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Lentiviruses allow widespread and conditional manipulation of gene expression in the developing mouse brain

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SUMMARY

Generation of transgenic mice, *in utero* electroporation and viral injection are common approaches to manipulate gene expression during embryonic development of the mammalian brain. While very powerful in many contexts, these approaches are each characterized by their own limitations: namely, that generation of transgenic mice is time-consuming and electroporation only allows the targeting of a small area of the brain. Similarly, viral injection has been predominantly characterized by using retroviruses or adenoviruses that are limited by a relatively low infectivity or lack of integration, respectively. Here we report the use of integrating lentiviral vectors as a system to achieve widespread and efficient infection of the whole brain after *in utero* injection in the telencephalic ventricle of mouse embryos. In addition, we explored the use of Cre-mediated recombination of *loxP*-containing lentiviral vectors to achieve spatial and temporal control of gene expression of virtually any transgene without the need for generation of additional mouse lines. Our work provides a system to overcome the limitations of retroviruses and adenoviruses by achieving widespread and high efficiency of transduction. The combination of lentiviral injection and site-specific recombination offers a fast and efficient alternative to complement and diversify the current methodologies to acutely manipulate gene expression in developing mammalian embryos.

KEY WORDS: Lentiviral injection, Brain development, Gene expression, Cre recombination

INTRODUCTION

Manipulation of gene expression is important to investigate gene function during embryonic development. In the context of the mammalian brain, there are three approaches commonly used: generation of transgenic mice, *in utero* electroporation and viral infection (De Vry et al., 2010; Glaser et al., 2005; Janson et al., 2001; Morozov, 2008), each characterized by specific advantages and limitations.

Generation of transgenic lines, in particular when combined with site-specific recombination, is the most versatile approach, allowing the manipulation of gene expression in a variety of contexts (Glaser et al., 2005), but it is laborious and time-consuming. Conversely, *in utero* electroporation is fast and simple (De Vry et al., 2010) but is limited by a low reproducibility with regard to size and location of the targeted area and by a transient expression of the transgene due to dilution of the ectopic DNA in mitotic cells. In addition, although electroporation can target a high proportion of cells, it does so only in a relatively small area, hampering the assessment of phenotypes at the level of the whole organ. Finally, viral approaches combine the ease of electroporation with, potentially, stable integration and manipulation of a high number of cells (Janson et al., 2001). However, the two advantages of stable integration and high infectivity were revealed to be mutually exclusive because the two genera of viruses mainly used until now, retroviruses (more specifically gamma retroviruses of the Murine leukemia family) and adenoviruses (or adeno-associated viruses), could only provide one or the other.

Specifically, retroviruses permanently integrate their genome into the host cell after mitosis, representing a useful system to carry long-term and stable transgene expression (Joyner and Bernstein, 1983;

Liu et al., 1998; Weiner et al., 2002; Gaiano et al., 1999; Jang et al., 2012; Matsukawa et al., 2003; Nanmoku et al., 2003; Shen et al., 2005; Stott and Kirik, 2006). However, infectivity with retroviruses was revealed to be modest relative to other viral vectors, and this is probably the reason why their first use in the developing mammalian brain has been exploited for clonal analysis (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988). By contrast, adenoviruses were reported to achieve higher infectivity, probably because they can be produced at very high titre and can transduce both dividing and nondividing cells (Leingärtner et al., 2003; Rahim et al., 2012; Shen et al., 2004). However, similarly to electroporation, manipulation of gene expression by adenoviruses is transient owing to lack of integration, leading to dilution as a result of cell divisions, which is particularly troublesome when targeting cells with high proliferative activity.

Lentiviruses have been extensively characterized in the adult brain (Baekelandt et al., 2002; Naldini et al., 1996a; Naldini et al., 1996b) for their ability to transduce dividing as well nondividing cells and their low inflammatory response (Baekelandt et al., 2003), which is relevant in the context of gene therapy (Dreyer, 2011). Surprisingly, lentiviruses are rarely used in studies during development, and no report has assessed their infectivity in the embryonic mouse brain. We hypothesized that lentiviruses could combine the advantages of both retroviruses and adenoviruses and decided to investigate the efficiency and pattern of lentiviral infection in a timecourse during corticogenesis. In addition, and perhaps more importantly, we investigated the use of site-specific recombination (Glaser et al., 2005) of transgenes delivered by lentiviruses as a means to achieve fast, widespread and spatiotemporal control of gene expression, almost exclusively accomplished until now by the generation of transgenic lines.

