*** Title page and abstract**

GAL4-NFkappaB Fusion Protein Augments Transgene Expression from Neuronal Promoters in the Rat Brain

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Running Title: Enhancement of the transcriptional activity of neuronal promoters

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Abstract

Targeted gene expression mediated by a mammalian cellular promoter is desirable for gene therapy in the brain where there are a variety of different neuronal phenotypes, several types of supportive cells and blood vessels. However, this approach can be hampered by weak activity of some cellular promoters. In view of the potency of the transcription factor NFκB in regulating neuronal gene expression, we have assessed whether it can be used to enhance the strength of neuron-specific promoters. Our approach was to use a neuronal promoter to drive expression of a chimeric transactivator, which consisted of a part of the transcriptional activation domain of NFκB p65 protein fused to the DNA-binding domain of GAL4 protein from yeast. The second copy of the neuronal promoter is modified by introducing the unique GAL4 binding sequences at its 5′ end and used to drive the expression of a transgene. Binding of the chimeric transcriptional activator upstream of the second promoter was expected to potentiate its transcriptional activity. In this study, the approach was applied to the platelet-derived growth factor β-chain and synapsin-1 neuron-specific promoters and tested *in vitro* and *in vivo* using plasmid, lentiviral and baculoviral vectors. We observed up to a 100-fold improvement in reporter gene expression in cultured neurons and 20-fold improvement in the rat brain *in vivo*. Moreover, the cell-type specificity of the two tested promoters was well preserved and restricted to neurons. Finally, the expression driven by the new lentiviral vectors with the p65-potentiated synapsin-1-promoter showed no signs of decline or cell damage 4 weeks after injection. This approach should be suitable for constructing powerful and stable gene gene expression systems based on weak cell-specific promoters in neuronal phenotypes.

Key words: Gene transfer, neuron-specific promoter, transcriptional activity, neurons, brain

Introduction

Cellular promoters with neuronal specificity have been used to restrict transgene expression to neurons for the purpose of gene therapy or neurobiology research [1]. The platelet-derived growth factor β-chain (PDGF) and synapsin-1 (SYN) promoters are two examples of such promoters. The human PDGF promoter is active in neurons throughout the brain and the spinal cord, but not in glial cells [2]. Similarly, the human SYN promoter can selectively drive neuronal expression in various regions in the brain [3,4]. In general, mammalian cellular promoters are relatively weak activators of transcription when compared to those derived from viruses, such as the cytomegalovirus immediate-early enhancer/promoter (CMV promoter), which is a commonly used strong promoter. This inherent weakness in driving transgene expression could compromise the efficacy of certain targeted gene therapy applications that require high-level expression of therapeutic genes confined to neuronal populations.

Several approaches have been developed in order to improve the transcriptional activity of weak cellular promoters. These include (1) designing promoters by eliminating from a natural promoter the elements that do not contribute to promoter strength or specificity and at the same time multimerizing the positive regulatory promoter or enhancer elements, (2) designing promoters containing activating point mutations, (3) constructing chimeric promoters by combining the regulatory elements from different promoters, (4) enhancement at the posttranscriptional level, and (5) using recombinant transcriptional activators to achieve transcriptional amplification [5,6]. A significant drawback of the first four approaches is that they might only be applicable successfully to a subset of promoters and the exact strategy has to be developed for each promoter. The fifth approach, a relatively generalizable method, referred to as recombinant transcriptional activation [5,7] or two-step transcriptional amplification [8-11] utilizes artificial chimeric transcriptional activators to enhance transgene expression from a cell type specific promoter. The basic principle of the method is to use a cell-specific promoter to drive the expression of a fusion protein containing a strong transcriptional activation domain and a DNA binding domain, which bind to a specific site in a modified promoter that drives transgene expression. The binding of the fusion protein is expected to promote the assembly of RNA

polymerase II complexes at the TATA box of the modified promoter and augment transgene expression. The most commonly used chimeric transcriptional activator is the herpes simplex virus transcriptional activator VP16 fused to the DNA binding domain of yeast transcriptional activator GAL4 [8-15]. This design takes advantage of the unique DNA binding sequence of GAL4 that exists in yeast but not in mammalian genomes. Thus, when included in a mammalian cellular promoter this sequence enables specific binding of artificial chimeric transcriptional activators. This results in augmented expression of the transgene while minimizing the chances of interference with the expression of other genes in mammalian cells.

Nuclear factor-kappaB (NFκB) denotes a group of dimeric transcription factors, with the p50/p65 dimer as the most common complex regulating the expression of mammalian genes. In the central nervous system (CNS) neurons NFκB activity is constitutive and relatively high [16,17]. This transcription factor plays a crucial role in the survival of neurons in a variety of physiological and pathological settings [17-19]. For example, NFκB is activated in neurons in response to excitotoxic, metabolic, and oxidative stress. Activation of NFκB in neurons increases the levels of anti-apoptotic proteins and provides strong neuroprotection, whereas inhibition of NFκB activity leads to cell death after neurotoxic insults [17-20]. NFκB also plays a crucial role in both neuronal ontogeny and establishment of synaptic plasticity by regulating genes encoding neurotrophic factors, neurotransmitter receptors and calcium-regulating proteins [18].

In view of the potent regulatory functions of NFκB in neuronal gene expression, we investigated whether it can be used to develop a transcriptional amplification strategy for potentiating the activity of weak neuronal promoters. We have used the transcriptional activation domain of the mouse NFκB p65 to construct a GAL4p65 fusion protein. This chimeric transcriptional activator was introduced into plasmid, lentivirus (LV) and baculovirus (BV) vectors accommodating either the PDGF or SYN promoter. Their activity was assessed in cultured neural cells and in the rat brain *in vivo* using two different reporters, luciferase and enhanced green fluorescent protein (EGFP).

Results

Neuronal promoters display lower activities than commonly used viral promoters

As a starting point, we compared levels of transgene expression from neuronal promoters with those from commonly used viral promoters in plasmid vectors. We cloned all the promoters into the same luciferase reporter vector, pGL3-basic vector (Promega), in order to reduce the effect of plasmid backbone on gene expression (Table 1). The neuronal promoters we examined were the PDGF and SYN promoters whereas the viral promoters included CMV, SV40, and RSV promoters. A potent hybrid CMV enhancer/chicken β-actin (CAG) promoter was also included for comparison. The plasmid vectors were compacted with polycation polyethylenimine 25kDa (PEI25k). *In vitro* transfection was carried out in PC12 and C17.2 neuronal cell lines and expression *in vivo* was assessed after injection of these vectors into the striatum of the rat brain. Expression levels were measured using a quantitative luciferase activity assay, essentially as described previously [21].

As shown in Figs. 1A and 1B, the neuronal promoters provided lower levels of luciferase activity compared to the viral promoters in cultured PC12 and C17.2 neuronal cell lines. For example, the PDGF promoter in C17.2 and the SYN promoter in PC12 neurons showed luciferase activity more than 100-fold lower than the CAG, CMV and SV40 promoters. Compared to the RSV promoter, the PDGF promoter displayed a lower activity in both C17.2 and PC12 neuronal cell lines whereas the SYN promoter showed lower activity in PC12 but stronger activity in C17.2 cells. These findings highlight the need for improving the strength of neuronal promoters, although activity differences between a neuronal promoter and a viral promoter might vary from one cell line to another.

