Dual promoter lentiviral vectors for constitutive and regulated gene expression in neurons

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Abstract

Gene transfer methods for efficient co-expression of exogenous proteins in neurons are crucial tools towards the understanding of the molecular basis of the central nervous system. Lentiviruses are retroviral vectors that can transduce a wide variety of cells including differentiated neurons. In this work, we have generated lentiviral vectors containing dual promoters that allow efficient co-expression of exogenous proteins in neurons. We show that insertion of two copies of a human synapsin promoter/WPRE cassette in a single lentiviral vector directs robust co-expression of cDNAs in cultured neurons, while excluding expression in the surrounding glial cells. Furthermore, insertion of the tetracycline-inducible system (Tet-off) controlled by the synapsin promoter results in tightly regulated expression of EGFP when used as a transgene in cultured neurons. Transduction of primary neurons with this inducible system leads to a 100-fold increase in EGFP mRNA levels in the absence of doxycycline. In transduced cultures, EGFP transcription is inhibited within 24 hours upon addition of doxycycline. The viral systems we developed here provide neuron-specific and regulated expression mediated by single lentiviral vectors and will prove valuable tools for the study of neuronal function.

Keywords: lentivirus, synapsin promoter, inducible expression, primary neurons.

1. Introduction

Expression of exogenous proteins in cultured neurons represents an important approach towards the understanding of the molecular basis of neuronal function in normal and pathological states. In addition to the expression of single genes, efficient expression of two cDNAs in the same neuron is often desired in several experimental approaches. For example, many proteins, such as ion channels require co-expression of different subunits for normal function, or often a reporter fluorescent protein needs to be co-expressed to monitorize neuronal morphology or function when assaying the role of an exogenous cDNA. A similar problem arises when systems for regulated expression are used, as most regulated expression systems require the co-delivery in the same cell of the target gene and a transcriptional transactivator or repressor (Yamamoto et al., 2000). Therefore, the generation of molecular tools that allow for the simultaneous delivery of cDNAs into neurons is of critical importance.

Viral gene delivery systems are powerful tools for the expression of exogenous cDNAs especially in neuronal cells, as they overcome problems with low efficiency of gene transfer. However, a general challenge for both, constitutive and regulated gene expression from viral vectors is the co-delivery of multiple cDNAs into neurons. In recent years, lentiviral vectors have become a widely used system for the expression of exogenous cDNAs in different cell types in

vivo and in vitro, (Lois et al., 2002; Pfeifer et al., 2002; Delenda, 2004). Compared to other gene transfer systems, lentiviral vectors provide several advantages. As retroviral vectors, the viral genome is integrated into the chromosome of the host cell after retrotranscription, allowing for long-term, stable expression of the transgene (Lois et al., 2002; Pfeifer et al., 2002). In contrast to other retroviruses, lentivirus can integrate into the genome of both, dividing and non-dividing cells (Naldini et al., 1996). These properties make lentiviral vectors an excellent vehicle to deliver exogenous genes in postmitotic neurons (Blomer et al., 1997; Wong et al., 2006). Most commonly, lentiviral vectors are pseudotyped with the Vesicular Stomatitis Virus Glycoprotein (VSVG) and therefore infect a broad range of cell types. This low cell type preference represents a challenge when cell type specific expression is desired. For example, in studies on neuron-specific signaling mechanisms it is often essential to avoid infection of surrounding astroglia in tissue or in cell culture. A strategy for circumventing such problems is to insert regulatory elements into the viral vectors that direct cell type specific or inducible gene expression. This is possible due to the relatively large packaging capacity of lentiviral particles, which can accommodate the incorporation of several transcriptional-regulatory units into the viral genome (Azzouz et al., 2002). In addition, the promoter activity within the viral long terminal repeat (LTR) is irreversibly disabled in self-inactivating (SIN) vectors, allowing expression of the transgene to be controlled by the activity of exogenous promoters (Lois et al., 2002). Among the different neuronal

promoters analyzed, the human synapsin (hSYN) promoter is very well suited for neuron-specific expression of transgenes in vitro and in vivo due to its small size and high cell type specificity (Kugler et al., 2001; Glover et al., 2002; Nakagawa et al., 2006; Hioki et al., 2007).

