

Noninvasive Monitoring of Long-Term Lentiviral Vector-Mediated Gene Expression in Rodent Brain with Bioluminescence Imaging

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Gene transfer into the central nervous system is an emerging therapeutic strategy for a range of neurological diseases, including neurodegeneration. This approach would benefit from imaging technologies that could determine the extent, magnitude, and duration of transgene expression. We have used bioluminescence imaging (BLI) to image lentiviral vector-mediated gene transfer into the mouse brain. We constructed human immunodeficiency virus type 1 lentiviral vectors that encode firefly luciferase and transduce cells in culture. After stereotactic injection of these vectors into the brain, we were able to detect luciferase expression in living mice and rats. We characterized the signal in mouse brain in terms of localization, kinetics, resolution, and reproducibility and demonstrated that it correlates with the level of firefly luciferase expression. Although the signal decreased gradually to about 20% of the initial value in the first month, the signal remained constant thereafter for more than 10 months. We demonstrated that the light signal can be used as a reporter by using a bicistronic vector. This is the first study to document noninvasive monitoring of long-term transgene expression in the adult mouse brain and provides the basis for applying BLI in the study of brain disease and gene therapeutic strategies.

Key Words: bioluminescence imaging, lentiviral vector, gene expression, luciferase, optical imaging, mouse, rat

INTRODUCTION

Noninvasive molecular imaging plays an increasing role in biomedical research (for a review see [1]). It allows one to follow an animal over time and to study molecular processes within a relevant milieu. Bioluminescence imaging (BLI) uses the production of visible light photons during the enzymatic oxidation of luciferin by luciferase [2]. When luciferase is expressed in an animal the tissue will absorb and scatter the photons. A fraction of these photons will leave the animal and can be detected externally by a high-sensitivity CCD-camera. BLI is a relatively low-cost, highly sensitive, high-throughput, and quantitative technique and is therefore used in oncology, cardiology, vectorology, infectiology and in transgenic animals [3]. For neurological research BLI has

been used to track *ex vivo*-transduced and implanted neuronal progenitor cells [4,5], to monitor the growth of implanted [6–8] and spontaneous [9,10] brain tumors, to determine gene expression after adenoviral vector injection in orthotopic brain tumors [11], and to monitor treatment of herpes simplex encephalitis [12]. The gene transfer studies into intracranially implanted tumors monitored gene expression for only 17 days [11]. Yoshimitsu *et al.* [13] used BLI to evaluate gene expression after intravenous injection of lentiviral vectors encoding luciferase in mouse neonates. Prior to our study, BLI had not been used to detect and follow-up viral vector-mediated gene transfer into the adult mouse brain. Lentiviral vectors (LV) transduce both dividing and nondividing cells and ensure stable and long-term trans-

gene expression because they integrate into the host genome [14]. Stereotactic injection of LV into the brain of mice, rats, or primates generates locoregional transgenic animals that are not affected by developmental plasticity. The contralateral side of the brain serves as an internal control. We and others have used this technology to create models of neurodegenerative diseases such as Parkinson disease [15], allowing functional genomic studies and evaluation of therapeutic strategies [16]. LV have great therapeutic potential for CNS disease since a wide range of neuronal and glial cell types are stably transduced, expression is long term, and inflammatory and immune responses are mild [17]. Preclinical therapeutic efficacy was achieved after local injection [18], after systemic delivery [19], or by retrograde axonal transport after peripheral injection [20]. LV have been engineered to encode short hairpins as precursors of siRNA to silence gene expression by RNA interference [21]. Promising therapeutic results have been obtained for diseases caused by “toxic gain-of-function” mutations such as amyotrophic lateral sclerosis [22,23]. The use of viral vector-mediated gene transfer into the brain is confined by the interindividual variation of gene expression and the lack of noninvasive assessment of the location, magnitude, and duration of transgene expression.

We have previously shown that LV transduction in mouse brain results in stable transduction of neurons and astrocytes for up to 1 year [24]. We have optimized lentiviral vector constructs and production methods for brain applications [25]. Here, we evaluate the feasibility of BLI monitoring of LV-mediated gene transfer in the mouse brain. We constructed LV encoding firefly luciferase and characterized the BLI signal after stereotactic injection in the mouse brain in terms of kinetics, reproducibility, correlation with gene expression, and long-term persistence. With a LV encoding green fluorescent protein linked to firefly luciferase by an internal ribosome entry site, we show that BLI can report gene expression in the brain. Finally, we provide proof-of-principle data for BLI in the adult rat brain.

RESULTS AND DISCUSSION

BLI Allows Quantification of Luciferase Expression in LV-Transduced Cells

We created a lentiviral vector construct encoding firefly luciferase (LV-Fluc) as well as a construct encoding eGFP (enhanced green fluorescent protein) and firefly luciferase separated by the internal ribosome entry site (IRES) of the encephalomyocarditis virus (EMCV) (LV-eGFP-I-Fluc) (Fig. 1). We confirmed the expression of firefly luciferase in 293T cells transduced with LV-Fluc or LV-eGFP-I-Fluc by Western blotting (Fig. 2A). We measured luciferase activity in cell culture after lentiviral vector transduction using BLI (Fig. 2B) and luminometry. We first validated BLI by comparison with the luminometry results obtained from