GAL4p65 improves the activity of neuronal promoters in cultured neurons

To test our strategy, we constructed two types of plasmid vectors: (i) either PDGF or SYN promoter was used to drive the expression of the chimeric transactivator GAL4p65 (pPDGF-GAL4p65 and pSYN-GAL4p65 in Table 1) and, (ii) 5×GAL4 binding sites were introduced upstream of one of the neuronal promoters that were used to drive the expression of either a

luciferase or EGFP reporter gene (pGBS-PDGF-Luc and pGBS-SYN-EGFP in Table 1). Effects of vectors expressing the chimeric transactivator were tested by combining them with one of the vectors driving expression of one of the reporters.

We first tested the effect of GAL4p65 on the activity of the PDGF promoter using luciferase as a reporter. Increasing the dose of pPDGF-GAL4p65 combined with a fixed dose of pGBS-PDGF-Luc increased luciferase activity in both PC12 and C17.2 neuronal cell lines (Fig. 2A). When the ratio of pPDGF-GAL4p65 vs. pGBS-PDGF-Luc was set at 1, the increase in transgene expression reached a plateau, being 97-fold higher in C17.2 cells and 20-fold higher in PC12 cells over the controls without the use of the chimeric transactivator (0 μg of pPDGF-GAL4p65 in Fig 2A). These levels of gene expression achieved by transcriptional amplification were compatible to that provided by the CMV promoter in PC12 cells and 25-fold higher than that from the CMV promoter in C17.2 cells (Figs. 1A and 1 B). We then examined the effect of GAL4p65 on the activity of SYN promoter using EGFP as a reporter. As shown in Fig 2B, GAL4p65 driven by the SYN promoter increased the number of EGFP-positive PC12 cells by ~8-fold, providing a level of gene expression comparable to that when GAL4p65 was expressed using the CMV promoter (Fig. 2B).

GAL4p65 improves the activity of neuronal promoters in the rat brain in vivo

Encouraged by the above *in vitro* results, we next tested the effects of GAL4p65 *in vivo*. Initially we used plasmid/PEI25k complexes. We have shown in our previous studies that these complexes, as well as BV vectors, can be taken up by nerve terminals and transported retrogradely along axons to regions remote from an injection site in the CNS [22,23]. Thus, in addition to the injection site (the striatum), we also analyzed the cerebral cortex where many neurons innervate striatal neurons. After the injection of pGBS-PDGF-Luc together with the pPDGF-GAL4p65, a significant increase in gene expression was observed in both regions examined, with 15- and 3-fold increase in the striatum and cerebral cortex respectively (Fig. 3A). These findings constitute a proof of principle for the potentiating effect of GAL4p65 *in vivo*.

However, the dual vector system tested above may potentially lead to sub-optimal transduction efficiency caused by differences in cellular uptake of the two different vectors. In addition, the requirement for two vectors results in an increased total dose for effective functioning. We therefore incorporated the two expression cassettes into a single viral vector. Taking advantage of the large cloning capacity and broad tropism for both dividing and nondividing cells both baculoviral and lentiviral vectors were used.

The baculoviral vector BV-2xPDGF-Luc (Table 1 & Fig. 3B) was constructed to accommodate both the GAL4p65 activator under the control of the PDGF promoter and the luciferase reporter gene driven by the PDGF promoter fused with 5 x GAL4 binding sites. A BV vector with the PDGF-luciferase cassette, BV-1xPDGF-Luc (Table 1) was constructed as a control. After injection of 10^8 pfu of viral particles into the striatum, BV-2xPDGF-Luc significantly increased gene expression by 9-fold at the site of injection and 21-fold in the cerebral cortex when compared with BV-1xPDGF-Luc (Fig. 3C). Even an injection with 10^7 pfu of BV-2xPDGF-Luc particles produced transgene expression 3-fold higher in the striatum and 4-fold higher in the cerebral cortex compared to that achieved using 10^8 pfu of BV-1xPDGF-Luc particles (Fig. 3C).

We also constructed three LV containing: (1) The EGFP reporter gene under control of the SYN promoter alone (LV-1xSYN-EGFP, Table 1); (2) Both the SYN-driven EGFP reporter gene and the SYN-driven GAL4p65 activator element in one viral vector backbone (LV-2xSYN-EGFP, Table 1 & Fig. 4A); and (3) Both the CMV-driven EGFP and the SYN-driven GAL4p65 in a single viral vector (LV-CMV/SYN-EGFP, Table 1, Fig. 4A). We tested their activity *in vivo* after injection into the hypoglossal motor nucleus. At 7 days postinjection, LV-2xSYN-EGFP and LV-CMV/SYN-EGFP produced significantly more EGFP-positive cells when compared to LV-1xSYN-EGFP (Fig. 4B). More than a 5-fold increase in the density of EGFP-positive cells was observed with LV-2xSYN-EGFP and LV-CMV/SYN-EGFP compared to those injected with LV-1xSYN-EGFP without the GAL4p65 activator (Fig. 4C). It was notable that the amount of EGFP fluorescence in the neurons targeted with GAL4p65-containing vectors was much higher (Fig. 4B) although we did not quantify this parameter.

In a separate experiment animals were allowed to survive for up to 4 weeks after LV-2xSYN-EGFP had been injected into the hypoglossal motor nucleus. In these experiments we did not observe any obvious changes over time in the number of EGFP-positive cells per field of view and the fluorescence intensity of these cells (Fig. 4D). The same brain sections collected at the end of the experiment were also immunohistochemically stained with antibodies against the glial fibrillary acidic protein (GFAP), a marker protein for astrocytes. An increased GFAP expression is a reliable marker for gliosis that is closely associated with neuronal damage. On both days 7 and 28, we observed no signs of reactive gliosis (Fig. 4D). These results suggest that the transgene expression driven by our GAL4p65 system was stable and did not lead to any obvious toxic effects.

Cell-type specificity of transcriptional amplification strategy using a neuronal promoter-driven GAL4p65

One of the critical issues in developing transcriptional amplification technology using a cell-type specific promoter is whether cell-type specific transgene expression could be achieved. To address this issue, we first performed *in vitro* experiments using either plasmid or BV vectors and compared the effects of GAL4p65 in both neuronal and non-neuronal cell lines. In the experiment using co-transfection of pPDGF-GAL4p65/pGBS-PDGF-Luc, significant improvement in gene expression was observed in neuronal lines such as C17.2 and PC12 but not in non-neuronal COS-7 and CHO cells (Fig. 5A). Similarly, the infection with BV expressing GAL4p65 demonstrated an improved gene expression in C17.2 and PC12 neurons but not in U251 and T98G glioma cells (Fig. 5B). Obviously, the PDGF promoter was active in these non-neuronal cell lines, as demonstrated by the use of pGBS-PDGF-Luc alone or BV-1xPDGF-luc (Figs. 5A and 5B). These results indicate that GAL4p65 might need the assistance of neuron-related transcriptional factors and regulators to augment the activity of the PDGF promoter or that the potentiating activity of GAL4p65 was suppressed in the non-neuronal cell lines we used.

To test the specificity of these vectors *in vivo*, we validated neuronal expression using a neuronal specific antibody, NeuN. Essentially all luciferase-positive cells transduced in the

striatum using co-transfection with plasmids pPDGF-GAL4p65 and pGBS-PDGF-Luc were also NeuN-positive, whereas none of them were stained positively for GFAP, indicating that the transgene was expressed exclusively in neurons and not glia (data not shown). We also injected BV-2xPDGF-luc into the CA1 region of the rat hippocampus. The luciferase-positive cells were located almost exclusively in the pyramidal cell layer (Fig. 6, left column). In an experiment using LV-2xSYN-EGFP in a single vector to target neurons in the hypoglossal motor nucleus, EGFP was completely co-localized with the neuronal NeuN marker (Fig. 6, middle column), whereas none of the EGFP positive cells were GFAP-positive (Fig. 6, right column) again demonstrating that the expression of EGFP in LV-2xSYN-EGFP remained completely restricted to neurons.

