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# Efficient *in vivo* expression of a reporter gene in rat brain after injection of recombinant replication-deficient Semliki Forest virus

Research Article

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## Summary

**Recombinant replication-deficient Semliki Forest virus (SFV) expressing bacterial β-galactosidase was injected into the amygdala and striatum of male Wistar rats. Reporter gene expression was detected up to 28 days post-injection. The maximal expression levels were obtained 1-2 days post-injection. *In situ* hybridization studies demonstrated high expression of LacZ mRNA until day 2, but no signal was detected 4 days post-injection. No significant change in body weight and temperature, exploratory locomotor behavior and forced motor performances were observed after SFV-LacZ injections. The neuronal gene transfer with SFV vectors did not trigger any major cell toxicity.**

## I. Introduction

A multitude of different methods and vehicles have been developed to increase the efficiency of delivery of recombinant genes *in vivo* for gene function and gene therapy applications. The non-viral delivery vehicles include naked DNA and a variety of liposome-DNA complexes consisting of cationic lipids (Filion and Phillips, 1997). Naked DNA is highly sensitive to degradation with a half-life of only 5 min when injected intravenously (Lew et al., 1995), whereas the lipid structures can offer an increased protection. However, the low delivery efficiency is a considerable drawback using these vectors (Boulikas 1996). Viral vectors have offered the possibility to achieve higher transfection frequencies. Retroviral vectors are capable of very high transduction rates and even retrovirus producer cell lines can be used for gene delivery (Markowitz et al., 1990). The retrovirus delivered transgene can be stably integrated into the host genome to provide long-term gene expression (Miller et al., 1990). The drawback with retroviruses is that only relatively low virus titers can be achieved and only dividing cells are infected. Adenovirus vectors are capable of infecting non-dividing as well as dividing cells and their transduction frequency is generally high (Haffe et al., 1992). The duration of expression is, however, limited due to cellular and humoral immune responses induced by the virus infection (Yang et al., 1994). Adeno-associated virus (AAV) are replication-deficient parvoviruses. They are nonpathogenic and nonimmunogenic, but can replicate in cell culture only in the presence of adenovirus or helper virus (Clark et al., 1995). AAV have only a limited packaging capacity of foreign DNA (<4.5 kb), but can integrate into the host genome. Herpes simplex virus (HSV) offers very good infectivity and allows large inserts of foreign DNA to be introduced (~30 kb). Virus infection can be maintained indefinitely in a latent state, but HSV infections generally show severe cytoxicity to cells. This effect has been reduced by deletion of some viral genes,

like ICP27 and ICP34.5 from the HSV genome (Howard et al., 1998). Lentiviruses offer good infectivity and long-term expression, and are therefore potential candidates as vectos for gene therapy (Verma and Somia, 1997). Despite the variety of gene delivery methods available, there are still needs for improvements and modifications of existing vectors as well as development of new vector technology.

Recently, Sindbis virus, a member of the Alphavirus family, was used for successful high level delivery and expression of β-galactosidase in mouse nucleus caudata/putamen and nucleus accumbens septi (Altman-Hamandzic et al., 1997). The goal of our study was to examine the ability of Semliki Forest virus (SFV) vectors (Liljeström and Garoff, 1991), a closely related Alphavirus, to infect neuronal cells *in vivo* by direct delivery to a desired location in rat brain. SFV has an extremely broad host range which allows efficient infection of many cell types, including post-mitotic cells. *In vivo* packaging results in high titer (up to $10^{10}$ infective particles/ml) replication-deficient recombinant SFV particles. Recombinant SFV-LacZ virus particles were injected into the amygdala and striatum of rat brain. These regions play important roles in controlling motor functions and in regulating emotional states, respectively. We have investigated the neuropathological consequences of the SFV inoculation at different time points. Macroscopical analyses were carried out using neurological and behavioral parameters and the microscopical studies were performed on fixed brain tissue.

