Citation: Molecular Therapy — Methods & Clinical Development (2014) **1,** 14047; doi:10.1038/mtm.2014.47

www.nature.com/mtm

ARTICLE

Comparative analysis of lentiviral vectors and modular protein nanovectors for traumatic brain injury gene therapy

María Luciana Negro-Demontel[1,2], Paolo Saccardo[3–5], Cecilia Giacomini[6], Rafael Joaquín Yáñez-Muñoz[7], Neus Ferrer-Miralles[3–5], Esther Vazquez[3–5], Antonio Villaverde[3–5] and Hugo Peluffo[1,2]

Traumatic brain injury (TBI) remains as one of the leading causes of mortality and morbidity worldwide and there are no effective treatments currently available. Gene therapy applications have emerged as important alternatives for the treatment of diverse nervous system injuries. New strategies are evolving with the notion that each particular pathological condition may require a specific vector. Moreover, the lack of detailed comparative studies between different vectors under similar conditions hampers the selection of an ideal vector for a given pathological condition. The potential use of lentiviral vectors versus several modular protein-based nanovectors was compared using a controlled cortical impact model of TBI under the same gene therapy conditions. We show that variables such as protein/DNA ratio, incubation volume, and presence of serum or chloroquine in the transfection medium impact on both nanovector formation and transfection efficiency *in vitro*. While lentiviral vectors showed *GFP* protein 1 day after TBI and increased expression at 14 days, nanovectors showed stable and lower *GFP* transgene expression from 1 to 14 days. No toxicity after TBI by any of the vectors was observed as determined by resulting levels of IL-1β or using neurological sticky tape test. In fact, both vector types induced functional improvement *per se*.

Molecular Therapy — Methods & Clinical Development (2014) **1**, 14047; doi:10.1038/mtm.2014.47; published online 15 October 2014

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of mortality and morbidity in both industrialized and developing countries, being of greater importance in poorer countries.[1,2] TBI is frequently referred to as the "silent epidemic" because beyond symptoms such as paralysis, additional complications affecting intellectual ability, sensation, language, and emotion may not be readily apparent. In fact, studies that included several European countries showed that TBI resulted in one of the highest injury burden pathologies due to permanent disability,[3] and among the highest costs for health systems.[4] Extensive efforts have been made to develop neuroprotective therapies for this devastating disorder, but despite interesting preclinical results, no successful outcomes have been observed in human clinical trials to date.[1,2] Following an initial mechanical insult, focal TBI results in complex delayed secondary progressive injury due to anatomical, neurochemical, metabolic, inflammatory, and cellular changes that account for many of the neurological deficits observed.[1,2,5]

The introduction of functional genes into an organism for treating or correcting a range of pathologies has emerged in the past few decades as a powerful tool.[6,7] Worldwide, over 1,800 gene therapy clinical trials are either ongoing or have been approved.[8] Thirty-six of these trials involve using different gene therapy vectors for treating chronic disorders of the central nervous system (CNS).[8,9] One main focus in gene therapy research is the development of appropriate delivery systems,[6,7,10] which can be complicated and is considered to be a bottleneck for achieving desired clinical outcomes. Viral vectors tend to be the most effective carriers of nucleic acids into foreign cells, as they are evolutionary optimized for this purpose. One of the most popular types of viral vectors being developed for treating CNS disorders are HIV 1-derived lentiviral vectors.[11,12] They have been tailored in recent years to have reduced biological risk and to have features that make them excellent delivery vehicles including applications for treating CNS disorders. These vectors are safe, have low immunogenicity and transduce postmitotic cells.[13–15] Nonintegrating lentiviral vectors, in particular, show promising features for use in treating CNS disorders.[16,17] Nonviral vectors have also gained attention,[6,7,10,18] and in particular, vehicles based on multifunctional proteins in DNA complexes. These vectors constitute a very versatile type of carrier for therapeutic nucleic acids, constructed by combining appropriate functional domains fused as a single polypeptide chain.[19] This approach has generated the first prototypes of modular recombinant protein nanovectors where the integrated domains enable the construct to mimic an infective viral cycle. In this way, delivery of nucleic acids is effectively targeted. This type of nanoparticle has also been termed "artificial

[1]Neuroinflammation and Gene Therapy Laboratory, Institut Pasteur de Montevideo, Montevideo, Uruguay; [2]Departmento de Histología y Embriología, Facultad de Medicina, UDELAR, Montevideo, Uruguay; [3]Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Barcelona, Spain; [4]Department de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Barcelona, Spain; [5]CIBER en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Barcelona, Spain; [6]Cátedra de Bioquímica, Departamento de Biociencias, Facultad de Química, UDELAR, Montevideo, Uruguay; [7]Royal Holloway University of London, Egham, UK. Correspondence: H Peluffo (hugo.peluffo@pasteur.edu.uy)
Received 2 May 2014; accepted 28 August 2014

virus".[6,10] The modular nature of such constructs allows for selection and incorporation of well-characterized peptides with different features so as to improve function by iteratively redesigning the construct.[18,20] Several nanovectors of this type can be successfully used to transfect cells *in vitro*[10,21–25] and to result in the expected therapeutic effect when used *in vivo*.[26–29] There is thus great interest in developing the potential of these nanovectors for use in a clinical context. One such modular nanovector, *i.e.*, NLSCt, is based on a tetrameric carrier protein including β-Galactosidase engineered with a polylysine K_{10} tail which binds to and condenses DNA, a NLS motif for nuclear localization and an prototypic integrin-interacting RGD domain, which binds to membrane integrins to promote cell internalization.[30] This vector was shown to induce biologically relevant concentrations of transgenic protein after acute excitotoxic brain injury.[26,28,31,32] The NLSCt protein RGD interacting motif could induce neuroprotection *per se* raising the interesting possibility of utilizing vector functional motif activities to rapidly direct neuroprotective actions on the specific target.[26,27] The modular principles underlying the NLSCt vector were further improved by generating two smaller nanovectors termed HKRN and HNRK. These polypeptides are based on alternative combinations in a single small polypeptide of domains RGD, NLS, and K_{10} and an additional poly-histidine domain H_6 that provides for endosomal escape and is also useful for purification of these constructs. These nanovector constructions could be used to achieve significant levels of transgene expression in cultured cells,[10] but testing *in vivo* transfection properties have not been done.

Importantly, the original hypothesis that a single very efficient gene therapy vector could be used for most applications has been revised to the idea that each particular pathological condition may require a specific vector. For instance, for treatment of acute traumatic CNS injuries, the vector should induce a rapid but not permanent induction of transgene expression, it should not be proinflammatory as inflammation is a key mediator of neuropathology and a specific cell type may or may not needed to be targeted depending on the mechanism of action of the transgene.[1,2] In addition, other less obvious variables have become important to consider: (i) which are the optimal levels of transgene expression? (ii) What is the ideal time window for expression? (iii) Should the vector induce widespread or a localized transduction? Moreover, the lack of detailed comparisons of different vector types in the same model under identical conditions continue to hamper selection of the most appropriate vector for particular pathological conditions.

In this context, the aim of the present study was to quantify, under similar conditions, the potential of lentiviral vectors versus the modular recombinant nanovectors NLSCt, HNRK, and HKRN as gene therapy vectors for TBI. We first describe the effect of several variables such as protein/DNA mass ratio, incubation volume, and presence of serum or chloroquine in the transfection medium on nanovector formation and transfection efficiency *in vitro*. Moreover, using the controlled cortical impact (CCI) model for TBI, we evaluated the level and time-window of transgene expression, induction of inflammation, and toxic effects induced by both types of vector.