the same transduced cells. Although a near-perfect correlation ($r^2 = 0.9994$) between both data sets was obtained, BLI displayed a higher dynamic range in the lower values (Fig. 2C). Luciferase activity measured with BLI in intact cells was ~300-fold lower than that in cell lysates with a near-perfect correlation ($r^2 = 0.9989$) (Fig. 2D). We then measured luciferase activity after transduction of SHSY5Y, PC12, N2a, and GI261 cells with serial dilutions of LV-Fluc or LV-eGFP-I-Fluc and compared these to 293T cells. The following results were obtained: (i) mean luciferase activities were 48, 20, 11, and 5%, respectively, of the activity in 293T cells for LV-Fluc (Fig. 2F) and similar values were obtained for LV-eGFP-I-Fluc (data not shown); (ii) in these cell lines, the mean luciferase activity of the LV-eGFP-I-Fluc vector was 5.2 ± 2.0 -fold lower than that of LV-Fluc (range 2.3–7.6) (Fig. 2G and data not shown); and (iii) a very good correlation ($r^2 = 0.99 \pm 0.02$) between luciferase activity (measured by BLI) and eGFP fluorescence (measured by FACS) was obtained for 293T, N2a, and GI261 cells transduced with LV-eGFP-I-Fluc (Fig. 2E and data not shown). Thus, our lentiviral vectors encoding firefly luciferase are able to transduce various cell types, including relevant neuroblastoma, pheochromocytoma, and glioma cell lines. These *in vitro* data validate our lentiviral vectors and support the use of the IVIS system to detect and quantify luciferase activity.

BLI of Luciferase Expression in LV-Transduced Mouse Muscle and Brain

Next, we injected 2 μ l of LV-Fluc (17 ng p24) into the gastrocnemius muscle of three NMRI mice. Seven days after injection we imaged the mice in lateral decubitus after ip injection of luciferin. The detected signal (photon flux: $3.6 \times 10^5 \pm 1.3 \times 10^5$ p/s) was significantly higher than in four uninjected control mice ($5.3 \times 10^4 \pm 3.4 \times 10^3$ p/s, $P < 0.05$). The signal measured 21 days after injection was higher ($2.3 \times 10^6 \pm 2.3 \times 10^6$ p/s), but this increase was not significant ($P > 0.05$) (Fig. 3A). *In vivo* detection of luciferase activity in muscle is in accordance with Wu *et al.* [26] who imaged luciferase expression after transduction with adenoviral vectors.

When we injected 2 μ l of LV-Fluc (17 ng p24) in the right striatum of four NMRI mice, we detected a signal projecting from the right side of the skull after ip injection of luciferin from day 5 onward. The signal was significantly higher than that observed in four control mice injected with LV-eGFP ($1.8 \times 10^6 \pm 7.2 \times 10^5$ vs $1.4 \times 10^5 \pm 8.2 \times 10^4$ p/s, $P < 0.05$). In these control mice the signal was predominantly located in the snout (Fig. 3B). Coronal sections of the brain of mice that were injected with luciferin and subsequently sacrificed displayed a BLI signal in the right striatum as expected (Fig. 3C), demonstrating that the photons are produced by endogenous cells within the brain. This conclusion was further corroborated by the detection of expression of luciferase protein by immunohisto-

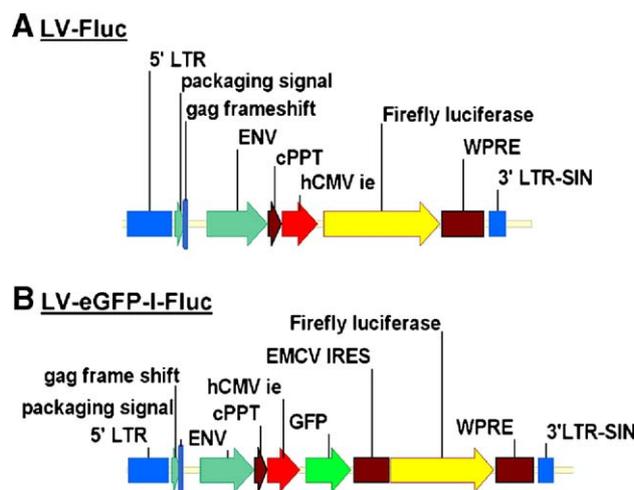


FIG. 1. Lentiviral vector constructs. (A) The LV-Fluc transfer plasmid with the firefly luciferase (Fluc) cDNA under control of the human cytomegalovirus immediate early promoter (hCMVie) followed by the woodchuck hepatitis posttranscriptional regulatory element (WPRE). The promoter is preceded by the polypurine tract (cPPT) and these elements are flanked by the 5' LTR and 3' SIN LTR. (B) In the LV-eGFP-I-Fluc plasmid the same promoter is controlling expression of enhanced green fluorescent protein (eGFP) and Fluc through an IRES derived from EMCV.

chemistry, revealing luciferase-positive cells in the right striatum (Fig. 3D).

Characterization of the BLI Signal after Stereotactic Injection of LV-Fluc into the Mouse Striatum

To study the influence of the fur on imaging, we imaged seven mice on day 28 postinjection (LV-Fluc), before and after shaving the skull. The BLI signal in the shaved mice was twofold higher ($P < 0.01$) (Supplementary Fig. S1A), in concordance with previously published data [26]. Henceforth we shaved the skin covering the skull of all mice prior to imaging. We evaluated the kinetics of the BLI signal in four mice 55 days after LV injection. We imaged the mice immediately after ip injection of luciferin with successive 2-min frames for more than 5 h. The signal increased during the first 20 min following luciferin injection, reaching a peak at 31 ± 3 min, and displayed an exponential drop afterward. From 140 min after injection on, the signal decreased below 10% of its maximum (Supplementary Fig. S1B). This pharmacokinetic profile allows three measurements per day and the rapid testing of the influence of experimental variables on luciferase expression. To assess the reproducibility of imaging, we scanned four individual mice on 4 successive days (day 47 to 50 after LV injection) (Supplementary Fig. S1C). The coefficient of variation ranged from 9 to 17% for individual mice and was 12% for the group as a whole (Supplementary Fig. S1D). A similar experiment in 10 different mice 365 days after injection yielded a range from 8 to 38% in individual mice and again 12% for the group (Supplementary Fig. S1E). We consider this reproducibility suffi-

ciently high and stable in time to address biological questions using BLI.