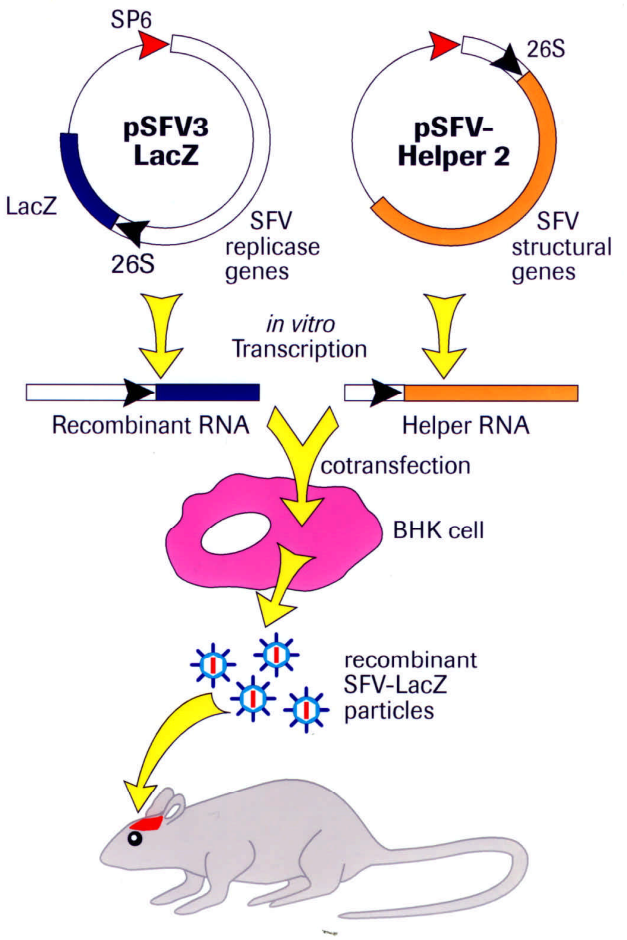


**Fig. 1. Semliki Forest virus vectors for *in vivo* gene delivery.** *In vitro* transcribed RNA from pSFV3-LacZ and pSFV-Helper2 were cotransfected into BHK cells to *in vivo* package recombinant SFV-LacZ particles. These were injected into the amygdala and striatum of rat brain.

## II. Results and Discussion

### A. Injection of SFV-LacZ into rat brain

High-titer recombinant SFV-LacZ virus was generated as described in the Experimental procedures and as schematically illustrated in **Fig. 1**. The infectivity of the SFV-LacZ virus was tested by infection of BHK cells in 6-well plate cultures followed by X-gal staining. 100% infectivity was achieved with a multiplicity of infection (MOI) of 4. To enable the infection of a reasonable large population of cells, 1 x $10^5$ SFV-LacZ particles were injected into the amygdala and striatum of male Wistar rats (**Fig. 2**), respectively, as described in the Experimental procedures.

### B. Behavioral studies

To study the effect of reporter gene expression based on virus vector delivery, rats receiving SFV-LacZ and control animals injected with sterile culture medium were subjected to behavioral studies. No significant change in body weight and temperature, exploratory behavior and forced motor performances was observed between the two groups at any time post-injection. Physical and behavioral parameters recorded 1, 7 and 14 days post-injection are described in **Table 1**. Body weights did not differ significantly and a similar gradual increase was recorded for both SFV-LacZ injected rats and control animals, which is indicative of a good general condition of the animals (normal food and water intake, healthy metabolism). No infection-induced hyperthermia was detected as the body

General health and behavior of rats at day 1, 7 and 14 following central injections of SFV or vehicle control

Average and s.e.m data are presented with P values for statistical significance using a Student t-test comparing Virus to Control

| | DAY 1 | | | | | Day 7 | | | | | Day 14 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | | Virus | | | Control | | Virus | | | Control | | Virus | | |
| | (n=4) | sem | (n=8) | sem | p | (n=4) | sem | (n=7) | sem | p | (n=4) | sem | (n=8) | sem | p |
| General condition: | | | | | | | | | | | | | | | |
| body weight (g) day 0 | 277 | 19.6 | 294 | 13.1 | 0.48 | 277 | 20.2 | 300 | 16.3 | 0.40 | 273 | 22.0 | 290 | 17.1 | 0.57 |
| body weight (g) day n | 278 | 23.4 | 282 | 14.4 | 0.86 | 288 | 24.8 | 295 | 16.4 | 0.82 | 312 | 30.3 | 318 | 16.2 | 0.85 |
| body temperature (°C) | 34.9 | 0.3 | 35 | 0.3 | 0.79 | 34.6 | 0.5 | 35.2 | 0.6 | 0.51 | 34.6 | 1.1 | 35.1 | 0.7 | 0.69 |
| Spontaneous exploration: | | | | | | | | | | | | | | | |
| total distance (cm) | 503 | 154.3 | 875 | 118.9 | 0.09 | 2093 | 288.7 | 1710 | 210.5 | 0.30 | 1694 | 363.9 | 2068 | 138.1 | 0.26 |
| vertical activity (score) | 238 | 50.7 | 264 | 49.7 | 0.76 | 588 | 42.8 | 929 | 256.7 | 0.32 | 1474 | 676.2 | 930 | 105.5 | 0.28 |
| Forced performance: | | | | | | | | | | | | | | | |
| rotarod performance (s) | 33.8 | 8.9 | 55.3 | 3.1 | 0.02 | 28.8 | 10.9 | 48.9 | 7.2 | 0.14 | 27.5 | 11.9 | 54.3 | 5.8 | 0.04 |
| grip strength (g) | 1098 | 75.7 | 956 | 90.2 | 0.33 | 1151 | 136.0 | 960 | 74.6 | 0.21 | 1104 | 138.1 | 1024 | 138.6 | 0.72 |