In this work, we have developed and tested new dual promoter lentiviral vectors for constitutive and regulated neuron-specific expression. First, we show that the insertion of two hSYN promoter/WPRE cassettes into a SIN-lentiviral vector efficiently drives simultaneous expression of two cDNAs in a neuron-specific manner. In a second system, we use expression of the tetracycline-dependent transactivator, tTA, under control of the hSYN promoter to drive inducible-expression of a transgene delivered on the same lentiviral vector. These dual promoter vectors should facilitate the analysis of neuronal morphology and function in normal and pathological conditions.

2. Materials and methods

2.1. Generation of lentiviral vectors.

All dual-gene lentiviral vectors are based on the LenLox3.7 plasmid (generously provided by L. Van Parijs) (Rubinson et al., 2003). LenLox3.7 vector carries a U6 promoter followed by a CMV-WPRE cassette that controls EGFP expression

(Rubinson et al., 2003). To insert two copies of the hSYN promoter, two XbaI-HpaI and NsiI-NheI DNA fragments containing a 480 bp region from the distal most 3' end of the human synapsin promoter were amplified by polymerase chain reaction (PCR) from the vector pMH4-I-hSYN (generously provided by M. Bahr, University of Göttingen, Germany) (Kugler et al., 2001). The amplification fragments were ligated into the LenLox3.7 plasmid from which the U6 and CMV promoters had been removed by XbaI-HpaI and NsiI-NheI digestions, respectively. From this vector, the WPRE sequence was amplified by PCR with NotI-NsiI sites and cloned downstream of the first synapsin promoter to generate the SYN-SYN-GFP vector. The plasmid SYN-DsRed-SYN-GFP (Fig. 1A) was generated by inserting the DsRed sequence obtained by PCR with NotI-XhoI sites from the vector pDsRed-N1 (Clontech) under the control of the first SYN-WPRE cassette. One-single synapsin promoter vectors coding for EGFP (SYN-GFP) or DsRed proteins (SYN-DsRed) were generated from SYN-DsRed-SYN-GFP by removing the SYN-DsRed or SYN-GFP cassettes using NotI-EcoRI and XbaI-NotI restriction sites, respectively.

Inducible lentiviral vectors were originated in several cloning steps from the SYN-DsRed-SYN-GFP vector. First, the TRE inducible promoter from the pTRE vector (Clontech) was amplified by PCR with NsiI-AscI ends and cloned in the SYN-SYN-GFP to replace the second hSYN promoter, after digestion with NsiI-AscI. Next, a 1008-bp fragment obtained by PCR from the pTet-Off plasmid (Clontech)

containing the tTA coding sequence was cloned in BamHI-NotI sites downstream the hSYN promoter to generate SYN-Tetoff-GFP (Fig. 1B). To construct the SV40-Tetoff-GFP vector, a 441-bp fragment containing the SV40 promoter was inserted in SYN-Tetoff-GFP vector after removal of the hSYN promoter by XbaI digestion. All constructs were verified by DNA sequencing.

2.2 Cell culture

Primary culture of embryonic cortical neurons was performed as described (Brewer et al., 1993) with some modifications. Plates were treated with poly-L-lysine (100 μg/ml) and laminin (4 μg/ml) overnight at 37°C before seeding. Cerebral cortices from 18-days old rat embryos (Wistar) were dissected and mechanically dissociated in culture medium (Eagle minimum medium supplemented with 22.2 mM glucose, 0.1 mM glutamine, 5% FBS, 5% donor horse serum and penicillin/streptomycin). Cells were seeded at a density of 50.000 cells/cm^2 in the same media and maintained at 37 °C and 5% CO_2.

