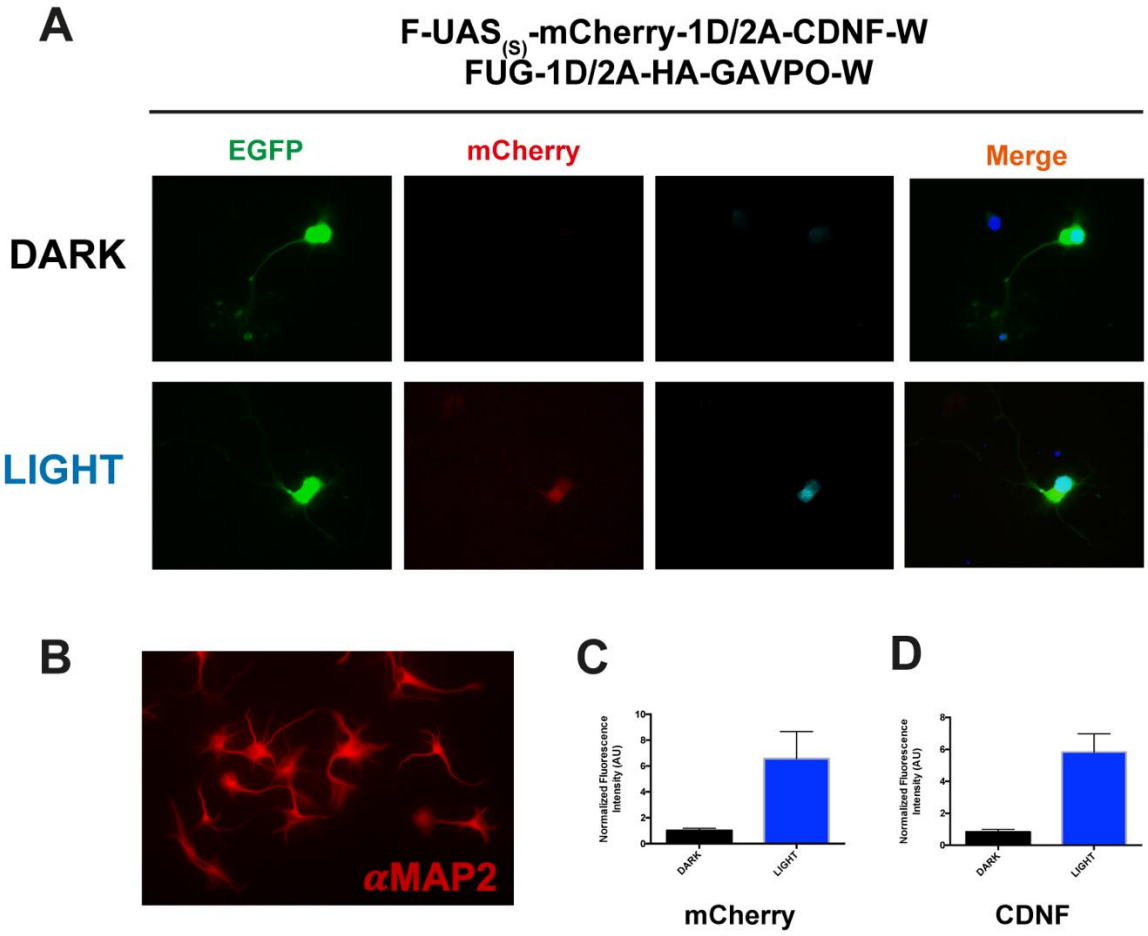
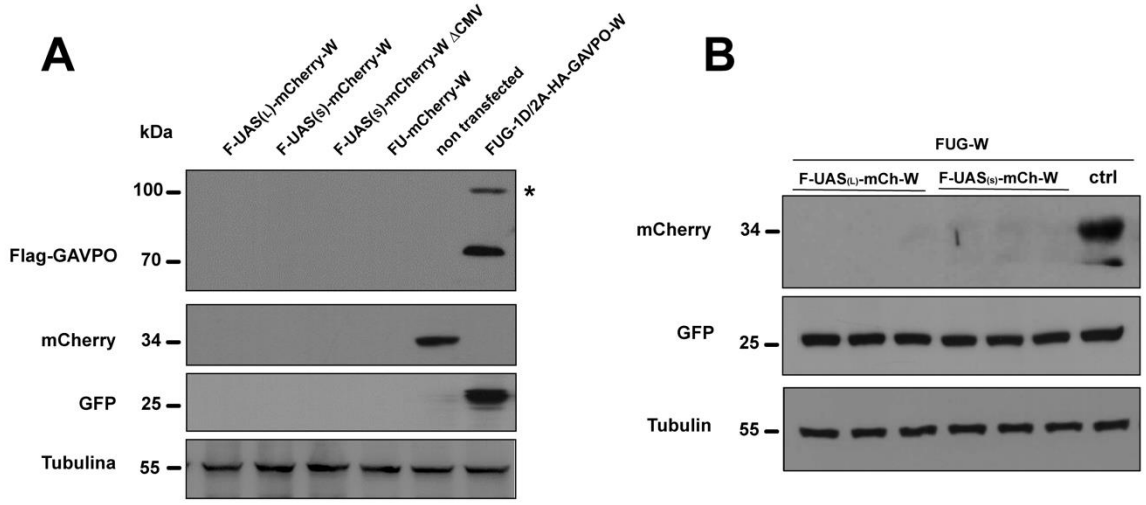