**Table 1.**

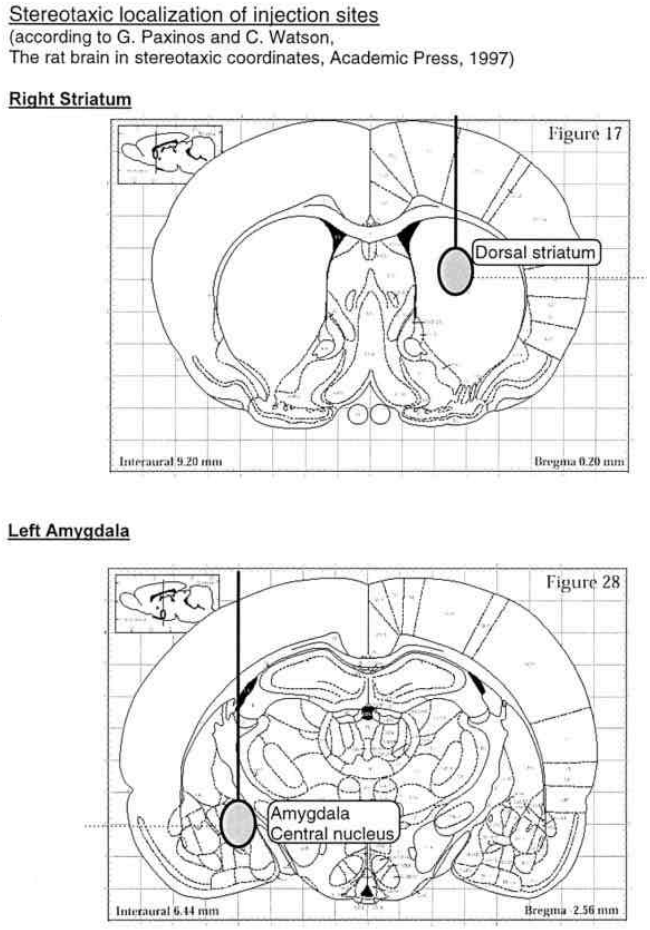


**Fig. 2. Stereotaxic localization of injection sites.** Injection cannulae were lowered into the right striatum (top) and left amygdala (bottom) for local delivery of SFV-LacZ virus or vehicle. Stereotaxic coordinates are from G. Paxinos and C. Watson (The Rat Brain in stereotaxic coordinates, Academic Press, 1997).

temperature remained normal. No difference between the groups in spontaneous exploration of a novel environment by the animals was recorded in measurement of total distance and vertical activity which is indicative of a state of normal emotional reactivity of the rats. As the animals were still recovering from surgery (1 day post-injection) scores were lower for both SFV-LacZ injected and control animals on day 1 when compared to 7 and 14 days post-injection. No impairment was seen at any post-injection date on forced motor performance; virus-injected animals even performed better (i.e. remained longer on the rotating rod) than control rats on some occasions (1 and 14 days post-injection). No statistically significant differences in muscular strength were detected at any time following injection. Equally, no differences were observed at 2, 4, 21 and 28 days post-injection in groups of smaller size (n = 2, controls; n = 3, virus injections; data not shown). This neurobehavioral evaluation suggests that central injection of recombinant replication-deficient SFV particles has no major consequences on the general health and on regular sensorimotor functions of male Wistar rats.