The Localization of the BLI Signal Reflects the Anatomical Site of Injection

To determine whether the resolution of BLI would allow anatomical localization in the mouse brain, we analyzed the relation between the injection site and the localization of the BLI signal on the skull. We injected mice with LV-eGFP-I-Fluc into the left and right striatum (L and R Str), the right olfactory bulb (OB), the right substantia nigra (SN), and the right medial globus pallidus (GP) ($n = 3$). On day 14 after injection, we performed BLI scans showing a focus on the left side of the head in the L Str mice and on the right side in the R Str mice (Fig. 4A). In the OB mice, the focus was located more anterior and medial compared to the striatal injection site, whereas the focus was more posterior in the SN mice. The focus in the GP was located between the R Str and the SN foci. These sites correspond to the expected locations based on the injection coordinates. Using the BLI images, the mice could be unambiguously classified according to injection site, except for the GP mice, which were hard to distinguish from the SN mice. The *in vivo* observations were validated by *ex vivo* BLI of coronal slices (Fig. 4B) and by immunohistochemistry for eGFP (Fig. 4C), which demonstrated specific transduction of the injection site. There were no significant differences in photon flux between the different sites (all $P > 0.3$, multiple two-sided *t* tests on log normalized values) (Supplementary Fig. S2).

We conclude that the spatial resolution of BLI in mouse brain is sufficient to localize the source of photon emission with an error lower than 3 mm. This will allow the use of BLI to study biological phenomena that involve migration of cells from one area to the brain to another, as has been done to study the migration of neuronal precursor cells [4,5].

BLI Allows Quantification of Luciferase Activity *in Vivo*

To confirm that the BLI signal is a good measure for luciferase activity *in vivo* we injected a dilution series of LV-Fluc (18, 6, and 2 ng p24) into the striatum of NMRI mice ($n = 4$ per titer). The signal was significantly higher than background in the groups injected with vector amounting to 18 or 6 ng p24 ($P < 0.01$ and < 0.05 , respectively) and decreased upon vector dilution. At the highest dilution (2 ng p24) the signal was not significantly higher than that from control mice injected with LV-eGFP corresponding to 6 ng p24 (Fig. 5A). We examined luciferase activity in individual mice that were injected with different titers (from 18 to 2 ng p24). We compared the *in vivo* BLI signal with (i) *ex vivo* BLI of 1-mm-thick brain slices and (ii) *in vitro* measurements of luciferase activity on tissue extracts from the striatum of the slice with the highest signal. There was a strong linear correlation between the *in vivo* BLI signal and the