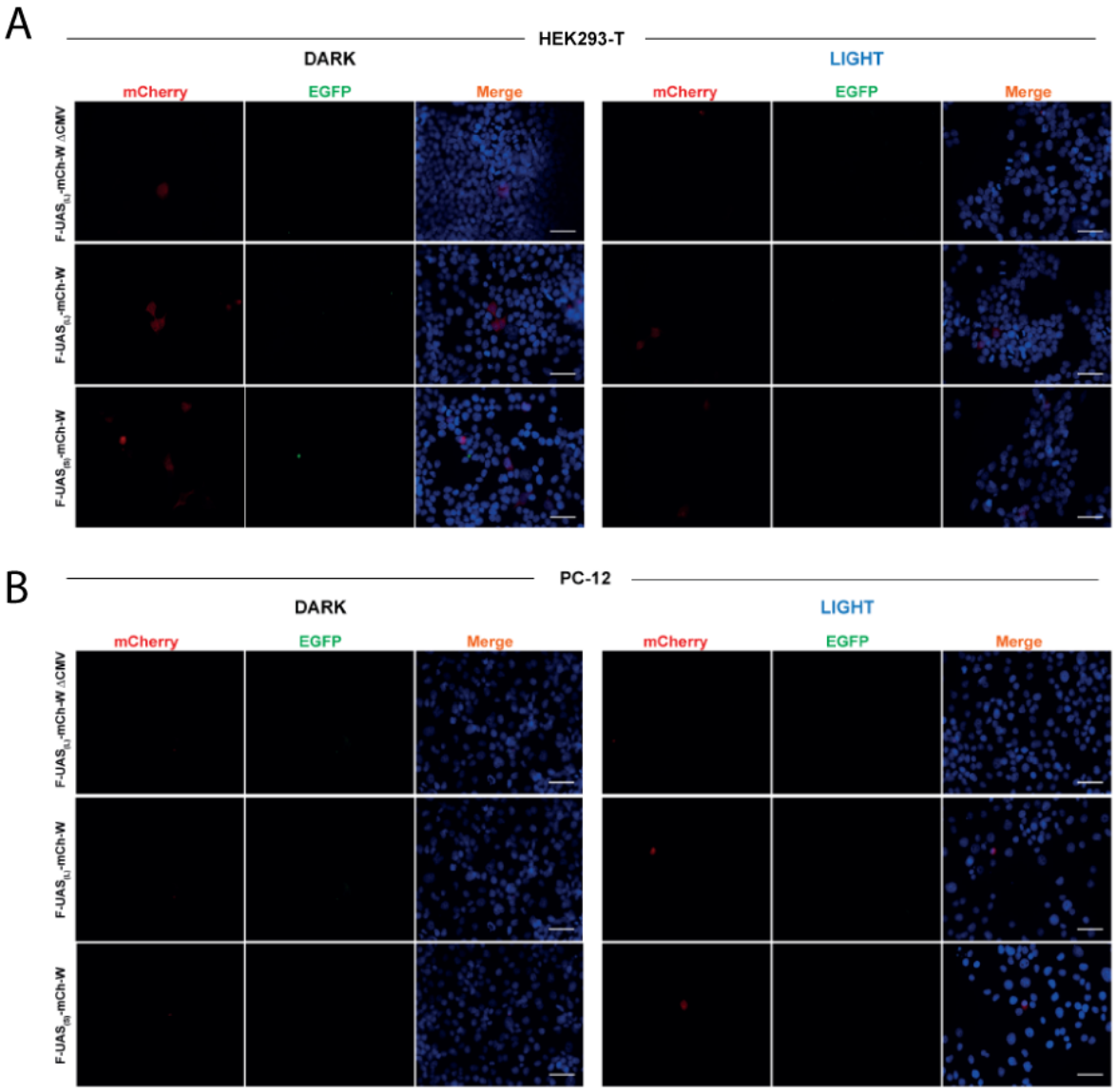
Figure 4. Transduction of primary hippocampal neurons with lentiviral particles. A) Lentiviral particles were packaging (see material and methods), and rat hippocampal neurons in the culture at 4 DIV were transduced with 150 μL of cell culture medium containing lentiviral particles. Then, neurons at 7 DIV were illuminated with blue light for 12 h and fixed. Finally, immunofluorescence against CDNF was performed, and fluorescence microscopy was used to observe neurons expressing EGFP (green), mCherry (red), and CDNF (purple). Scale Bar: 20 μm.

SUPPLEMENTARY FIGURES

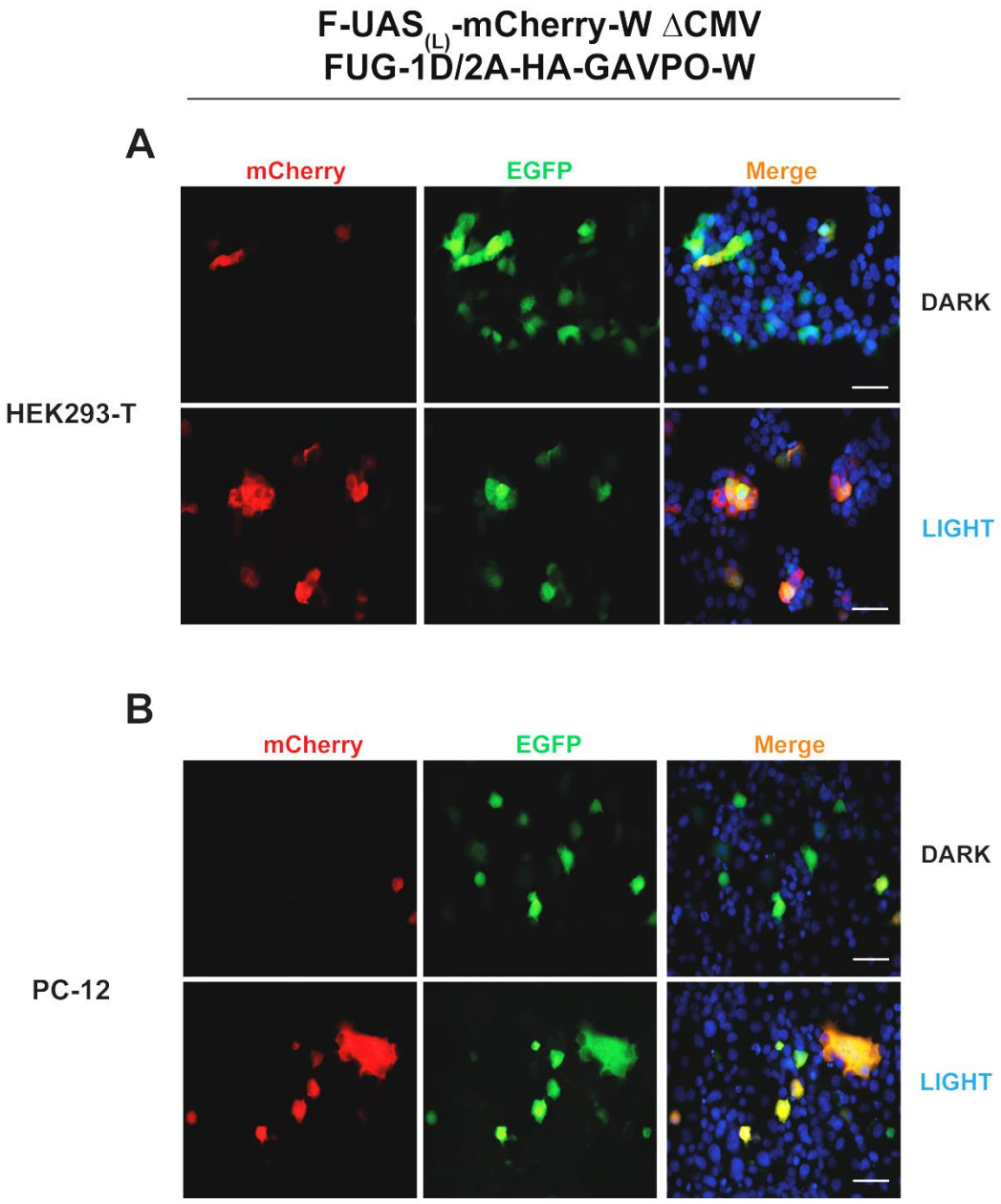
(Of the previous scientific article: Optimization of the Light-On system in a lentiviral platform to control the expression of genes in neurons)