RESULTS

Modular protein nanovectors HNRK and HKRN were incubated with plasmid DNA at room temperature, to generate self-organized nanoparticles of ca. 80 nm.[10] Since different protein/DNA mass ratios can result in different transfection efficiencies,[10] we analyzed transfection efficiency of HNRK and HKRN vectors formed at different protein/DNA mass ratios. This was done taking into account that protein/DNA mass ratios of 2.5 and 1.5 of HNRK and HKRN, respectively, result in complete retardation of total DNA in the sample in gel retardation assays.[10] HEK293T cells were incubated with nanovectors loaded with 2 µg of a GFP-expressing plasmid transcriptionally controlled by a CMV promoter. Percentage of cells transfected and the relative fluorescence intensity/cell were analyzed 24 hours after transfection. The HNRK and HKRN vectors purified from bacterial inclusion bodies had increased transfection efficiency when compared to the same vectors purified from soluble bacterial fractions (HNRKs), or when compared to the similar vector NLSCt or to naked DNA (Figure 1a). Increased protein/DNA mass ratios in nanocomplexes formed resulted in increased transfection efficiency with optimal ratios of 4.5–5 (Figure 1a), and declining efficiency at higher ratios thereafter (not shown). Interestingly, although a higher percentage of cells were transfected with lipofectamine than with HNRK or HKRN nanocomplexes, relative fluorescence intensity/cell was similar for the three groups at 4.5–5 protein/DNA mass ratios (Figure 1b). Also, there was no decrease in cell viability after treatment with nanocomplexes (not shown). We next transduced HEK293T cells with third-generation lentiviral vectors pseudotyped with VSV envelope protein. These vectors carried the same expression cassette used in the nanovector transfection assay, and were analyzed 1 and 3 days post-transfection/transduction (dpt) for percentage of cells transduced and for relative fluorescence intensity/cell. Transfection of cells with HNRK or HKRN resulted in maximal transgene expression at 24 hours (not shown), however and as previously reported lentiviral vectors required at least 3 days for full transgene expression (Figure 1c,d).[16] Surprisingly, GFP was detected as early as 1 dpt for all multiplicities of infection (MOIs) tested. Cell toxicity was observed at 3 dpt for the highest MOI tested (*i.e.*, MOI = 4.4, data not shown). HEK293T cell transduction efficiency, however, was higher for lentiviral vectors (at MOI 1, there are 1.4×10^6 to 1.4×10^7 copies of the *gfp* gene in transduced cells according to QPCR data derived calculations in ref. 33) compared with the nanovectors (the 2 µg DNA used per well corresponds to 2.4×10^{11} copies of *gfp* gene) in terms of percentage of transduced cells (Figure 1a–d). In terms of relative fluorescence intensity/cell, qualitative microscopic observations confirmed the increased intensity after lentiviral transduction when compared to nanovector transfection. Comparative quantitative data are shown in Table 1.

Transfection efficiency of nanovectors for applications in gene therapy relies on their ability to escape endosomes after being endocytosed. We show that transfection efficiency with both HNRK and HKRN can be increased, especially the HNRK vector, using the prototypical endosome destabilizing agent chloroquine (Figure 2a,b). Serum proteins are another factors known to affect transfection efficiency for different vectors.[34] We show that the presence of 10% serum during transfection of HEK293T cells results in an ~40 and 35% decrease in the transfection efficiency for HNRK and HKRN, respectively (Figure 2c,d).

Another limiting factor for *in vivo* administration of these vectors is the volume that can be injected into CNS parenchyma due to very limited extracellular space. Thus, we evaluated the effect of nanocomplex formation in a reduced volume. A reduction in nanocomplex formation volume, *i.e.*, from 200 to 30 µl OPTIPRO medium, however, resulted in 52 and 42% decreases in HEK293T cell transfection efficiency for HNRK and HKRN nanovectors, respectively (Figure 3a,b). Another important variable that can affect nanocomplex formation and transfection efficiency is protein/DNA incubation time. Analysis of HNRK/DNA nanocomplex particles formed using dynamic light scattering (DLS) and transmission electron microscopy (TEM) indicated particle size tended to be less variable when produced in 200 µl OPTIPRO medium compared with being

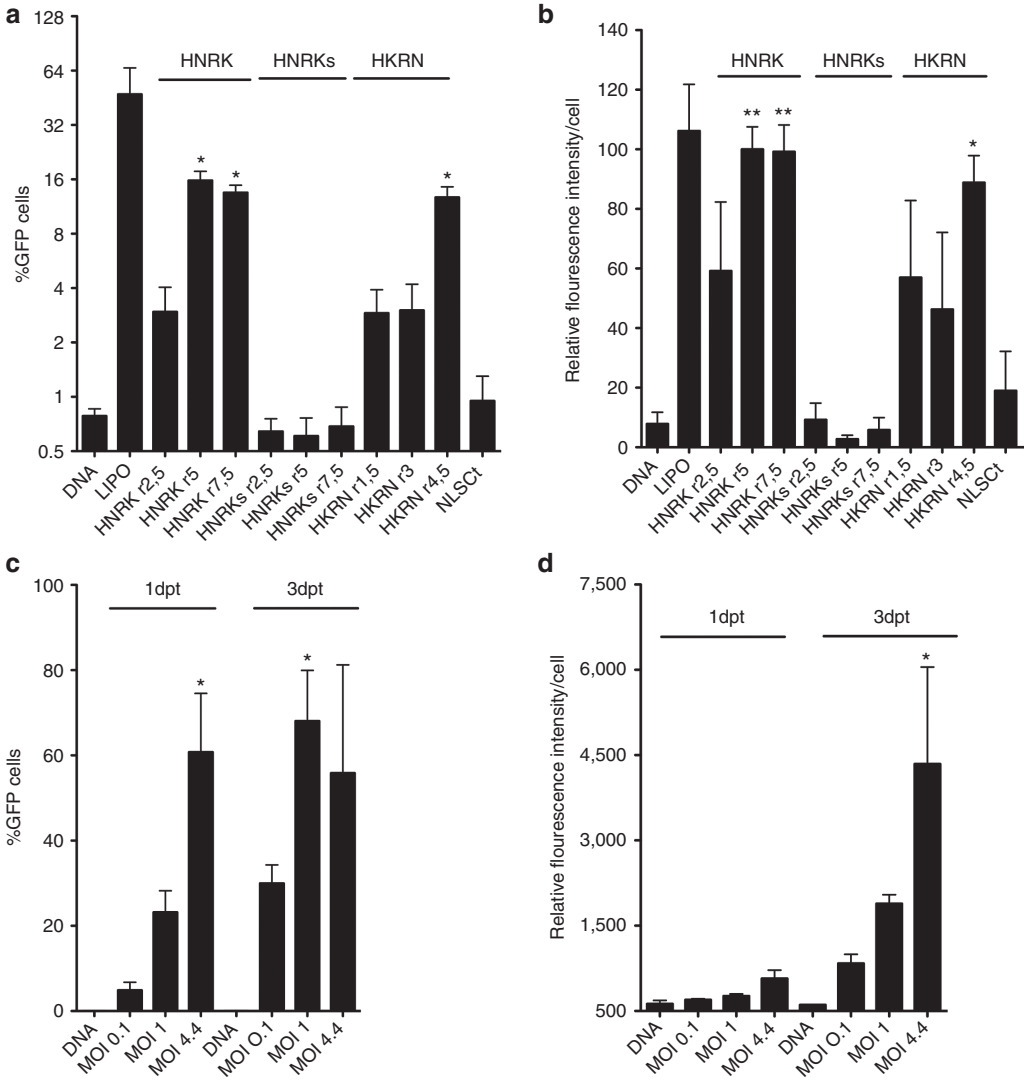

Figure 1 *In vitro* transfection and transduction efficiency of vectors. HEK293T cells were incubated for 4 hours with DNA, lipofectamine (LIPO), or modular protein nanovectors at different protein/DNA mass ratios (ratios from 2.5 to 7.5 are shown), and transfection efficiencies (**a**) and relative fluorescence intensity per cell (**b**) were quantified 1 day later by flow cytometry. DNA concentrations used (2 µg/well, 2.4×10^{11} copies of *gfp* gene) were the same in all cases so as to compare transfection efficiencies. HNRK and HKRN vectors were purified from insoluble bacterial fractions and compared with HNRK obtained from soluble bacterial fractions (HNRKs). In parallel experiments, HEK293T cells were incubated with lentiviral vectors at different multiplicities of infections (MOIs) (at MOI 1, there are 1.4×10^{6} to 1.4×10^{7} copies of the *gfp* gene) and transduction efficiencies (**c**) and relative fluorescence intensity per cell (**d**) were quantified at 1 and 3 days post-transduction (dpt). **a, b**: **P* < 0.05 when compared with NLSCt, DNA, or HNRKs; **c, d**: **P* < 0.05 when compared with DNA.