2.3. Lentivirus production and infection of primary cell cultures

Recombinant lentiviral particles were produced as previously described (Lois et al., 2002). Briefly, HEK293T cells were triple transfected using the calcium phosphate method with the lentiviral expression vector and two packaging

vectors: pCAG-VSVg, a plasmid expressing the VSV-G envelope gene, and pCMVΔR8.91, a plasmid expressing the gag/pol genes. The supernatants containing the viral particles were collected 48-60 hours after transfection and concentrated using filtration columns (Centricon Plus-20, molecular weight cutoff 100 kDa; Millipore) as described (Reiser, 2000). Viral titers were determined by serial dilution on PC12 pheochromocytoma cells by FACScan analysis as the synapsin promoter is active in this cell line (Thiel et al., 1991). Primary cell cultures were transduced at the indicated DIV by adding concentrated lentivirus at a multiplicity of infection (m.o.i.) of 0.5 to the growing media. When indicated, doxycycline (Calbiochem) was added at 2 μg/ml to the culture media.

2.4. Fluorescence activated cell sorting analysis

PC-12 cells were seeded at a density of 50.000 cells per well in 12-well plates. Twenty-four hours later the cultures were transduced with LV-hSYN-DsRed, LV-hSYN-GFP or LV-hSYN-DsRed-hSYN-GFP at m.o.i. 0.5. Seventy-two hours later the cells were resuspended in 500 μl of PBS solution and analyzed by FACScan (B-9320 Becton Dickinson). Filter sets of 515-545 and 564-606 nm wavelength band pass were used for the detection of EGFP and DsRed proteins, respectively. The 488 nm wavelength line of a mercury lamp was used to excite both fluorescent proteins. BD CellQuest Pro software was used for quantitation analysis. The threshold for gating was determined as the fluorescence value

above which less than a 3% of the control non-infected cells were considered as positive events.

2.5. Fluorescence microscopy

Neuronal cultures were fixed with 4% paraformaldehyde in phosphate buffer containing 4% sucrose. For immunofluorescence, fixed cells were permeabilized and blocked with 10% normal goat serum in PBS containing 0.1% Triton X-100. Cells were incubated with primary and secondary antibodies for 1-2 hours in the same blocking solution. The following monoclonal antibodies were used as neuronal or glial markers: neuronal nuclei protein (NeuN) (Abcam, Cambridge, UK), neuronal class βIII-tubulin (Tuj-1, Covance, Verkeley, CA) and glial fibrillary acidic protein (GFAP) (Chemicon, Temacula, CA). Immunoreactivity was detected with secondary antibodies conjugated to Alexa Fluor 647 or Alexa Fluor 594 (Invitrogen, Carlsbad, CA). Cells were mounted with Fluoromount-G (SouthernBiotech). Confocal images were acquired on a Leica TCS SP2 or on a Radiance 2000 confocal (BioRad) coupled to an inverted Axiovert S100 TV microcope (Zeiss) with a 63x Plan-Apochromat oil immersion objective.

2.6. Immunoblot analysis

For biochemical experiments, primary neuronal cultures growing on 12-well plates were transduced at 1 DIV with LV-SYN-Tetoff-GFP at m.o.i. of 0.5. Twenty-four hours after infection, the lentivirus-containing media were removed and new conditioned media added into the wells. Media-containing DOX (2 μg/ml) was added to the cultures to inhibit the expression of the transgene. Culture media were refreshed every 72 hours. When indicated, cultures were lysated in lysis buffer (50 mM Tris-HCl pH8, 150 mM NaCl, 1% Triton X-100, 1mM DTT) containing protease inhibitors (1 mM PMSF, 0.2 mM 1,10-phenanthroline, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 mM benzamidine). Protein determination was performed using the BCA reagent, and equal amounts of protein (25-50 μg) were fractioned by polyacrylamide gel electrophoresis and transferred to PVDF membranes (Pall, Life Sciences). Immunodetection of proteins was performed by standard procedures using rabbit anti-GFP (Invitrogen, Carlsbad, CA) and mouse anti-βactin (Sigma Co., St. Louis, MO) antibodies. Immunoreactivity was detected with secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA).

