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Neurotoxicity of the Synuclein Proteins in Cell-based Models of Parkinson's Disease

Development of a regulated Synuclein expression system

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1. INTRODUCTION

1.1. PARKINSON'S DISEASE: A NEURODEGENERATIVE DISORDER

Parkinson disease (PD) is the second most common neurodegenerative disorder worldwide, since it affects approximately 1% of the population older than 60 years, and up to 4% of those older than 80 years.[1, 2] PD is pathologically characterized by a cell loss in the substantia nigra pars compacta (SNc).[1, 2] The SNc is located within the midbrain[2] and composed of mesodiencephalic dopaminergic neurons, which are responsible for the synthesis of dopamine (DA).[3] DA is a neurotransmitter involved in many physiological pathways, including cognitive and motor functions.[3, 4] Therefore, when DA input is depleted, PD symptoms arise[3], affecting not only the CNS but also peripheral and autonomic nervous system, and the function of different organs.[5]

The disease presents a late onset and specific symptoms (bradykinesia, rigidity, tremor at rest, etc.) are detectable when half of the dopaminergic neurons are already lost, and 60 to 80% of the dopamine levels have been reduced.[1, 6, 7]

Numerous genes have been identified to be directly related with the development of either familial or idiopathic/sporadic PD.[8, 9] Particularly, *SNCA* (PARK1 locus) encodes for a 140 amino acid protein termed α-synuclein (α-Syn).[8] This gene, composed of 6 exons, is located on chromosome 4q22.1/4q21 and at least three missense mutations (A53T, A30P, E46K), duplications and triplications have been identified as strong causes of both types of PD.[8, 10]

1.2. THE ROLE OF α-SYN

The term proteinopathies involves a range of neurodegenerative diseases characterized by the formation of insoluble aggregates of misfolded proteins.[10] α-Synucleopathies are proteinopathies caused by the aggregation of the protein α-Syn[10], and within which PD is classified[11]. In a healthy brain, α-Syn is expressed as a soluble 14kDa protein in the soma and synapses of neurons belonging to the CNS.[5] Pathologically, it has been proved to be the major component of Lewi Bodies (LBs) and Lewi Neurites (LNs), which are hematoxylin-eosine positive staining

intracytoplasmic inclusions found in the brain of PD patients.[8, 10, 12] LBs and LNs also constitute the main molecular hallmarks of the disease.[5, 6]

Aggregation of α-Syn has been proved to be not only triggered by mutations in the *SNCA* gene, but also by dysfunction of protein degradation systems which normally regulate intracellular α-Syn levels (especially the ubiquitin-proteasomal system, UPS, and the autophagy-lysosome pathway, ALP).[10] Posttranscriptional modifications, such as phosphorylations and ubiquitinations, dysfunction of chaperones and oxidative stress have been also found to be involved in α-Syn aggregation.[7]

α-Syn presents a high conformational plasticity and its conformation depends on different factors, such as its own concentration and the presence of other molecules which the protein interacts with.[10] Several studies support the idea that α-Syn's toxicity remains in its lipid-binding ability[7], since the α-helical structure it adopts when it interacts with membranes has been demonstrated to enhance protein aggregation.[5] Moreover, several studies both *in vivo* and *in vitro* have shown that α-Syn aggregates (composed of β-sheet fibrils) trigger protein aggregation among the neuronal network in a prion-like fashion, thus suggesting a possible mechanism for the disease's spreading.[10]

However, neither the physiological role nor the exact mechanisms by which α-Syn and/or its aggregates are toxic to the cells, especially to the dopaminergic neurons from the SNc, are completely clear.[7]

1.3. PROTEIN OVER-EXPRESSION SYSTEMS

Since it is not possible to observe cells die in the human brain, there is a strong need of developing experimental models to confirm α-Syn's toxicity.[7] PD models have been specially focused on the overexpression of α-Syn in animals' brains and dopaminergic neurons.[11, 13, 14, 15, 16, 17] With this propose, different ways to overexpress the protein have been developed, from transgenic mice, *C.elegans* or *D.melanogaster*, to recombinant expression of human α-Syn in mammals.[18, 21] Adeno-associated virus (AAV)[19, 20] are among the most widely methods used for

cell/animal transfection with protein expression systems, which roughly consists on the induction of a protein expression into the cells by infection with a viral vector.[22]

1.3.1. AAV-mediated gene expression

AAV are non-enveloped, single-stranded DNA vectors with a T=1 icosahedral capsid of 25 to 26nm in diameter that provide a long-term, persistent gene expression.[19, 24] AAV have been reported to infect and transduce both dividing and non-dividing cells, including neurons, with a minimal cellular toxicity or host immune response.[23] Briefly, the viral infection is as follows: first, AAV particles attach to cell surface sugars present on proteoglycans (such as sialic acid, galactose or heparin sulfate) and to specific primary cell-surface receptors (which vary depending on the serotype of the AAV). Then, secondary receptors mediate endocytic uptake of the particles.[19]Once internalized, the AAV escapes the endosome and transports to the nucleus, where it releases the viral genome. Inside the host's nucleus, the single-stranded DNA is converted into double-stranded DNA by host DNA polymerases, initiating this way, a productive viral infection (expression of viral proteins).[14]

1.3.1.1. tetOFF Expression System

One of the most used protein expression systems in combination with the AAV technique is the tetracycline (Tet)/doxycycline (Dox)-controlled transgene expression (tetOFF) system.[25] This expression system is based on two main components: (i) the tetracycline trans-activator (tTA), a fusion protein between the tetracycline repressor DNA binding domain of *E.coli* (tetR) and the C-termial transcriptional activator domain of the VP16 protein from herpes simplex virus, and (ii) the tetracycline response element (TRE), upstream the gene of interest, which consists of seven copies of the tetracycline resistance operator (tetO) binding site and a minimal cytomegalovirus (CMV) promoter region, which contains the TATA box and transcription start sites.[26] In the absence of Dox, the tTA can bind the tetO sequence located upstream the minimal promoter and stimulate gene expression. Dox binds to the tTA2, disabling it from binding to the tetO sequences, thus the addition of antibiotic abolishes transcription of the target gene.[23] Expression of tTA2 is controlled by a promoter that can be used to confer cell specificity.[26] However, since

the size of the capsid it rather small, genome material is meant to be composed also by rather shot sequences. The small human synapsin promoter 1 (hSYN1) has been shown to be of especial interest and adequate due to its relatively small size (470-480bp) and high neuron specificity.[24, 28]

1.4. OBJECTIVES

The work here exposed was based on the cloning and testing of AAV-tetOFF vectors previously designed by Dr. S. Kügler's laboratory. The main objectives of this work were:

(i) To determine whether the protein expression of the tetOFF system used in this work has a greater, lower or equal protein expression in comparison to a constitutive expression system.

(ii) To determine if separating the two components of the tetOFF system into two different vectors (thus co-transducing the cells with two different AAV vectors) raises better transducing and protein expression results than transducing the cells with just one AAV containing the two components in a single vector.

(iii) To check if the protein expression is effectively controlled by the antibiotic Dox.