MATERIALS AND METHODS

Constructs and viral preparation

HIV-1-derived viral constructs were generated as previously described (Artegiani et al., 2011). Briefly, green fluorescent protein (GFP) cDNA was

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amplified and fused to a nuclear localization signal (nls) by PCR and cloned in the p6NST90 plasmid containing HIV-1 derived 3' and 5' long terminal repeats (LTRs) (Artegiani et al., 2011) to generate the transfer vector GFP^{nls}. To obtain the GFP^{lox} plasmid, overlapping GFP-*lox* and *lox*-nls fragments were mixed to produce the GFP-*loxP*-nls sequence, a second *loxP* site added at the 3' by PCR, and the resulting GFP-*loxP*-nls-*loxP* cassette cloned in the p6NST90 plasmid. Primers (supplementary material Table S1) were designed to express the GFP in frame with the nls, and stop codons were added to terminate translation in both the recombined and not-recombined construct. Viral preparations, as previously described (Artegiani et al., 2011; Artigiani et al., 2012), were further optimized to increase viral concentration. GFP^{nls} or GFP^{lox} vectors together with pCD/NL-BH (Mochizuki et al., 1998) and pczVSV-G (Pietschmann et al., 1999) plasmids (coding for HIV-1 Gag/Pol and VSV-G, respectively) were co-transfected (5 µg each) in 293T cells (5 × 10⁶ in a 10 cm dish, total of 45 dishes). Induction with 10 mM Na Butyric acid (Sigma-Aldrich) was performed after 24 hours and the following day supernatants were filtered, ultracentrifuged (1.5 hours, 120,000 g, 4°C), and pellets washed with PBS, ultracentrifuged again and incubated on ice for 3 hours before being resuspended in 75 µl PBS and stored at -80°C. To determine the viral titre, 1.5 × 10⁴ 293T cells were plated in D-MEM, 10% heat-inactivated fetal bovine serum and 100 U/ml pen/strep. A few hours later, when cells have adhered to the plates, serially diluted (from 1:100 to 1:10,000) viral suspensions were added. Forty-eight hours later, cells were washed with PBS, fixed for 30 minutes with 4% paraformaldehyde in 120 mM phosphate buffer pH 7.4 (PFA) and the percentage of GFP+ cells quantified on digital images. Alternatively, cells were treated with 0.05% Trypsin-EDTA and resuspended in PBS for flow-cytometry quantification. Titre was calculated considering the first dilution that led to nonsaturated infection (i.e. 1:10,000) using the formula (C × D × P) / V = transducing unit (TU) / V, in which C = number of plated cells, D = dilution factor, P = percentage of GFP+ cells and V = volume of the viral suspension. The viral titer was in the range of 2.5–6.0 × 10⁷ TU/µl.

Animals and surgery

Animal experiments were approved by local authorities (24D-9168.11-1/2008-16 and 2011-41). Time-pregnant wild-type C57/Bl6 or *Emx1*^{Cre/+} (Iwasato et al., 2000) mice were isoflurane-anesthetized at embryonic day (E) 12.5 and ca. 1 µl of viral suspension mixed with 0.05% Fast Green was injected through the uterine walls into the embryonic lateral ventricle as described for *in utero* electroporation (Artegiani et al., 2012; Lange et al., 2009). Brains were collected at E15.5, E18.5 and postnatal day (P) 2 and 10. Mice were PCR-genotyped (Iwasato et al., 2000) and both *Emx1*^{Cre/+} and *Emx1*^{Cre/Cre} were included in our analysis. Eventually, 1 mg BrdU was injected intraperitoneally every 2 hours for 4 hours before the mice were killed.

Immunohistochemistry, microscopy and analysis

Brains were dissected and fixed in PFA overnight at 4°C, cryoprotected in 30% sucrose, embedded in TissueTek optimal cutting temperature (OCT) compound and 30 µm thick coronal cryosections from the entire brain were stereologically collected and analyzed. Samples were permeabilized and blocked in 0.3% Triton X-100 in PBS (IHC buffer) containing 10% Normal Donkey Serum for 2 hours and incubated overnight with rabbit or goat polyclonal GFP antibody (Rockland, 1:1000) in IHC containing 3% serum. After washing with PBS, sections were incubated with DyLight-conjugated secondary antibodies (Jackson Laboratories, 1:1000) in IHC overnight at 4°C, washed with PBS and mounted. DAPI was used to stain nuclei. Fluorescence composite pictures of individual focal planes were acquired with an ApoTome microscope (Zeiss) and processed using AxioVision software (Zeiss) to obtain a Z-stack reconstruction picture. Analyses and quantification of infected cells, their distribution and number were performed on digital images taken from at least three animals for each time point.