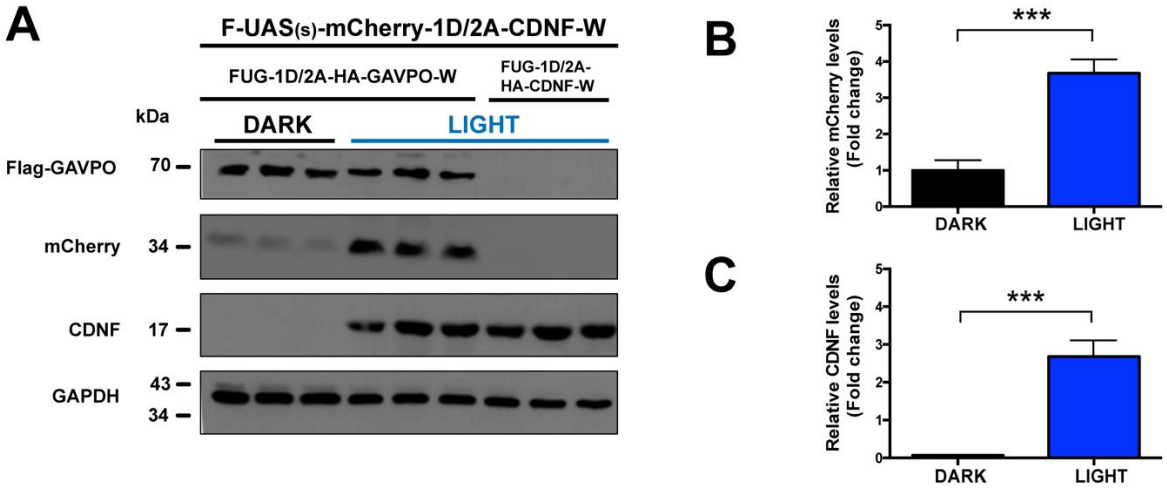
Supplementary Figure 1. Characterization of the expression of LightOn lentiviral vectors. (A) HEK293-T cells were transfected for 24 hours with F-UAS$_{(L)}$-mCherry-W or F-UAS$_{(s)}$-mCherry-W or FU-GAVPO-W or FUG-1D/2A-HA-GAVPO-W vectors, and western blot was performed to evaluate the expression of GFP, mCherry, and Flag-GAVPO. FU-mCherry-W transfected cells were used as mCherry expression control, and GAPDH was used as load control. B) HEK293-T cells were co-transfected with F-UAS$_{(L)}$-mCherry-W or F-UAS$_{(s)}$-mCherry-W light-responsive vector and FUGW (as transfection control). Western blot was performed to evaluate the expression of GFP and mCherry. FU-mCherry-W was used as a control (ctrl), and ß-Tubulin was used as load control.



Supplementary Figure 2. Expression of different versions of UAS light-responsive promoter in cells that do not express GAVPO. HEK293-T (A) and PC-12 (B) cells were transfected only with F-UAS$_{(S)}$-mCherry-W or F-UAS$_{(L)}$-mCherry-W or F-UAS$_{(L)}$-mCherry-W ΔCMV. Cells were maintained in darkness conditions (DARK) or blue light illumination (LIGHT) for 24 hours. mCherry was visualized by epifluorescence microscopy. Scale Bar: 20 µm.



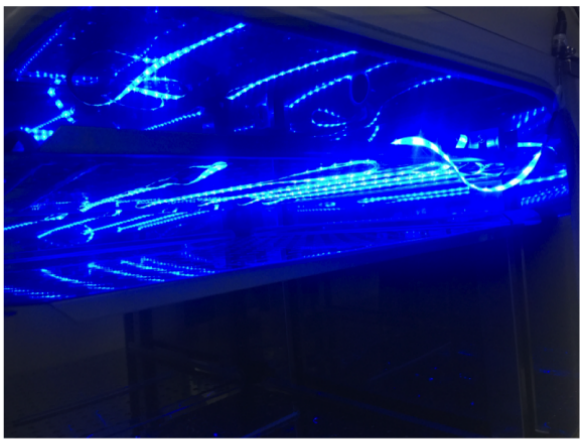
Supplementary Figure 3. Co-transfection of the F-UAS$_{(L)}$-mCherry-W ΔCMV light-responsive and the FUG-1D/2A-HA-GAVPO-W vectors. To evaluate the possible contribution of CMV promoter upstream LTR sequences, HEK293-T (A) and PC-12 (B) cells were transfected with F-UAS$_{(L)}$-mCherry-W ΔCMV and FUG-1D/2A-HA-GAVPO-W. Cells were maintained in darkness conditions (DARK) or blue light illumination (LIGHT) for 24 hours. mCherry and GFP were visualized by epifluorescence microscopy. Scale Bar: 20 µm.



Supplementary Figure 4. Expression of mCherry and CDNF dependent on GAVPO. A) HEK293-T cells were co-transfected with F-UAS$_{(s)}$-mCherry-1D/2A-HA-CDNF-W and FUG-1D/2A-HA-GAVPO-W or FUG-1D/2A-HA-CDNF-W (the control without GAVPO). Cells were exposed to darkness or blue light illumination conditions. After 24 hours, cells were homogenized, and western blot was performed to detect Flag-GAVPO, mCherry, CDNF, and GAPDH proteins. GAPDH was used as load control. B, C) Quantification of relative mCherry and CDNF protein levels.

A

B



Supplementary Figure 5. Home-made blue light-modulated expression system used in the cell culture incubator. A sterile house-made blue light system was generated. This system consisted of a blue LED tape with 294 blue LEDs.

Construction of lentiviral optogenetic vector OPTO-CDNF

We previously demonstrated that we could regulate the expression of CDNF by blue light, based on an optogenetic system comprising two vectors. A vector to express the light-regulated transcription factor GAVPO and another vector that expresses the gene of interest controlled by the UAS promoter. A more efficient and less invasive way to achieve the same goal is to have a single vector with both functions. For this, we generated the bicistronic OPTO vector that, on the one hand, encodes CDNF under the UAS control and on the other hand, encodes GFP and GAVPO under a ubiquitin promoter (Figure 5).

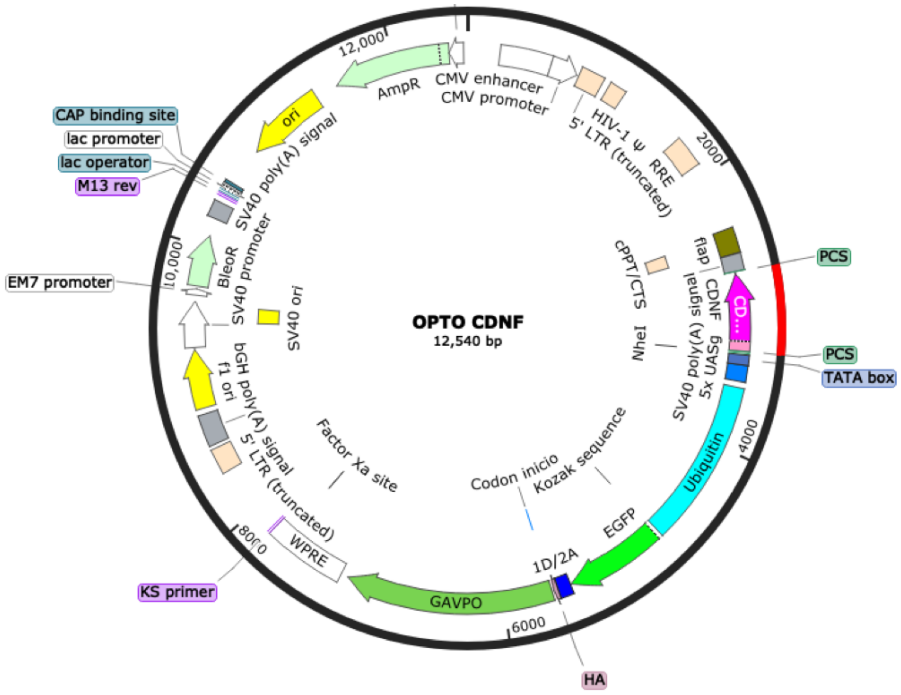


Figure 5. The schematic draw of the blue-light inducible lentiviral expression vector OPTO-CDNF. The OPTO vector expresses bicistronically GFP and GAVPO (green), under ubiquitin promoter (light blue), in one direction and on the other hand, expresses the gene of interest (purple) under UAS promoter (blue), in another direction.