Table 1 Comparative efficiency of transfection/transduction of HEK293T cells at 3 dpt with lentiviral vectors and HNRK nanovectors

Treatment	*Estimated gfp gene copy number*	*% of cells expressing GFP compared to lentiviral vectors*	*Median GFP Fluorescence intensity/cell*	*Mean GFP Fluorescence intensity/cell*
Lentiviral vectors MOI 1[a]	1.4×10^{6}–1.4×10^{7}	100.0 ± 19.0	456.1 ± 16.2	1,593.0 ± 24.7
HNRK 10 µg + 2 µgDNA	2.4×10^{11}	11.3 ± 0.4	17.1 ± 0.8	646.0 ± 31.8
HNRK 1 µg + 0 .2µgDNA	2.4×10^{10}	0.4 ± 0.1	14.9 ± 1.2	240.8 ± 201.2
HNRK 0.1 µg + 0.02 µgDNA	2.4×10^{9}	0.2 ± 0.0	11.5 ± 0.7	89.0 ± 77.5

[a]The multiplicities of infection (MOI) 1 titer obtained in HeLa cells as described in Materials and Methods and used throughout this work corresponds to titer MOI 20 in HEK293T cells, and the latter has been used to calculate the number of *gfp* gene copy number.

produced in 30 µl phosphate-buffered saline (PBS), the vehicle used for *in vivo* administration of vectors (Figure 3c,d). There were no significant differences in nanocomplex formation with either 20 minutes or 5 hours incubation times for either condition (200 µl OPTIPRO or 30 µl PBS). Accordingly, no differences were observed in HEK293T cell transfection efficiency of HNRK/pDNA nanovectors

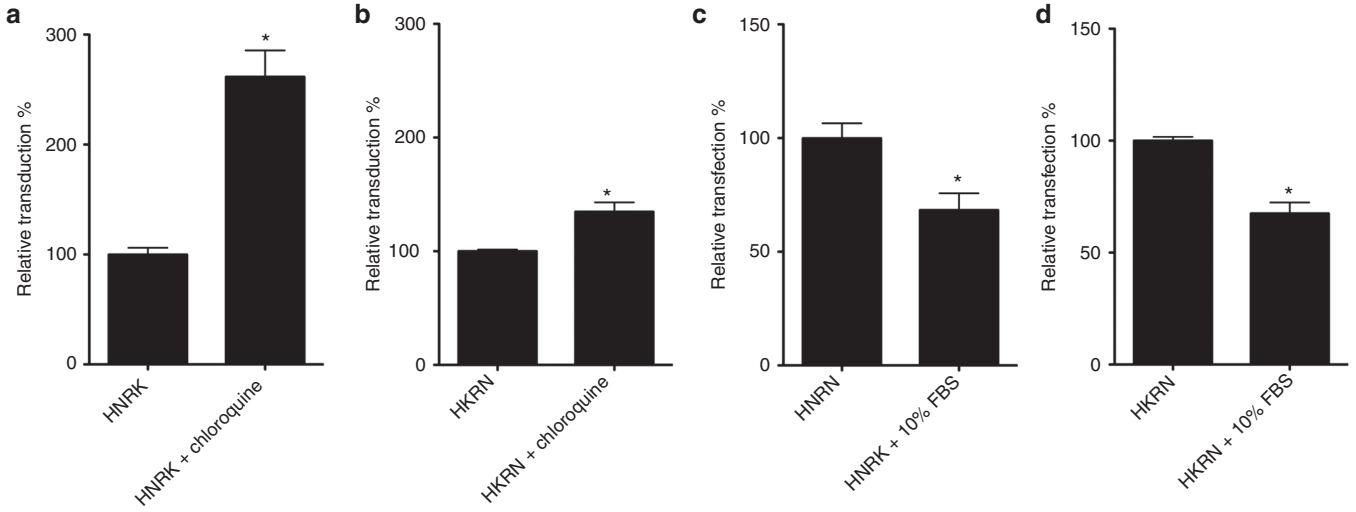

Figure 2 Effects of chloroquine and fetal bovine serum (FBS) on transfection efficiency of protein nanovectors. HEK293T cells were incubated for 4 hours with the modular protein nanovectors HNRK (**a, c**) or HKRN (**b, d**) in the absence or presence of the endosome disrupting agent chloroquine (**a, b**), or in absence or presence of 10% FBS (**c, d**). One day later, transfection efficiencies were quantified by flow cytometry. *$P < 0.05$ when compared to vectors in the absence of chloroquine or FBS.

formed in 200 µl OPTIPRO over 20 minutes versus those formed over 5 hours (Figure 3e). Nanocomplex size observed by TEM and hydrodynamic diameter observed by DLS differed. While TEM images showed particles to be between 20–200 nm, DLS indicated particles to be between 1–2 µm suggesting clustering or aggregation of particles.

Selection of an appropriate vector for a given gene therapy application is critical. In order to determine which vector is most suited for treatment of TBI, we compared several important variables of HNRK/pDNA nanovectors and third-generation lentiviral vectors under identical conditions using a CCI TBI injury model. The previously described nanovector, NLSCt/pDNA, was also included as an internal standard. This vector was shown to be able to induce transgene expression after acute excitotoxic brain injury, and to result in producing significant biological effects.[26,28,32,35] Comparisons of relative levels of GFP protein after CCI lesion were made by enzyme-linked immunosorbent assay (ELISA) of samples from untreated control rats and those receiving a 4 hours delayed intraparenchymal injection of nanovectors NLSCt/pDNA or HNRK/pDNA or lentiviral vectors. GFP protein was detected in all three cases at 1 and 14 days postlesion (dpl) (Figure 4a). The GFP level at 1 dpl was similar in NLSCt/pDNA and HNRK/pDNA injected animals, tending to decrease in NLSCt/pDNA-treated animals but to increase in HNRK/pDNA treated-animals at 14 dpl. The GFP level at 1 dpl after lentiviral injection tended to increase compared with both NLSCt/pDNA- and HNRK/pDNA-treated animals. There was a 100-fold increase in GFP protein levels comparing 1 and 14 dpl after lentiviral injection. Remarkably, there was also a 100-fold increase in GFP expression in animals injected with the lentiviral vectors compared with HNRK/pDNA-treated animals at 14 dpl. GFP levels in different animals of the same treatment groups at 14 dpl showed significant differences despite carefully controlled injection procedure and use of same vector batches. In the case of nanovectors, due to experimental reasons, incubation time for self-assembly of protein and pDNA varied from 20 minutes to 4 hours. However, there was no correlation for time of assembly and *in vivo* transgene expression (data not shown). Detection of HNRK vector by western blot at 1 dpl showed the presence of the protein in only one of four animals analyzed, indicating that the vector may be rapidly degraded *in vivo* (not shown).

Many gene therapy vectors induce inflammation, which may result in toxicity due to the treatment. It is very important to avoid these effects in treating TBI, where inflammatory reactions are key responses in development of subsequent neuropathology. Determination to what extent our vectors elicits inflammatory responses is thus critical for their development. We analyzed levels of IL-1β, the prototypic proinflammatory marker, after CCI and injection of the different vectors. No significant differences were observed comparing vehicle-injected and nanovector-injected animals (Figure 4b). To further evaluate possible toxic effects of vectors, the sticky tape functional test was performed to evaluate motor and sensory function (Figure 4c,d).[36] As previously described, when this test was done with both nonlesioned animals or on the ipsilateral forepaw of lesioned animals, a very rapid (2–5 seconds) detection and removal of the tape results (not shown). After CCI, animals show increased time for detection and removal of the sticky tape placed on the contralateral forepaw. In accordance with studies on neuroprotection after excitotoxic brain lesions,[26,27] NLSCt/pDNA-treated animals in fact displayed less pronounced functional compromise at 10 days after CCI (Figure 4c). Administration of the nanovector HNRK/pDNA also resulted in less pronounced functional compromise at 10 dpl. Interestingly, animals subjected to CCI spontaneously recovered from the neurological deficits measured by this test over time, hindering further evaluation after 14 dpl (not shown). A very similar profile was observed after lentiviral vector injection, showing less pronounced functional compromise at 14 dpl (Figure 4d).

DISCUSSION

Detailed comparative studies for evaluating the potential of different vectors for gene therapy treatment of specific pathologies are urgently needed in the current gene therapy scenario. Here, we evaluate two promising vector types, namely modular recombinant nanovectors and lentiviral vectors, for treatment 4 hours after TBI using a CCI model. The gene therapy strategy involves a protocol similar to that used used for Parkinson's disease clinical trials, including carefully controlled stereotaxic injection of a given vector into the brain lesion penumbra. Vectors administered in this study exhibited significant brain GFP protein levels 1 day after treatment