RESULTS AND DISCUSSION

Lentiviral infection allows efficient gene transfer during embryonic brain development

In order to evaluate the efficiency of lentiviruses to transduce cells in the mouse developing brain, we used an HIV-1 based viral vector

encoding for ubiquitin-driven nuclear GFP (GFP^{nls}) (Artegiani et al., 2011) as an easy and fast readout of transduction efficiency in individual cells. VSV-G-pseudotyped viruses were produced, paying particular attention to maximize the titer, which reached levels comparable to those typically obtained with adenoviruses in the range of 2.5–6 × 10⁷ TU/µl (supplementary material Fig. S1). To assess infectivity, C57/Bl6 embryos were injected in the lateral ventricle with GFP^{nls} HIV-1 at E12.5, and their brains collected at different embryonic or postnatal stages (i.e. E15.5, E18.5, P2 or P10) (Fig. 1A).

Manipulated embryos were viable and could complete gestation without any evident impairment. The analysis of brain sections revealed a general tissue morphology and cellular composition undistinguishable from nonmanipulated brains (Fig. 1B,C and data not shown). Although lentiviral vectors are generally known for a late onset of transgene expression, we observed a strong and widespread GFP^{nls} fluorescence at the earliest time point analyzed, E15.5 (Fig. 1B, left), with proportion and distribution of GFP^{nls}+ cells as well as their fluorescence intensity being essentially identical at any other time point (Fig. 1).

Even if the viral suspension was injected only in one of the two lateral ventricles we found a bilateral and widespread distribution of GFP^{nls}+ nuclei in both hemispheres from the most rostral to caudal portions of the brain, confirming that viruses can spread over the whole ventricular system. Of nearly 50 injected embryos, over 70% displayed a similar distribution and intensity of GFP fluorescence on whole-mount brains and sections. The proportion of infected cells was quantified on cryosections through the E15.5 and E18.5 cerebral cortex, ganglionic eminence, striatum, septum, hippocampal formation, diencephalon as well as midbrain and hindbrain (Fig. 1C,D). This proportion was remarkably constant, ranging from 20 to 30% (Fig. 1D, left). Considering cell density (Fig. 1D, right) and volume of brain areas obtained by morphometric analyses (Wong et al., 2012), we calculated a total of about 1.3 × 10⁶ transduced cells as a sum of cortex, ganglionic eminence and hippocampal formation, 5 × 10⁵ in the midbrain, and 2 × 10⁵ in the hindbrain of a single E15.5 brain. This number of transduced cells provides an advantage for certain studies, e.g. proteome or transcriptome analyses after genetic manipulation.

Percentage of transduced cells was similar at E15.5 and E18.5 in all regions analyzed. In particular, for a well-studied germinal layer of the brain, the ventricular zone of the lateral cortex, we found that the proportion of GFP^{nls}+ cells, and their fluorescence intensity, was virtually unchanged over time (Fig. 1D, left), supporting the notion that lentiviruses allowed us to overcome the dilution of transgenes in proliferating cells typical of electroporation. Exposure to BrdU for 4 hours before sacrifice yielded a proportion of cells in S phase that was similar in GFP+ and GFP- cells at both developmental times (Fig. 1E). This proportion was also in line with previous measurements of cell cycle parameters (Takahashi et al., 1995), indicating that viral transduction per se did not alter cell proliferation.

Site-specific recombination by lentiviral injection

Temporal and spatial control of gene expression by site-specific recombination is perhaps the most versatile approach currently available to investigate gene function and to perform lineage tracing of progenitor cells in virtually any organ (Glaser et al., 2005; Joyner and Zervas, 2006; Morozov, 2008). However, this type of manipulation is laborious and time-consuming, requiring the generation of at least two transgenic mouse lines by which one provides tissue-specific expression of the recombinase (e.g. Cre or