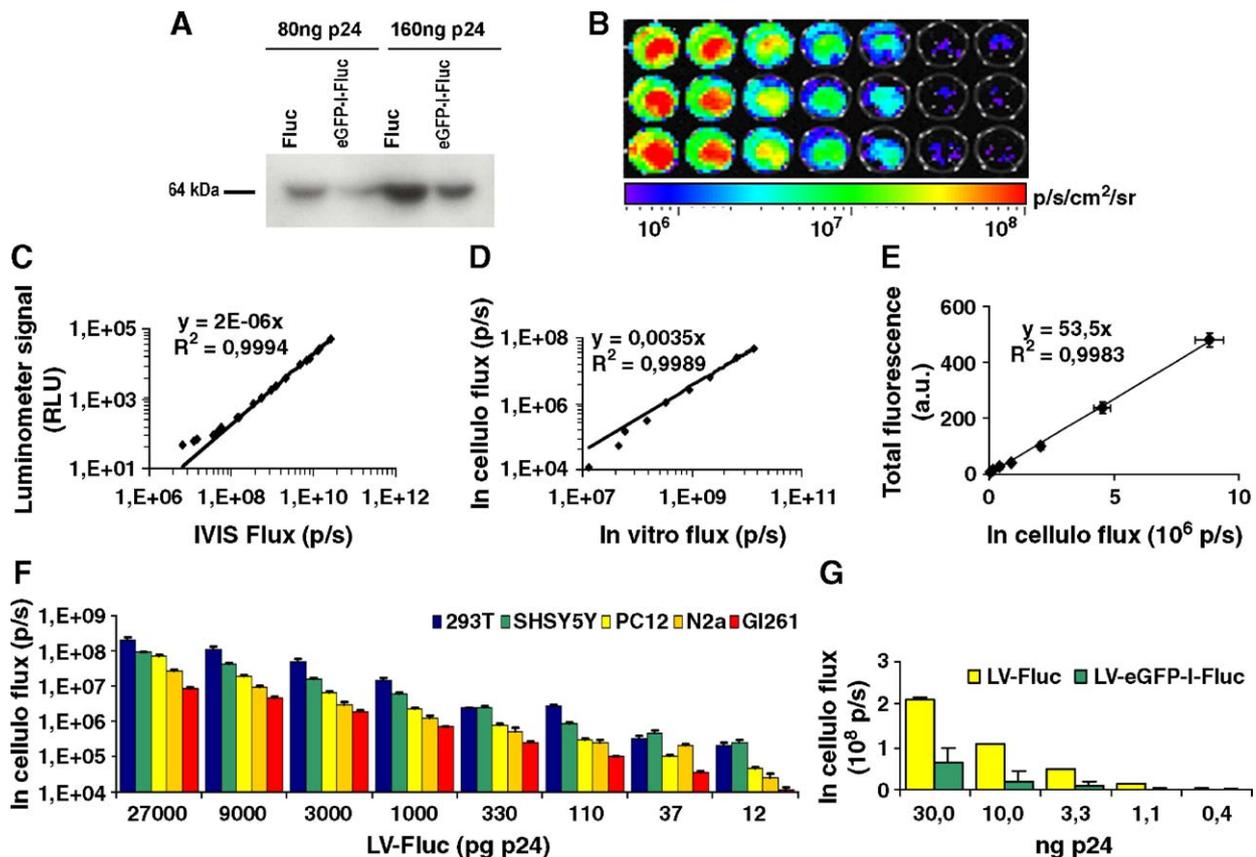


FIG. 2. BLI allows quantification of gene expression in cell culture. (A) Western blot detects firefly luciferase expression in 293T cells transduced with 80 and 160 ng p24 of LV-Fluc and LV-eGFP-I-Fluc. (B) BLI of 293T cells transduced with serial threefold dilutions of LV-Fluc. (C) External calibration of the IVIS signal against a luminometer shows a very good linear correlation. (D) Strong linear correlations between the photon flux measured in living cells and the luciferase activity measured in cell extracts. (E) Strong linear correlation between eGFP fluorescence measured by FACS and luciferase activity in GI261 cells transduced with different amounts of LV-eGFP-I-Fluc. (F) BLI-based quantification of luciferase activity in 293T, SHSY5Y, PC12, N2a, and GI261 cells transduced with serial threefold dilutions of LV-Fluc. (G) Five- to sixfold decrease in luciferase activity in 293T cells transduced with equal amounts of LV-eGFP-I-Fluc compared to LV-Fluc.

maximum pixel of the slices ($r^2 = 0.84$, $P < 0.001$) (Fig. 5B), between the *ex vivo* BLI values and the *in vitro* luciferase activity ($r^2 = 0.89$, $P < 0.001$) (Fig. 5C), and between the *in vivo* BLI signal and the *in vitro* luciferase activity ($r^2 = 0.76$, $P < 0.001$) (Fig. 5D). The fact that the *in vivo* BLI values correlate better with the values from the brain slices than with those from the brain extracts is probably due to the experimental error associated with creating the brain extract. We conclude that the bioluminescence signal as measured in the living mice is a reliable measurement of luciferase gene expression in the brain, in groups as well as in individual mice.

Imaging of Long-Term Lentiviral Vector-Mediated Luciferase Expression in the Mouse Brain

We followed a group of animals injected with LV-Fluc ($n = 10$) and another group with LV-eGFP as control ($n = 4$) for more than 1 year with weekly to monthly BLI scans (Fig. 6). After reaching a maximum at day 8 pi, the BLI signal

decreased continuously during the first 37 days until the value reached about 16% of the peak value. From day 42 onward the signal remained stable for more than 300 days. The signal remained significantly higher (by a factor of ~17) than in control mice that were followed during 150 days ($P < 0.01$ for all time points, unpaired *t* test of log normalized values) and this during the entire experiment. Stable gene expression after lentiviral vector transduction is in accordance with the literature [24,27]. However, most studies thus far relied on invasive methods to establish persistence of gene expression, requiring multiple cohorts of animals sacrificed at different time points. Yoshimitsu *et al.* [13] used BLI to evaluate luciferase expression after intravenous injection of lentiviral vectors encoding luciferase in mouse neonates, transducing the brain among other organs. They also observed a signal that remained constant at 12 and 24 weeks after transduction but did not provide data for other time points. Shai *et al.* [28] observed a relatively stable luciferase expression in mouse salivary glands after LV

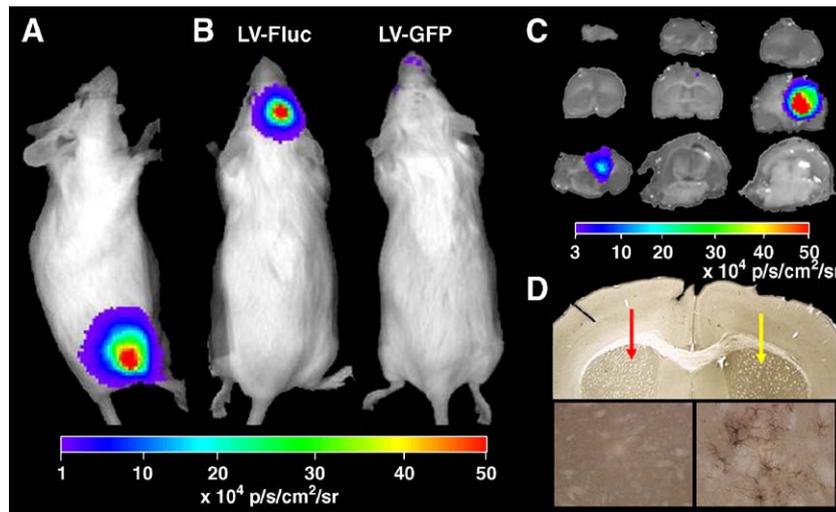


FIG. 3. BLI of luciferase expression in LV-transduced mouse muscle and brain. (A) An intense BLI signal was detected in the right leg of a mouse 21 days after intramuscular injection of 2 μ l LV-Fluc. (B) A BLI signal located on the right side of the skull was detected in a mouse that received a stereotactic injection of 2 μ l LV-Fluc into the right striatum. Note the absence of specific BLI signal in the mouse injected in the striatum with a control vector (LV-eGFP); only some faint signal comes from the nose. (C) BLI of coronal sections of a mouse brain 14 days after LV-Fluc vector injection into the right striatum. (D) Immunohistochemistry confirms the localized expression of firefly luciferase in the right striatum (yellow arrow) without expression on the contralateral side (red arrow) at low magnification ($\times 1.6$). The insets show positively stained individual neurons in the right striatum at high magnification ($\times 40$) that are not found on the contralateral side.

transduction monitored on a weekly basis for 3 months. In many other studies, stable gene expression has been assessed by measuring secreted protein in plasma, which cannot provide data about the local evolution of gene expression. The decrease in signal during the first month warrants further characterization. The decrease could be due to progressive transgene silencing or increased activation of gene expression driven from the hCMV promoter in the early postoperative phase. Although no evidence for gene silencing or gene activation after LV transduction of brain has been reported before, the sensitivity and reproducibility of BLI in individual mice may be required to detect these phenomena. BLI monitoring of kinetics of luciferase expression is therefore probably the method of choice to study transgene silencing in different neuronal cell types and to compare different promoters and insulators in the viral vector constructs. A transient increase in the passage of luciferin through an injured blood–brain barrier (BBB) after stereotactic injection is unlikely since it has been demonstrated that luciferin does not penetrate an intact blood–brain

barrier, albeit at lower levels than in other tissues [29]. Since luciferin is administered at a high dose, its concentration in the brain will likely be saturating the limiting amount of luciferase enzyme present. Studies using luciferase-transgenic animals [30] or after gene delivery without BBB disruption [13] have also demonstrated the feasibility of imaging luciferase expression with an intact BBB.