Characterization of lentiviral optogenetic vector OPTO-CDNF

We tested the efficiency of OPTO-CDNF in HEK293-T cells by analyzing the expression of transactivator inducible element HA-GAVPO, GFP, and CDNF, by western blot and immunofluorescence. The OPTO-CDNF vector expressed HA-GAVPO and CDNF correctly in the weight expected. HA-GAVPO was expressed in both conditions, blue light, and darkness. On the other hand, CDNF was expressed only in blue-light conditions. Also, the expression of CDNF was assessed by immunofluorescence and only was presented in cells that express GFP.

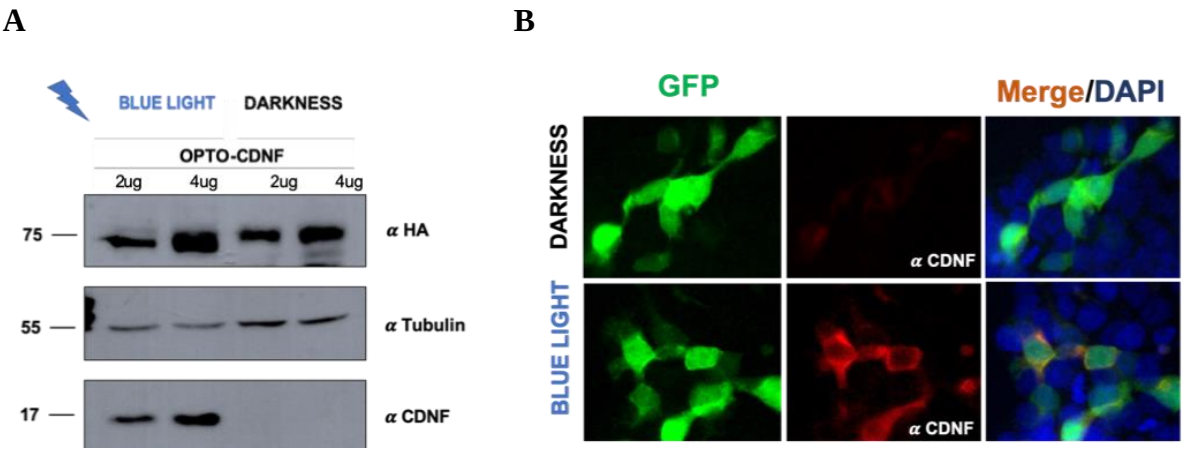


Figure 6. OPTO-CDNF vector expresses HA-GAVPO and CDNF correctly. HEK293-T cells were transiently transfected with OPTO-CDNF vector in conditions of blue light and darkness. Twenty-four hours later, cells were harvested to quantify the expression of HA and CDNF. Tubulin was used as a loading control (A). Also, in the cells expressing OPTO-CDNF, IF was performed for CDNF (red) in both conditions.

Blue light-dependent expression of CDNF increases UPR markers

Corroborating the correct expression of the OPTO-CDNF vector, we transfected HEK293T with the OPTO-CDNF vector and induced the expression of CDNF by blue-light for twenty-four hours (BLUE LIGHT). The control was transfected with the OPTO-CDNF vector but was not induce with blue light (DARKNESS). We were able to induce CDNF by blue-light, not so in darkness condition. Also, the blue light-regulated expression of CDNF induces UPR markers such as BiP, ATF4, ATF6, and XBP1. Therefore, this result confirms our previous finding and strengthens our hypothesis. Future studies will determine if CDNF regulated by blue light can protect against ER stress.

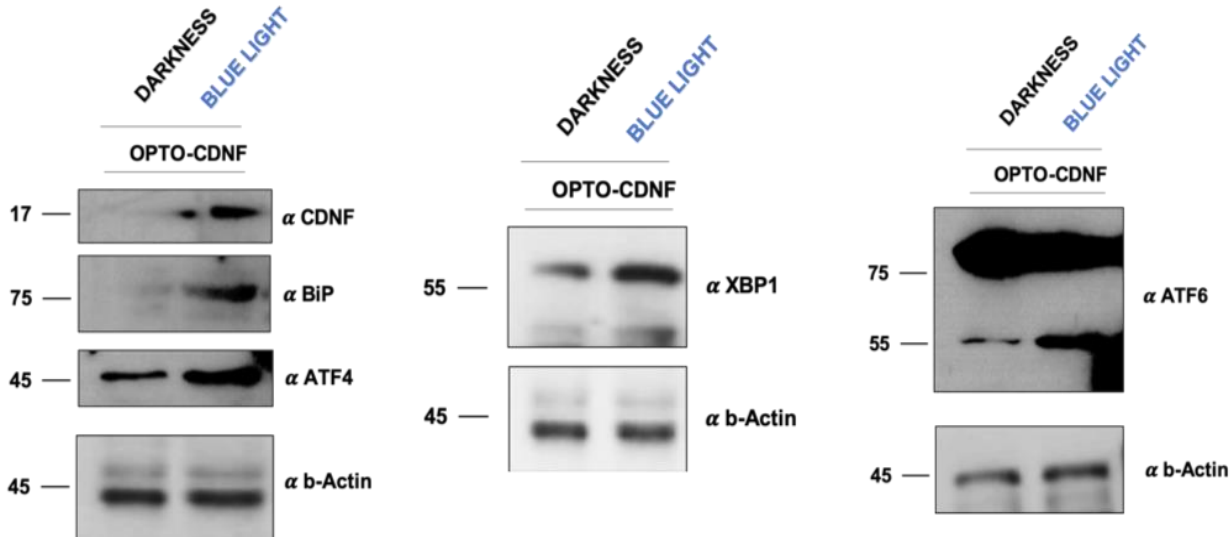


Figure 7. Expression of CDNF regulated by blue light can induce UPR markers such as BiP, ATF4, ATF6, and XBP1. HEK293-T cells were transfected with OPTO-CDNF vector for 24h in two conditions per separate: darkness, or illumination with blue light. Then, the cells were harvested, and western blot was performed against CDNF, BiP, ATF4, ATF6, and XBP1. b-actin was used as a loading control.

GENERAL DISCUSSION

The molecular mechanism of the cytoprotective role of CDNF would be associated with ER stress and UPR pathways.

The ER is a fundamental organelle for proper cell function and required to maintain a correct balance between a load of protein synthesis, it is adequate folding, and posterior Golgi export in a process called proteostasis. Specifically, the ER has a quality control system to eliminate unfolded or misfolded proteins from the secretory pathway and exports only the properly folded proteins to their final destinations. If this balance is altered, the proteostasis is affected, and as a consequence, pathological states could emerge. Several studies have suggested that the perturbation of the proteostasis is directly related to an ER stress, where there is an altered UPR (Ron & Walter, 2007; Walter & Ron, 2011; Varma & Sen, 2015; Hetz & Saxena, 2017; Hetz & Papa, 2018).