(iv) To test if the expression system here described is useful for expression of recombinant human α-Syn in primary neuron cultures, and test the cells degrading capacity.

2. MATERIALS AND METHODS

2.1. pAAV VECTOR PRODUCTION

Bicistronic AAV serotype 6 (recombinant AAV2/6: AAV serotype 2 genome in AAV serotype 6 capsid) vectors were produced in order to transduce neuron cultures with plasmids that contained the tTA gene (pAAV-t-TA2-IB/AAV-t-TA2-B) and a recombinant human α-Syn gene (pAAV-TRE-αSyn); the enhanced green fluorescent protein (EGFP, pAAV-TRE-EGFP) was used as a reporter. EGFP and α-Syn were also cloned together in a fourth plasmid (pAAV-TRE-αSyn-WB-TRE-GFP-WB). All

vectors produced are depicted in **Figure 1.B**. The recombinant viral genome consisted mainly in a tetOFF system and an ampicillin resistance gene (AMP). Two

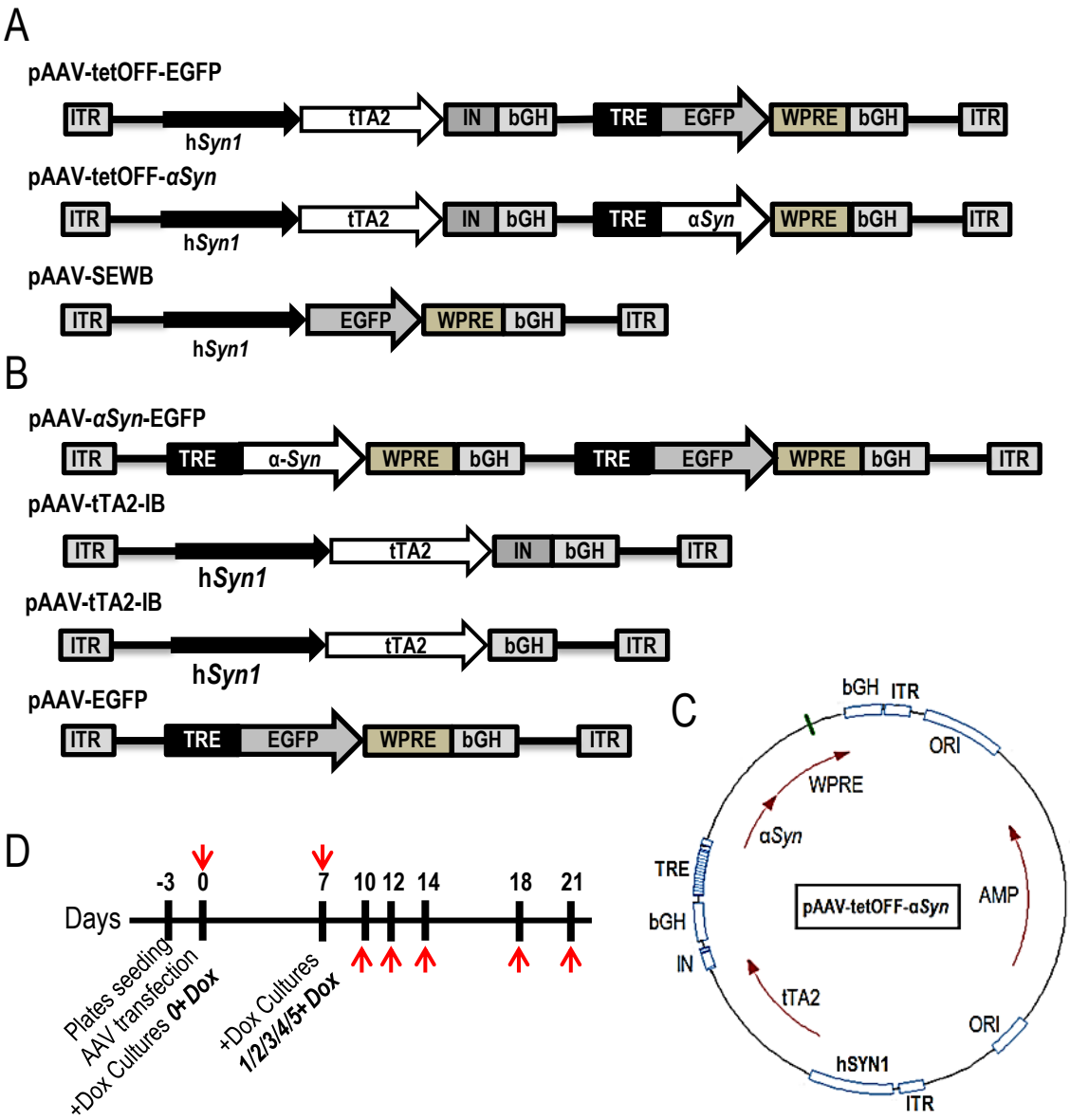

Figure 1. (A) AAV vector plasmids used for new vector construction/cloning of the double adenoviral tetOFF protein expression system vectors. (B) AAV vector plasmids produced for co-infection of tetOFF protein expression system. (C) Schematic representation of the pAAV-tetOFF-*αSyn* plasmid (AMP, ampicillin resistance gene; ORI, origin of replication; ITR, inverted terminal repeats; SYN1, human synapsin promoter; tTA2, tetracycline transactivator 2; IN, intron; bGH, bovine growth hormone poly-A signal; TRE, tetracycline response element; *α-Syn*, human wild type *α-Syn*; WPRE, woodchuck post-transcriptional control element). (D) Schematic schedule for Western Blot experiments given in days. Red arrows indicate time point when culture samples were harvested.

origins of replication (ORI) allowed plasmid replication in bacteria, and two short inverted terminal repeats (ITR), flanking the tetOFF system cassette, were needed for viral genome recognition and encapsidation (**Figure 1.C**).

The tetOFF system used in this work was based on: hSYN1 which triggers gene expression exclusively in neurons[24], the tetracycline trans-activator 2 (tTA2) gene, followed by a small chimeric intron (IN) and a bovine growth hormone (bGH) poly (A) signal, which enhance expression and stabilize tTA2 mRNA, a TRE domain, composed of seven repeats of the tetracycline operator (*tetO* x7) upstream the gene of either the EGFP or the recombinant human α-Syn. Finally, a woodchuck hepatitis post-transcriptional control element (WPRE) stabilizes mRNA, resulting in a 2 to10-fold higher expression rate of target protein.[27]

Previous to experimental procedures, all plasmids were virtually produced and assayed using the computer program Clone Manager® (Scientific & Educational Software, USA) in order to determine restriction sites and DNA fragments size.

pAAV digestion: Different restriction enzymes (**Table 1**) were used to digest the DNA plasmids pAAV-tetOFF-EGFP and pAAV-tetOFF-αSyn. These plasmids were previously designed and produced by Dr. S. Kügler's research group (**Figure 1.A**).

All digestions were carried out in a final volume of 100µl in 0,5ml Eppendorf tubes, containing 10-5µg DNA, 10x Cut Smart® Buffer (50mM Potassium Acetate, 20mM Tris-acetate, 10mM Magnesium Acetate, 100µg/ml BSA pH 7.9) (New England BioLabs® Inc., NEB, Germany) and 100U of the corresponding restriction endonuclease (NEB, Germany). Time and temperature parameters of digestion reactions depended on the restriction enzymes used.