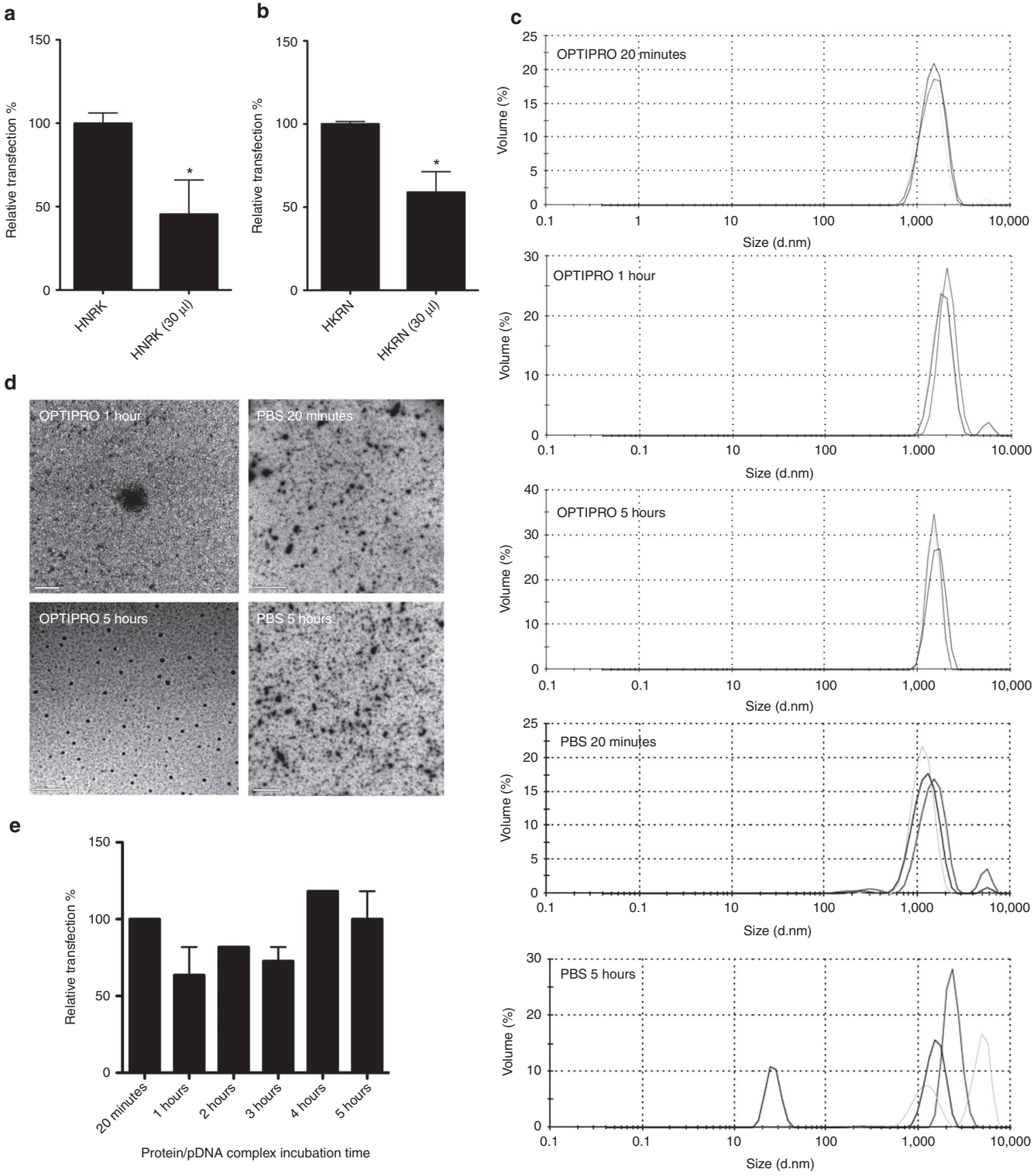

Figure 3 Effect of incubation volume and medium on the protein nanovector complex size and transfection efficiency. HNRK and HKRN modular protein nanovectors were allowed to self-assembly during a 20-minute incubation of HNRK and HKRN proteins with plasmid DNA in either 200 µl OPTIPRO medium or 30 µl PBS. Assembled nanovectors were then used to transfect HEK293T cells and transfection efficiencies were quantified 1 day later by flow cytometry (**a, b**). HNRK nanovector stability was analyzed after self-assembly in 200 µl OPTIPRO medium or 30 µl phosphate-buffered saline, and for 20 minutes to 5 hours incubation periods. The products formed were analyzed by dynamic light scattering (**c**) or transmission electron microscopy (**d**) as indicated. Transfection efficiency of HNRK nanovector after self-assembling in 200 µl OPTIPRO medium for 20 minutes to 5 hours was analyzed by flow cytometry 1 day after transfection (100% represents transfection efficiency for nanovectors formed in the 20 minutes incubation time). *$P < 0.05$ when compared to vectors allowed to self-assembly in 200 µl OPTIPRO.

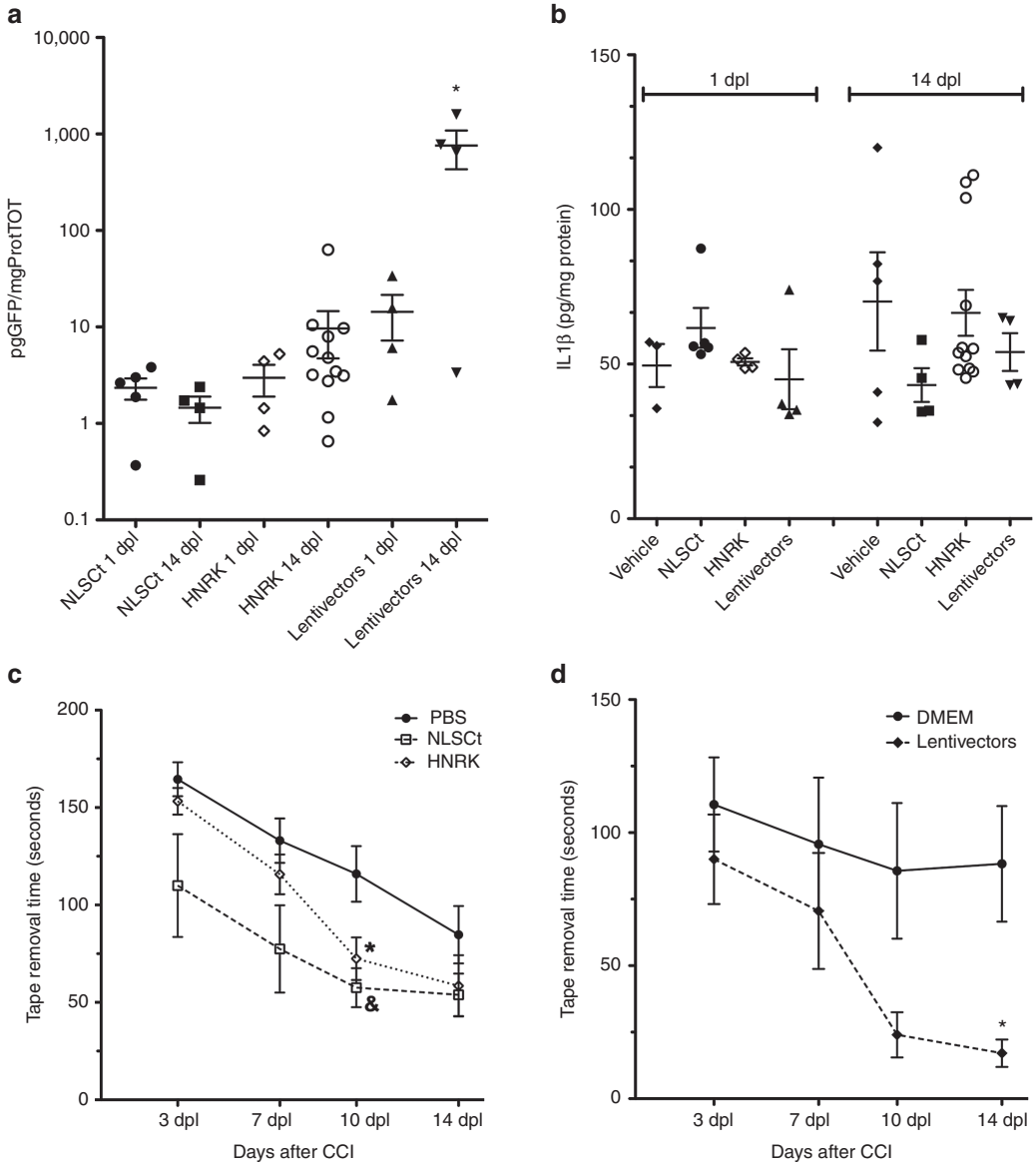

Figure 4 Comparative efficiency and toxicity of protein nanovectors and lentiviral vectors after a controlled cortical impact (CCI). Four hours after a CCI, animals were intracortically injected with either protein nanovectors or lentiviral vectors at three locations in the lesion border (see Materials and Methods). At 1 and 14 days dpl, animals were sacrificed and levels of GFP protein analyzed by enzyme-linked immunosorbent assay (ELISA) (**a**, $^*P < 0.05$ when compared to all other groups). The effect of vectors on proinflammatory responses after CCI treatment was analyzed by measurement of IL-1β expression levels by ELISA and compared with vehicle-treated CCI animals (**b**), and no significant differences were observed. The putative toxicity of the vectors after a CCI was evaluated by the sticky tape functional test (**c, d**). The mean time elapsed for removal of the sticky tape on the lesioned forepaw is shown for each group from 3 to 14dpl. Phosphate-bufffered saline (PBS) $n = 12$, Dulbecco's modified Eagle medium (DMEM) $n = 8$, NLSCt $n = 7$, HNRK $n = 13$, lentivector $n = 9$. $^*P < 0.05$ when compared to PBS at 10 dpl (**c**); $^*P < 0.05$ when compared to DMEM at 14 dpl (**d**).

and for at least 2 weeks. Transgene expression levels were much higher and more variable using the lentiviral vectors, and no apparent increase in inflammation or neurodegeneration was observed after treatment with any of the vectors. In fact, the injection of either vector type *per se* induced functional recovery of the animals.