### C. β-galactosidase expression

Brain sections from rats injected with SFV-LacZ particles and medium, respectively, were stained with X-gal at the different time points (1, 2, 4 , 7 , 14 and 28 days post-injection) and *in situ* hybridization with a LacZ gene specific probe was carried out at 1 h, 24 h, 48 h and 4 days post-injection. Twenty-four hours after injection into striatum and amygdala, both LacZ mRNA and β-galactosidase were detected at the site of injection as well as in ventricular ependymal cells throughout the brain (**Fig. 3**). The β-galactosidase expression was restricted to the infected cells and their processes and was not observed in other brain regions. LacZ mRNA was restricted to perikarya and no hybridization signal was found in cell processes. Whereas both transcript and recombinant protein were detected at 48 h post-injection (mainly at the injection sites), no mRNA was present at later time points. β-galactosidase, on the other hand, was detected, albeit in ever decreasing amounts, 4, 7, 14 (**Fig. 4e**) and even 28 days (**Fig. 4f**) post-injection.

In order to determine the presence or not of a toxic effect of the viral infection, adjacent sections were also stained with Toluidine Blue. Using the vehicle-injected animals as controls, virus-induced inflammation (in the form of local glioses) at the injection site could be observed 1-4 weeks after administration. Experiments are in progress with marker protein staining for astroglioses (GFAP) and microglioses (OX-42).

Over all, our findings suggested that a reporter gene can be delivered to the caudate putamen and central amygdala for local expression in the infected cells, mainly neurons, and does not appear to spread into neighbouring regions, except via the ventricular system into ependymal cells of the lateral third and fourth ventricles. Modifications of the injection procedure (i.e. decrease in injection speed, volume or virus concentration) might further reduce or eliminate this spread. Whereas LacZ transcripts were only detected in the first 48 h after injection, β-galactosidase could still be found after 4 weeks. This is most probably due to the high stability of this particular enzyme.

## III. Conclusions

Our results clearly demonstrate the feasibility of using SFV vectors for efficient infection of neuronal cells in different regions of the rat brain. We could obtain local expression of β-galactosidase, mostly due to the replication-deficient nature of the recombinant SFV particles. The infection rate at the injection site was extremely high and the duration of the recombinant protein expression at least 28 days. This is comparable to the duration of bacterial β-galactosidase expression obtained with other viral vectors, like adenovirus (Neve 1993) and herpes virus (Fotaki et al., 1997). A further suggestion of the exceptionally high stability of the recombinant β-galactosidase came from our *in situ* hybridization experiments, where we demonstrated that no LacZ mRNA could be detected after 48 h post-injection. Similar observations have been demonstrated *in vitro* in BHK cells infected with SFV-LacZ virus by RT-PCR techniques, where LacZ mRNA disappears approximately 65 h post-infection (Lundstrom, unpublished data). The kinetics of other recombinant proteins might be different and could result in faster degradation of the gene product. However, the transient nature of the protein expression is evident from our results. Although this will exclude the use of SFV vectors, at least in their present form, for long-term expression, the lack of neuronal cell damage caused by the SFV infection should allow efficient transient gene expression in short term studies. Fast generation of site-specific knock-in and knock-out gene expression studies should be possible. Our behavioral studies also demonstrated that the SFV injections did not trigger any widespread inflammatory response or extensive cell destruction, although a local inflammatory response was evident at 14 and 28 days post-injection. There were no change in the animals' exploratory locomotor behavior or forced motor performance, further indications of intact neuronal cells, compared to control animals.