The BLI Signal Reports Expression of a Transgene of Interest

The usefulness of BLI monitoring of gene expression in brain would increase considerably if the signal could be used as an indirect measurement of expression of a transgene of interest. To verify whether BLI can report the expression of a gene of interest, we studied the relationship between the BLI signal and eGFP expression in mice injected with various amounts of LV-eGFP-I-Fluc (180 and 45 ng p24, $n = 3$). We compared the *in vivo* BLI signal with the eGFP-transduced volume as determined by immunohistochemistry and stereological counting

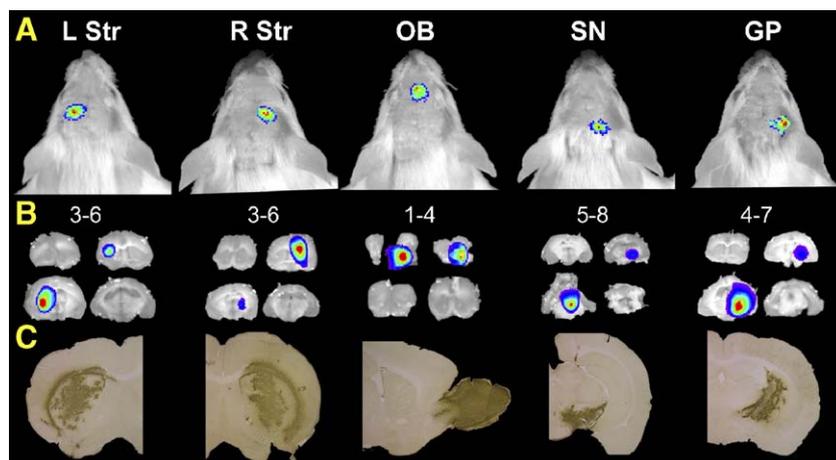
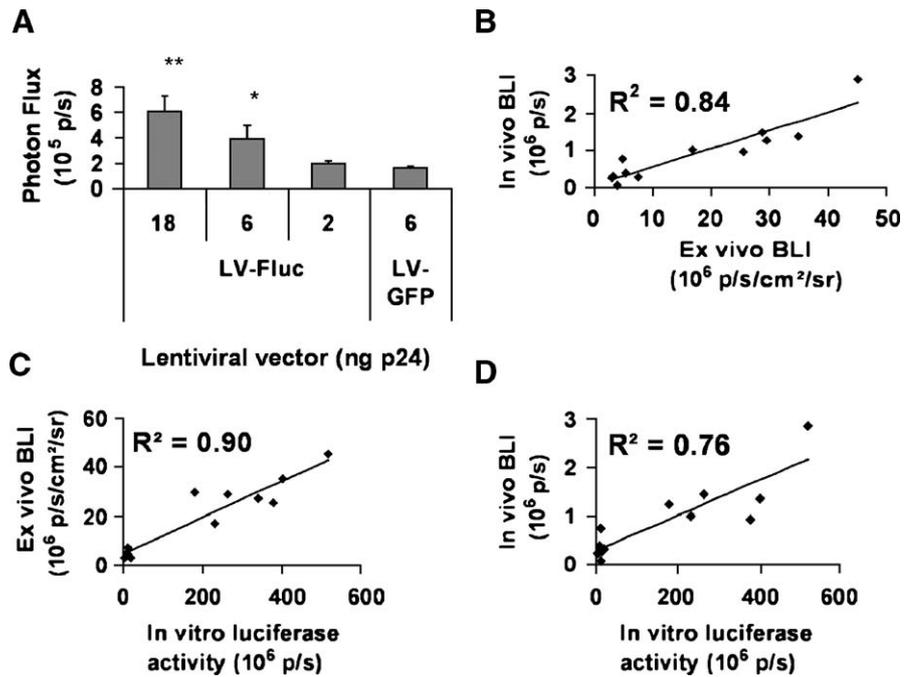


FIG. 4. The localization of the BLI signal reflects the anatomical site of injection. (A) The location of the BLI signal focus depends on the site of injection. BLI scans 14 days after injection of LV-eGFP-I-Fluc show a focus that is located above the injection site: caudal to the eyes and on the left side of the head for the left striatum (L Str), caudal to the eyes and on the right side for the right striatum (R Str), between the eyes for the olfactory bulb (OB), near the caudal edge of the skull for the substantia nigra (SN), and intermediate between R Str and SN for the globus pallidus (GP). These sites correspond to the expected locations based on the injection coordinates. (B) *Ex vivo* BLI of 1-mm-thick coronal slices show the localization of the signal at the site of injection. The slices are numbered in the anteroposterior direction from bulbus olfactorius (1) to cerebellum (9). (C) Immunohistochemistry for eGFP confirms the site of injection.

FIG. 5. Strong correlation of the *in vivo* BLI signal with *ex vivo* luciferase activity. (A) BLI signal 14 days after injection of 2 μ l of various titers of LV-Fluc into the right striatum. The signal decreased with decreasing titers. $**P < 0.01$, $*P < 0.5$ compared to control mice injected with LV-eGFP. Twelve other mice were injected with varying titers of LV-Fluc. BLI was performed 14 days postinjection. *Ex vivo* brain imaging on coronal slices was performed the same day. *In vitro* luciferase activity was determined on tissue extracts from these slices. A very strong correlation was observed between: (B) the *ex vivo* maximum pixel and the *in vivo* photon flux ($r^2 = 0.84$), (C) the *in vitro* luciferase activity and the *ex vivo* maximum pixel ($r^2 = 0.89$), and (D) the *in vitro* luciferase activity and the *in vivo* photon flux ($r^2 = 0.76$).