There remain several obstacles that need to be better understood for the development of such vectors for use in therapeutic applications and other *in vivo* conditions. Direct injection of vectors into the brain parenchyma limits their delivery to a relatively small volume of tissue due to the very limited CNS extracellular space. Direct intraparenchymal injection of vectors into the human brain by stereotactic methods, as in Parkinson's disease clinical trials, on the other hand can be accomplished without important permanent side effects or intracerebral hemorrhages, and also have resulted interesting clinical outcomes.[37–40] In the human brain, up to 80 µl can be injected at a given site.[37] No more that 5 µl, however, is normally injected per injection site in rat brain. To increase amount and distribution of vector administered in our study, three injections of 2 µl each were administered in the anterior, lateral, and posterior lesion penumbra regions. In an attempt to further concentrate vector administered for a given injection, we conducted our standard NHRK nanovector self-assembly normally done in a 200 µl volume in a smaller volume of 30 µl. Self-assembly of vectors into nanoparticles by combining modular protein with pDNA is a very interesting and useful phenomenon enabling assembled vectors to act as efficient vehicles for nucleic acid transport to the cell nucleus.[10,24]

Furthermore, and consistent with other studies,[10,24] we found an optimal protein/pDNA mass ratio for nanovector self-assembly and transfection efficiency. The optimal protein/pDNA mass ratio for assembly and transfection efficiency was ca. 5 for both HNRK and HKRN protein nanovectors. There are 2,284 HNRK or HKRN proteins/plasmid molecule, resulting in a predicted 7,529 net positive charges/nanovector molecule at physiological pH. Kenny and colleagues have shown that a similar anionic Neurotensin receptor targeted nanovector showed increased diffusion in noninjured brain parenchyma when compared to its cationic counterpart.[41] This suggests the interesting possibility that a similar approach with our nanovector could increase its brain distribution. However, one has to consider if a widespread distribution is always desired, as under some conditions a specific nuclei or region of the brain may need to be targeted. Moreover, after acute brain injury significant edema occurs, potentiating diffusion of molecules in the entire lesioned area. Accordingly, diffusion of vector in the entire lesioned area has been shown for the 249AL vector, which is almost identical to the NLSCt vector used here.[42]

HNRK/pDNA or HKRN/pDNA nanocomplex formation in 30 µl PBS rather than 200 µl OPTIPRO resulted in the formation of complexes with about 50% decreased transfection efficiency using the same conditions for transfection of cultured HEK293T cells. This decrease in transfection efficiency for particles formed in the smaller volume correlated with higher variability in particle size as determined by TEM or DLS. These changes in apparent particle size may affect transfection efficiency and may reflect differences in pDNA compaction, altered protein functional domain exposure, particle clustering or pDNA release from particles inside the cell. Increased particle size may also affect the diffusion in the lesioned brain. How variation in particle size may affect their transfection efficiency will require further study. Understanding processes and mechanisms of nanocomplex formation might allow for refining efforts to form more uniform particles in smaller volumes and that have improved transfection efficiency. Lentiviral particle preparations do not aggregate when added to cell cultures,[43] however comparing possible aggregation after intraparenchymal injection should be analyzed.

HNRK and HKRN modular proteins display a cell-targeting domain (RGD), a DNA attaching/condensing domain (K_{10} tail), a nuclear localization domain (NLS) and a poly-histidine tail that can stimulate endosomal escape. However, endosomal escape may still need to be improved further to increase transfection efficiency. In this regard, we included endosome-disrupting agent, chloroquine, in the cultures to evaluate putative increases in transfection efficiency. Chloroquine addition indeed resulted in increased transfection efficiency for both HNRK and HKRN nanovectors, with more pronounced effects for the HNRK vector. This suggests engineering the HNRK vector can be further improved using other known endosome escaping motifs.[18] Alternatively, chloroquine could be co-injected with assembled nanovector *in vivo* to increase transfection efficiency, as chloroquine has been approved for use in human subjects. Finally, instead of performing an intracerebral injection, protein nanovectors could be administered by an intravenous route and may reach the brain after TBI where the blood–brain barrier has been altered. Many vectors fail to induce significant transgene expression levels when combined with serum, including lentiviral vectors.[34] Lentiviral vectors are inactivated by the serum complement system[34] and vector activity decreased ~2 logs within 45 minutes after intravenous administration.[44] This problem can be overcome using lentivectors pseudotyped with the VSV-G protein subjected to directed evolution,[45] or by conjugating the VSV-G envelope protein with poly(ethylene) glycol.[44] Nonviral vectors may also show decreased transfection efficiency *in vivo* due to serum interactions, and the addition of different shielding molecules, *e.g.*, *N*-succinyl-chitosan/polyethylenimine can be included to contribute to enhanced stability in the presence of serum.[46] Here, we show that the presence of 10% serum induced a 35–40% decrease in HEK293T cell transfection efficiency for both nanovectors. This suggests that intravenous administration may need further protection of the vectors as described above to be an effective way to induce efficient transgene expression in target brain cells. Unpublished results from our laboratory indicate that intravenous administration of the NLSCt and the HNRK vector induced *gfp* transgene expression in several organs, but this issue needs further *in vivo* testing.

Only few reports have focused on gene therapy applications for acute CNS injuries like TBI. In some studies, expression of a protective transgene is induced prior to TBI. Minnich *et al*. [47], e.g., overexpressed GDNF with adenoviral vectors (AdV) 1 week before CCI, and observed neuroprotection for up to 2 weeks after.[47] However, when GDNF AdV vectors were injected immediately after CCI, no neuroprotection was observed unless L-arginine was included in the injection administrated.[48] A comparative gene therapy study using a fluid percussion injury TBI model was recently reported by Siddiq *et al*.[49], were overexpression of a transactivator of vascular endothelial growth factor A using AdV and adeno-associated viral vectors was performed. Interestingly, the two vector types have different temporal transgene expression profiles. AdV vectors show high initial levels of transgene expression but that is attenuated at longer time point,[50] while adeno-associated virus (AAV) exhibit slower but long-lasting transgene expression and as well as fewer proinflammatory responses.[51] Siddiq *et al*. injected identical pfu/ml of either vector in 2 µl per injection site 20 minutes after the fluid percussion injury and observed that overexpression of VEGF-A using both vectors had beneficial effects, and included a 30-day functional improvement mediated by AVV. In this study, different parameters for AdV and AVV were analyzed in treated animals precluding a comprehensive comparison of the two vectors.

AdV expression of a transgene under the control of a CMV promoter in the CNS was shown to peak at 4–8 days after injection and decline by 14 days.[50] This decline in expression was found to be a consequence of immunological responses directed against cells with transgenes.[52] The use of CMV promoter driven integration proficient and deficient lentiviral vectors[16] as well as CBA promoter driven AVV[51] for CNS transduction were shown to induce persistent transgene expression in immunocompetent animals, and should thus be useful as vectors for targeting CNS tissues. Here, we show that even after TBI, where inflammation is greatly potentiated by lesion progression, transgene expression did not decline for at least 14 days using any of the vectors. In fact, we also found that there was a significant increase in transgene expression mediated by lentiviral vectors at 14 dpl. There was also a trend to increased expression with use of the HNRK vector compared with use of the NLSCt vector. An increase in transgene expression was also evident from 1 to 3 days in lentiviral transduced HEK293T cultured cells. Dorsal root ganglion neurons also showed both an increase in % GFP positive cells and in GFP fluorescence/cell from 2 to 8 days after transduction using the same lentiviral system.[16] In our study, however, GFP protein was detected at 1 dpt both *in vitro* and *in vivo*. Transgene expression by lentiviral vectors has not been documented previously at 1 dpt, and then GFP protein observed after lentiviral transduction might be due to carry-over during vector production or pseudotransduction. Independently of the origin of GFP, its presence at 1 dpt may contribute to neuroprotection after TBI, were the therapeutic window is narrow and thus a rapid treatment is essential.