Discussion

The activity of native promoters in mammalian cells, in particular those operating in relatively transcriptionally inactive CNS neurons, is lower than that of the viral promoters, which have evolved to hijack the cellular protein-producing machinery (Fig. 1). Obviously, low promoter activity presents an obstacle for gene therapy and experimental applications when delivered genes need to be expressed at high levels. In the current study we demonstrate the feasibility of using a GAL4p65 fusion protein to improve the strength of two neuronal promoters, without affecting their neuron specificity. To our knowledge, this study is the first to use the transcriptional amplification strategy to augment transgene expression from neuron-specific promoters in the brain *in vivo*.

The construction of a recombinant transcriptional activator GAL4p65 is based on the well-identified modular structures of two transcriptional factors, the murine NFkB p65 and yeast GAL4. As with other Rel family members, murine p65 contains an N-terminal ~300 amino acid conserved region known as the rel homology domain [24]. This region is responsible for DNA-binding and dimerization. It also contains a nuclear localization sequence, which is essential for the transport of active NFκB complexes into the nucleus. Like RelB and c-Rel, the C-terminal portion of p65 contains potent transactivation domains. The most active part of the transcription activation domain of p65 is located between amino acids 364–549, which has been used for

generation of the mammalian Two-Hybrid system by Stratagene™. The yeast GAL4 gene expression system is one of the most widely studied eukaryotic transcriptional regulatory systems. Of the 881 amino acids that constitute the transcriptional activator of GAL4, the fragment compromising amino acids 1-147 contain the DNA binding domain and also acts as a nuclear localization signal [25]. In this study, we fused the transcriptional activation domain of murine p65 (364-550 amino acids) to the DNA-binding domain of GAL4 (1-147 amino acids) to form GAL4p65. As demonstrated, the generated chimeric protein works as a strong artificial transcriptional factor.

By appending multiple GAL4 binding sites upstream of the PDGF and SYN promoters, we have artificially introduced the binding motifs for p65 close to the promoters. The mechanism underlying the potentiating effect of p65 is not fully understood, but probably is related to a favorable interaction between p65 and other transcription factors and regulators normally attracted by PDGF and SYN promoters. In mammalian genomes, promoters containing potential NFκB responsive elements often contain binding sites for other transcription factors, such as AP-1, NF-IL6, STAT1 and IRF-1 [26], the interactions of which are crucial for the optimal regulation of gene expression. In the case of GAL4-VP16, it has been postulated that this artificial transactivator may synergistically enhance transcription levels by stabilizing the pre-initiation complex [27,28]. Analysis of the recognition of transcriptional regulation elements by RNA polymerase, transcription factors and auxiliary proteins in our GAL4 binding site-fused neuronal promoters would be helpful in understanding the enhancement mechanism and in determining whether the approach demonstrated in this study would be universally applicable to other cellular promoters.

We have used two copies of a neuronal promoter either in separate constructs or within a single viral gene transfer vector. One of the copies is used to drive the expression the GAL4p65 fusion protein and another, with GAL4 binding sites inserted at its 5′ end, for transgene expression. The two copies of the same cellular promoter provide dual control of cell specificity, ensuring transgene expression only in targeted cells, such as neurons. Another commonly used approach that can improve the strength of a cellular promoter is the use of viral enhancers, for

example the CMV enhancer fused 5′ to a cellular promoter. Including the CMV enhancer in this way increases the number and diversity of transcriptional factor-binding sites in the 5′ flanking region of a transgene, thus in some case improving transgene expression. We had previously used this approach to modify three promoters that regulate tissue-specific proteins in the nervous system. We successfully improved the transcriptional activity without affecting cell specificity in two of the three promoters, the PDGF promoter [21] and the glial fibrillary acidic protein promoter [29], but failed with the third one, the neuron-specific enolase (NSE) promoter (unpublished observation). In the brain, CMV promoter activity is remarkably different in different groups of cells, especially when it is incorporated in adenoviral and LV vectors [4, 30]. While it is highly active in glial cells, only some neuronal phenotypes exhibit high level of expression when targeted with CMV-based constructs. Thus, it is likely that the incorporation of the control elements of CMV enhancer into another promoter may bias gene expression in favor of certain cellular phenotypes and distort the expression profile of the original promoter. Moreover, in some constructs and cellular systems, CMV enhancer actually fails to improve transgene expression [31]. The transcriptional amplification strategy used in the current study does not modify transcriptional factor binding sites and thus should be less likely to affect the specificity of the promoter upon which is based. Our immunostaining data using both neuron- and glial-specific antibodies are supportive of this claim. The cell/tissue specificity of a cellular promoter could be affected by viral vectors that accommodate the promoter in their vector backbone. For example, after a 1.4-kb PSA promoter was placed into a first-generation adenoviral vector, the tissue specificity of the promoter was decreased up to 400-fold [32]. Similarly, a NSE promoter placed into herpes simplex virus type 1 vector [33] and a glial cell-specific JC virus promoter placed into a retroviral vector [34] lose their cell specificity. One possible reason of the loss of desired specificity could be the powerful endogenous viral transcriptional controls overriding the cellular promoters [35]. This did not occur in the two types of viral vectors tested in the current study. LV used in this study is a self-inactivating HIV-1 based vector with most of the U3 region of the 3′ LTR deleted, which eliminates essentially all transcriptional functions of the HIV genome. The genomic viral DNA is therefore inserted into the target genome as a promoter-less sequence. The

lack of active viral promoter avoids detrimental interference between the inserted viral promoter elements and endogenous genes [36, 37]. As for insect baculoviral vectors, their infection in mammalian cells does not result in expression of any viral genes [38-40]. Even though certain sequences of BV could function as promoters or enhancers, they will be silent in mammalian cells due to the absence of supporting transcriptional factors, thus being less likely to influence the cell-type specificity of an inserted mammalian promoter.

In conclusion, we have demonstrated that GAL4p65 is a potent and specific transactivator, which can boost gene expression driven by two target neuronal promoters *in vitro* and *in vivo* in different gene delivery systems and brain areas. To make the transcriptional amplification approach more robust, several variables can be optimized. These include (1) the number of GAL4-binding sites; (2) the space between the GAL4-binding sites and the downstream promoter; (3) the number of activator domains; (4) the orientation of the transgene and the transactivator-expressing cassettes and (5) the distance and insulator sequence between the two gene expression cassettes. Also, modifications of some regulatory sequence elements such as introns, translation initiation sites, the polyadenylation signal and posttranscriptional regulatory elements can be tested to further potentiate our system. We believe that our approach may be applied for generation of other powerful viral vector gene delivery systems with high level of specificity for different cell types.

Materials and Methods

Construction of pGL3-based vectors for promoter strength comparison

For comparison of promoter activities, we cloned 6 promoters, 3 viral, 2 neuronal and 1 hybrid promoters, into pGL3-basic vector from Promega (Madison, WI, USA). The 3 viral promoters are the 657 bp CMV promoter from pRc/CMV2 (Invitrogen, Carlsbad, CA, USA), the 572 bp Rous sarcoma virus long terminal repeat promoter (RSV) from pREP4 (Invitrogen), and the 203 bp Simian virus 40 promoter (SV40) from pGL3-Control Vector (Promega). The 2 neuronal promoters are the 1.48 kb human PDGF promoter from psubPDGF-EGFP, kindly provided by Prof. H Büeler (University of Zurich, Switzerland), and the 495 bp human SYN promoter from

pSYN1 kindly provided by Dr. S. Kügler (University of Göttingen, Germany). The 1.74 kb hybrid CAG promoter (CMV enhancer fused to chicken β-action promoter) was from pCAGLuc kindly provided by Dr. Yoshiharu Matsuura (National Institute of Infection Disease, Japan). Except the PDGF promoter, which was PCR amplified and inserted between *Sac*I and *Hind*III of pGL-3 basic vector, all the other five promoters including CMV, RSV, SV40, SYN and CAG were PCR cloned between *Kpn*I and *Hind*III sites.