(Fig. 7). The BLI signal was $4.3 \pm 2.1 \times 10^6$ and $4.0 \pm 2.4 \times 10^5$ p/s in the high- and low-titer group, respectively. The corresponding transduced volume was 6.6 ± 1.8 and 3.8 ± 0.27 mm³ (Supplementary Fig. S3). This represents a 91% decrease in BLI signal and a 42% decrease in transduced volume in the low-titer group compared to the high-titer one.

In cell culture there is thus a very strong correlation between BLI signal and fluorescence in cells transduced with different amounts of vector. In mouse brain, however, the decrease in BLI signal is more pronounced than the decrease in eGFP-transduced volume. This is likely due to the fact that stereological quantification of the transduced volume measures only the number of positive cells, not taking into account the level of eGFP expression in transduced cells, whereas the bioluminescent signal integrates the number of positive cells and the amount of luciferase expressed. Based on these data BLI results in a better quantification of gene expression than immunohistochemistry, providing data faster (as soon as 4 days after transduction) and more reproducibly, without invasive actions, which allows long-term follow-up.

BLI Can Detect Luciferase Expression in the Rat Substantia Nigra

To evaluate if BLI monitoring of locoregional transgene expression would also be feasible in the rat brain, we performed a bilateral injection of LV-eGFP-I-Fluc targeting the substantia nigra in three rats. Seven days after injection, we could detect a signal projecting in two foci on the left and right caudal parts of the skull, whereas there was no signal above background in control rats

(Supplementary Figs. S4A and S4B). Quantitative analysis revealed a photon flux of $1.9 \pm 0.8 \times 10^6$ and $5.2 \pm 1.8 \times 10^4$ p/s in the LV-eGFP-I-Fluc and control group, respectively (37-fold increase, $P < 0.005$) (Supplementary Fig. S4D). Immunohistochemistry for eGFP showed that expression was localized in and above the pars compacta of the substantia nigra, where nearly all cells were positive (Supplementary Fig. S4C). We conclude that despite an increase in thickness of overlying structures, the BLI signal is still sufficient to allow detection of firefly luciferase expression in deep structures of the rat brain, expanding the utility of this technique to this widespread model animal for neurological research. As signal can be detected from the substantia nigra, which is one of the deepest brain structures in the rat, it is likely that expression in any region of the rat brain can be detected.

BLI has a number of advantages compared to other molecular imaging techniques such as positron emission tomography (PET), single photon computed tomography (SPECT), magnetic resonance imaging (MRI), and fluorescence tomography. The hardware is commercially available and is relatively inexpensive and the operating costs are low compared to radioisotope-based techniques. The sensitivity is good as we were able to detect luciferase expression after injection of only 6 ng p24 of LV-Fluc and the background signal is relatively low. Although the background signal derived from the snout is higher than that in oncology or cardiology studies, this is still many times lower than the typical fluorescent background encountered in fluorescent-based imaging strategies [31]. The major limitations of BLI (and fluorescence imaging) are the limited penetration through living

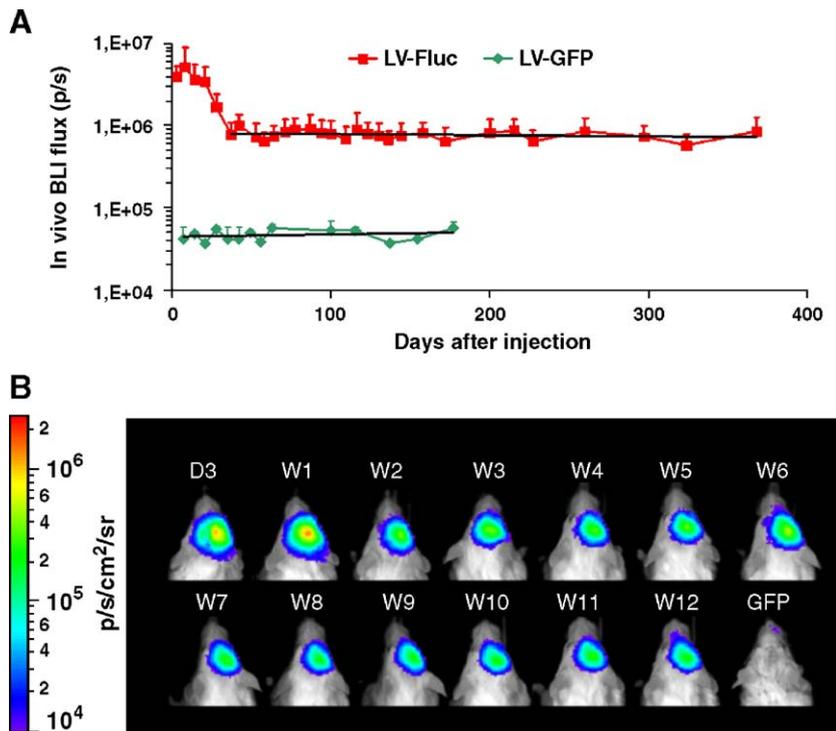


FIG. 6. Time course of BLI signal after LV transduction of mouse brain. (A) Long-term evolution of the BLI signal in a group of mice ($n = 10$) injected with 17 ng p24 of LV-Fluc and in a group injected with 8.4 ng p24 control vector (LV-eGFP, $n = 4$). After a peak at day 8 to 14, the signal declined during the first month to 16% of the maximum value at day 37 and then remained constant at $17.5 \pm 2.3\%$ of the maximum value from day 42 to 365. A linear regression line is drawn from day 37 to 365 ($R^2 = 0.027$) for LV-Fluc and for all time points for LV-eGFP ($R^2 = 0.041$). (B) BLI of a representative animal show an initial rise in signal at week 1 followed by a decrease and thereafter a stabilization of the signal. The control animal shown represents the highest signal seen in a control animal.

tissue, restricting its use to mice and rats. SPECT, PET, and MRI have low background and are tomographical in nature, enabling three-dimensional localization of the signal. Although mathematical approaches have been worked out [32], there are currently only limited data available regarding BLI tomography, limiting current BLI applications to planar imaging. Another limitation of BLI is the need to inject luciferin substrate, which has been overcome by genetically encoded fluorophores for fluorescence imaging and by magnetic proteins such as ferritin for MRI [33].