When digesting with *SpeI* and *SphI, DNA polymerase I, large (Klenow) Fragment* enzyme was used in order to produce blunt ends. *Klenow* reaction mixture contained the DNA (previously precipitated) resuspended in 100µl NEB II Buffer, 10U *Klenow*® enzyme (NEB, Germany), 5µl of each desoxynucleotide at a final concentration of 33µM (dATP, dGTP, dCTP, dTTP) (NEB, Germany), and raised up to150µl with TE Buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). Reaction was

Table 1. AAV plasmids and enzymes used for the cloning of the new viral vectors. Plasmid abbreviations correspond to constructs depicted in Figure 1. The fragment *TRE-α-Syn* corresponds to the fragment containing the α-Syn gene introduced into the linearized pAAV-EGFP in order to produce the construct pAAV-α-Syn-EGFP.

pAAV	Restriction endonuclease	DNA fragment obtained
pAAV-tetOFF-EGFP	EcoRI; MfeI	pAAV-TRE-GFP
	SpeI; SphI	pAAV-tTA2-IB
pAAV-tTA2-IB	StuI	pAAV-tTA2-B
pAAV-tetOFF-αSyn	SpeI; SphI	TRE-αSyn

carried out at 25^{o}C during 20 minutes. To stop the polymerase reaction tubes were kept at 4^{o}C (ice).

For production of pAAV-αSyn-EGFP, plasmids pAAV-EGFP and pAAV-tetOFF-αSyn were digested with *SpeI* and *SphI*, in order to open the plasmid and obtain the TRE-αSyn-WB DNA fragment, respectively. Subsequent dephosphorylation of the linear pAAV-EGFP blunt ends was necessary for ligation of both fragments: 5µl Antartic Phosphatase Buffer 10x® (50 mM Bis-Tris-Propane-HCl, 1 mM MgCl2, 0.1 mM ZnCl2, pH 6) (NEB, Germany) and 2.5U *Antartic Phosphatase*® (NEB, Germany) were added to the total volume of Klenow reaction mixture. Dephosphorylation was carried out at 37^{o}C, for 15 minutes. Reaction was stopped by heating the sample up at 70^{o}C for 5 minutes. All digestion steps were checked by analytic electrophoresis in acrylamide gels.

DNA purification: DNA fragments obtained by restriction enzyme digestion were separated by electrophoresis in acrylamide gels, and purified using a QIAquick® Gel Extraction Kit protocol (QIAGEN, Germany). DNA concentration was measured spectrophotometrically, using an Eppendorf BioPhotometer® ($1E_{260}$=47µg DNA/ml; A_{260}/A_{280} equal or above 1.70).

DNA ligation: Ligation of linear plasmids was carried out in a total volume of 20µl containing 100-150ng DNA, 2µl Ligase reaction Buffer 10x (50mM Tri-HCL, 10mM $MgCl_2$, 1mM ATP, 10mM DTT, pH 7.5) (NEB, Germany), 0.5U *T4 DNA Ligase*® (NEB, Germany), and adjust up to 20µl with distilled water. Before addition of the Buffer and the enzyme, the mixture was heated 3 minutes at 45^{o}C to undo any

possible bindings between DNA fragments. Ligation mixture for production of pAAV-αSyn-EGFP contained 300ng pAAV-TRE-GFP-WB and 600ng TRE-αSyn-WB fragment in order to increase insertion probability. Reaction was carried out at 16^{o}C during 10-15 minutes, and another subsequent 5-10 minutes at room temperature.

In case of DNA modified with *Klenow* enzyme, reaction buffer had to be previously changed from NEB II Buffer® to Ligase reaction Buffer. DNA was thus purified following the QIAquick PCR Purification Kit Protocol® (QIAGEN, Germany).

Cell electroporation and culture: Before cell electroporation, the total volume of ligation reaction was dialyzed against 100ml dialysis buffer (10% glycerol, 1mM EDTA) using a Nitrocellulose MF-Millipore^{TM} Membrane Filter (0.05μm cut-off) (Merck Millipore, Germany) in order to dilute salt concentration.

30μl of Sure Cells (electrocompetent *E.coli*, 2-3x10^{10}cells/ml, kept at -80^{o}C in 10% glycerol, 0.125% yeast extract and 0.25% tryptone) were electroporated against 1-2μl of the dialyzed ligation reaction. Electroporation was performed setting an electric field of 1.70kV (Gene Pulser® II, BioRad). A time constant above 4.0ms was determined as optimal for good cell electroporation results. Immediately after electric discharge, cells were resuspended in 1ml Medium (++) (20mM Mg^{2+}, 20mM Glucose, 100ng/ml ampicillin) and incubated for 1 hour at 37^{o}C, 600-700rpm. Then, the total volume of each culture was divided and spreaded on three agar plates containing 100ng/ml ampicillin. Plates were kept overnight at 37^{o}C. After 14-16 hours, grown colonies were picked up using a pipette tip and put directly into 50ml Falcon tubes with 6-7ml Medium (++); one colony per tube. Then tubes were slightly bortexed and incubated for 12-15 hours at 37^{o}C and 800rpm.

DNA extraction: Recombinant plasmids were obtained from Sure Cell cultures using a QIAprep® Spin Miniprep Kit (QIAGEN, Germany). DNA concentration was always determined spectrophotometrically. In order to check plasmids' integrity, purified DNA was always digested with *SmaI* and fragments sizes were determined by electrophoresis. If the DNA fragments showed an equal size to the ones obtained artificially with the Clone Manager® program, plasmids were considered correctly

cloned, and subsequently used for AAV production. AAV particles were produced following the protocol described by D. Grimm *et al.* 1998.

2.2. PRIMARY NEURON CULTURES AND IN VITRO INFECTION

Primary neuron cultures were prepared samples of E17.5 Wistar rat embryos (P0) as previously described.[31] Cells were cultured in 24-well plates (where just the upper and the lower raw were used) and kept at 37^{o}C in LB medium (700µl/well). Cell density was estimated by correlating the acidification of the LB medium between tested and control cultures.

***In vitro infection*:** Viral vectors were added to primary neuron cultures when reached a confluence of ~50-60%. The pAAV-SEWB plasmid was used as positive control (constitutive construct). pAAV-EGFP was used as negative control (no protein expression). Virus concentration was expressed in transducing units/microliter (tu/µl) and virus titer in transducing units (tu); 3x10^{7}tu was considered as the patron titer.

Different ratios pAAV-tTA2-IB/pAAV-tTA2-B to pAAV-EGFP were tested. Viral vectors containing the EGFP gene were added at the fixed patron titer, while serial dilutions of AAV containing tTA2 gene were used to set ratios 3:1, 1:1, 1:3, 1:10, 1:30, 1:100, 1:300 and 1:1000: 1x10^{8}, 3x10^{7}, 1x10^{7}, 3x10^{6}, 1x10^{6}, 3x10^{5}, 1x10^{5} and 3x10^{4}tu of, respectively.