Numerous pathological conditions and toxicants, such as thapsigargin and tunicamycin, are implicated in the disruption of ER functions and consequently can lead to the activation of ER stress response in the cells (Chen et al. 2000; Gu et al. 1995; Jiang et al. 2007; Thastrup et al. 1990). Among stressful stimuli are: decreased oxygen levels, viral infections, deprivation of nutrients, changes in redox homeostasis, increased level of protein synthesis, and reduced levels of calcium ions in the ER lumen (Chan et al. 2015; Krebs et al. 2015; Kupsco and Schlenk 2015; Halbleib et al. 2017). These disturbances rapidly lead to the aggregation of unfolded and misfolded proteins in the lumen of the ER, further activating the

UPR signaling pathways as a cellular adaptive program to cope with stress conditions. Abnormal activation of the UPR has been observed in several neurodegenerative diseases. Therefore, the efforts are aimed to understand the mechanisms that maintain the integrity of the ER under stress, as an approach to develop future therapies against neurodegenerative disorders. Here, we studied the neurotrophic factor CDNF, which has emerged as a potential therapy against Parkinson's disease. The main findings of this thesis propose that the intracellular action of CDNF is mediated by the modulation of the UPR pathways to inhibit ER stress-induced cell death.

The CDNF/MANF form an evolutionarily conserved protein family with a cytoprotective role in neurons and other cell types. CDNF was originally identified and characterized more than ten years ago, along with its homologous protein MANF. A single homologous gene for mammalian MANF/CDNF is also found in invertebrate animals, such as *D. melanogaster* and *C. elegans*, in which no other neurotrophic factors of this family have been identified so far. The above suggests that CDNF appeared late in evolution, probably as gene duplication in vertebrates that happened in fish. However, whether MANF and CDNF have a similar or distinct role in vertebrates has not been established.

Regarding their function, increasing evidence indicates that CDNF and MANF, when applied as extracellular proteins or delivered by viral vectors, can protect and repair midbrain dopamine neurons in vivo (Airavaara et al., 2012; Back et al., 2013; Cordero-Llana et al., 2015; Lindholm et al., 2007; Voutilainen et al., 2011, 2009). Unlike MANF, little is known about the possible role of CDNF in other pathological contexts. For example, the

neuroprotective effects of MANF have also been shown in rodent models of cerebral ischemia and spinocerebellar ataxia (Airavaara et al. 2010; Yang et al. 2014). Also, the immune modulation of MANF promotes tissue repair and regeneration in the retina (Neves et al. 2016). It should be noted that the cytoprotective effects of CDNF and MANF are not restricted to neurons.

Structure analysis of MANF and CDNF revealed a two-domain protein with an N-terminal domain homologous to saposin-like proteins (SAPLIPs) and a C-terminal domain containing a CXXC motif and a non-canonical like-KDEL C-terminal sequence (Parkash et al. 2009). Human CDNF contains an N-linked glycosylation site (Apostolou et al. 2008) and an O-linked glycosylation site (Sun et al., 2011), and both glycosylated and non-glycosylated forms of CDNF are detected in overexpressing cells (Apostolou et al., 2008). Regarding MANF, this was not glycosylated when it was expressed in cell lines (Apostolou et al. 2008; Lindholm et al. 2008). In this study, we expressed rat CDNF, which is not glycosylated in the Golgi apparatus. However, two studies reported that glycosylation is not required for its neuroprotective activity or its secretion (Lindholm et al., 2007; Sun et al., 2011).

One of the main described biological function of CDNF/MANF is its neuroprotective effect in dopamine neurons when it is expressed intracellularly or applied exogenously. This last action suggests the presence of an extracellular receptor for these neurotrophic factors, but despite extensive research efforts to dilucidated cell surface receptors for CDNF and MANF, they have not been identified. Henderson and colleagues suggested that cell surface localized KDEL receptors could be the receptors for MANF, where its translocation to the cell surface

in ER-stressed cells could mediate cell surface binding of MANF, and possibly also CDNF (Henderson et al. 2013). Previously, it has been discussed about the MANF/CDNF family and the role that could play the lipid-mediated interactions with the cell surface (Lindholm and Saarma 2010). Regarding MANF, it seems plausible that lipid-binding could mediate its initial cellular membrane interaction and internalization. Supporting this view, binding to sulfatide (also known as 3-O-sulfogalactosylceramide), was recently suggested to mediate internalization and cytoprotective effects of extracellular MANF (Bai et al. 2018). If CDNF is being internalized and if acts by a similar mechanism (or interacting with other lipids), remains to be studied. It should be noted that there are no studies that report a membrane receptor or a lipid-mediated interaction.

In this work, we studied the role of intracellular CDNF on UPR, and our findings suggest that CDNF could be a key regulator of ER stress. Another group and we have reported that CDNF protects against ER stress (Arancibia et al. 2018; Liu et al. 2018), but where can CDNF exert its cytoprotective role? CDNF can be destined to ER by two possible mechanisms: Firstly, after synthesis, CDNF is mostly retained in the ER, due to its non-canonical ER retention signal KTEL (human) and QTEL (rat). In this work, we used rat CDNF with its QTEL sequence, and we observed by immunofluorescence that CDNF is located and retained in the ER. However, it has been previously reported that CDNF can be released to the extracellular milieu (Sun et al. 2011). In our study, we observed that endogenous CDNF was found mostly inside the cell, although a small fraction of CDNF was detected in the media (Supplementary Figure 5), suggesting that the intracellular role of CDNF could be of great importance to explain its neuroprotective functions.

On the other hand, we engineered a CDNF mutant version without the QTEL sequence and evaluated if CDNF is retained in ER or secreted to the extracellular milieu. Our results showed that CDNF Δ-QTEL was at similar levels to CDNF wild type, given that the GFP levels in both cases were similar. However, CDNF Δ-QTEL was detected mostly in the extracellular milieu, showing that the CDNF that lacks the QTEL sequence is not retained in the ER. More importantly, the action of this CDNF mutant on UPR was abolished (Figure 9). These findings suggest that the mechanism of action of CDNF on UPR is mainly mediated through its localization at the ER.

Regarding the second mechanism, the action of CDNF could be mediated by a cell membrane receptor. Nevertheless, it has been more than ten years since CDNF was discovery and a receptor for CDNF has not been identified. In this context, there is a report that CDNF can be internalized, which suggests the presence of an extracellular receptor. In this study, Malik and colleagues showed that when CDNF was injected directly in the striatum and was analyzed by electron microscopy, the results revealed that the internalized CDNF was present mainly inside endosomes and multivesicular bodies and did not reach the ER (Mätlik et al. 2017). Therefore, our finding along with this report, suggests that the contribution of an extracellular action of CDNF probably is not relevant to the regulation of the UPR pathways under ER stress conditions.