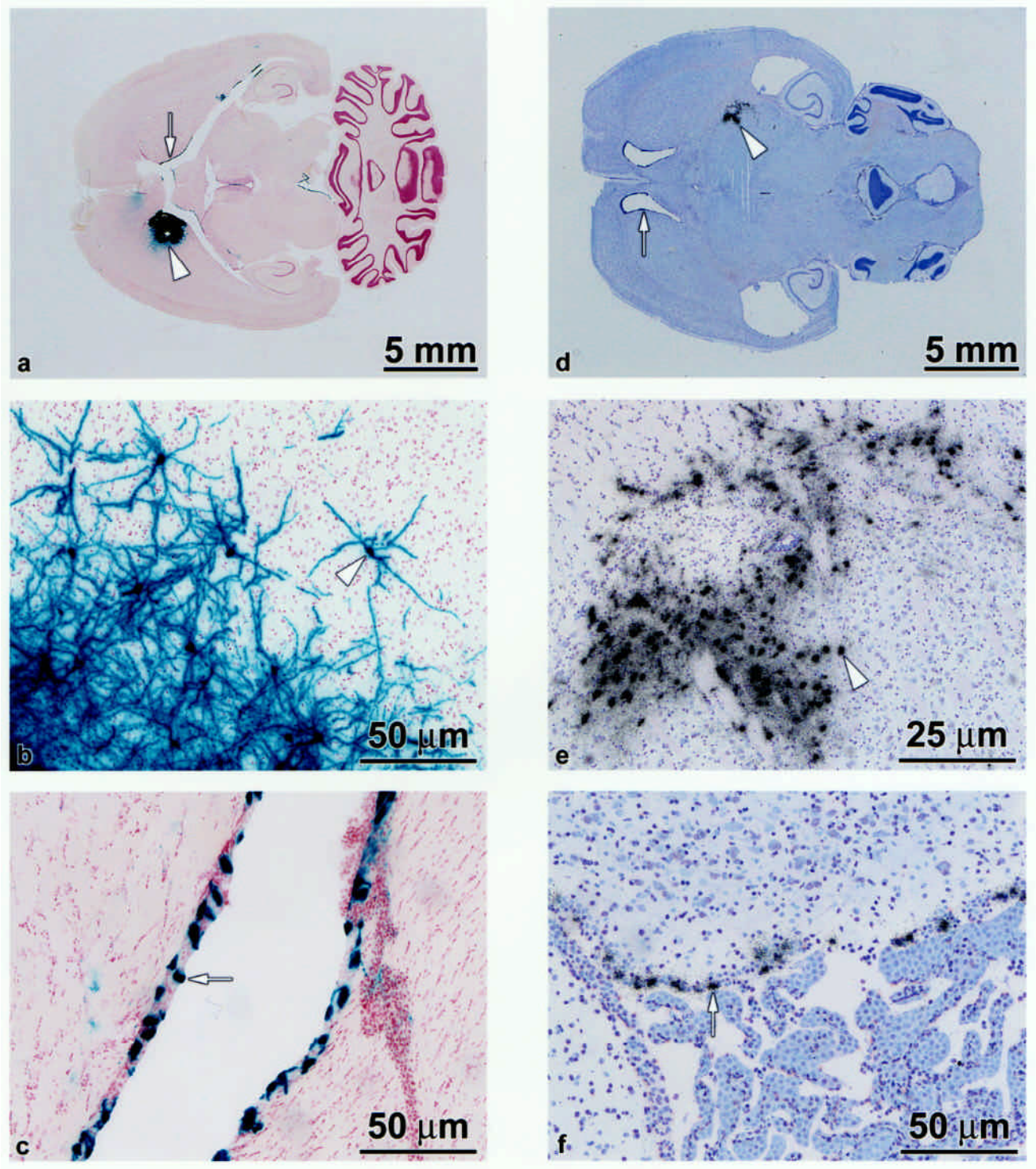


**Fig. 3. Regional distribution and cellular localization in rat brain of LacZ mRNA and β-galactosidase 24 h post-injection.** β-galactosidase (blue precipitate) is detected not only at the striatal injection site but also in the ependyma throughout the ventricular system (arrowhead and arrow, respectively in **a**). Note the expression not only in the neuronal cell bodies (**b**) and ependymal cells (**c**), but also in presumptive neuronal processes (**b**). LacZ mRNA is also detected at the site of injection and in the ependyma (arrowhead and arrow, respectively, in **d**). The cellular sites of synthesis of LacZ in presumptive neurons and ependymal cells (of the fourth ventricle) are illustrated in **e** and **f**, respectively.