Construction of PDGF-promoter containing vectors for transcriptional amplification

Plasmids pFR-luc, pCMV-AD and pCMV-BD were purchased from Stratagene (La Jolla, CA, USA). To construct pPDGF-GAL4p65, a plasmid vector was generated first by inserting the PCR product of the transcriptional activation domain of the mouse NFκB p65 gene (amino acids 364-549 [24] amplified from pCMV-AD into pCMV-BD that contains a CMV promoter and the yeast GAL4 DNA binding domain at *Bam*HI and *Xba*I sites. By replacing the CMV promoter in this vector with a PDGF PCR fragment promoter amplified from psubPDGF-EGFP, pPDGF-GAL4p65 was obtained.

To construct pGBS-PDGF-Luc, the PDGF promoter was amplified from psubPDGF-EGFP and inserted into pGL3-basic vector between *Sac*I and *Hind*III sites. Five tandem GAL4 DNA binding sites (17 bp each) in pFR-Luc were then PCR amplified and inserted upstream of the PDGF promoter between *Kpn*I and *Sac*I sites.

The transfer vector pFastBac1 from Invitrogen was used for generation of baculoviral vectors. To construct BV-1xPDGF-Luc, a fragment containing the 5×GAL4 DNA binding sites, the PDGF promoter, the luciferase gene and the SV40 polyA signal was excised from pGL3-GBS-PDGF-Luc with *Kpn*I/*Bam*HI and placed into pFastBac1 cut with the same restriction enzymes. To construct BV-2xPDGF-Luc, a 1.7 kb fragment containing the luciferase gene was first excised from pFB-CMV E-PDGF-Luc [23] with *Xho*I/*Hind*III and directionally cloned into pFastBac1. Fragments containing the 5×GAL4 DNA binding sites/the PDGF promoter and the PDGF promoter/GAL4p65 were then PCR amplified from pGBS-PDGF-Luc and pPDGF-GAL4p65, respectively, and inserted into the *Not*I/*Xho*I and *Bss*HII/*Sal*I sites of pFastBac1.

Construction of SYN-promoter containing vectors for transcriptional amplification

This group of vectors was generated based on the recently modified lentiviral shuttle vector pTYF-Sw-linker [30]. The plasmid pTYF-Sw-linker incorporates two restriction sites for I-*Sce*I, a site-specific homing endonuclease, to flank multiple cloning sites in both adenoviral shuttle vector and lentiviral backbones. Thus, the whole expression cassette can be directly swapped between adenoviral vectors and lentiviral vectors.

To construct pGBS-SYN-EGFP, a 1.56 kb I-*Sce*I fragment from pXcX-GBS-SYN-EGFP (previously prepared, unpublished) was inserted into the I-*Sce*I sites of pTYF-Sw-linker. This I-*Sce*I fragment contains 5×GAL4 DNA binding sites, the SYN promoter and the EGFP gene. Three cloning steps were necessary to generate pXcX-GBS-SYN-EGFP. First, the SYN promoter fragment was excised from pSYN1 with *Mlu*I/*Xha*I, ligated into *Mlu*I/*Hind*III digested pXcX-Sw-linker (the redesigned adenoviral shuttle vector, [30]) via a synthetic linker with restriction overhangs for *Xba*I and *Hind*III. Then, the 5×GAL4 binding sites were PCR amplified from the pFR-luc and cloned into the *Nhe*I/*Mlu*I sites upstream of the SYN promoter. Finally, an EGFP PCR fragment, amplified from pEGFP-C1 (Clontech, Palo Alto, CA, USA) was inserted into *Spe*I/*Not*I sites downstream to the SYN promoter.

To construct pCMV-GAL4p65, a plasmid vector was generated first by inserting the PCR product of the transcriptional activation domain of the mouse NFκB p65 gene amplified from pCMV-AD into pCMV-BD. Then a PCR fragment containing the CMV promoter, GAL4p65 and SV40 polyA signal was amplified from the vector and inserted into *Mlu*I/*Spe*I digested pTYF-Sw-linker.

To construct pSYN-GAL4p65, we first generated a plasmid by inserting the PCR product of the transcriptional activation domain of the mouse NFκB p65 gene amplified from pCMV-AD into pCMV-BD. The CMV promoter in this vector was then replaced with the SYN promoter PCR fragment between *Ase*I/*Nhe*I sites. The plasmid was digested with *Ase*I, filled in with Klenow enzyme, followed by *Mlu*I digestion. The fragment encoding the SYN promoter, GAL4p65 and the SV40 polyA signal was then isolated and introduced into *Eco*RV/*Mlu*I digested pTYF-SW-linker.

pGBS-SYN-EGFP was used to generate the LV-1xSYN-EGFP viral vector. To generate LV-2xSYN-EGFP shuttle vector pTYF-2xSYN-EGFP, pXcX-GBS-SYN-EGFP was first digested by *Nhe*I, filled in with Klenow enzyme, followed by *Not*I digestion. A blunt end-*Not*I fragment encoding 5×GAL4 binding sites, SYN promoter and EGFP was then isolated and cloned into *Mlu*I/blunt/*Not*I treated pSYN-GAL4p65. Similarly, LV-CMV/SYN-EGFP shuttle vector pTYF-CMV/SYN-EGFP was constructed by introducing the *Nhe*I/blunt/*Not*I fragment of the 5×GAL4 DNA binding sites, the SYN promoter and the EGFP gene into the plasmid pCMV-GAL4p65 previously treated with *Mlu*I/blunt/*Not*I.

Production of viral vectors

To construct recombinant BV vectors, pFastBac1 plasmid (Invitrogen) was used. BV vectors containing PDGF-promoter-based constructs were produced and propagated in Sf9 insect cells according to the manual of the Bac-to-Bac baculovirus expression system (Invitrogen). Budded viruses were collected with the insect cell culture medium, filtered through a 0.45-μm pore size filter (Millipore, Bedford, MA, USA) to remove contaminating particles, and concentrated by ultracentrifugation at 28,000g for 60 min. Viral pellets were re-suspended in appropriate volumes of 0.1 M phosphate-buffered saline (PBS) and their infectious titers (plaque-forming units, pfu) were determined by plaque assay on Sf9 cells.

The LV system used in this study is derived from HIV-1 and pseudotyped with vesicular stomatitis virus coat. LV stocks were produced by transient cotransfection of the shuttle plasmids, the packaging vector pNHP and the envelope plasmid pHEF-VSVG in HEK293FT cells. Viral concentration and titration were carried out as described earlier [41].