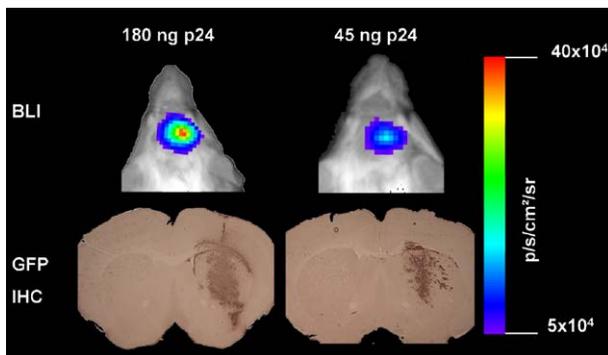


FIG. 7. BLI allows monitoring of gene-of-interest expression. Example of the BLI signal and the eGFP-transduced volume in representative mice injected with 180 or 45 ng p24 of LV-eGFP-I-Fluc. The BLI signal was measured 14 days after injection and the mice were subsequently sacrificed. The eGFP-positive volume was determined by anti-eGFP immunohistochemistry and stereological counting.

In conclusion, this is the first report on BLI monitoring of viral vector-mediated transduction of the adult brain in living mice. We were able to detect stable gene expression for more than 1 year. Our work opens perspectives for the use of BLI in the study of brain disease beyond malignancies and infectious diseases. BLI can be used for optimizing novel viral or nonviral vectors for gene transfer into the central nervous system, yielding data on the levels of gene expression as well as on the persistence of gene expression over time. Furthermore, by combining the luciferase reporter with a gene inducing neurodegenerative disease, neurodegeneration may be followed quantitatively over time and potential therapeutic strategies may be evaluated noninvasively.

MATERIALS AND METHODS

Lentiviral vector construction and production. We constructed a lentiviral transfer plasmid encoding firefly luciferase. The firefly luciferase fragment was obtained by PCR amplification with a Fluc forward primer (5'-CGGGATCCATGGAAGACGCGCAAAAAC-3') and a Fluc reverse primer (5'-TCCCCCGGGTTACACGCGCATCTTCC-3') from CSCMV-sr39tk-IRES-Fluc (a kind gift from Dr. Sanjiv Sam Gambhir, Stanford University). The unique restriction sites *Bam*HI and *Xma*I were added to the 5' and 3' ends, respectively. After amplification, the Fluc PCR product was digested with *Bam*HI and *Xma*I and inserted into the transfer plasmid pCHMWS [24] to yield pCHMWS-Fluc (LV-Fluc). We also constructed a bicistronic lentiviral vector encoding eGFP and Fluc separated by an EMCV IRES sequence [34]. The IRES-Fluc fragment was removed from CSCMV-sr39tk-IRES-Fluc using *Bsa*I and *Eco*RI and inserted into the *Pst*I and *Eco*RI site of the pBKRSV shuttle plasmid. The IRES-Fluc fragment was excised using *Xho*I and *Bss*HII and cloned into pCHMWS-eGFP, digested with *Mlu*I and *Xho*I, resulting in pCHMWS-eGFP-IRES-Fluc (LV-eGFP-I-Fluc). Luciferase

expression from both transfer plasmids was confirmed by Western blot analysis of extracts from transfected 293T cells. Luciferase activity was measured in a luminometer by using the Luciferase Assay System (Promega, Madison, WI, USA). Highly concentrated lentiviral vectors were produced as described previously [25].

Cell culture and transduction. 293T (human embryonic kidney), N2a (murine neuroblastoma), and SHSY5Y (human dopaminergic neuroblastoma) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Gibco BRL, supplied by Invitrogen, Merelbeke, Belgium) supplemented with 10% heat-inactivated fetal calf serum (Harlan Sera-Lab Ltd., International Medical, Brussels, Belgium) and 1% penicillin (10,000 U/ml)/streptomycin (10 mg/ml) (Gibco BRL). PC12 (rat pheochromocytoma) cells were maintained in the aforementioned medium supplemented with 1% essential amino acids (Gibco BRL). G1261 (mouse glioma) cells were maintained in the aforementioned medium supplemented with 2 mM glutamine (Cambrex, East Rutherford, NJ, USA) and 500 μ l β -mercaptoethanol (Sigma-Aldrich, Steinheim, Germany). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. The day before transduction, cells were seeded in a 96-well plate at 15,000 cells per well. On the day of transduction, medium was replaced by DMEM containing serial dilutions of the vector and incubated for 5 h. Five hours after transduction, medium was replaced, and 48 h after transduction the cells were assayed.

FACS analysis of eGFP expression. Cells were transduced with specific amounts of lentiviral vector as described. Two days posttransduction eGFP expression was analyzed by FACS. Cells were trypsinized and subsequently fixed in 2% paraformaldehyde prior to analysis with a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). The data obtained were analyzed using the CellQuest software package provided with the instrument.

Bioluminescence measurement of live cells. Cells were plated as described previously in a 96-well plate with black-tinted walls. D-Luciferin-containing medium was added to a final concentration of 150 μ g/L medium. After 5 min incubation, the cells were imaged for 1 to 5 s in the IVIS system.