Variability in transgene expression levels despite using highly controlled procedures is a concern that remains difficult to resolve. However, data showing individual expression levels are normally not reported and thus further underscore the importance of this problem.[53] We observe that lentivectors and to a lesser extent protein nanovectors can show significant variation in the levels of transgene expression. Each animal received three independent 2 µl injections using a Hamilton syringe and an automatic nanoinjector at 0.4 µl/minute and leaving the syringe in place for 5 additional minutes, making it highly unlikely that, *e.g.*, blockage of the syringe or reduced diffusion would account for such variation in transgene expression. Another potential contribution to this variability could be the TBI procedure or the evolution of delayed secondary progressive injury. However, injection of either integrative proficient or integrative deficient lentiviral vectors into nonlesioned spinal cord of mice and rats also resulted in some animals having high levels of transgene expression and others with very low levels of expression. This was seen despite use of a carefully controlled injection procedure and use of identical vector batches,[16] as in the present study. Individual variations in immune responses against the transgene or the vectors, or fluctuation of injections near or farther away from blood vessels may account for differences in GFP expression levels observed between animals. Taken together, this indicates that variability may be due to unknown technical issues or to the vectors, but not to TBI-related variables. Other methods like convection enhanced delivery associated with different catheters used in clinical trials delivering nanoparticles or viral vectors may provide the means for more reproducible delivery of vectors.[41,54] In addition, no correlation was found between self-assembly reaction times for protein and pDNA and resulting GFP transgene expression *in vivo* (not shown). Also, no significant variation in transfection efficiency for HEK293T cells was found for either 20 minutes or 5 hours self-assembly reaction times, suggesting that this variable does not account for the *in vivo* transgene level expression variations. Further studies need to be performed to establish to what extent variations in *in vivo* transgene expression levels are due to experimental conditions in the overall gene therapy procedure in small animals or associated to other variables such as immune response.

The time frame for TBI treatment is short in most neuroprotective strategies, and thus using a gene therapy approach should involve vectors that rapidly express a transgene. In the fluid percussion injury TBI model mentioned above, where vectors were administered 20 minutes after by intraparenchymal injection,[49] AdV and AVV were shown to induce transgene expression at 3 days (first time studied) and not before 14 days, respectively. There are no other studies in which transgene expression response time has been analyzed at shorter times after acute brain injury. In this study, we show that both NLSCt and HNRK nanovectors and lentiviral vectors when administered 4 hours after CCI result in significant GFP protein levels at 24 hours after CCI. The therapeutic strategy used in this study includes a 4-hour interval between CCI and treatment which approximates the time it make take from time of TBI cranial injury to that of diagnosis and possible treatment in a well-organized clinical context. Thus, rapid GFP transgene expression observed with HNRK nanovector and the presence of GFP protein after lentiviral vector injection induced by carry-over or pseudotransduction suggests that they could provide an appropriate expression profile for treatment of at least the initial phases of TBI. Further comparative studies are needed to determine if these vectors can effectively provide neuroprotection after a CCI. Interestingly, nanovector NLSCt was reported to induce neuroprotection under different acute excitotoxic conditions and with different transgenes when injected intracortically 4 hours after lesion generation.[26,28] Moreover, the expression of toxic transgenes with the same vector increased excitotoxic lesion.[31,32] We report here that the HNRK vector tends to produce a stable level of transgenic protein until 14 dpl, while the lentivector induced a significant increase in the levels of transgenic protein up to 14 dpl. The HNRK vector could be reinjected to prolong the transgene expression over time, but a careful evaluation of the putative adaptive immune response elicited toward the vector itself should be evaluated. Taken together, these results suggest that both nanovectors and lentiviral vectors can result in expression of biological relevant concentrations of transgenic protein *in vivo* after a CCI, and provides flexibility in selection of the most appropriate vector for neuroprotection after TBI.

Neuroinflammatory cascades are major contributors to secondary progressing injury,[5] and thus additional inflammatory reactions promoted by gene therapy vectors may constitute a problem. We show here that no increase in levels of prototypical proinflammatory cytokine IL-1β was observed after injection of any of the vectors, suggesting that no further inflammation is produced. Moreover, no toxic effect for any of the vectors used were noted by the functional sticky tape test. In addition, both NLSCt and HNRK nanovectors exhibited functional neuroprotective effects after CCI. The NLSCt vector alone (not harboring pDNA) was also previously shown to be neuroprotective after acute excitotoxic brain injury.[26] The mechanism involved its RGD integrin-interacting motif and interaction with glial cells.[27] In addition, the HNRK and HKRN vectors without pDNA were also shown to be neuroprotective for cultured PC12 cells.[55] Thus, data presented here confirm the neuroprotective potential of these nanovectors *per se* in a clinically relevant TBI model. Surprisingly, the lentiviral vectors preparation also showed a significant functional neuroprotection using the sticky tape test, and the beneficial effects of these vector preparations without expressed transgenes should be studied in detail.

The lack of extensive comparative studies using several vectors under the same experimental TBI conditions hampers the selection of putative ideal vectors for clinical applications. Our study aimed to contribute to this important clinical need by comparing two of the most promising prototypes, *i.e.*, modular nanovectors and lentiviral vectors. Our data indicates that both the HNRK protein nanovector and the lentiviral vectors are very useful prototypes for gene therapy applications for treatment of TBI due to the rapid and non-toxic and non-proinflammatory expression of a transgene. As already stated, this is not the case for AAV which need at lest 14 dpt to show the presence of the transgene.[49] However, they observed a beneficial functional improvement at 30 dpt by overexpressing VEGF-A, suggesting that the selection of the ideal vector for the treatment of TBI must be considered in the context of the neuroprotective transgene of choice and its timing of expression for effective neuroprotection. HNRK nanovector showed rapid and prolonged stable expression while lentiviral vectors show slower and increasing levels of expression with time. As inferred from the limited data available for AAV in TBI models, they show a similar profile of time-course transgene expression as lentiviral vectors, with variable expression levels at different time points. The regulation of expression of the transgene is critical for adjusting the dosage for treatment. This could potentially be achieved using inducible promoters for the expression from lentiviral vectors and AAV as reported.[56] Dosage of transgene expression driven by modular nanovectors would be more simple. Adenoviral vectors induce significant inflammatory reactions leading to the rapid elimination of the transgene expression in the brain.[49] For this reason and due to the fact that the inflammatory cascades are an important part

of the physiopathology of TBI, these vectors may not be adequate for the treatment of TBI. Neither of the vectors tested here contributed strongly to the neuroinflammation provoked by CCI, a profile probably shared by AAV, suggesting that they are all suitable for the treatment of TBI. Vectors can also be expected to differ in terms of cell tropism, which again should be carefully considered in context of the mechanism of action of the selected transgene. All three vectors have a broad tropism that can in addition be modulated by surface protein domains, and are for thus useful vectors. Some AAV serotypes have been shown cross the blood brain barrier, and thus having good potential for TBI treatment by intravenous administration.[57] On the other hand, local intraparenchymal injection of the vectors for TBI treatment may be a wiser choice to avoid intravenous injection of high titers of vectors that may have a negative impact. Taken together, one or more of these vectors may prove to be suitable for the treatment of TBI, but its choice should consider the specific neuroprotective mechanism of the transgene used. Importantly, critical properties for development of vectors as clinical formulations have to be taken into account. The ability of these nanovectors to readily self-assemble at room temperature self-assembly with no effect of incubation times on nanovectors transfection efficiency is an important step toward this goal. Also these vectors are noninfective, and the protein can be lyophilized with no loss of activity. In this regard, these vectors would be of better choice over the viral vectors if the mechanism of action of the neuroprotective transgene involves rapid, short-term and low expression levels. However, if a long-term and high-level expression is needed to achieve neuroprotection, both non-integrated lentiviral and AVV may prove to be more suitable. Finally, the functional improvement observed by the modular nanovector and the lentiviral vector preparations *per se* opens the way for the design of additional combinatorial tools for neuroprotection.

MATERIALS AND METHODS

Nanovector production and purification

The production of both chimerical proteins HKRN and HNRK, cloned in plasmid pET-28a (+)TEV, was induced with addition of 1 mmol/l Isopropyl β-D-1-thiogalactopyranoside (IPTG) to plasmid-containing BL21 (DE3) *Escherichia coli* cell cultures (at $OD_{600\ nm}$ = 0.4–0.6) growing in Luria–Bertani medium (Sigma-Aldrich, Chemie Gmbh Munich, Germany) at 37 °C. After 4 hours, cells were harvested by centrifugation, washed with PBS, and stored at −80 °C. The pellet was suspended in lysis buffer, 20 mmol/l Tris-HCl pH 8.0, 500 mmol/l NaCl, 6 mol/l guanidinium chloride, and cells were disrupted by sonication in the presence of ethylenediaamene tetra acetic acid (EDTA)-free protease inhibitor cocktail tablets (Sigmafast Cat. Nº s8820, Sigma-Aldrich). The soluble fraction was obtained by centrifugation at 15,000 g for 45 minutes at 4 °C and further purified using 0.22 µm filters. Proteins were purified in a single step using Ni^{2+}affinity chromatography by ÄKTATM FPLC (GE Healthcare, Uppsala, Sweden). After loading samples onto the column in 20 mmol/l Tris-HCl pH 8.0, proteins were eluted using a 20 CV linear gradient to 100% elution buffer 20 mmol/l Tris-HCl pH 8.0, 500 mmol/l NaCl, 6 mol/l guanidinium chloride, and 1 mol/l imidazole. Protein-containing fractions were pooled and passed through a PD-10 desalting column (GE Healthcare) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline and quantified by bicinchoninic acid method. Production and purification of the soluble form of HNRK was performed as described above but using the soluble bacterial fraction and in the absence of guanidinium chloride. The NLSCt vector production was induced with the addition of 1 mmol/l IPTG to plasmid-containing BL21 (DE3) *E. coli* cells (at $OD_{600\ nm}$ = 0.4–0.6) growing in Luria–Bertani medium at 37 °C. After 4 hours, cells were harvested by centrifugation, washed with PBS, and stored at −80 °C. The bacterial pellet was suspended in TM buffer (0.44% m/v Tris + 0.4% m/v $MgCl_2$ pH 7.5), and cells were disrupted by sonication in the presence of EDTA-free protease inhibitor cocktail tablets. The soluble fraction was separated by centrifugation at 15,000 g for 30 minutes at 4 °C and filtered through 0.22 µm filters. The vector was purified by column chromatography using 4% p-aminobenzyl 1-thio-β-D-galactopiranoside immobilized to agarose (Sigma-Aldrich, Nº A0414), which was washed and equilibrated with 20 CV TM buffer. Elution was done using tetraborate buffer pH 10 (3.81% m/v $Na_2B_4O_7$) supplemented with β-mercaptoethanol (0.2% v/v). Protein-containing fractions were identified using β-galactosidase activity, pooled and desalted on a PD-10 column (GE Healthcare) using 0.05 mol/l PBS pH7.5 and quantified by the bicinchoninic acid method. Finally, all purified proteins were stored at −80 °C until use.