Fluorescence analysis: The ability of the tetOFF expression system tot transfect neurons in vitro and to mediate regulatable transgene expression was assessed by measuring EGFP fluorescence. Fluorescence was monitored over time in situ, on a Zeiss Axiovert Microscope equipped with a CCD camera and Multi Vision Image Analysis Program (exposure time of 600ms). Cell cultures were photographed and analyzed every 2-3 days during 3 weeks after transduction, using ImajeJ® Analysis Software (National Institutes of Health, USA). Statistical analysis was assessed by Windows Excel® and pairwise comparisons of values were performed by paired, two-tail Student's *t*-test.

2.3. DOXYCYCLINE TEST

To test the efficiency of doxycycline (Dox) in silencing protein expression, cultures were exposed to the antibiotic on different culture time points. Doxycycline was added to half the wells on the same day (Dox0), three (Dox3) and ten (Dox10) days after transfection, at a final concentration of 0,1ng/µl (7µl/wel of 10ng/µl stock Dox). Negative controls were those with no addition of antibiotic, where expression was assumed to not be suppressed. Ratios tested were 1:10, 1:100 and 1:1000.

2.4. WESTERN BLOT ANALYSIS

To test the effect of α-Syn expression and its cell degrading capacity, a combination of plasmids pAAV-tTA-B and pAAV-αSyn-EGFP at a ratio 1:3 was used to transduce cortical neuron cultures. Viral vectors were added 3 days (day 3) after cell seeding (day 0) (**Figure 1.C**, Appendix). Doxycycline (0,1ng/µl) was added to some wells 1 week after transduction in order to stop protein expression. Negative controls were cultures where Dox was added the same day of transduction (day 0). Cells were harvested for western blotting on day 0, 7, 10, 12, 14, 18 and 21 after transduction. Proteins were transferred to a PVDF membrane and probed with the following antibodies: a mouse monoclonal antibody against recombinant human α-Syn (Syn 211, 32-8100, Invitrogen), an antibody against constitutive (both human and endogenous rat) α-Syn (610787 BD Transduction Laboratories^{TM}), and antibodies against EGFP (anti-EGFP, Roche) and against tubulin (anti-Tubilin, Sigma).

3. RESULTS

3.1. TEST AND COMPARISON OF pAAV CONSTRUCTS.

3.1.1. pAAV-SEWB vs pAAV-tetOFF-EGFP

Expression rate between the constitutive system (pAAV-SEWB) and the tetOFF system (pAAV-tetOFF-EGFP) was studied in first place. As shown in **Figure 2**, differences in fluorescence intensity were significant already 3 days postransduction.

Cell cultures transduced with the constitutive vector doubled their fluorescence values every 7 days, showing an increase of around 20% per day between day 7 and

day 17 after the addition of AAV vectors (pictures B, C and D from **Figure 2.A**). Cultures remained with a cell density comparable to the control cultures (non-transduced cultures) until 21 days after transduction (longest period of time checked) (data not shown).

By contrast, cell cultures transduced with pAAV-tetOFF showed much lower fluorescence values. The fluorescence triggered by this expression system was half the one triggered by the constitutive one already on day 7 postransduction. In this case, fluorescence remained low and almost invariable between day 7 and day 17. These cultures also showed an unhealthy aspect if compared with control cultures or cultures transduced with pAAV-SEWB since they showed lower cell density (data not shown): mediums from cultures transduced with the tetOFF system remained with higher pH whereas the ones from pAAV-SEWB-transduced cultures or control cultures lowered the pH more quickly. On day 20 postransduction differences in fluorescence between these two expression systems were remarkable. Same results were observed when increasing pAAV-tetOFF titer from $3x10^7$ to $1x10^8$tu (data not shown).

3.1.2. Different ratios pAAV-tTA2-B to pAA-EGFP

Protein expression differences between pAAV-tTA2-IB and pAAV-tTA2-B appeared to be non-significant (data not shown). Cultures transduced with ratio 3:1 and 1:1 showed comparable fluorescence values to pAAV-tetOFF-transduced cultures ($3x10^7$tu) (data not shown). Number of fluorescent cells, culture aspect and cell density were also equivalent.

When transducing neuron cultures with a 1:10 ratio, significant differences in fluorescence values were already remarkable on day 3 postransduction in comparison with both, constitutive and tetOFF systems (**Figure 2**). The fluorescence values of this ratio doubled the values triggered by the pAAV-SEWB, and this remained until 12 days after transduction. From day 14 on, these differences were triplicated. Fluorescence triggered when transducing neuron cultures with a ratio 1:10 increased an average of 183.5% per day. On day 20, the fluorescence values of cultures trans-