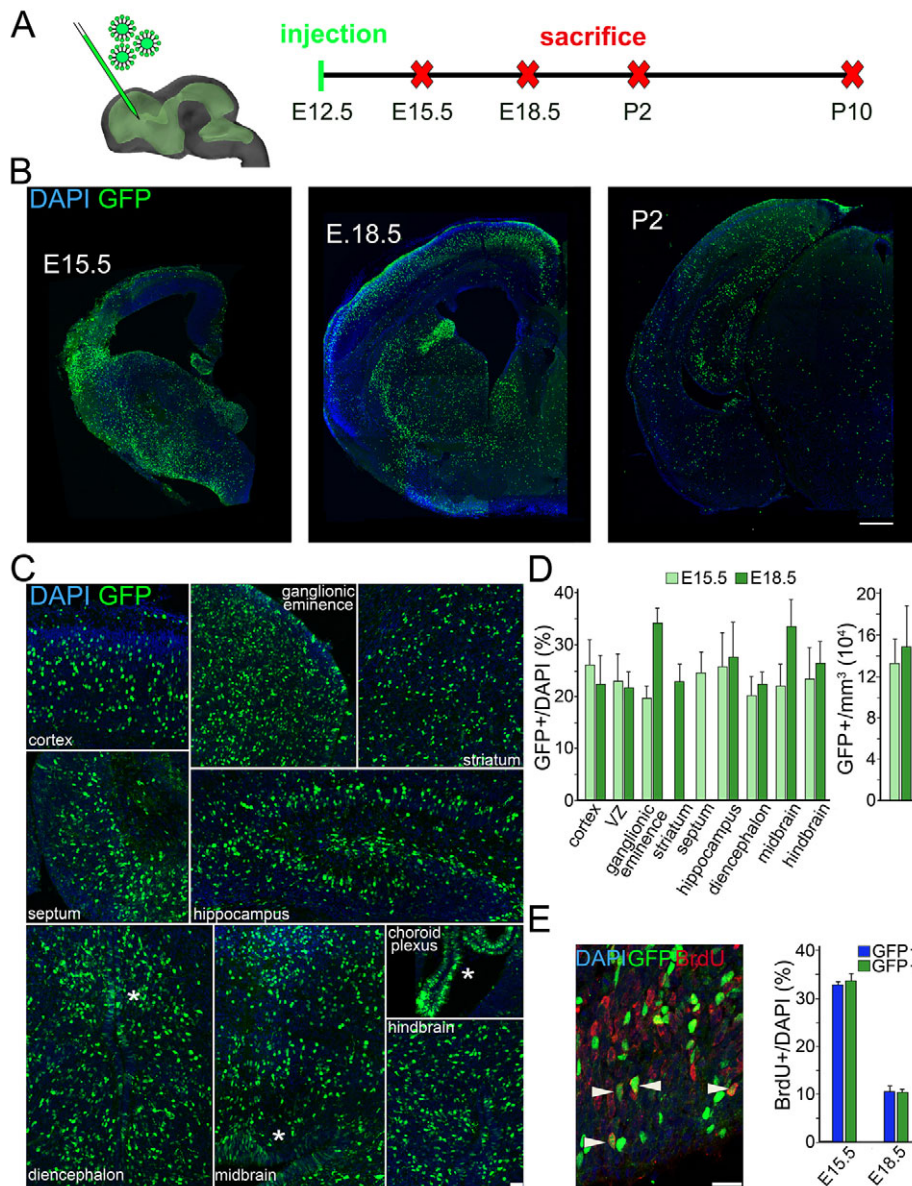


Fig. 1. Lentiviruses mediate widespread gene expression in the developing brain.

(A) Experiment layout with lentiviral injections performed at E12.5 and brains analyzed at E15.5, E18.5, P2 or P10. (B) Fluorescence pictures (top) of 30 μ m thick coronal sections of E15.5, E18.5 and P2 GFP^{nls}-injected brains (as indicated) showing GFP expression (green) and DAPI counterstaining (blue). Rostral, medial and caudal sections were chosen (from left to right). (C) High magnification-fluorescence pictures as in B showing GFP expression in specific brain areas (as indicated) at E18.5. (D) Proportions (left) of GFP^{nls}+ cells in different brain areas and developmental times as indicated. The right panel indicates the average density of cells in all areas. (E) Fluorescence picture of the E15.5 ventricular zone (left) and quantification (right) of BrdU+ cells (red) in GFP+ (white arrows) versus GFP- progenitors (red, green and blue, respectively). Scale bars: 300 μ m in B; 200 μ m in C; 30 μ m in E. Error bars indicate s.d.; $n > 3$ embryos. The brain cartoon was taken from the Allen Brain Atlas (www.brain-map.org). The choroid plexus and the third and fourth ventricles (asterisks in C) reproducibly displayed higher (70-80%) infectivity. VZ, ventricular zone.