2.7. Quantitative real-time PCR

Cortical neurons cultured on 12-well plates were transduced at 1 DIV with LV-hSYN-Tetoff-GFP (m.o.i of 0.5). Twenty-four hours later the lentivirus-containing

media were replaced by conditioned media. In control cells, the cultures were grown for additional 14 days in the absence of DOX. In DOX-treated cells, doxycycline (2 μg/ml) was added to the culture media 9 days after infection. cDNA was prepared from cultures grown for 0, 4, 9, 10, 12, 14 DIV using the "cell to cDNA" kit following manufacturer's indications (Roche). PCR reactions (20 μl) contained 2-5 μl of cDNA, 0.05U Taq polymerase (Biotools), 0.2 mM dNTPs, 0.2 μM amplification primers, 1:40000 Sybr Green, and 3 mM $MgCl_2$. The PCR reactions were performed using the Mx3000P QPCR system (Stratagene), as follows: a denaturalization step at 95ºC for 2 min, followed by 40 cycles of 95°C for 20 s, 68°C for 20 s and 72°C for 30 s. The forward (5'-CTGCTGCCCGACAACCACTA-3') and the reverse (5'-CGTCCATGCCGAGAGTGATC-3') EGFP primers amplify a 124 bp product. EGFP transcript amplification was normalized against GADPH using the forward (5'-TTGCCATCAACGACCCCTTC-3') and the reverse (5'-GCCTTGACTGTGCCGTTGAA-3') primers. EGFP transcript amplification was normalized against GADPH according to the Comparative C_t Method (delta-deltaCt method).

3. Results

3.1. Dual promoter lentiviral vectors for constitutive neuron-specific co-expression

In order to drive constitutive expression of two exogenous cDNAs specifically in neurons, we inserted two copies of a 0.5 kb fragment of the proximal part of the hSYN promoter into a single SIN-lentiviral vector backbone (Fig. 1A). To analyze the functionality of this dual vector, the cDNAs of two reporter fluorescent proteins, DsRed and EGFP, were cloned downstream of the first and second hSYN promoter respectively, generating the SYN-DsRed-SYN-GFP vector (Fig. 1A). Previous reports have shown that the insertion in viral vectors of the woodchuck hepatitis virus posttranscriptional control element (WPRE) increases the expression levels of the transgenes in a promoter-independent manner (Zufferey et al., 1999; Brun et al., 2003). In accordance, we observed no significant expression from the first hSYN promoter in dual promoter vectors lacking an internal WPRE (data not shown). Thus, two WPREs were incorporated into the dual promoter vector, one downstream of each hSYN promoter (Fig. 1A).

3.2. Characterization of cDNA expression in PC12 cells

To characterize expression of DsRed and EGFP cDNAs from the dual promoter vector, we produced recombinant lentiviruses and infected the neuronal-like PC12 rat pheochromocytoma cell line. Analysis by fluorescence-activated cell sorting revealed robust expression levels of DsRed and EGFP proteins driven by the SYN-DsRed-SYN-GFP vector (Fig. 2). Removal of either of the two hSYN

promoters from the dual vector yielded similar mean fluorescence values for each cDNA, as compared to the dual promoter vector (Fig. 2A). Within the transduced population of PC12 cells, EGFP and DsRed expression showed a linear correlation, indicating efficient co-expression of both cDNAs in single cells (Fig. 2B). Confocal images of cultures of PC12 cells transduced with each lentiviral vector further indicated co-expression of the reporter proteins at similar levels (Supplementary Fig. 1A). These results suggest that both hSYN promoter/WPRE cassettes are equally active in the single and dual promoter configuration and that simultaneous presence of the two promoters does not result in a significant loss of promoter activity.