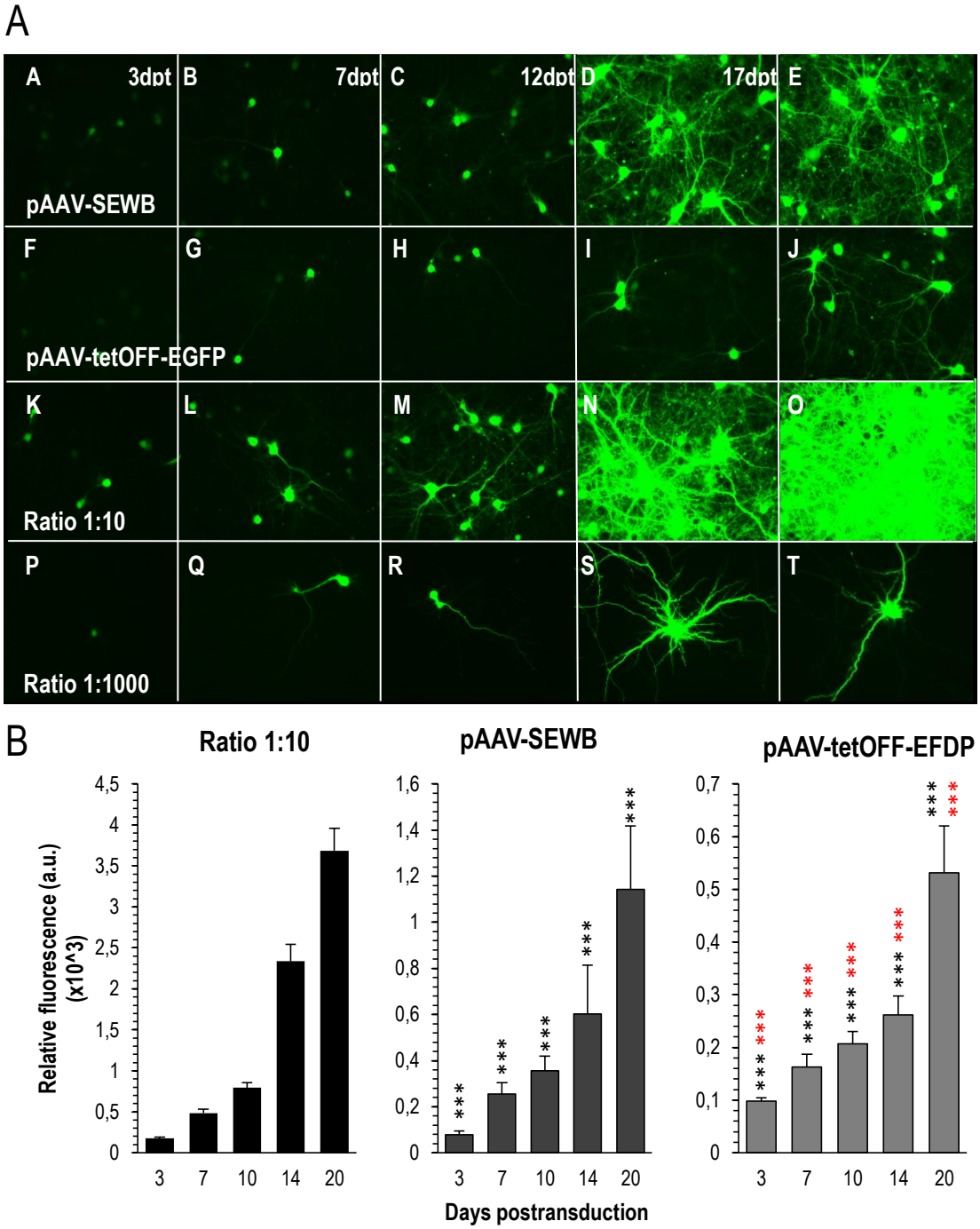

Figure 2. Fluorescence analysis of neuron cultures transduced with the different expression systems until 21 days after vector supply. (A) Fluorescence microscope images from cultures transduced with the constitutive construct (pAAV-SEWB) where EGFP expression is directly under the control of the hSYN1 promoter (A-E). Cultures transduced with the tetOFF-EGFP construct (pAAV-tetOFF-EGFP), where EGFP expression is under the control of the tTA2 interaction with the TRE sequence (F-J). Cultures transduced with different tTA2 versus TRE-EGFP ratios: ratio 1:10 ($3x10^6$tu pAAV-tTA2-B; $3x10^7$tu pAAV-EGFP) (K-O) and ratio 1:1000 ($3x10^4$tu pAAV-tTA2-B; $3x10^7$tu pAAV-EGFP) (P-T). (B) Statistical results of fluorescence analysis. Bars presented as mean values ± s.e.m. Black stars above bars represent significance difference between the constitutive and the tetOFF expression systems as compared with the fluorescence triggered by Ratio 1:10; red stars represent significance differences between the tetOFF system and the constitutive system (***P<0.05). (*"pdt", days postransduction).* Pictures K-O correlate with pictures A-E from Figure 4.A.

duced with ratio 1:10 surpassed the values from cultures transduced with the constitutive system in a 322.5%, and in a 693.6% if comparing it with the tetOFF system (**Figure 2.B**). Furthermore, the acidification of the cultures' medium was comparable to the ones from pAAV-SEWB and control cultures, indicating no remarkable loss in cell density (data not shown). Similar results were obtained when transducing cultures with ratio 1:3 (data not shown).

When looking at cultures transduced with ratio 1:1000, two main observations were made: (i) the fluorescence observed was comparable, or even higher, to the one from ratio 1:10-transduced cultures (**Figure 3.B**), and (ii) in contrast to the cultures transduced with the constitutive system or ratio 1:10, here, distinct single neurons could be seen (**Figure 3.A**). The eye-catching difference in fluorescence means may be due to background fluorescence levels differences. Neuron somas, axons and dendrites could be also well identified. However, the number of fluorescent neurons was much lower than the one obtained with any of the other expression systems. Cultures' aspect and cell density were comparable to the control, constitutive and ratio 1:10-transduced cultures.

Data obtained from cultures transduced with ratios 1:30, 1:100 and 1:300 also sho wed a higher protein expression in comparison with the constitutive construct (data not shown). In general, differences between ratios were assessed not to fluorescence values but mostly to fluorescent cell numbers. Maximum number of fluorescent cells was reached when transducing cultures with either 1:3 or 1:10 ratios, whereas this number decreased when lowering the pAAV-tTA2 titer in smaller ratios. This observation determined which vector ratio to use in subsequent experiments.

3.2. DOXYCYCLINE TEST

After proving that the new expression system worked for protein expression, doxycycline was added to transduced cell cultures in order to check if the protein synthesis could be stopped. Results from this test are shown in **Figure 4**.

Cultures where Dox was added at the moment of transduction showed no appreciable fluorescence during the whole experiment. Cultures where Dox was added 3 days postransduction showed an increasing fluorescence until day 7 (**Figure 4.B**). After