tamoxifen-inducible Cre) and the second contains its substrate sequences (e.g. *loxP* sites). Nevertheless, the versatility of the Cre/*loxP* (or similar) systems (Glaser et al., 2005; Morozov, 2008) has led to the generation of an impressive number of mouse lines in which Cre is selectively expressed in the desired cell type, tissue, portion of an organ or entire organ systems (Heffner et al., 2012; Murray et al., 2012) (www.creportal.org) and genome wide, genetrap lines were generated to perform conditional mutagenesis (Branda and Dymecki, 2004; Hardouin and Nagy, 2000; Glaser et al., 2005) (www.genetrap.org; www.eucomm.org). Yet, while these mouse lines are available, any additional study based on the conditional manipulation of a new functional or reporter gene would necessarily require the generation of an additional *loxP*-containing line.

Site-specific recombination after local injection with viruses containing *loxP* sites in Cre-expressing mice has already been reported for certain areas of the adult brain (Artegiani et al., 2011; Cardin et al., 2010; Zhang et al., 2010b). Therefore, we investigated the use of lentiviral injection in the embryonic telencephalic

ventricle as a proof-of-principle of a practical alternative to transgenic mice to achieve widespread site-specific recombination in the whole brain.

To this aim we used a lentiviral construct encoding for a GFP whose nls was flanked by *loxP* sites (GFP^{lox}) (Artegiani et al., 2011) and injected viruses in E12.5 *Emx1*^{Cre} embryos in which Cre expression is restricted to the dorsal telencephalon (Iwasato et al., 2000). The proportion, distribution and fluorescence intensity of cells infected with GFP^{lox} viruses were assessed at E18.5, resulting in essentially identical results to previous experiments (Figs 1, 2). With regard to the subcellular localization of GFP, we observed that brain regions where *Emx1* is known not to be expressed (Gulisano et al., 1996) were characterized by cells with exclusively nuclear fluorescence whereas in dorsal telencephalic areas, including the cerebral cortex and the hippocampal formation, virtually all infected cells displayed cytoplasmic GFP (Fig. 2). Thus, lentiviral injection in currently available Cre-expressing lines allows widespread site-specific recombination in developing mouse embryos.

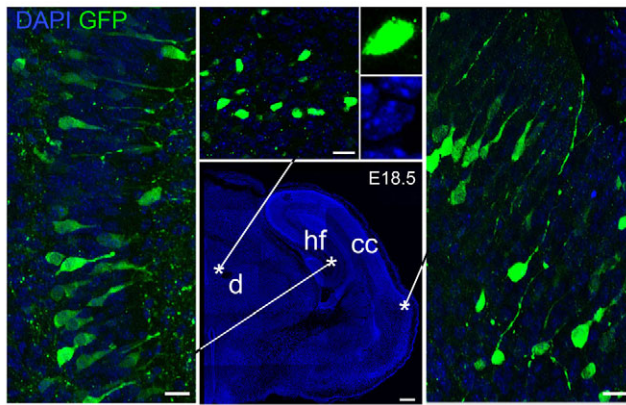


Fig. 2. Cre-mediated conditional mutagenesis by lentiviral injection. Fluorescence picture of a 30 µm thick coronal section of a E18.5, GFP^{lox}-injected *Emx1*^{Cre} brain showing the whole, DAPI-stained cerebral hemisphere (blue). Higher magnifications of the hippocampus (left), telencephalon (right) and diencephalon (top) are shown to appreciate cytoplasmic versus nuclear distribution of GFP (green). Cre-mediated redistribution of the GFP from the nucleus to the cytoplasm occurred exclusively in areas known to express *Emx1* (Gulisano et al., 1996). Scale bars: 200 µm and 25 µm in low and high power views, respectively. *n*>73 embryos. cc, cerebral cortex; d, diencephalon; hf, hippocampal formation.

Lentiviruses provide an additional powerful resource to manipulate gene expression during development

Here we have characterized the use of lentiviral injection as an alternative to the more commonly used adenoviral and retroviral carriers and reported the first use of site-specific recombination of a viral-delivered gene during embryonic development.