3.3. Co-expression of cDNAs in primary neurons

To determine whether neuron-specificity is preserved in our lentiviral vectors containing two hSYN promoter-WPRE cassettes, we analyzed the expression of DsRed and EGFP proteins in mixed cultures of primary neurons and glia cells transduced with LV-SYN-DsRed-SYN-GFP (Fig. 3 and Supplementary Fig 1B). Mixed cortical cultures were grown for 6-7 days in vitro (DIV) and incubated with LV-SYN-DsRed-SYN-GFP at a multiplicity of infection (m.o.i.) of 0.5 to favor a single integration per cell. The expression of the exogenous proteins was analyzed 7 days after transduction by observing DsRed and EGFP fluorescence. As shown in Figure 3, co-expression of DsRed and EGFP proteins was detected

in neurites and cell bodies of cultured neurons (lower magnification fields are shown in Supplementary Fig 1B). Quantitative analysis of transduced cultures showed that all DsRed-expressing neurons were found to be positive for EGFP expression (200 analyzed cells, 3 independent experiments). In addition, neurons that showed relatively high expression levels of one transgene, also expressed high levels of the other transgene (Fig. 3, arrows). In some cases, neurons expressing low EGFP levels show barely detectable DsRed levels (arrowheads), further confirming comparable gene expression levels of each transgene within single neurons. Quantitation of cultures stained with the neuronal marker NeuN, showed that the EGFP-expressing cells were identified as neurons. By contrast, staining with the astrocyte specific marker GFAP showed virtually no expression of EGFP in these glial cells (Fig. 3). Similar results were obtained in mixed cultures of hippocampal neurons (data not shown). Together, these data indicate efficient and specific co-expression of two cDNAs in cultured neurons mediated by a single dual-promoter lentiviral vector.

3.4. Generation of dual promoter lentiviral vectors for regulated expression

After establishing the dual promoter vectors for neuron-specific expression we adapted this system for inducible expression of transgenes, using the previously established tetracycline (Tet)-based regulation system (Gossen and Bujard, 1992). In the Tet-off system, the tTA transactivator mediates transcription of a

transgene controlled by the TRE inducible promoter. Transcriptional activation is negatively regulated by the presence of tetracycline or its analog, doxycycline (DOX). The presence of DOX blocks the binding of tTA to the TRE promoter, thereby inhibiting transgene expression. Therefore, regulated expression with the Tet-off system requires the delivery in the same target cell of both the tTA transactivator and the transgene. To direct inducible transgene expression in cultured neurons with a single vector, we combined in a dual promoter lentiviral vector the neuronal-specificity of the hSYN promoter and the inducibility of the Tet-off system (Fig. 1B). In this vector, the hSYN promoter mediates the expression of the tTA transactivator, whereas the TRE promoter drives expression of the cDNA of interest, e.g. EGFP in the SYN-Tetoff-GFP vector (Fig. 1B). Therefore, hSYN-mediated expression of tTA would in turn regulate EGFP expression in neurons in a DOX-dependent manner. We also generated a second lentiviral vector, SV40-Tetoff-GFP, where the ubiquitous SV40 promoter controls tTA expression (Fig. 1B).

3.5. Doxycycline-regulated expression of EGFP in mixed neuronal cultures

To study inducible transgene expression, dissociated cortical cultures were transduced with LV-SYN-Tetoff-GFP at 1 DIV and EGFP fluorescence was analyzed 7-8 days later. As shown in Figure 4, EGFP expression was clearly detected in cultured neurons grown in the absence of DOX (“on” state). Staining

of the transduced cultures with antibodies against the neuronal marker βIII-tubulin, showed that over 90% of EGFP-expressing cells were identified as neurons, whereas only a 5.5 % were co-stained with the glial marker GFAP (Fig. 4C). The opposite situation was found for vector LV-SV40-Tetoff-GFP, where transgene expression was mainly detected in glial cells and in only few neurons (92% glia, 8% neurons). These data confirm that the VSVG-pseudotyped lentiviral particles efficiently infect both, neuronal and non-neuronal cells but that the hSYN promoter restricts expression to neurons (Fig 4A and B). When transduced parallel cultures were grown in the presence of DOX for 7 days ("off" state), EGFP expression was inhibited irrespectively of whether the cultures were incubated with LV-SYN-Tetoff-GFP or LV-SV40-Tetoff-GFP viruses (Figs. 4 and 5). Addition of DOX does not alter neuronal density as compared with untreated controls, suggesting that neuronal viability is not affected by the presence of the drug (Fig. 4A). These results indicate that the hSYN promoter directs inducible gene expression in cultured neurons mediated by a dual promoter vector containing the Tet-off system.