Gene transfer in cultured cells

In vitro experiments were carried out in non-neuronal cells, including African green monkey kidney cells COS-7, Chinese hamster ovary cell line CHO, human glioma cell lines U251 and T98G, and neuronal cell lines, including rat pheochromocytoma PC12 cells and C17.2 neonatal mouse cerebellum stem cells. Cos-7, CHO, U251, T98G, undifferentiated C17.2 cells were grown

in Dulbecco's modified Eagle's medium (DMEM) supplemented at 37°C with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO_2. Undifferentiated PC12 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 5% horse serum. Neuronal differentiation of C17.2 was carried out by plating the cell onto poly-L-lysine (50 mg/ml)/laminin (20 mg/ml) coated 24-well plates at a density of 5×10^4 per well in serum-free DMEM/F12 (Gibco BRL, MD, USA) with 1% N2 supplements (Gibco). After 2 days, cultures with over 90% of cells with neuronal phenotype were used for transfection experiments. For the induction of PC12 cells to neurons, the cells were plated in 24-well plates coated with 0.2 mg/ml rat-tail collagen at a density of 5×10^4 per well. The medium was replenished every 2 to 3 days for 7-10 days. More than 90% of the cells were induced to neuronal phenotype and subsequently used for cell transfection.

For *in vitro* transfection of plasmid vectors containing the luciferase reporter gene, polycation/DNA complexes were used. Plasmid DNA was diluted in 5% glucose and PEI25k (Sigma-Aldrich, San Diego, CA, USA) was prepared as an aqueous stock solution containing 10 mM nitrogen. Complexes were formed at the ratio of 10 equivalents of PEI nitrogen per DNA phosphate by adding the appropriate amount of PEI solution into the DNA solution, briefly mixing by vortexing and incubating for 30 min at room temperature. Ten μl of the PEI/DNA complexes containing 0.5 μg plasmid DNA was used to transfect cells in 100 μl of Opti-MEM (Invitrogen) for 3 h at 37°C. The medium was then replaced with fresh growth medium and the cells were further incubated at 37°C for 24 h before being collected for luciferase activity assay. For *in vitro* transfection of plasmid vectors containing the EGFP reporter gene, the Superfect transfection reagent (Quiagen, Hilden, Germany) was used according to the manufacturer's instructions. Cells were treated for 3 h and the transfection medium was then replaced by fresh growth medium. The cells were further incubated at 37°C for 48 h before being analyzed for EGFP expression. For BV infection of cultured cells, appropriate amounts of BV vectors were added in 100 μl of serum free DMEM, and incubated with the cells at 37°C for 1 h. After the incubation, the serum free DMEM containing the viruses was replaced by fresh growth medium, and the cells were collected for luciferase activity assay 1 day after infection.

In vivo gene transfer in the rat brain

For striatum injection, male Wistar rats (~250–300 g) were anaesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg of body weight). Five µl of PEI25k/DNA complexes or BV vectors was then injected stereotaxically at two sites (AP ±1.0mm, ML +2.5mm, and DV -5.0mm from Bregma and the dura) using a 10 µl Hamilton syringe connected with a 30-gauge needle, at a speed of 0.5 µl/min. The needle remained in place for another 5 min before being slowly retracted. For injections into the hippocampus, 1 µl of baculoviruses was injected into a site 4.4 mm posterior to bregma, 3.2 mm lateral to the central suture and 2.5 mm ventral to the dura. Two days after injection, rats were terminally anaesthetized (sodium pentobarbital, 100 mg kg^{-1}, i.m.). Some of the anesthetized rats were perfused intracardially with 4% formaldehyde in 0.1 M PBS (pH 7.4). In the handling and care of animals in this part of the study, *the Guidelines on the Care and Use of Animals for Scientific Purposes* issued by National Advisory Committee for Laboratory Animal Research, Singapore was followed. The experimental protocol was approved by Institutional Animal Care and Use Committee (IACUC), Nationals University of Singapore and Biological Resource Center, the Agency for Science, Technology and Research (A* STAR), Singapore.

For injections into the hypoglossal motor nucleus, male Wistar rats (~250-300 g) were anaesthetized using an intramuscular injection of a combination of medetomidine (250 µg/kg) and ketamine (60 mg/kg). A total of six bilateral microinjections of LV at a dose of 1.2 x 10^6 transducing units (TU) per rat were used. Injections were made at the level of calamus scriptorius and 400 µm rostral and caudal to it, 300–500 µm from the midline and 450-550 µm ventral to the dorsal surface of the medulla, as described previously [30]. Seven days after injection, rats were terminally anaesthetized (sodium pentobarbital, 100 mg kg^{-1}, i.p.) and perfused intracardially with 4% formaldehyde in 0.1 M PBS, pH 7.4. All procedures in this part of the study were carried out according to the Home Office Animals Scientific Procedures Act 1986, UK.

Luciferase assay

Cultured cells were washed and permeabilized with 100 µl of reporter cell lysis buffer (Promega). Brain tissue samples were homogenized in PBS (100 µl PBS per 50 mg tissue) by sonication for 10 sec on ice. Homogenized tissues were then centrifuged at 13,000 rpm for 10 min at 4°C. Ten µl of cell extract or supernatant of homogenized tissues was used for luciferase assay with a luciferase assay kit (Promega) in a single-tube luminometer (Berthold Lumat LB 9507, Bad Wildbad, Germany) for 10 sec. The total protein concentration of each sample was determined using the DC Protein Assay (Bio-Rad, Hercules, CA, USA).

Immunofluorescence analysis

For analysis of immunofluorescence, the brains of transfected animals were removed and post-fixed in 4% formaldehyde in 0.1 M PBS, pH 7.4 for 2-4 h. Cryostat sections were cut at 20 or 30 µm thickness for free-floating immune staining. A polyclonal anti-luciferase (Promega, dilution 1:150), a monoclonal anti-GFAP protein (Chemicon International, USA; dilution 1:150) or a monoclonal anti-neuron-specific nuclear protein (NeuN; Chemicon International, dilution 1:150) were used as primary antibodies. Anti-rabbit IgG TRITC conjugate (Sigma–Aldrich, dilution 1:500) and anti-mouse IgG FITC conjugate (Sigma–Aldrich, dilution 1:500) were used as secondary antibodies. Mounted sections were analyzed using a laser scanning confocal microscope.

Statistical Analysis

Paired and unpaired t-test was applied, as appropriate. The differences were considered significant at $p<0.05$. All values in the text and figures refer to mean ± SD.

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Figure legends

Figure 1. Comparison of promoter strength in neuronal cells and the rat brain *in vivo*. The levels of luciferase gene expression from neuronal PDGF and SYN promoters, viral gene regulatory elements, including CMV, SV40, and RSV promoters, and the hybrid CAG promoter in C17.2 (A), PC12 (B) neuronal cell lines. Plasmid vectors were compacted with PEI25k. For transfection, 0.5 μg of DNA was used per well in 24-well plates. Luciferase activity assays were preformed one day after cell transfection. The results are expressed in relative light units (RLU) per milligram of total cell protein for the cultured neurons. In this and the following figures the error bars represent standard deviation.

Figure 2. GAL4p65 augments gene expression from neuronal promoters *in vitro*.
(A) Dose-dependent effects of GAL4p65 on the luciferase expression from the PDGF promoter in C17.2 and PC12 neuronal cell lines. The indicated amount of pPDGF-GAL4p65 was co-transfected with 0.5 μg of pGBS-PDGF-Luc per well in a 24-well plate using PEI25k. Luciferase activity assays were preformed one day after transfection in quadruplicate. The results are expressed in total RLU per mg protein.
(B) Effect of GAL4p65 on the EGFP expression from the SYN promoter in PC12 cells. The cells were transfected with 0.5 μg pGBS-SYN-EGFP, either alone or together with the equal quantity of pSYN-GAL4p65 or pCMV-GAL4p65 using Superfect transfection reagent. EGFP positive cells were counted 2 days posttransfection in triplicate using 100 × magnification and six fields per well selected randomly. The average values from a representative experiment out of 3 repeated experiments are shown.