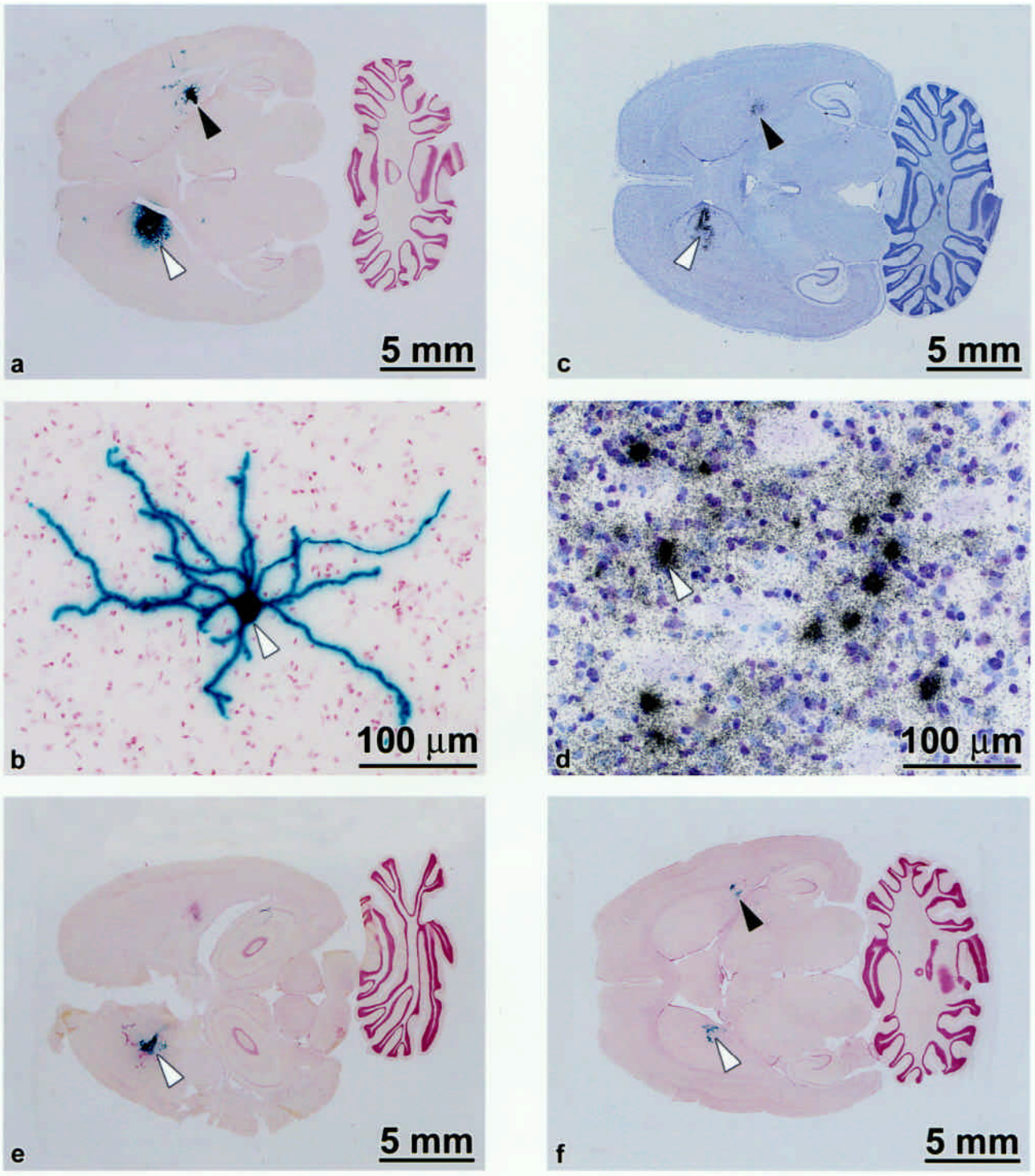


**Fig. 4. Regional distribution and cellular localization in rat brain of LacZ mRNA and β-galactosidase 2, 14 and 28 days post-injection, respectively.** β-galactosidase (blue precipitate) is detected at the striatal and amygdala injection sites (white and black arrowheads, respectively). Note the expression not only in the neuronal cell bodies, but also in their processes (**b**). LacZ mRNA is also detected at the striatal and amygdala injection sites (white and black arrowheads, respectively, in **c**). The cellular sites of synthesis of LacZ in presumptive neurons are illustrated in **d**. **a-d** illustrate 2 days post-injection, **e** and **f** the regional distribution of β-galactosidase at 14 and 28 days post-injection, respectively.

## IV. Experimental procedures

### A. Cell cultures and recombinant SFV production

BHK-21 cells were grown in a mixture of F12-MEM/Iscove (1:1) in 10% FCS (Gibco-BRL) for *in vivo* packaging of recombinant SFV particles (Lundstrom et al., 1994). Briefly, *in vitro* transcripts from pSFV3-LacZ (SFV replicase genes + LacZ gene) and pSFV-Helper 2 (SFV structural genes) (Berglund et al., 1993) were co-electroporated into BHK-21 cells (**Fig. 1**). *In vivo* packaged recombinant SFV particles were collected 24 hours later by harvesting the medium from the cell cultures. The SFV particles were activated with α-chymotrypsin and the titer of the virus stocks determined by infection of defined numbers of BHK-21 cells with different dilutions of recombinant SFV-LacZ followed by X-gal staining. The titers were generally in the range of $1 \times 10^9$ infectious particles / ml. The virus stocks were filter sterilized through 22 μm filter (Millipore) and no further purification or concentration was necessary. The virus stocks were diluted to $1 \times 10^8$ infectious particles / ml prior to use.