Several studies suggest that MANF is crucial for ER homeostasis since knockdown of MANF in cultured cells and animal models, such as mice and fruit fly, results in the abnormal activation of UPR (Apostolou et al. 2008; Palgi et al. 2009; Lindahl et al. 2014). Like MANF,

CDNF could be essential to maintain ER homeostasis; however, to our knowledge, a CDNF knock-out animal model has not yet been reported. For this reason, it has not been possible to study the role of CDNF in ER proteostasis in a full KO or conditioned animal model. Future studies of loss of function may help assess whether CDNF has a role in the UPR and if it is essential to maintain ER homeostasis. The focus of this thesis was to study the gain of function of CDNF in an ER stress cell model.

Role of CDNF on UPR

In this study, we report the cytoprotective role of CDNF against thapsigargin-induced ER stress in HEK-293T cells and primary hippocampal neurons, exploring the mechanism associated with the modulation of adaptive UPR signaling during ER stress. CDNF represents a novel class of neurotrophic factors that are important for the maintenance of the ER proteostasis, which is altered in neurodegenerative diseases. CDNF could be controlling UPR and apoptosis pathways in order to protect cells in different ER stress contexts. Growing evidence from clinical studies and studies in animal models of Parkinson's and Alzheimer's diseases indicate that ER stress is a common feature of neurodegenerative diseases and contributes to neuron loss. The above is supported by studies that show a correlation between an exacerbated UPR and neurodegenerative diseases, where high levels of phosphorylated PERK and eIF2α (associated with a terminal UPR) were found in the brains of post-mortem patients of Parkinson and Alzheimer diseases (Hoozemans et al., 2005, 2007). Also, it has been reported that the treatment with 6-OHDA and MPP+, in the dopaminergic MN9D cell

line, increases the protein levels of CHOP, which has been associated with the apoptotic pathway in late stages of the UPR (Holtz & O'Malley, 2003).

Several studies show that CDNF delivered by transduction or transfection has a neuroprotective effect on different cell lines, neurons and animal models of Parkinson´s disease treated with 6-OHDA or MPP+ (Bäck et al. 2013; Ren et al. 2013; Mei and Niu 2014; Nadella et al. 2014; Latge et al. 2015; Liu et al. 2017; Wang et al. 2017). The use of 6-OHDA or MPP+, widely used to mimic a parkinsonian phenotype, has been related to ER stress (Ryu et al. 2002; Hara et al. 2011; Luo et al. 2012; Cai et al. 2016). Thus, we established a similar ER stress cell model treating HEK293-T cells with Thapsigargin (Tg), which depletes ER calcium and induces cellular stress (Chen et al. 2000). The HEK293-T cells line has been widely used in several studies that involve thapsigargin-induced ER stress (Rao et al. 2002; Li et al. 2008; Smith et al. 2008; Samali et al. 2010; Oslowski and Urano 2011) and has high rates of transfection in comparison with other cell lines. In this cell line, we found that exposure to Tg decreased cell viability, as previously was reported in human hepatoma cells (Gu et al. 1995). However, cells overexpressing CDNF show improved cell viability regarding cells that express the mock plasmid, revealing that it protects against the Tg-induced ER stress. The above is similar to the report from Liu and colleagues, where they demonstrated that CDNF protects cardiomyocytes against Tunicamycin-induced ER stress (H. Liu et al. 2018). Also, and supporting our hypothesis, Zhang and colleagues showed that CDNF has a neuroprotective effect against cerebral ischemia, and they proposed that its role could be related to the ER stress pathways (Zhang et al. 2018).

In the context of the expression of CDNF, a study reported that in h2c9 cells, the treatment with tunicamycin, an ER stressor with a different mode of action from Tg, induces the expression of CDNF. Thus, CDNF could be considered as an ER stress response gene (ERSR) like its homolog MANF, which also induces a similar response when the cells are treated with Tg (Glembotski et al. 2012). Also, MANF is induced by the expression of ATF6 and XBP1 (Tadimalla et al. 2008). Nevertheless, in our experimental conditions, we found that intracellular and extracellular levels of CDNF in HEK-293T cells remain similar to control during a thapsigargin-induced ER stress. Therefore, although MANF and CDNF are located in the ER, our finding suggests that CDNF has a different mechanism of how is induced by an ER stress, indicating a possible divergent control compared to MANF.

Given the location of CDNF in ER due to the non-canonical ER retention sequence QTEL, or possibly by its interaction with BIP, we propose that CDNF can modulate the signaling pathways of the UPR. In the case of MANF, it has been reported that this neurotrophic factor reduces cell apoptosis via upregulating ER-resident protein BiP in SH-SY5Y cells (Huang et al. 2016). Also, MANF has a similar expression pattern to BiP in mouse tissues (Mizobuchi et al. 2007) and is retained in the ER through interaction with BiP (Glembotski et al. 2012; Norisada et al. 2016). In our study, we observed that overexpression of CDNF in HEK293-T cells induces BiP expression. Furthermore, pulldown proteomic and co-immunoprecipitation experiments show that CDNF interacts with BIP (Zamorano et al., personal communication). Also, CDNF co-localizes with BiP protein in the ER (data not shown). So, could the BiP protein be an intermediary for the action of CDNF? As indirect evidence, a study reported that BiP inhibition blocks exogenous MANF-mediated cell

survival (Huang et al. 2016). Thus, despite the differential induction of MANF and CDNF expression by ER stressors, our results suggest that the molecular mechanism action of CDNF could be mediated by BiP protein.

Earlier studies suggested that the UPR was explained by the observation that overexpression of misfolded protein induces the chaperon BiP (Kozutsumi et al. 1988). This dependency of BiP to trigger UPR defined BiP as a master regulator of UPR, and it was proposed that activation of UPR was given by BiP dissociation of IRE1, a unique and evolutionary conserved transmembrane UPR sensor. In this model, BiP controls its own expression, and ER stress is monitored by the concentration of free chaperone (Shamu, Cox, and Walter 1994).

Considering the above, it could be proposed that CDNF, by increasing its contents within the ER may interacts with BiP, although the affinity of this interaction is unknow, we suspect that it may be high, since in proteomic studies is the major protein being identified. The binding of CDNF to BiP could displace the binding of BiP with the UPR transmembrane sensors ATF6, IRE1, and PERK, inducing the activation of the three downstream signaling pathways, which eventually will end up in a posterior increase in the expression of BiP, as a positive regulation mechanism. Futures studies of the complex between CDNF and BiP and temporal studies of BiP induction could give light about the affinity and the context of this interaction during ER stress.

The observation that the UPR is related to the amount of BiP available in the ER is given by 1) BiP overexpression diminished UPR signaling given that presumably, BiP is binding to UPR sensors; 2) decreasing the concentration of BiP in the ER the UPR is activated, which means there could be an activation of the UPR sensors due to the lack of BiP; and 3) accumulated misfolded proteins in the ER that do not interact with BiP failed to induce the UPR (Hardwick et al. 1990; Dorner et al. 1992; Kohno et al. 1993; Morris et al. 1997; Leonard et al. 2014). Our results show that CDNF increases the expression of ATF4, cleaved ATF6 and spliced XBP1, down-stream proteins of three transmembrane UPR sensors, PERK, ATF6, and IRE1, respectively, indicating an activation of the UPR. Therefore, it is plausible that CDNF could be activating the UPR in conditions of non-ER stress, not mediated by misfolded proteins that ultimately actives IRE1 and triggering UPR, as previously was reported (Gardner and Walter 2011; Zhou et al. 2006), making of CDNF a true "hormetic factor", regulating the hormesis zone of the UPR.