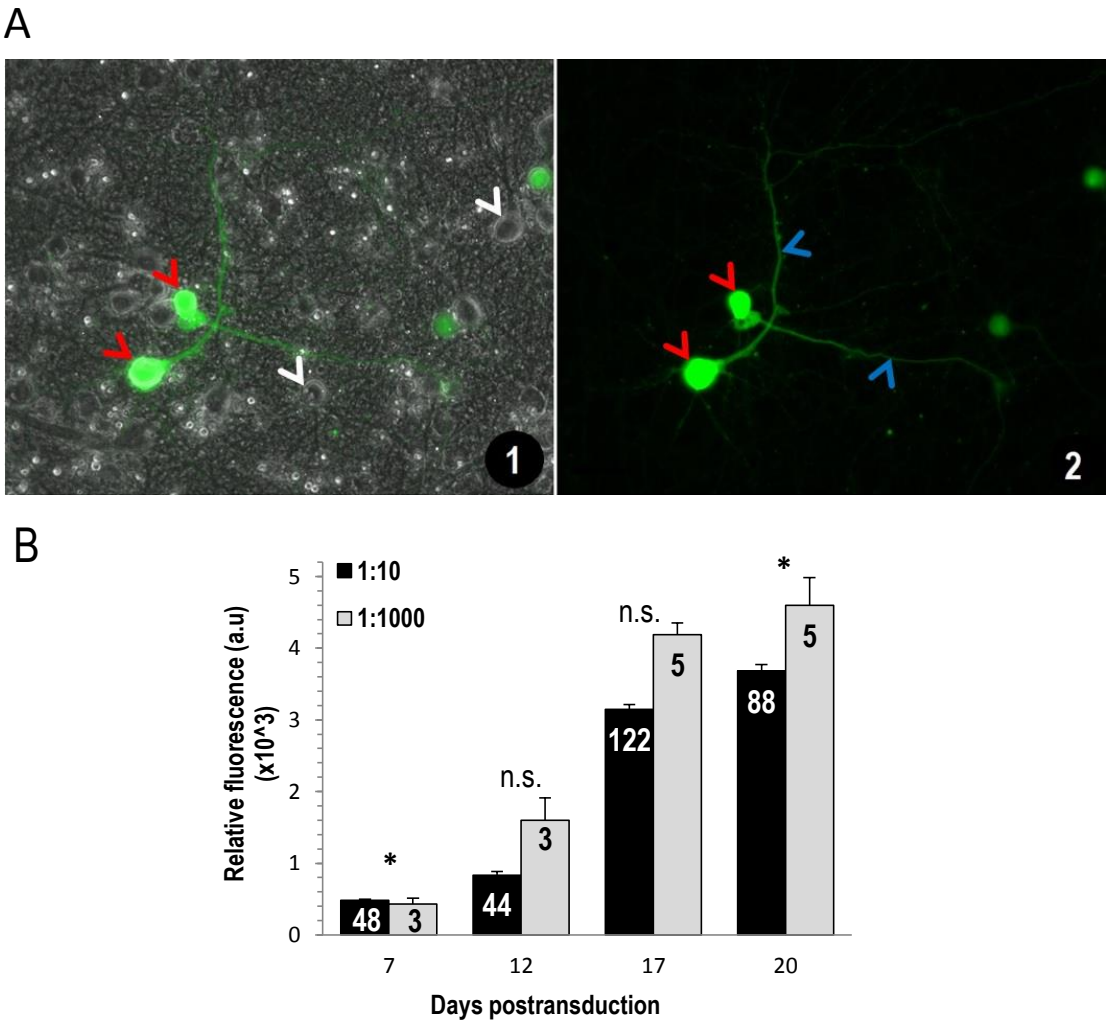

Figure 3. Cultures transduced with ratio 1:1000 showed single fluorescent neurons but high protein expression. (A) Superposition of fluorescence and phase contrast images: (1) shows punctual fluorescent neurons (red arrows) between non-shining cells (white arrows). When analysing only EGFP expression (2), clear axons and dendrites could be distinguished (blue arrows). (B) Fluorescence values of neuron cultures transduced with ratios 1:10 and 1:1000 were significantly different after 12 days postransduction (**P*<0.5, n.s., non significant). Bars represent mean values ± s.e.m. The number of fluorescent neurons counted is specified in each bar.

this time point, fluorescence values decreased rapidly, and already one week after the addition of the antibiotic (day 10), values were comparable to those obtained on the +Dox day0 cultures. Cultures where Dox was added 10 days after virus addition showed an increase in fluorescence until day 12. Two days after antibiotic supply the fluorescence started to lower, up to a decay of the 70% within the next seven days. Same results were obtained with cultures +Dox day10 transduced with ratios 1:100 and 1:1000, where fluorescence also started to decrease two days after the addition of

Dox (**Figure 4.C**). Lower numbers of fluorescent cells were also appreciable here when looking at smaller ratios than 1:10, even though the cell density was equal (data not shown).

At day 3 postransduction, there was not any significant difference between the fluorescence shown by cultures –Dox and +Dox day3, or between cultures +Dox day3 and +Dox day10. There was, however, a discrepancy between –Dox and +Dox day10 values, even though this difference disappeared four days later. At this same time point, fluorescence values between cultures where Dox was added on day 3 and cultures –Dox and +Dox day10 (in which Dox was not added yet), appeared to be already significant.

The depletion in fluorescence of cultures +Dox day10 can be seen when comparing pictures R, S ant T from **Figure 4.A**. 12 days postransduction, neat neurons, axon and dendrites could be easily identified. On day 17 postransduction, fluorescence decayed greatly, and neuron morphology was apparently lost: fluorescence lowered until axons and dendrites were barely appreciable in comparison with cultures without Dox. Only somas were recognizable. However, culture aspect and cell density remained comparable to control cultures (data not shown). On day 20, only neuron somas were detectable while dendrites and axons had lost almost all their brightness, thus, their EGFP content.

3.3 WESTER BLOT ANALYSIS

Alpha-synuclein showed an approximate size of 16Kb when stained with either the antibody against constitutive (anti-αSyn BD) or the one against human α-synuclein (anti-αSyn 211) (**Figure 5**).

Figure 5.A shows a clear increase in α-Syn amount along the following days after transduction of samples –DOX. This is explained by the fact that the protein expression remained active since Dox was not added to these neuron cultures. Same happened with the expression of EGFP.

On the other and, samples +DOX show exactly the opposite. After the addition of Dox on day 7 postransduction, the protein concentration started to decrease. When