Figure 3. Plasmid- and baculoviral vector-mediated GAL4p65 expression augments gene expression from the PDGF neuronal promoter in the rat brain *in vivo*.
(A) Plasmid-mediated transfection. pGBS-PDGF-Luc (1 μg), either alone or together with pPDGF-GAL4p65 (1 μg), was compacted with PEI25k and injected into the rat striatum. Four rats were used for each group. Two brain regions were collected 2 days after injection for luciferase activity assay. Values are expressed as RLU per region. ** $p<0.01$ and *** $p<0.001$, compared with pGBS-PDGF-Luc.
(B) Schematic diagram of the expression cassette of a baculoviral vector with both GAL4p65 and GAL4 DNA binding sites. GAL4 BD: GAL4 DNA-binding domain. GAL4 BS: GAL4 DNA binding sequence.
(C) Baculovirus-mediated transduction. Viral particles were injected into the rat striatum. Four rats were used per group. Two brain regions were collected 2 days after injection for luciferase activity assay. * $p<0.05$ and *** $p<0.001$, compared with BV-1xPDGF-Luc.

Figure 4. Lentivirus-mediated GAL4p65 expression augments gene expression from neuronal promoters in the rat brain in vivo.

(A) Schematic diagram of the expression cassettes of two lentiviral vectors with both GAL4p65 and GAL4 DNA binding sites. LTR: Long terminal repeat. SYN: Human synapsin I promoter. CMV: CMV enhancer and promoter. GAL4 BD: GAL4 DNA-binding domain. GAL4 BS: GAL4 DNA binding sequence.

(B) EGFP expression in the rat hypoglossal motor nucleus 7 days after injection of LV-1xSYN-EGFP, LV-2xSYN-EGFP or LV-CMV/SYN-EGFP at the dose of 1.2 x 10^6 TU per rat. Images are the average intensity projection confocal stacks of ~ 20 μm.

(C) Quantification of EGFP positive cells. Three rats were used per group. For each rat, four sections surrounding the injection tract and three fields in each section were selected randomly for cell counting.

(D) EGFP expression in the rat hypoglossal motor nucleus 4 weeks after injection of LV-2xSYN-EGFP at the dose of 1.2 x 10^6 TU per rat. The same section was also immunohistochemically stained with antibodies against GFAP. Images are the average intensity projection confocal stacks of ~ 20 μm. Note no signs of degraded neurons replaced by glia, indicating that the transgene is not detrimental for the cells.

Figure 5. GAL4p65 enhances the activity of the PDGF promoter in a neuron-specific manner.

(A) Plasmid-mediated transfection. pGBS-PDGF-Luc (0.2 μg) either alone or together with pPDGF-GAL4p65 (0.2 μg) was compacted with PEI25k and used for transfection in neuronal PC12 and C17.2 and non-neuronal COS-7 and CHO cell lines in 48-well plates. Luciferase activity assays were preformed one day after transfection in quadruplicate. The results are expressed in RLU per milligram of total cell protein. *** $p<0.001$, compared with pGBS-PDGF-Luc.

(B) Baculovirus-mediated transduction. Neuronal PC12 and C17.2 cells and glioma U251 and T98G cells were infected in quadruplicate with either BV-1xPDGF-Luc or BV-2xPDGF-Luc at an MOI of 200. Luciferase activity assays were preformed one day after transfection. The results are expressed in RLU per milligram of total cell protein. * $p<0.05$ and *** $p<0.001$, compared with BV-1xPDGF-Luc.

Figure 6. Neuronal specificity as demonstrated by immunohistochemical analysis of rat brains. BV-2xPDGF-Luc (1 x 10^9 pfu) was injected in the rat hippocampus and LV-x2SYN-EGFP (1.2 x 10^6 TU) into the rat hypoglossal motor nucleus. Tissues were collected 2 days after baculovirus and 7 days after lentivirus injection. Frozen transverse sections were cut and used for Luc, NeuN and GFAP immunostaining.

Table 1: Vectors used in the current study.

Name	Vector Backbone	Promoter	Transgene Product
Vectors for promoter strength comparison			
pGL3-CMV-Luc	pGL3 plasmid	CMV	Luciferase
pGL3-SV40-Luc	pGL3 plasmid	SV40	Luciferase
pGL3-RSV-Luc	pGL3 plasmid	RSV	Luciferase
pGL3-CAG-Luc	pGL3 plasmid	CAG	Luciferase
pGL3-PDGF-Luc	pGL3 plasmid	PDGF	Luciferase
pGL3-SYN-Luc	pGL3 plasmid	SYN	Luciferase
PDGF-promoter containing vectors			
pPDGF-GAL4p65	pCMV-BD plasmid	PDGF	GAL4p65
pGBS-PDGF-Luc	pGL3 plasmid	PDGF with GAL4 binding sites	Luciferase
BV-1xPDGF-Luc	Baculovirus	PDGF with GAL4 binding sites	Luciferase
BV-2xPDGF-Luc	Baculovirus	PDGF & PDGF with GAL4 binding sites	GAL4p65 Luciferase
SYN-promoter containing vectors			
pSYN-GAL4p65	pTYF plasmid	SYN	GAL4p65
pGBS-SYN-EGFP	pTYF plasmid	SYN with GAL4 binding sites	EGFP
LV-1xSYN-EGFP	Lentivirus	SYN with GAL4 binding sites	EGFP
LV-2xSYN-EGFP	Lentivirus	SYN & SYN with GAL4 binding sites	GAL4p65 EGFP
CMV-containing vectors used for comparison			
pCMV-GAL4p65	pTYF plasmid	CMV	GAL4p65
LV-CMV/SYN-EGFP	Lentivirus	CMV & SYN with GAL4 binding sites	GAL4p65 EGFP