3.6. Doxycycline-regulated switching of transgene expression

To analyze the time-course kinetics of transgene expression, mixed cultures of cortical neurons were incubated with LV-SYN-Tetoff-GFP at 1 DIV and the expression of EGFP analyzed by Western blotting at different time points in

culture. In the absence of DOX, EGFP protein started to accumulate at 4 DIV, reaching maximal expression levels at 9-12 DIV (Figs. 5A,C). These maximal levels were maintained until the end of the culture period (18 DIV). Addition of DOX to the media at the time of transduction led to negligible EGFP levels in cultures grown for as long as 18 DIV (Fig. 5A).

To study the kinetics of switching off transgene expression, we transduced cortical neurons with LV-SYN-Tetoff-GFP at 1 DIV, grew cells for 9 DIV to allow expression, and then added DOX to the media. As shown in Figure 5B, EGFP levels decreased three days after addition of the drug, reaching minimal protein levels 9 days after DOX addition (Fig. 5C). Due to the long half-life of the EGFP protein that can lead to discrepancies in the repression kinetics of protein and mRNA levels (Kafri et al., 2000), we performed quantitative real-time PCR experiments in cortical neuronal cultures transduced with LV-SYN-Tetoff-GFP. An approximately 100-fold induction in EGFP mRNA levels was obtained in cultures grown for 9-12 days in the absence of DOX. Addition of DOX to cultures grown for 9 days in the activation state (absence of DOX), drastically inhibited EGFP mRNA levels within the first 24 hours (Fig. 5D). Three days after addition of DOX, EGFP mRNA was barely above background levels, indicating efficient inhibition of transgene expression.

4. Discussion

Many basic research and therapeutic applications require co-expression of different cDNAs in one population of target cells. In this study, we have generated and tested dual promoter lentiviral vectors that drive constitutive, neuron-specific expression of cDNAs. We then applied this system for regulated neuron-specific expression under the control of the tetracycline-mediated transactivator. These new vectors provide rapid and tightly controlled expression and are easy to use.

An alternative strategy for co-expression of exogenous proteins is the use of bicistronic mRNAs containing an internal ribosomal entry site (IRES). However, due to the significantly reduced expression of the cDNA placed downstream of the IRES, this approach often does not yield efficient co-expression of two cDNAs (Yu et al., 2003). Moreover, bicistronic constructs are less easily adapted for regulated expression without the need of introducing additional transgenes. Dual promoter viral vectors have been used previously in non-neuronal cells (Yu et al., 2003; Amendola et al., 2005; Osti et al., 2006). We integrated into viral vectors two hSYN promoters which direct neuron-specific expression of transgenes in vivo and in vitro (Kugler et al., 2001; Kugler et al., 2003a; Nakagawa et al., 2006). Bähr and colleagues used a similar approach for neuron-specific co-expression of two cDNAs in adeno-associated viral vectors (Kugler et al., 2003b). Our studies show co-expression at similar levels of two

cDNAs in primary neurons using a dual promoter lentiviral system. The two copies of the hSYN promoter/WPRE cassette each yield similar expression levels for the two cDNAs. Moreover, the hSYN promoters restricted expression of both cDNAs to neurons despite efficient infection of glial cells. Finally, the expression levels achieved in cultures infected at low m.o.i were sufficiently high to allow direct fluorescence detection of reporter proteins in living neurons, without the need for immunostaining. Therefore, our dual promoter lentiviral vectors should greatly facilitate imaging studies with genetically encoded reporters of neuronal function (Granseth et al., 2006).