Lentiviral vector production and concentration

Third-generation self-inactivating HIV-1-based vectors were produced by transient co-transfection of four endotoxin-free plasmids (Endo Free plasmid Maxi kit cat. Nº 12362, Qiagen, SP, Brazil) in 293T cells as described.[16] The transfer plasmid (pRRLs in_PPT_CMV_GFP which includes an H1-driven shRNA empty cassette that is incorporated in virus particles, 7,657 bp) was based on the SIN-18 pRRL backbone. This incorporates minimal HIV elements and produces self-inactivating (SIN) vectors through 3′-LTR truncation. The packaging plasmids were pRSV-rev (Addgene, Cambridge, MA, cat. Nº 12253) and pMDLg/pRRE (Addgene, cat. Nº 12251). Vectors were pseudotyped with a standard vesicular stomatitis virus G glycoprotein (VSV-G; plasmid pMD2.G) (Addgene, cat. Nº 12259). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (Invitrogen, San Diego, CA), penicillin (100 IU/ml), and streptomycin (100 mg/ml) in a 37 °C incubator with 5% CO_2. The medium was changed 30 minutes prior to transfection using a standard calcium phosphate transfection protocol. After 17 hours incubation, the medium was replaced by fresh DMEM supplemented with 10% fetal bovine serum and 4 mmol/l caffeine to increase viral titer. At 36 and 60 hours post-transfection, medium was harvested, cleared by low-speed centrifugation (2,500 rpm, 10 minutes, 4 °C), and filtered using 0.22 µm pore filters. Vector particles were concentrated by ultracentrifugation at 50,000 g for 2 hours at 4 °C. Pellets obtained were suspended in serum-free DMEM medium supplemented with 10 mmol/l $MgCl_2$ and 5 U/ml RNAse free DNAse I (Promega, Madison, WI), incubated 30 minutes at 37 °C and stored at −80 °C until use. Vectors were titrated by transducing HeLa cells with serial dilutions of vector stock in the presence of 8 µg/µl of Polybrene (Sigma-Aldrich), and GFP expression was analyzed by flow cytometry analysis 3 days post-transduction. The lentiviral titer obtained was 3.12×10^7 Tu/ml.

Dynamic light scattering

The size of protein/DNA complexes, formed at different incubation times, was determined using a DLS analyzer (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) at 633 nm. Samples were examined in triplicate and data analyzed using Malvern Zetasizer software v7.03.

Transmission electron microscopy

Protein/DNA complexes were also analyzed by TEM. Briefly, 10 µl of sample was put on a carbon coated grid and after 2 minutes negatively stained with 10 µl 2% uranyl acetate (w/v). Analysis was done using a JEM-1400 transmission electron microscope (Toyoko, Japan).

Nanovector self-assembly and transfections

The pRRLs in_PPT_CMV_GFPpre endotoxin-free vector described above and having the GFP marker to monitor transfection efficiency, was also used for construction of nanovectors. HKRN/pDNA and HNRK/pDNA nano complex self-assembly was done using individual 5 minutes incubations with different amounts of protein in 100 µl OPTIPRO medium (GIBCO Life Technologies, GrandIsland, NY) and 2 µg plasmid DNA in 100 µl OPTIPRO medium. Protein/DNA complexes were generated by mixing 100 µl DNA and 100 µl protein at specified ratios, and incubating at room temperature for 20 minutes or indicated times.[10] The 200 µl mixture was then administered to Hek293T/17 cells (Invitrogen, Carlsbad, CA) in 24-well plates grown to 70–80% cell confluence for 4 hours in the absence of serum. Subsequently, the medium was changed to include serum and cells were incubated for an additional 20 hours. Chloroquine 200 mmol/l (C6628, Sigma Aldrich) or 10% of fetal bovine serum (GIBCO Life Technologies) were added to the cells at the moment of incubation with the protein/pDNA nanocomplexes to test the ability of complexes to escape endosomes and to transfect cells in the presence of serum respectively. Transfection efficiency of nanocomplexes formed in smaller volumes was also tested. Complex self-assembly in these cases was done with identical ratios and quantities of protein and pDNA

as used in the 200 µl final volume experiments above but in 30 µl PBS. The transfection efficiency was quantified 24 hours after transfection using GFP reporter gene expression analyzed by flow cytometry in the FACSCalibur system using CellQuest software (BD Biosciences, Franklin Lakes, NJ). Results obtained when samples were visualized by fluorescence microscopy were consistent with flow cytometry gene expression analysis and negative controls (untreated cells and cells with plasmid only).

Animals, controlled cortical impact model, and vector injections

All experimental work was approved by Comisión Nacional de Experimentación Animal and the CHEA-UDELAR ethical commission, and conducted according to directives of the Federation of Laboratory Animal Science Associations. Adult male Wistar rats (280–300 g) were submitted to CCI traumatic lesions under 3% isoflurane anesthesia and O_2 (Abbott, Abbott Park, IL) as described previously.[36] Briefly, rats were mounted in an injury device stereotaxic frame in prone position and secured by ear and incisor bars. The head was held in horizontal plane, and a midline incision was made aseptically in which soft tissues were reflected, and a 4 mm round craniotomy was made over the right primary motor and somatosensory cortex (coordinates to the center of the craniotomy: AP: Bregma, L: −1.7 mm). A stereotaxic mounted cortical impactor (PinPoint PrecisionCortical ImpactorModel PCI3000 Hatteras Instruments, Cary, NC) was used with a 3-mm diameter tip (velocity: 1.5 m/s, contusion depth: 4-mm, contusion time: 120 ms). Four hours later, 6 µl of vehicle (PBS or DMEM/10 mmol/l $MgCl_2$), lentivector (titer: 3.12×10^7 Tu/ml, in DMEM/10 mmol/l $MgCL_2$), and protein vectors accommodating an expression plasmid assembled in PBS (NLSCt protein/DNA ratio 33.3, 0.14 µg DNA per animal, or HNRK ratio 5 both, 1.44 µg DNA per animal) were injected using a stereotaxic mounted nanoinjector (Quintessential Stereotaxic Nanoinjector, Stoelting CO, Wood Dale, IL) at 0.4 µl/minute in three locations (2 µl per site) corresponding to the anterior, right, and posterior lesion border (in all cases, ventral: −2mm) using a 5 µl Hamilton syringe. The needle was left in place for an additional 5 minutes to allow diffusion into the brain parenchyma. Studies were designed to randomize treatments and to include two or three different treatments for each surgical procedure.

Functional behavioral outcome measure: sticky tape test

The behavioral studies were performed at 3, 7, 10, and 14 days after CCI as described previously.[36] All tests were performed and analyzed by individuals blind to the experimental conditions. A 0.5-inch diameter round adhesive tape was placed on the heel of each forepaw and the time it took the animal to remove the tape from each paw was recorded. Three trials were performed and the average value was calculated for each rat. Individual trials were timed out at 3 minutes.

Sample processing and ELISA

GFP and IL-1β protein were quantified using commercially available ELISA kits: GFP ELISA kit (Abcam, cat. Nº #ab117992, Cambridge, MA, USA) and rat IL-1β ELISA kit (Invitrogen, cat.Nº #KRC0011) according to the manufacturer's instructions. Rats were sacrificed at the indicated days post lesion (dpl) by decapitation under deep pentobarbital anesthesia. Ipsilateral brain hemispheres were immediately removed and dissected to obtain fragments of $7 \times 8 \times 9$ mm containing the injured area, frozen in liquid nitrogen and stored at −80 °C. Homogenization was done in a Potter homogenizer in ice-cold 50 mmol/l Tris-50, 1 mmol/l EDTA, pH 8.0 containing a cocktail of protease inhibitors (Roche, Manheim, Germany) and a phosphatase inhibitor (10 mmol/l sodium orthovanadate, Sigma-Aldrich). The preparation was then cleared by centrifugation. Total protein concentration of samples was determined using the bicinchoninic acid method and identical protein concentrations were used for all ELISA measurements.