Parallel to the actions of ATF6 and IRE1, phosphorylated PERK also blocks general mRNA translation. Despite this block, the transcription factor ATF4 is produced in an attempt to restore ER homeostasis, or in a late response to induce the expression of CHOP to trigger apoptosis. Our results show that CDNF induces the early expression of ATF4 and CHOP, and possibly enhanced an early UPR to prevent an apoptotic response induced by ER stress. Previously, it has been reported that the increase of CHOP during early ER stress could also be contributing to neuroprotection in Parkinson's diseases (Bouman et al. 2011; Sun et al. 2013) and the early expression of ATF4 is associated with the induction of autophagy and antioxidant response (B'chir et al. 2013). Besides, the above is supported by the work of

Colla and colleagues, where Salubrinal, an enhancer of eIF2α phosphorylation of the ATF4/CHOP pathway, showed a neuroprotective effect in animal models that mimic Parkinson disease phenotype (Colla et al. 2012).

Here, we show that CDNF is a protector against ER stress due to an enhancement of UPR signaling. However, it is plausible to hypothesize that luminal CDNF overexpression in the ER induces UPR by increasing the protein load in the ER. We excluded this possibility by showing that overexpressed mRFP, which is retained in the ER by the retention signal KDEL. did not induce UPR markers. Thus, the increase in early UPR markers cause by CDNF is selective and is not a response to toxic protein levels at the ER. Moreover, we also assessed the expression levels of PDI protein, and these levels remain constant, either with CDNF overexpression or due to induction of ER stress by Tg, as reported by Peters and colleagues (Peters and Raghavan, 2011).

It is increasingly clear that exposure to low levels of ER stress can be beneficial in causing hormesis, the adaptive cellular response through which protective mechanisms are activated, making the cell resistant to later challenges (Mattson 2008). Interestingly, early induction of UPR showed to be beneficial in the cell and promotes cell viability against several insults (Zhang and Xu, 2018). Also, it has been reported that the hyperphosphorylation of Tau attenuates ER stress by upregulation of UPR genes (Liu et al. 2012). According to the above, our data suggest that CDNF induction of UPR markers could contribute to hormesis and thus exert a preemptive cytoprotective effect to a posterior ER stress. Futures studies are necessary to clarify this point.

Taken together, our results show that CDNF can alleviate Tg-induced ER stress and reduce cell death due to prolonged ER stress. Therefore, we propose that the expression of CDNF stimulates adaptive UPR by increasing and capturing BIP, which would then enhance the splicing of XBP1, the cleavage of ATF6, and the phosphorylation of PERK. The activation of PERK can subsequently stimulate the phosphorylation of eIF2a, and therefore increase the expression of ATF4. Early activation of the UPR could stimulate protein degradation pathways and increase chaperone levels, which would partially restore homeostasis and with it, cell survival in the face of prolonged ER stress.

Therapeutic challenges for Parkinson's disease: Proper CDNF delivery

In the context of future therapies, the MANF/CDNF family has become potential therapeutics due to the protective role of dopamine neurons in animal models of Parkinson's disease. In these ways, MANF/CDNF family are added to other neurotrophic factors that have already been studied previously, such as BDNF, GDNF, and NRTN (see Figure i). Compared to CDNF, the mechanism of function of these factors is known, and their receptors are fully characterized. However, they have shown modest effects on potential therapies against Parkinson's disease.

One of the main drawbacks of the therapeutic use of these factors is their peptidic nature that requires a continual dosification directly at the CNS since systemic access is restringed by the blood-brain barrier (BBB). Therefore, several approaches to the delivery of neurotrophic factors by gene therapy have been evaluated. Interestingly, our data suggest that CDNF could

be an attractive potential target for the treatment of neurodegenerative diseases given that its cytoprotection could be mediated by an intracellular action at the ER, where there is no need to be secreted.

A therapeutic interest in neurotrophic factors has been emerging for a long time, particularly in neurodegenerative diseases. In the brain, the blood-brain barrier acts as a barrier that prevents the diffusion of specific proteins, such as neurotrophic factors, forcing them to be administered intracranially. Given the difficulty of delivering these neurotrophic factors and the permanent effect that they can induce, efforts are directed to implement new delivery methods, more innocuous and ideally adjustable, so that the proper dose can be delivered in the necessary quantities.

Several neurotrophic factors have been tested for PD, and the results with GDNF and NTRN were not as expected and their effects as a therapeutic potential for Parkinson's disease were limited. However, the discovery of CDNF in 2007 raised the new hopes for a possible therapy against Parkinson's disease. CDNF in murine and primates models has shown a potent protective and restorative effect in animal models using 6-OHDA and MTPT. In Titi monkeys (*Callithrix jacchus*), when CDNF was infused intrastriatally, previous to a lesion with 6-OHDA, the PET images showed a significant increase in the binding activity of the dopamine transporter ligand compared to controls, suggesting neuroprotection of dopamine neurons (Garea-Rodríguez et al. 2016). These results prompt the study of several delivery methods to administer CDNF. One of them, in the context of cell therapies, consists of encapsulated CDNF secretory cells that could eventually be implanted in the specific zone

of the *substantia nigra*, so that CDNF is constantly produced and delivered to the dopamine neurons (Galli et al. 2019). Another approach that has been studied is to combine the delivery of CDNF with deep stimulation of the subthalamic brain, where it was shown that both have a synergistic effect in animal models of Parkinson's disease (Huotarinen et al. 2018).

The clinical studies carried out with intracranial administration of growth factors indicate that the method of drug delivery and the pharmacokinetic profiles of the therapeutic compound are critical determinants for its final neurorestorative effects. Several variables have to be appropriately managed to deliver neurotrophic factors in the CNS; within them are the number of doses required, the control of the dose temporarily, the safety of the delivery method, etc. The first approach to the delivery of CDNF at the CNS was the intranigral injections of recombinant viral vectors carrying CDNF. In a rat model of Parkinson's disease using 6-OHDA, gene therapy with AAV2 prevents the deterioration of dopamine neurons (Bäck et al. 2013; Ren et al. 2013). Also, the lentiviral delivery of CDNF was used in a study that shows that CDNF promotes nerve regeneration and functional recovery after sciatic nerve injury in adult rats (Cheng et al. 2013). Even bicistronic lentiviral vectors have been used to tracking the expression of CDNF in transduced cells (Fernández et al. 2014). Thus, gene therapy seems to be appropriate to regulate the delivery of CDNF. However, all previously viral vectors of CDNF use a constitutive promoter, which cannot be regulated. For this reason, efforts are focused on establishing better vectors for the delivery of CDNF, especially those that allow regulation of the expression, a great challenge in the future of gene therapy.

Over the years, several systems have been developed for the control of the expression of genes that are used as therapeutics drugs (Braselmann, Graninger, and Busslinger 1993; Gossen et al. 1995). However, these systems have presented certain disadvantages, such as free diffusion, be difficult to remove, toxic, and even present pleiotropic effects. Optogenetic has emerged as a promising methodology to evade the obstacles that these gene expression systems have presented so far. Optogenetic is the set of techniques that involves optical phenomena, such as light, for the control of genetic or molecular elements. It has been proposed to work with light as an inductor due to its beneficial properties, among which have been developed, a high sub-cellular resolution, be highly adjustable and have no toxicity. These properties will allow spatiotemporal control of the expression of a gene of interest.