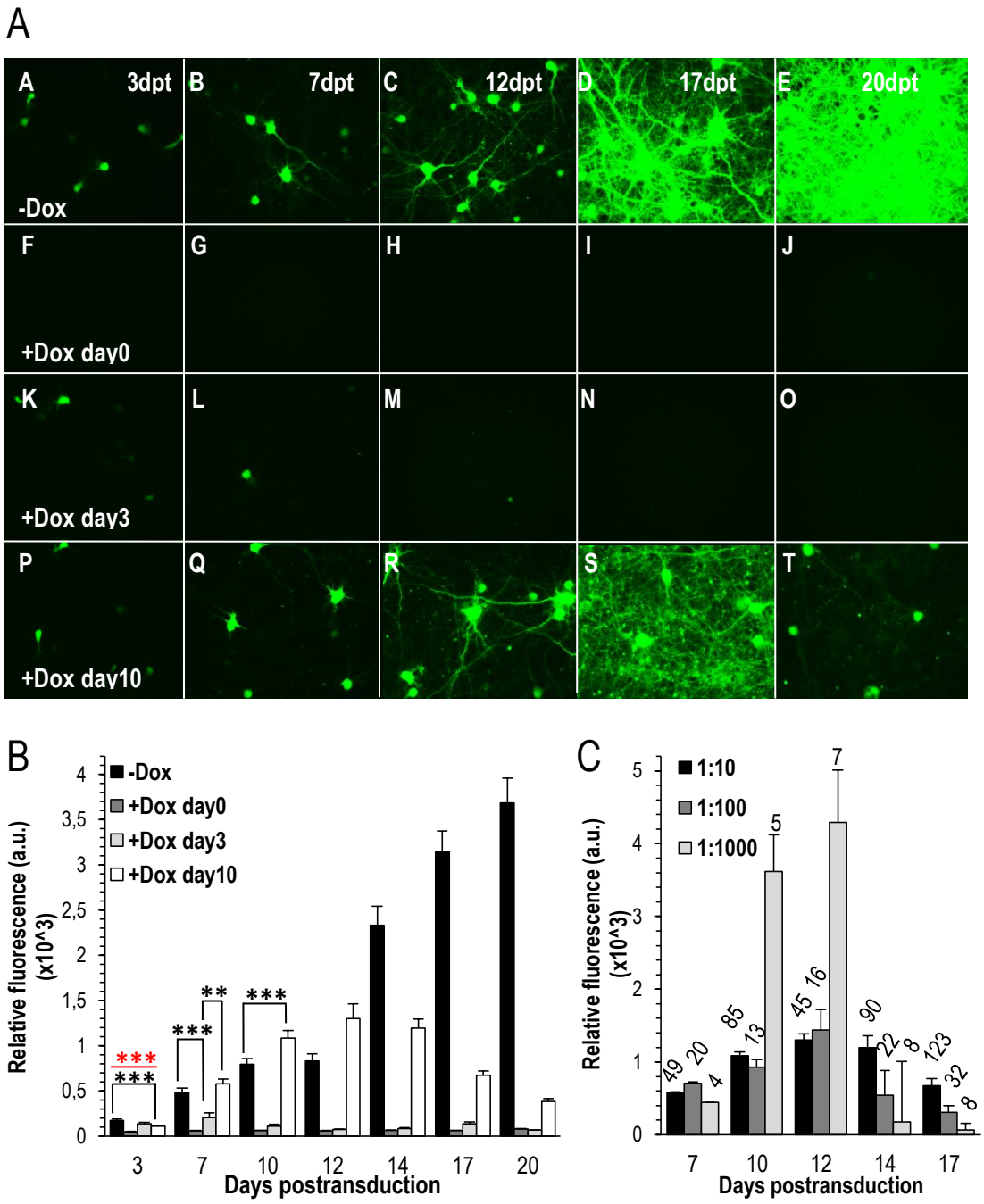

Figure 4. Treatment of transduced cultures with Dox showed a negative effect in EGFP expression and cultures' fluorescence. (A) Fluorescence microscope images from cultures transduced with Ratio 1:10 (3x106tu pAAV-tTA2-B; 3x107tu pAAV-EGFP). Cultures without Dox (-Dox) showed a continuous increase in fluorescence (A-E) whereas cultures where Dox was added on the same day of transduction (+Dox day0) expressed background fluorescence levels (F-J) during all the experiment. Cultures where Dox was added 3 days after transduction (+Dox day3, K-O) and cultures where Dox was added 10 days after transduction (+Dox day10, P-T). (B) Fluorescence values along days after transduction of Ratio 1:10-transduced cultures. Bars represent mean values ± s.e.m (****P*<0.05, ***P*<0.1). Red stars represent a significant difference between cultures where Dox was added on the moment of transduction in comparison with cultures where Dox was added at later time points. (C) Fluorescence values along days after transduction of Ratio 1:10, 1:100 and 1:1000-transduced cultures; numbers above each bar represent the number of fluorescent cells counted. Pictures A-E correlate with pictures K-O from Figure 2.A.

comparing day 10 and day 18 postransduction, the depletion in protein amounts was clearly obvious. Finally, on day 21, samples +DOX showed hardly detectable amounts of recombinant human α-Syn, and this was comparable to the negative controls (sample +DOX day 0), which were assumed to not have had any recombinant protein expressed. The same sequence of events could be seen with EGFP, even though, in this case, the protein concentration did not disappear completely. However, a clear depletion on its concentration was eye-catching when comparing samples +DOX on day 10 and 21 after transduction. When looking at **Figure 5.B**, by contrast, synuclein levels did not disappear completely on day 21. Sample +DOX on day 7 postransduction also showed little amount of synuclein protein. The latter observation is explained by the fact that the antibody used here stained not only the recombinant synuclein but also the rat's endogenous α-Syn expressed by the cells. In both blots it could be observed that, whereas EFGP and α-

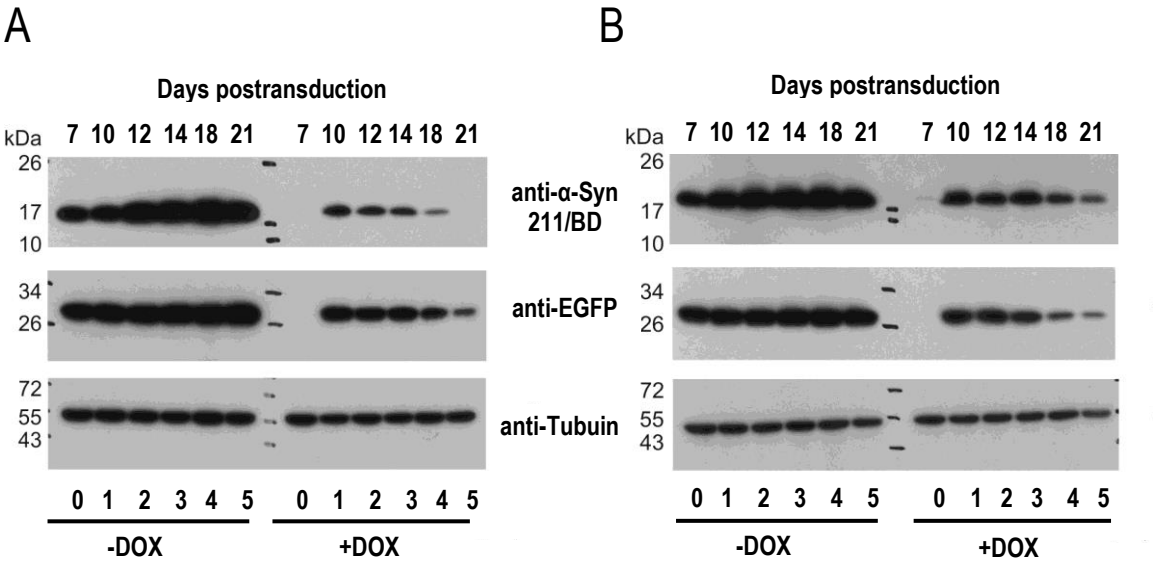

Figure 5. Immunoblots of whole cell protein lysates from neuron cultures transduced with a ratio 1:10 (3x106tu pAAV-tTA2-B; 3x107tu pAAV-EGFP) during 21 days in presence (+DOX) and absence (-DOX) of doxycycline. Dox (0,1ng/µl) was supplied at the same moment of transduction (+DOX 0) and seven days after the addition of viral vectors (+DOX 1-5). In (A), an antibody specific for human α-Syn (anti-α-Syn 211) was used to detect only the α-Syn expressed by the plasmids. In (B), total cellular α-Syn levels were assessed using an antibody recognizing both human and rat α-Syn (anti-α-syn BD). Samples without Dox show an increase in both EGFP and α-Syna mounts. A decrease of these same protein concentrations is appreciable when adding Dox on day 7 after transduction. Tubulin amounts remained invariable along days and showed an equal expression either in presence or absence of Dox.

Syn were not detectable on samples where Dox was added at the moment of transduction (with exception of α-Syn in **Figure 5.B)**, tubulin amounts were always present. Its concentration remained stable among days and neither the presence nor the absence of antibiotic seemed to have any effect on its expression rate. Tubulin is assumed to be physiologically expressed in the neurons, thus the addition of Dox is not predicted to cause any change on its synthesis.