We further modified our dual promoter system for regulated gene expression. Insertion of the Tet-off system into a single lentiviral vector controlled by the hSYN promoter mediated expression of EGFP in cultured neurons. Absence of DOX from the cultures induced expression of the transgene by increasing EGFP mRNA levels up to 100-fold, whereas addition of DOX to transduced cultures efficiently inhibited transcription within 24 hours. Single lentiviral vectors that allow DOX-regulated expression of transgenes controlled by ubiquitous promoters have been previously generated and their use in the CNS reported (Kafri et al., 2000; Vogel et al., 2004; Blesch et al., 2005; Szulc et al., 2006) Our data with the SV40-Tet-off-GFP vector suggest a preference of this ubiquitous promoter for glial cells in mixed neuronal cultures, although it has been previously shown that lentiviral vectors containing the SV40 promoter can

transduce neuronal cell lines (Brun et al., 2003). While the use of non-specific promoters might raise concerns when regulated expression in neurons is desired, the dual-gene lentiviral vector we have developed offers the possibility of targeting specific cell populations (neurons versus glial cells) by using appropriate tissue-specific promoters.

In comparison to gene delivery systems where the transfer genome is split in two plasmids, the use of dual promoter lentiviral vectors offers several advantages. All the molecular components are contained in a unique vector, which guarantees expression of the two cDNAs in the target cells. As a direct consequence, the viral titer and the number of integrations into the host genome are decreased, which reduces the chances of insertional mutation. In addition, the use of short regulatory units, such as the hSYN promoter and WPRE, results in vectors that remain well below the packaging capacity of a recombinant lentivirus. The modular design of the dual promoter vectors developed in this study allows for an easy replacement of the transgenes. We believe that the systems described here may prove an important tool for the understanding of neuronal function in normal and pathological conditions.

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Figure legends

Fig 1. Schematic drawings of the dual promoter lentiviral vectors used in this work. (A) SYN-DsRed-SYN-GFP. A vector containing two hSYN promoter/WPRE cassettes for the constitutive expression of two cDNAs in neurons. (B) Single lentiviral vectors carrying the entire Tet-off system controlled by heterologous promoters. For regulated expression of the transgene, the cDNA of the tetracycline-dependent transactivator tTA is inserted downstream of the neuronal hSYN promoter (SYN-Tetoff-GFP) or the ubiquitous SV40 promoter (SV40-Tetoff-GFP). In both vectors EGFP expression is regulated by the TRE inducible promoter. In the target cells, tTA protein binds and activates the inducible promoter to generate EGFP expression. The tetracycline analogue doxycycline (DOX) prevents the binding of the tTA transctivator to the TRE promoter blocking transgene expression. hSYNp, human synapsin promoter; WPRE, woodchuck

hepatitis virus posttranscriptional control element; SV40p, SV40 promoter; TREp, TRE inducible promoter; tTA, tetracycline-dependent transactivator; LTR, long terminal repeats. Unique restriction sites are shown: Ag, Age I; B, BamH I; Bs, BsrG I; Eco, EcoR I; Nh, Nhe I; No, Not I; Sm, Sma I; Xm, Xma I.

Fig 2. Efficient co-expression of EGFP and DsRed proteins in PC12 cells mediated by a dual promoter lentiviral vector. (A) Fluorescence-activated cell sorting analysis of PC12 cells transduced with LV-SYN-DsRed-SYN-GFP, LV-SYN-DsRed and LV-SYN-GFP (m.o.i. 0.5), as indicated. Red and green channels were detected separately 4 days after transduction. Non-infected PC12 cells are shown in each panel as a solid line. The number of positive cells, represented as the percentage of gated cells, and the mean intensity of fluorescence are indicated per each condition. Regions above threshold are shown as a horizontal grey bar in each panel. Fluorescence intensity (in a logarithmic scale) and number of events are represented in the *x* and *y* axis, respectively (B) Biparametric plots reveal a linear correlation of DsRed and EGFP expression in PC12 cells infected with LV-Syn-DsRed-Syn-GFP virus, non infected cells are shown as control. Fluorescence intensity per each protein is represented as a density plot (light grey points indicates a higher number of events).

Fig 3. Co-expression of exogenous proteins in cultured neurons. Mixed cultures of cortical neurons transduced with LV-SYN-DsRed-SYN-GFP were stained with antibodies against the neuronal marker NeuN (upper panels, blue in the colocalization) or with GFAP antibody to label glial cells (lower panels, blue in the colocalization). DsRed (red in the colocalizations) and EGFP expression (green in the colocalizations) were detected by direct fluorescence. Cells expressing relatively high levels of EGFP proteins show comparable high DsRed levels (arrows). Scale bar = 10 μm.