Injection of lentiviruses in the lateral ventricle during development led to highly reproducible, efficient and widespread infection comparable to the use of adenoviruses while using an integrating vector, thus overcoming: (1) the low infectivity of retroviruses; and (2) the lack of integration of adenoviruses. These two limitations constitute a disadvantage, particularly during embryonic development, when a substantial proportion of cells are characterized by a high proliferative activity. In fact, the genome of non-integrating adenoviruses, similarly to electroporated plasmids, is diluted by consecutive cell cycles, and although the ectopic protein may persist over a long period of time in postmitotic cells [1 year in the case of β -galactosidase (Shen et al., 2004)], the ectopic transgene will soon disappear in dividing cells, thus limiting the use of Cre-mediated recombination. However, the rather low infectivity of integrating retroviruses seems unlikely to be sufficient to induce major morphological changes at the level of whole organs. This low infectivity may be due to retroviruses targeting only dividing cells, and even if essentially all cells lining the ventricles divide during development, only a fraction is in mitosis at any given moment. In this context, retroviruses may also introduce bias in terms of targeting a synchronized population of mitotic cells.

Certainly, several limitations remain in the use of viral approaches: biosafety, limited packaging size of vectors, and the possibility that even integrated viral transgenes may be silenced over time. The latter has been reported to depend on methylation and can in turn be overcome by the use of UCOE-sequences (Zhang et al., 2010a). Moreover, infection will always be limited to a subpopulation of cells, and organs lacking a cavity may be more difficult to reach and efficiently target. Nevertheless, the possibility

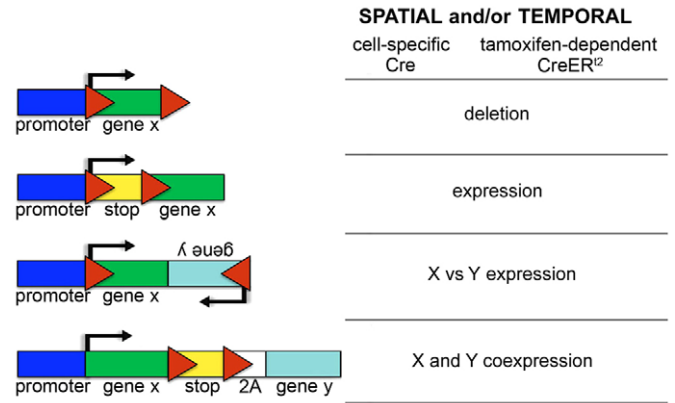


Fig. 3. Possible applications of conditional mutagenesis by lentiviruses. Diagrams (left) and triggered effect (right) of *loxP*-containing (triangles) lentiviral constructs to obtain spatial and/or temporal control of gene expression by (from top to bottom): (1) deletion of a transgene; (2) its expression; (3) switched expression from gene x to y; or (4) their co-expression by 2A peptides.

of obtaining high and widespread infectivity and stable integration provides a practical and fast alternative to the generation of transgenic mice to investigate gene function during mammalian development. In fact, our laboratory has manipulated the same genes in neural stem cells by all three overexpression systems discussed in this study, with electroporation allowing the fast assessment of cellular effects in a small brain area, generation of transgenic mice extending this effect to the whole organ, and viral injection leading to qualitatively identical cellular effects with, in our conditions, phenotypes of even a greater magnitude than transgenic mice (Lange et al., 2009; Nonaka-Kinoshita et al., 2013).

It is conceivable that our approach could be further diversified and optimized in future studies. For example, we used VSVG pseudotyped HIV-1 viruses characterized by a high tropism but the use of different envelope proteins, including those fused to single-chain antibodies (Lei et al., 2010), may allow the targeting of specific tissues and cell types. Whereas the use of *loxP*-containing lentiviruses in available Cre-expressing lines allows the use of our approach in applications as diverse as the *Cre/loxP* (or similar) system can provide (Fig. 3), spatial and/or temporal expression (or deletion) of one (or more) exogenous transgene(s) could be extended to the silencing of endogenous genes by expression of short hairpin RNAs triggering RNAi (Couto and High, 2010) or, eventually, to perform genome editing by Zn-finger nucleases (Urnov et al., 2010). By no means replacing conventional transgenic lines, our approach may thus serve as a proxy for widespread manipulation of gene expression in the entire brain with a timetable of weeks instead of years.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

Both authors conceived the project, analyzed the data and wrote the manuscript. B.A. executed all experiments.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.093823/-/DC1>

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