4. DISCUSSION

4.1. TRANSFECTION AND CO-INFECTION OF NEURON CULTURES

Many basic research and therapeutic applications require the use of effective transducing vectors and quick and high-rate protein expression systems. The tetOFF system has already been reported to be a reliable tool for an efficient protein expression both *in vivo* and *in vitro*.[23, 37, 38, 39, 40] Furthermore, the integration of a hSYN1 promoter has been shown to specifically drive high plasmid expression in neurons.[24, 28, 40] The first aim of this study was to determine the suitability of the presented tetOFF protein expression system in primary neuron cultures, constitutively controlled by a hSYN1 promoter and transferred into the cells using an AAV serotype 6 viral vector. AAV have been reported to efficiently transduce neurons[13, 24, 38] and single plasmid tetOFF vectors have been shown to trigger net fluorescence in the same type of cells.[33] Results here obtained indicate, in accordance with previous results of the host laboratory, an unsuccessful protein expression and cell toxicity when transducing primary neuron cultures with the single pAAV-tetOFF-EGFP construct, whereas, as previously reported[34], the constitutive construct reaches to trigger great protein synthesis within few days after viral supply when using a hSYN1 promoter. Indeed, the promoter chosen for recombinant expression is also of major importance. The use of an hSYN1 promoter may be expected to trigger a higher protein expression when comparing it with other constitutive of neuron-specific promoters.[28, 29, 30] L. Jiang *et al*. (2004), for example, used a similar tetOFF recombinant AAV vector to express humanized GFP *in vitro*. Their results showed a high protein synthesis, but with the difference that the tTA expression was under the

control of a cytomegalovirus (CMV) promoter. On the other hand, it has been reported that one molecular drawback of the tetOFF system is the fact that high amount of tTA may result toxic sometimes[23]: morphological changes and cellular growth rate attenuation are two of the most remarkable effects of high amounts of this protein.[22] Judging from the fluorescence experiments and taking this last reports into account, the idea of a neurotoxicity triggered by great amounts of tTA protein in the present study may be a possible explanation to these first negative results.

In order to overcome the problem observed, the expression system was separated into two pieces. Cultures would require to be co-transduced with two different vectors since the tTA protein is not expected to diffuse transcellularly.[38] The splitting of the tetOFF system has already been reported to work as well as, or sometimes better than, a constitutive construct founded of just a promoter and the target gene: H. Hoioki and colegues experiments in vivo showed that, under the control of the hSYN1 promoter, co-infection with a double lentiviral tetOFF vector triggered higher protein expression than the constitutive construct, composed of the GFP gene under the control of the hSYN1 promoter.[33] Results from the present study show that separating the tetOFF system and transducing primary neuron cultures with two different AAV vectors not only trigger protein expression efficiently, but also at a higher fashion than the simpler construct. This way, a viral titer of tTA2 vector of $3x10^7$tu seems to be the cut point between toxic and non-toxic tTA concentration when using a hSYN1 promoter, since ratio 1:1 showed very close results to those from cultures transduced with AAV-tetOFF vector. These results together with the previous observations, may corroborate the hypothesis that the toxicity of the tTA2 depends on both, the viral titer employed and the cellular affinity for the promoter. By contrast, ratio 1:3 or 1:10 seem to represent the best equilibrium between the tTA expressed, the number of tetO sequences (pAAV-EGFP titer) and neuron toxicity.

Finally, results show that between pAAV-tTA2-B titers of $1x10^6$ and $3x10^4$tu, the number of fluorescent cells is dose-dependent, since results show that lowering the tTA gene titer is translated in a smaller number of fluorescent cells, but not in fluorescence depletion. These observations indicate that: either the small amount of tTA2 produced is enough to bind to very higher amounts of tetO sequences within

the cell and trigger the expression of EGFP, or that the affinity for the hSYN1 promoter is high enough to trigger the synthesis of high amounts of tTA2. These results couldn't be compared, since, as far as the research made for this work has gone, no other experiments studying the expression triggered by different ratios of a double AAV vector tetOFF system have been reported to date.[22, 32, 36]

4.2. CONTROL OF PROTEIN EXPRESSION: DOXYCICLINE EXPERIMENTS

Efficient On/Off control of protein expression in a tetOFF system using the antibiotic Dox (or Tet) has been widely reported already.[22, 23, 25, 34, 37, 38] The performed experiments show a clear inhibition of protein expression by both, fluorescence and Western blot analysis. The addition of Dox to the medium at the time of transduction led to negligible EGFP levels in cultures grown for as long as 21 days postransduction. Same results, also in neuron cultures, have been observed by S. Gascón and colleagues: the addition of Dox to the medium at the moment of lentiviral transfection could suppress EGFP expression during the whole experiment.[39]

Previous reports indicate that the addition of antibiotic, either Tet or Dox, triggers an almost immediate loss in protein expression.[23, 37, 38] However, observations made in the present study show that fluorescence continues increasing until two to three days after the antibiotic supply. Flow cytometry experiments and quantitative, real time PCR carried out by L. Jiang *et al.* (2004) demonstrated that the addition of Dox efficiently stopped reporter gene expression but did not alter tTA amounts.[23] However, the WPRE element has been reported to target powerful, long-term transgene expression in a promoter-independent manner, even when supplied in low adenoviral titers.[35, 39] This way, since the Dox is meant to bind to all tTA2 molecules, the slight increase in EGFP concentration and fluorescence shown during the next two to three days after the Dox supply may be explained by the high content in EGFP mRNA that is produced. On the other hand, experiments also carried out by S. Gascón *et al.*(2008), in which they used double-vector tetOFF expression system with WPRE sequences, show that the addition of Dox silences mRNA transcription within the first 24 hours and reduces EGFP mRNA efficiently down to background

levels, even though the fluorescence does not start to decrease until 3 days after the addition of the drug.[40] Further experiments may be performed to determine whether the increase in fluorescence intensity seen in these presented results it's a matter of slow mRNA degradation or slow tTA2 inhibition.

In addition, EGFP degradation rate shows differences with α-Sy's. After the addition of Dox, synuclein amounts are lowered to almost zero levels within the next 14 days, whereas EGFP is still appreciable on day 21 postransduction. S. Gascón *et al.*(2008) demonstrated that when combining two target protein genes (GFP and DsRed, two reporter proteins) within a single plasmid, each one preceded by a TRE sequence and with a downstream WPRE element, protein expression rates of these genes are equivalent.[39] Thus, in this case, assuming the α-Syn and the EGFP are equally expressed, the difference in degradation rate may be explained by the well-known intracellular stability of the GFP protein, which is mostly due to its β-can-like structure.[36]

Finally, in accordance to previous observations[39], doxycycline is demonstrated to restrictively affect the expression of the tetOFF system, and does not interfere in other cell's pathways, since the addition of the antibiotic does not seem to provoke morphological or cell survival changes.

5. CONCLUSIONS

The conclusions out of this study are:

(i) When transducing primary neuron cultures with AAV vectors ($3x10^7$tu) that contain a single plasmid tetOFF system with a small hSYN1 promoter and a WPRE element, tTA2 synthesized reaches neurotoxic levels.

(ii) When splitting the expression system into two different plasmids and supplying them in different AAV vector ratios, toxicity is avoided. Ratios 1:3 to 1:10 of the dual-plasmid tetOFF construct, in combination with the hSYN1 promoter, seem to be optimal since fluorescence triggered in

primary neuron cultures triplicates the expression obtained with the constitutive system.

(iii) The number of fluorescent neurons is directly proportional to AAV-tTA2-B titers (between ratios 1:3 and 1:1000), whereas fluorescence intensity remains invariable.

(iv) Lowering AAV-tTA2-B titer down to 1:1000 ratios allows the identification and visualization of net single neurons within primary neuron cultures.

(v) The dual-plasmid tetOFF system seems to be successfully controlled by Dox since the addition of this antibiotic inhibits protein expression, enabling the cells (three weeks old primary neuron cultures) to efficiently degrade both recombinant human α-Syn and EGFP.

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