Fig 4. Doxycycline-regulated expression using dual-gene lentiviral vectors coding for the Tet-off system. (A) Representative fluorescence images of cortical cultures transduced with LV-SYN-Tetoff-GFP (upper panels) or LV-SV40-Tetoff-GFP (lower panels) at 1 DIV and grown for 7 additional days in the absence or presence of DOX, as indicated. Cultures were stained with the neuronal marker βIII-tubulin (red in the colocalizations) or with the glial marker GFAP (blue in the colocalization). In cultures transduced with LV-SYN-Tetoff-GFP, expression of the transgene is detected in neuronal cells. Few EGFP-positive neurons are found in cultures transduced with LV-SV40-Tetoff-GFP. As indicated, incubation with DOX inhibits the expression of EGFP in both situations. Scale bar = 10 μm. (B) Lower magnification fields showing EGFP expression from VSVG pseudotyped LV-SYN-Tetoff-GFP and LV-SV40-Tetoff-GFP virus. Cultures were grown in the absence of DOX and stained with the gial marker GFAP. Note that

EGFP is expressed in glial cells in cultures incubated with LV-SV40-Tetoff-GFP. Scale bar = 50 μm (C) Percentage of neuronal or glial EGFP-positive cells. Cultures were transduced with LV-SYN-Tetoff-GFP or LV-SV40-Tetoff-GFP and grown in the absence of DOX. The number of EGFP-expressing cells labeled with neuronal or glial markers was determined using specific antibodies as in **a**. In LV-SYN-Tetoff-GFP-transduced cultures, an average of 94 % of EGFP-positive cells were identified as neurons. In cultures transduced with LV-SV40-Tetoff-GFP only 8.4 % of EGFP-positive cells were found to be neurons. 1300 analyzed cells per condition, 3 independent experiments.

Fig 5. Time course kinetics of doxycycline-regulated EGFP expression. (A) Cortical cultures transduced with LV-SYN-Tetoff-GFP at 1 DIV were grown in the presence or absence of DOX for 18 days. Cell lysates were obtained at the indicated time points and EGFP expression analyzed by Western-blot. In the absence of DOX (upper panels), EGFP starts to accumulate at 4 DIV, reaching a maximum at 9-12 DIV. Incubation with DOX (lower panels) inhibits EGFP expression in transduced cultures grown in the presence of the drug for the same time period. (B) Doxycycline-mediated switch-off. Cultures were transduced with LV-SYN-Tetoff-GFP as in **a**, grown in the absence of DOX for 9 DIV and then incubated with DOX for additional 9 days. (C) Quantitation of EGFP protein levels. Neuronal cultures were transduced with LV-SYN-Tetoff-GFP and grown for 18 DIV in the absence of DOX or in the presence of DOX for the last 9 days.

Levels of EGFP were established by densitrometric analysis of immunoblots using NIH Image software. Percentage of normalized EGFP levels at 18 DIV are shown. The mean + standard deviation (SD) of three independent experiments are represented. (D) mRNA levels analyzed by quantitative real-time PCR from transduced cultures with LV-SYN-Tetoff-GFP. Data were normalized with GAPDH mRNA levels. The mean + SD of three independent experiments are shown.

Supplementary Fig 1. (A) Confocal images of PC12 cells transduced with LV-SYN-DsRed-SYN-GFP, LV-SYN-DsRed and LV-SYN-GFP (m.o.i 0.5) as indicated, showing co-expression of two reporter proteins in neuronal cells. Cells were stained with Hoescht to label the nuclei. Scale bar = 50 μm. (B) Confocal images at low power magnification of cortical neurons infected with LV-SYN-DsRed-SYN-GFP. DsRed and EGFP proteins were detected by direct fluorescence. Due to the low magnification of the images, fluorescence of DsRed and GFP proteins was mainly detected in the cell body of transduced neurons where the exogenous proteins concentrate at higher levels. Scale bar = 20 μm .