Figure
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Fig. 1

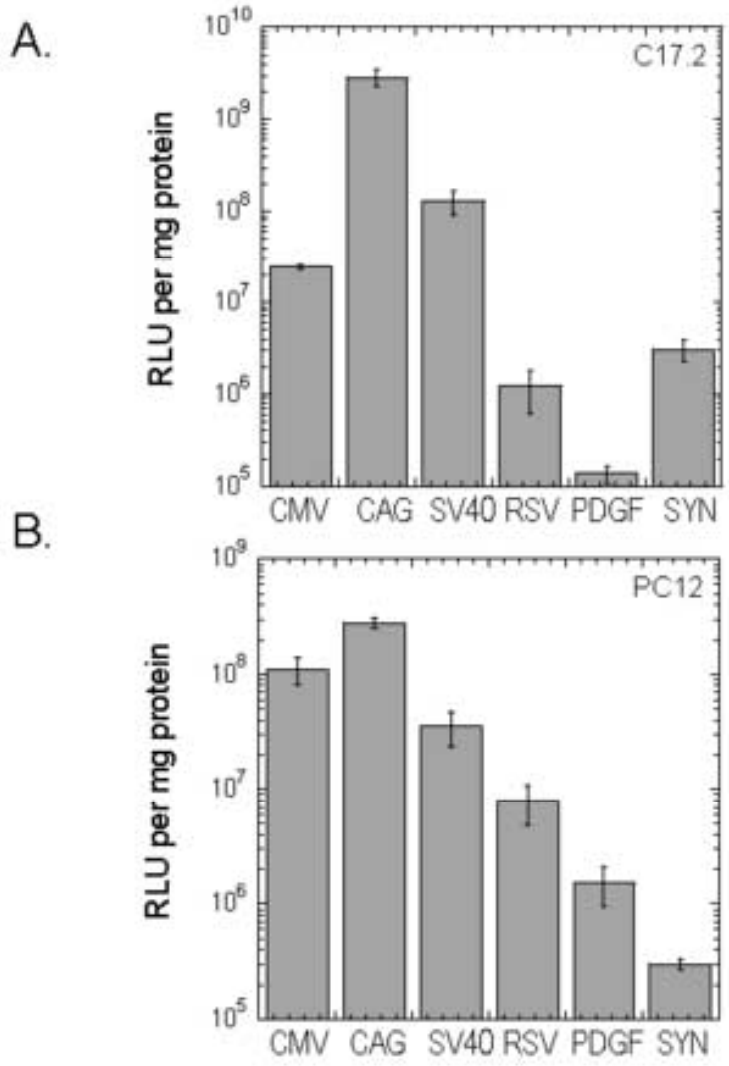

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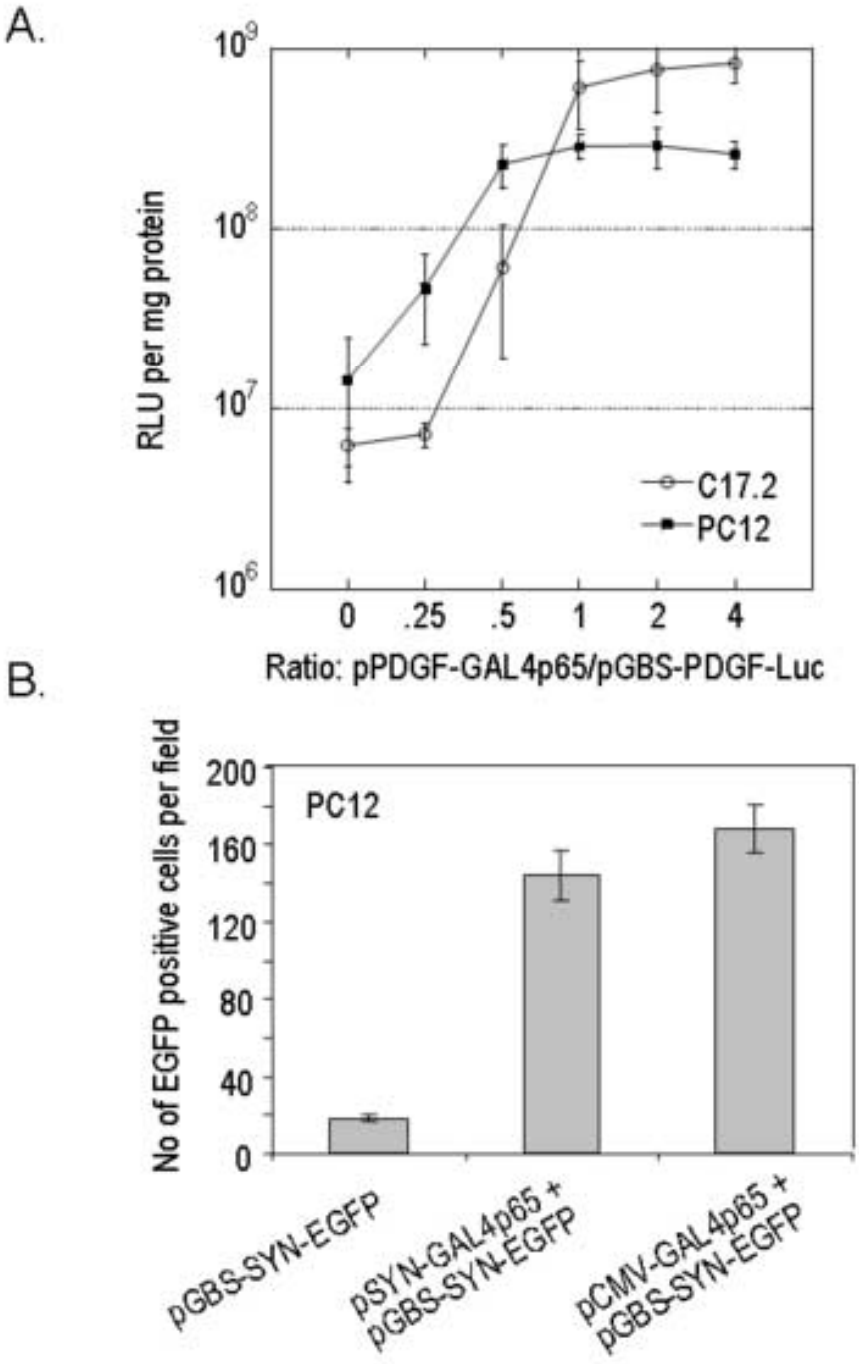
Fig. 2



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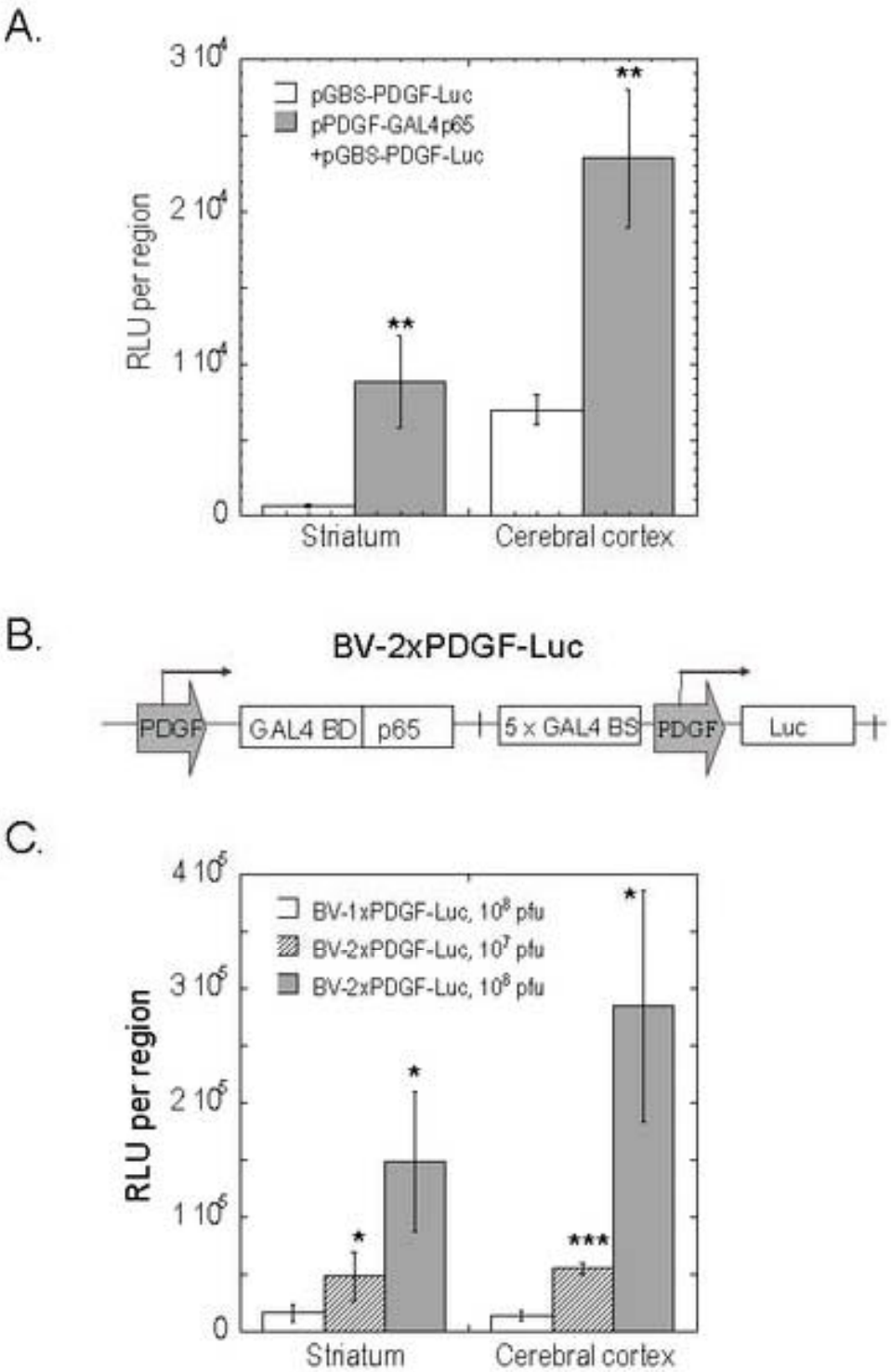