### B. Injections of recombinant SFV into rat brain

Male Wistar rats (lbm RoRo, SPF, Biological Research Labs Ltd, Switzerland) were housed individually under controlled laboratory conditions (temperature 20± 2°C, relative humidity 50-60%) with *ad libitum* access to food and water and were maintained on a normal 12 h light-12 h dark cycle (6 am-6 pm). Rats weighed 250-300 g at the time of surgery. They were stereotaxically microinjected under general anesthesia with Ketamine/Xylazine (200/10 mg/kg ip) in physiological saline under thermoregulatory control and oxygen supplementation. Craniotomy was performed using a fine dental drill for injection at one site located over the right striatum (0.2 mm anterior and 2.6 lateral to bregma; Paxinos and Watson, 1997) and at another site located over the left amygdala (2.6 mm posterior and 4.0 mm lateral to bregma). Stainless steel injectors attached to a stereotaxic holder were then lowered 5.0 mm ventral to the skull surface in the striatum and 8.0 mm in the amygdala (**Fig. 2**). These were connected via polyethylene tubing containing viral or control solutions to a 10 μl Hamilton syringe on a microinfusion pump (Harvard PHD 2000). Solutions were infused in a volume of 1 μl over 2 min (0.5 μl/min). The injection needle was left in place for 2 additional min before being slowly withdrawn over 1 min. The wound was then sutured and animals kept warm for 3-4 h after surgery. Post-operative buprenorphine (0.05 mg/kg) analgesic treatment (sc) was given for the next day.

A group of 21 rats were stereotaxically injected with the SFV-LacZ virus ($10^5$ particles/ μl) and 12 control rats received sterile vehicle (culture medium). Animals were carefully evaluated on day 1, 2, 4, 7, 14, 21 and 28 for consequences of viral injections on general health (global appearance, measures of body weight and rectal temperature), sensorimotor coordination, muscular capacity and exploration of a novel environment.

### C. Behavioral studies

**1. Sensorimotor function** was evaluated using a rotarod paradigm in which animals were required to walk on a rotating bar. The bar was 10 cm wide, 5 cm in diameter, 40 cm above the bench and rotated twice per minute. Trained animals were able to follow the slow regular movement of the bar for several minutes. Mild sedation or motor impairment translates into incoordination on the rotating rod and the animals fall off the bar. Time spent on the rotating rod is measured in seconds and maximal cut-off time is 60 s (non-impaired animal).

**2. Muscular capacity** was evaluated using a grip strength procedure consisting of a quantitative assessment of forelimb grip strength. A triangular bar, 2 mm in diameter, 5 cm wide was connected to a digital strain gauge. This device was used to measure graded changes in the forelimb grip strength of rats. Animals held by the tail grasped the bar and were then gently pulled away from the bar with a smooth steady pull until they released the triangle. The strain gauge remained fixed at its maximum deflection, which was the force required to break the animal's grip. Three readings were taken for each animal and the maximum of 3 permissible readings was recorded as the grip strength score (in g).

**3. Exploration of a novel environment** in a test of free exploratory activity was measured in activity monitors (40 x 40 x 30 cm, Omnitech Electronics) placed in a sound-proof room with central light. Locomotion was monitored via a grid of invisible infrared light beams. Horizontal and vertical activity were used in this study to describe the dynamic picture of rats. A vertical sensor monitoring rearing and jumping activity was attached 8 cm above the cage floor. An analyzer constantly collected the beam status information from the activity monitor and activity detected by the horizontal sensors was expressed as total distance run during the 30 min test.

The experimental procedures used in this study received approval by the local ethics committee and were performed in accordance with international and Swiss federal regulations and guidelines on animal experimentation.