In 2012, Wang and colleagues developed an optogenetic system for the expression of transgenes, named the "Light-On" system, which is based on a photosensitive transactivator called GAVPO (Wang et al., 2012). One of the components of the GAVPO is a photosensitive domain called Vivid (VVD) from the Neurospora crassa. The GAVPO protein responds to blue light (465 nm), where the VVD domain is responsible for the homodimerization of GAVPO (Zoltowski and Gardner 2011). In the presence of blue light, the homodimeric state of GAVPO can recognize a minimal promoter by using its DNA binding domain (Hong et al. 2008) and trigger the expression of a gene of interest. In the darkness, GAVPO does not dimerize and, consequently, there is no activation of the system (Wang et al. 2012).

In this work, we demonstrated that the LightOn system adapted to the lentiviral platform can be inducible by blue light. The original version of Wang and co-workers has a PolyA region upstream of the UASGx5 sequence as an insulator presumably to stop any transcription from upstream elements. For the lentiviral platform, the PolyA region is incompatible due to it could interfere with the complete synthesis of viral transcript during the viral particle production (Lois et al. 2002). We establish this methodology in a lentivirus system given that it can be inserted in the host genome, a cell or neuron, and express the transgene stably (Lois et al. 2002). For this reason, we constructed a bicistronic light-reported plasmid that expresses mCherry and CDNF considering the short version of UASGx5 inducible promoter (UAS(S)) since our interest was to evaluate this system by transduction. The results show that the UAS(S) promoter is functional as the original version ($UAS_{(L)}$ promoter) both in HEK293-T and PC-12 cells. However, we reported a background expression of the system given that we observed expression of the mCherry reporter by western blot and fluorescence microscopy. There are two options to explain this background expression: i) the background is caused by the activation of a CMV promoter, which is located upstream the 5'LTR in the lentiviral vector (see Figure 1) or ii) there is no strict condition of darkness so that GAVPO is expressed. For the first option, we transfected the reporter plasmid UAS-mCherry alone, and we almost did not observe expression. Then, we generated a lentiviral vector without CMV promoter, which was co-transfected with the GAVPO expression vector and exposed to blue light. Despite this modification, we were still able to observe the expression of mCherry, suggesting that CMV promoter does not contribute to the expression of reporter mCherry. For the second option, we co-transfected the UAS-mCherry plasmid with the GAVPO expression vector or with another plasmid without the GAVPO sequence. We did

not observe mCherry expression in the absence of GAVPO. Thus, we conclude that the background expression is triggered by GAVPO, presumably, due to a dimerization trigger by the lack of total darkness in the laboratory conditions. Since this vector is designed to deliver a CDNF in the CNS, it was important to determine the background expression in the absence of light, which was minimal.

When the bicistronic lentiviral vector that expresses mCherry and CDNF was tested over time (see Figure 1), mCherry and CDNF protein levels were detected from 30 minutes and continues to increase through the blue light induction time. At time 0, we observed the endogenous CDNF expression in HEK293-T cells (Arancibia et al. 2018), and a 1 hour of induction, it was ∼10-fold, which indicates that this lentiviral platform can respond rapidly after light induction, a feature highly desirable in an inducible system. In transcript levels, our induction of 30-40-fold was lower than those reported by Wang and co-workers. These differences are probably due to technical and methodological differences in the quantification of transcripts. Wang and co-workers evaluated transcript levels of a stabilized reporter gene by RT-qPCR and obtained 200-300-fold induction levels. The above could be explained due to the different mRNA degradation rates due to we used non-stabilized reporter genes, cell systems used, and also differences in the promoter and plasmid used. The induction level obtained in our experiment is more aligned with the use of this type of inducible promoter, where gene induction is around one order of magnitude. The induction levels obtained in our vector make this platform amenable to be used in CDNF delivery at the CNS.

It is important to note that the lentiviral platform is functional as lentiviral particles carrying the UAS(S) promoter. We transduced hippocampal neurons and induce expression of CDNF by blue light, obtaining similar results to transfection experiments in HEK293-T. Furthermore, to prove our optogenetic system functionality, we induced CDNF expression by blue light and evaluated its effects on several UPR markers as previously reported (Arancibia et al. 2018). In order to decrease the variability due to the two vectors system and to develop a potential single vector therapeutic system, we generated an all-in-one vector, where we include the bicistronic expression of GFP and GAVPO under ubiquitin promoter and the CDNF mini-gene driven by the UAS promoter in the opposite direction, to clearly evaluate its expression induced by blue-light. The resulting all-in-one vector system shows that CDNF expression is modulated by blue-light and can induce several UPR markers (Figures 6 and 7, second part doctoral thesis).

In summary, we demonstrated that the LightOn system adapted to the lentiviral platform is inducible by blue light. With the induction levels obtained in this work, we suggest that this lentiviral platform could be used to regulate the expression levels of CDNF or other peptides in the CNS. The system developed here, using light as inducer, permits an easily regulation of gene expression with a high spatial and temporal resolution offering several advantages over the traditional vector used for gene therapy in the CNS. Thus, we expect that combining this lentiviral platform with new technologies of illumination such as nanoparticles (Chen et al. 2018; Wang et al. 2017), it could be possible to develop new peptide-based therapies oriented to CNS.

SUMMARY

1) CDNF protects HEK293-T cells against Thapsigargin-induced ER stress.

2) CDNF induces BiP and downstream UPR signaling pathways in HEK293-T cells and neurons.

3) CDNF attenuates the expression of proteins related to cell death induced by ER stress in HEK293-T cells and neurons.

4) CDNF without the QTEL sequence is not retained in ER and is constitutively secreted into the extracellular space.

5) CDNF without the sequence QTEL is not able to protect HEK293-T cells against an ER stress.

6) CDNF without the sequence QTEL does not induce proteins related to UPR nor attenuates proteins related to cell death induced by ER stress (CHOP).

7) The Expression of CDNF was modulated by blue light in HEK293-T cells by transfection, and in neurons by transduction.

8) The expression of CDNF modulated by blue light induces the expression of UPR markers.

CONCLUSION

In this thesis, we study the possible role of CDNF in the regulation of UPR signaling. We observed that the expression of CDNF protects HEK293-T cells against thapsigargin-induced ER stress. Our results suggest that cytoprotection was possible because CDNF expression increases the adaptive UPR and decreased ER stress-induced apoptotic pathways. On the other hand, in experiments with a truncated version of CDNF without non-canonical ER retention sequence QTEL, we show that the protective effect and the induction of adaptive UPR pathways require that CDNF be located in the ER.

With these results, CDNF has become an attractive therapeutic target to treat neurodegenerative diseases such as Parkinson's and Alzheimer's diseases, and diabetes, where an alteration in the proteostasis of the ER is observed. These findings gave an immense opportunity to develop tools to delivered CDNF in the CNS. For this purpose, we were able to design a new single vector (or all-in-one) lentiviral platform for an optogenetic regulated expression of CDNF in neurons, making this an alternative and powerful tool to open the gates to the possible new treatments for Parkinson's and other neurodegenerative diseases.

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