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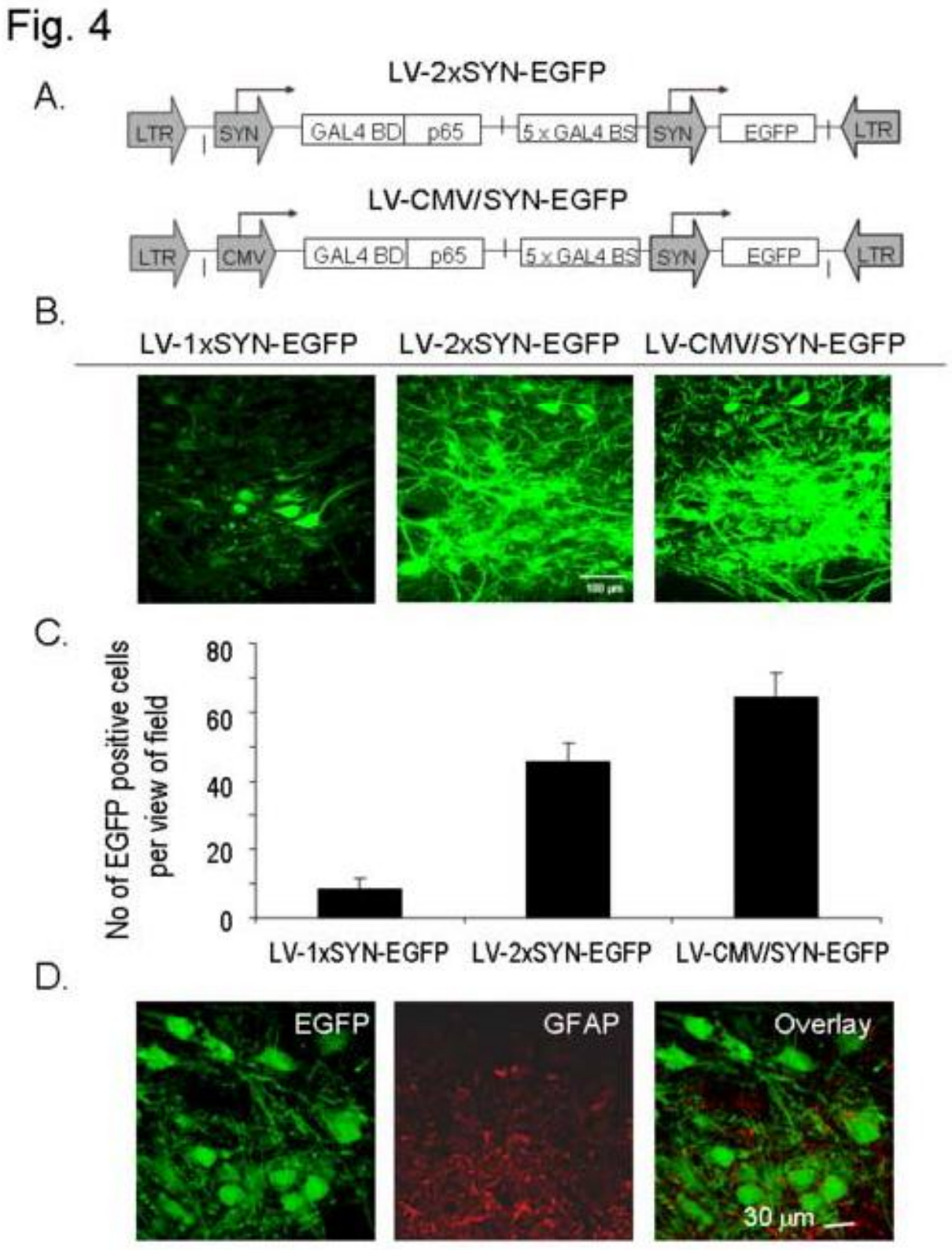

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Fig. 5

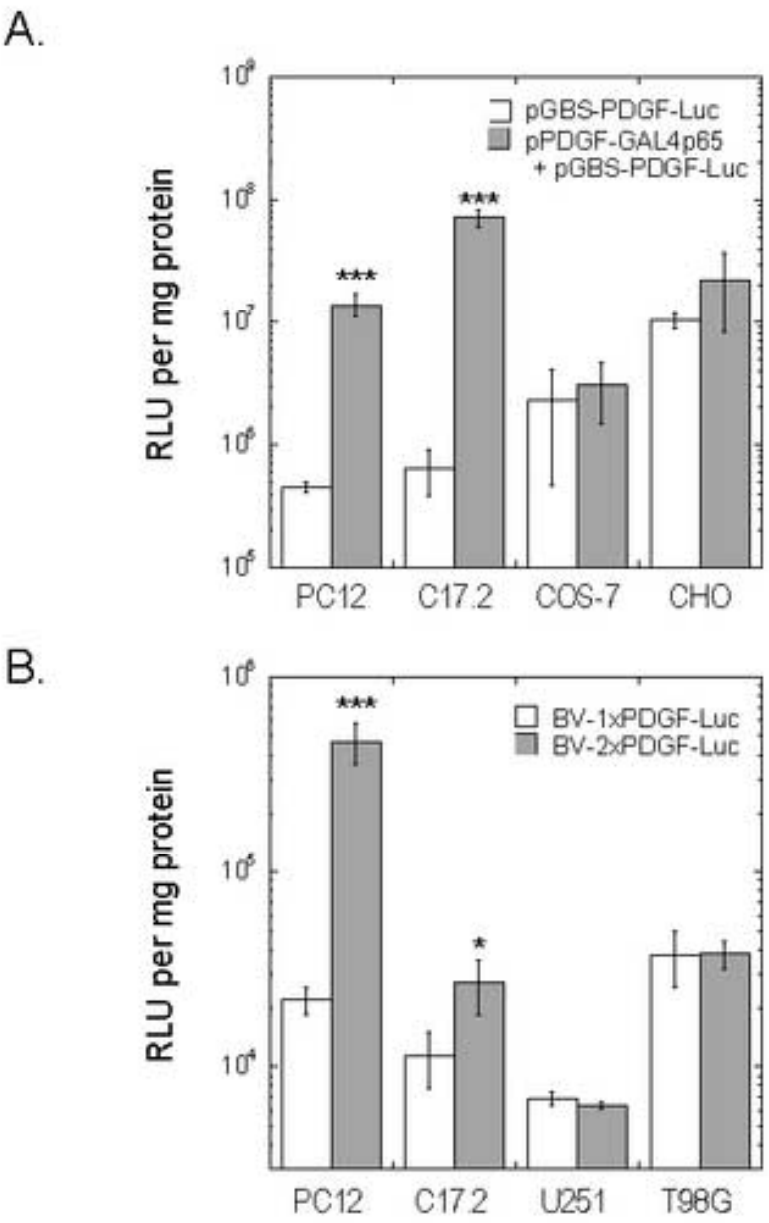

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Fig. 6