### D. Histological analyses

On each test day (1, 2, 4, 7 , 14, 21 and 28 days post-injection) two to three animals in each group (virus and vehicle) were sacrificed for histological analysis. Directly following $CO_2$ inhalation euthanasia, animals were transcardially perfused (clamped dorsal aorta) with 20 ml of 4% paraformaldehyde (PFA) for fixed brain extraction. Brains were stored for 4 h in the same PFA solution, then cryoprotected in 30% sucrose at 4°C overnight and stored at -80°C until sectioned. Free-floating sections were cut on a freezing microtome at 40 μm then reacted for β-galactosidase, as well as LacZ mRNA. Some sections were stained for 0.5-1.0 min in 0.5% Toluidine Blue (Fluka 89640) in 0.2 M acetate buffer pH 4.5.

### E. Enzyme histochemistry of β-galactosidase

Oxidation solution was prepared as follows. 80 µl Nonidet P-40 and 0.04 g sodium deoxycholate were added to the oxidation stock solution (40 ml 10x PBS, 360 ml 2x distilled $H_2O$, 0.65 g potassium ferricyanide ($K_3Fe(CN)_6$), 0.84 g potassium ferrocyanide (3 $H_2O$) ($K_4Fe(CN)_6$ 3 $H_2O$), 0.16 g $MgCl_2$ 6 $H_2O$) and stirred thoroughly and filtered (45 µm). The resulting bright yellow solution was stored at room temperature under light-tight conditions. β-galactosidase was visualized by adding 10 mg of X-gal substrate (Boehringer Mannheim 1680293) to 0.25 ml DMSO. Once dissolved, 10 ml oxidation solution was added with careful mixing to avoid the formation of air bubbles. Tissue sections were rinsed ( 2 x 15 min) in PBS, then reacted with the X-gal solution overnight at 31-33°C in a dark box. The reacted sections (an insoluble blue indoyl precipitate reaction for β-galactosidase) were then rinsed again (2 x 15 min) in PBS, post-fixed in ice-cold 4% PFA for 15 min, rinsed in PBS, mounted on precleaned glass slides, counterstained with 1% Neutral Red (Sigma), dehydrated and coverslipped with DePeX.

### F. *In situ* hybridization

For selected time points we also investigated the regional and cellular expression of LacZ transcripts using a 60-mer oligonucleotide probe (nucleotides 3001-3060) selective for the LacZ gene (Casadaban et al., 1983). The hybridization procedure has been previously described (Saura et al., 1996). Briefly, 12 µm cryostat sections of fresh-frozen rat brains (1 h, 24 h, 48 h and 4 days post-injection) and 30 µm freezing-microtome sections of perfusion-fixed rat brains (1, 2, 4, 7, 14 and 28 days post-injection) were used. The cryostat sections were mounted on Superfrost Plus™ slides then fixed in 4% PFA in PBS, pH 7.4 for 20 min followed by three 5 min washes in PBS.

The oligonucleotide was ordered from Genosys Biotechnologies and labeled at the 3' end with terminal deoxynucleotidyl-transferase (BRL) and [$^{35}$S] dATP (New England Nuclear). The labeled probe was separated from unincorporated nucleotides with a Biogel P30 spin column (twice 4 min at 1600 x g, Sorvall SW24). Sections were hybridized with 50 µl of a solution with the following composition: 4 x SSC, 20% dextran sulfate, 0.25 µg/ml herring sperm DNA (denatured) , 50% deionized formamide (BRL), 0.1 M dithiotreitol (DTT) (Fluka), 0.5 x Denhard's solution and the $^{35}$S-labeled probe (3 x $10^5$ cpm). Sections were covered with strips of Fujifilm™ and incubated in moist chambers at 43°C overnight. Following removal of the strips, the sections were washed twice in a solution containing 1 x SSC and 10 mM DTT for 15 min at 55°C, then in 0.5 x SSC with 10 mM DTT once for 15 min at room temperature. After a dip in 2 x distilled $H_2O$, sections were dehydrated in ethanol, exposed (for up to 4 weeks) to sheet film (Hyperfilm™, β-Max, Amersham) or dipped in Ilford K5 nuclear emulsion to reveal the regional and cellular localization of the mRNA, respectively. The film or emulsion was developed in Kodak PL12 or Kodak D19, respectively, then transferred to Kodak Rapid Fix. Nissl- or Neutral Red-counterstained sections were examined with brightfield optics using a Zeiss Axiophot.

### G. Imaging

The regional and cellular distribution of X-gal and LacZ mRNA were recorded as digital images using a ProgRes high resolution color camera and Adobe Photoshop software.

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