Vector injections. Adult female NMRI mice were used. The animals were housed under 14 h light/10 h dark cycle with free access to food and water. All animal experiments were approved by the bioethics committee of the Katholieke Universiteit Leuven. For intramuscular delivery, 250 μ l of a ketamine/medetomidine mixture was injected intraperitoneally before the right lower leg was shaved and a 5-mm incision was made in the skin. Using a 10- μ l Hamilton syringe, 2 μ l of LV-Fluc (17 ng p24) was injected within 30 s into the right gastrocnemius muscle. The skin was sutured and after subcutaneous injection of 25 μ l of ampicillin (200 mg/ml), a dose of 250 μ l of atipamezole antidote was administered ip. The animals were kept in individually ventilated cages until sacrifice at day 21, at which time both gastrocnemius muscles were excised and stored at -80°C. For brain injections, anesthetized animals were placed in a stereotaxic head frame (Stoelting, Wood Dale, IL, USA). A midline incision of the skin was made and a small hole drilled in the skull in the appropriate location, using bregma as reference. The coordinates used for striatal injection were anteroposterior (AP) 0.5, lateral (L) 2.0, and dorsoventral (DV) 3.0-2.0 mm. Two microliters of highly concentrated vector supplemented with Polybrene (4 μ g/ml) was injected at a rate of 0.25 μ l/min with a 30-gauge needle on a 10- μ l Hamilton syringe. After 4 min of injection (1 μ l), the needle was raised slowly in the dorsal direction over the distance indicated by the two dorsoventral coordinates. After the injection, the needle was left in place for an additional 5 min to allow diffusion before being slowly withdrawn from the brain. The coordinates used to target different anatomical sites were substantia nigra, AP -3.1, L -1.2, and DV -4.0 mm; medial globus pallidus, AP -1.3, L -1.8, and DV -4.0 mm; olfactory bulb, AP 4.7, L -1.0, and DV -1.5 mm. Injections were performed similar to the striatal injection except for the fact that there was only one injection site and that we used only 1 μ l for injection in the olfactory bulb.

Histology and stereological counting. To assess lentiviral transduction, the mice were deeply anesthetized with pentobarbital and perfused transcardially with saline followed by ice-cold 4% paraformaldehyde in PBS for 15 min. The brain was removed from the skull and postfixed overnight in the same fixing solution. Coronal brain sections (50 μ m thick) were cut with a Vibratome and stored at 4°C in PBS buffer containing 0.1% sodium azide. A polyclonal antibody against firefly luciferase (Promega) was used for immunohistochemistry. The sections were treated with 3% hydrogen peroxide and incubated overnight with the primary goat anti-Fluc antibody (diluted 1:500) in 10% normal rabbit serum and 0.1% Triton X-100. The sections were then incubated in biotinylated rabbit anti-goat secondary antibody (Dako, Glostrup, Denmark), followed by an incubation with Strept-ABC-HRP complex (Dako). Detection was with diaminobenzidine, using H₂O₂ as a substrate. A similar procedure was used to detect eGFP expression with a primary rabbit anti-eGFP antibody (diluted 1:10,000) and a secondary swine anti-rabbit antibody (Dako). The transduced volume was determined by stereological counting based on the Cavalieri method with Stereoinvestigator software (MicroBrightfield, Magdenburg, Germany) as described previously [21].

In vivo bioluminescence imaging. The mice were imaged in an IVIS 100 system (Xenogen, Alameda, CA, USA). Anesthesia was induced in an induction chamber with 2.5% isoflurane in 100% oxygen at a flow rate of 1 L/min and maintained in the IVIS with a 1.5% mixture at 0.5 L/min. The mice were injected with D-luciferin (126 mg/kg) dissolved in PBS (15 mg/ml) by either the intraperitoneal or the intravenous route. Subsequently, they were placed in the prone position in the IVIS and consecutive 1- to 5-min frames were acquired until the maximum signal was reached or until the signal returned to background level in the pharmacokinetic studies. Each frame depicts the bioluminescence signal as a pseudocolor image superimposed on the gray-scale photographic image. The data are reported as the photon flux (p/s) from a 1.23-cm² circular region of interest around the head.

Ex vivo brain bioluminescence imaging. The mice were injected with the anesthetic mixture as described and injected in the lateral tail vein with D-luciferin (126 mg/kg). Mice were placed in the IVIS and two consecutive scans of 1 min were acquired. Immediately afterward the mice were sacrificed by cervical dislocation and decapitated and the brain was dissected. The brain was placed in an acrylic brain matrix (Harvard Apparatus, Holliston, MA, USA) and sliced into 1.0-mm-thick coronal sections. These were imaged for 1 min in the IVIS before both striata from the section emitting the most light were removed and stored at -80°C. The data are reported as the maximum pixel (p/s/cm²/sr) from the most intense slice.

In vitro luciferase activity assay. For the cell culture experiments, the cells were lysed using 20 μ l of Luciferase Cell Culture Lysis Reagent (Promega) per well of a 96-well plate. They were subsequently incubated with 100 μ l Luciferase Assay Reagent (Promega) and luciferase activity was measured for 2 s in both a Luminocount luminometer (Perkin-Elmer, Milan, Italy) and the IVIS system. To determine luciferase activity on brain slices, proteins were extracted from brain tissue. The tissue was first frozen at -80°C and then pulverized with tissue grinders in Luciferase Cell Culture Lysis Reagent. Samples were centrifuged at 12,000 g and supernatant was collected afterward, while the pellet was discarded. Luciferase activity was measured by incubating 20 μ l of extract in 100 μ l Luciferase Assay Reagent (Promega) and by measuring for 2 s in the IVIS system.

Statistical analysis. Results are expressed as means \pm standard error of the mean. Linear regression analysis was performed to obtain the Pearson's correlation coefficient. Unpaired Student's *t* tests were used to compare the mean values of different groups of mice unless stated otherwise.

Western blot analysis, stereotactical injection, in vivo bioluminescence imaging, and histology in rats. These procedures are reported in the online supplementary information.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymthe.2006.05.007.

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