Data processing and statistical analysis

All results are expressed as mean ± standard error of the mean. Student's *t*-test was used to determine significant differences ($P < 0.05$) between two experimental groups after determination of the normal distribution of the samples and variance analysis. Two-way analysis of variance followed by Bonferroni's multiple comparison test was used to determine significant differences ($P < 0.05$) for experimental settings with more than two experimental groups or time points.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank Fundació Marató TV3 (110533), Catalunya, Spain, Comisión Sectorial de Investigación Científica (CSIC-UDELAR), Uruguay, Agencia Nacional de Investigación e Innovación (ANII), Uruguay, PEDECIBA, Uruguay, FOCEM (MERCOSUR Structural Convergence Fund), COF 03/1111 (to H.P. and M.L.N.), and Genoma España (Project GENAME, to R.J.Y.-M.) for financial support. We thank Pau González for critical comments on this manuscript, Agustín Correa, Gonzalo Obal and Sabina Victoria for technical assistance, and Paul Gill for English language editing service. A.V. received support from from Ministerio de Ciencia e Innovación (MICINN) (ACI2009-0919), Agència de Gestió d'Ajuts Universitaris i de Recerca (2009SGR-108), and Centro de Investigación Biomédica en Red (CIBER) de Bioingeniería, Biomateriales y Nanomedicina, with assistance from the European Regional Development Fund. A.V. has been distinguished with an ICREA ACADEMIA award.

REFERENCES

1. Maas, AI, Stocchetti, N and Bullock, R (2008). Moderate and severe traumatic brain injury in adults. *Lancet Neurol* **7**: 728–741.
2. McConeghy, KW, Hatton, J, Hughes, L and Cook, AM (2012). A review of neuroprotection pharmacology and therapies in patients with acute traumatic brain injury. *CNS Drugs* **26**: 613–636.
3. Polinder, S, Meerding, WJ, Mulder, S, Petridou, E and van Beeck, E; EUROCOST Reference Group (2007). Assessing the burden of injury in six European countries. *Bull World Health Organ* **85**: 27–34.
4. Polinder, S, Meerding, WJ, van Baar, ME, Toet, H, Mulder, S and van Beeck, EF; EUROCOST Reference Group (2005). Cost estimation of injury-related hospital admissions in 10 European countries. *J Trauma* **59**: 1283–90; discussion 1290.
5. Kumar, A and Loane, DJ (2012). Neuroinflammation after traumatic brain injury: opportunities for therapeutic intervention. *Brain Behav Immun* **26**: 1191–1201.
6. Mastrobattista, E, van der Aa, MA, Hennink, WE and Crommelin, DJ (2006). Artificial viruses: a nanotechnological approach to gene delivery. *Nat Rev Drug Discov* **5**: 115–121.
7. Kay, MA (2011). State-of-the-art gene-based therapies: the road ahead. *Nat Rev Genet* **12**: 316–328.
8. Ginn, SL, Alexander, IE, Edelstein, ML, Abedi, MR and Wixon, J (2013). Gene therapy clinical trials worldwide to 2012 - an update. *J Gene Med* **15**: 65–77.
9. Eberling, JL, Jagust, WJ, Christine, CW, Starr, P, Larson, P, Bankiewicz, KS *et al.* (2008). Results from a phase I safety trial of hAADC gene therapy for Parkinson disease. *Neurology* **70**: 1980–1983.
10. Domingo-Espín, J, Vazquez, E, Ganz, J, Conchillo, O, García-Fruitós, E, Cedano, J *et al.* (2011). Nanoparticulate architecture of protein-based artificial viruses is supported by protein-DNA interactions. *Nanomedicine (Lond)* **6**: 1047–1061.
11. Dull, T, Zufferey, R, Kelly, M, Mandel, RJ, Nguyen, M, Trono, D *et al.* (1998). A third-generation lentivirus vector with a conditional packaging system. *J Virol* **72**: 8463–8471.
12. Naldini, L, Blömer, U, Gallay, P, Ory, D, Mulligan, R, Gage, FH *et al.* (1996). *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**: 263–267.
13. Raoul, C, Abbas-Terki, T, Bensadoun, JC, Guillot, S, Haase, G, Szulc, J *et al.* (2005). Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. *Nat Med* **11**: 423–428.
14. Ralph, GS, Radcliffe, PA, Day, DM, Carthy, JM, Leroux, MA, Lee, DC *et al.* (2005). Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nat Med* **11**: 429–433.
15. Yip, PK, Wong, LF, Pattinson, D, Battaglia, A, Grist, J, Bradbury, EJ *et al.* (2006). Lentiviral vector expressing retinoic acid receptor beta2 promotes recovery of function after corticospinal tract injury in the adult rat spinal cord. *Hum Mol Genet* **15**: 3107–3118.
16. Peluffo, H, Foster, E, Ahmed, SG, Lago, N, Hutson, TH, Moon, L *et al.* (2013). Efficient gene expression from integration-deficient lentiviral vectors in the spinal cord. *Gene Ther* **20**: 645–657.
17. Yáñez-Muñoz, RJ, Balaggan, KS, MacNeil, A, Howe, SJ, Schmidt, M, Smith, AJ *et al.* (2006). Effective gene therapy with nonintegrating lentiviral vectors. *Nat Med* **12**: 348–353.
18. Peluffo, H (2011). Modular multifunctional protein vectors for gene therapy. In: Yuan, X (ed). *Non-Viral Gene Therapy*. InTech: Rijeka, Croatia. pp. 597–614.
19. Arís, A and Villaverde, A (2004). Modular protein engineering for non-viral gene therapy. *Trends Biotechnol* **22**: 371–377.
20. Ferrer-Miralles, N, Vázquez, E and Villaverde, A (2008). Membrane-active peptides for non-viral gene therapy: making the safest easier. *Trends Biotechnol* **26**: 267–275.
21. Vázquez, E, Cubarsi, R, Unzueta, U, Roldán, M, Domingo-Espín, J, Ferrer-Miralles, N *et al.* (2010). Internalization and kinetics of nuclear migration of protein-only, arginine-rich nanoparticles. *Biomaterials* **31**: 9333–9339.

22. Vazquez, E, Roldán, M, Diez-Gil, C, Unzueta, U, Domingo-Espín, J, Cedano, J *et al.* (2010). Protein nanodisk assembling and intracellular trafficking powered by an arginine-rich (R9) peptide. *Nanomedicine (Lond)* **5**: 259–268.
23. Xavier, J, Singh, S, Dean, DA, Rao, NM and Gopal, V (2009). Designed multi-domain protein as a carrier of nucleic acids into cells. *J Control Release* **133**: 154–160.
24. Arís, A and Villaverde, A (2000). Molecular organization of protein-DNA complexes for cell-targeted DNA delivery. *Biochem Biophys Res Commun* **278**: 455–461.
25. Arís, A, Feliu, JX, Knight, A, Coutelle, C and Villaverde, A (2000). Exploiting viral cell-targeting abilities in a single polypeptide, non-infectious, recombinant vehicle for integrin-mediated DNA delivery and gene expression. *Biotechnol Bioeng* **68**: 689–696.
26. Peluffo, H, Acarin, L, Arís, A, González, P, Villaverde, A, Castellano, B *et al.* (2006). Neuroprotection from NMDA excitotoxic lesion by Cu/Zn superoxide dismutase gene delivery to the postnatal rat brain by a modular protein vector. *BMC Neurosci* **7**: 35.
27. Peluffo, H, González, P, Arís, A, Acarin, L, Saura, J, Villaverde, A *et al.* (2007). RGD domains neuroprotect the immature brain by a glial-dependent mechanism. *Ann Neurol* **62**: 251–261.
28. Peluffo, H, Alí-Ruiz, D, Ejarque-Ortíz, A, Heras-Alvarez, V, Comas-Casellas, E, Martínez-Barriocanal, A *et al.* (2012). Overexpression of the immunoreceptor CD300f has a neuroprotective role in a model of acute brain injury. *Brain Pathol* **22**: 318–328.
29. Gonzalez, P, Peluffo, H, Acarin, L, Villaverde, A, Gonzalez, B and Castellano, B (2012). Interleukin-10 overexpression does not synergize with the neuroprotective action of RGD-containing vectors after postnatal brain excitotoxicity but modulates the main inflammatory cell responses. *J Neurosci Res* **90**: 143–159.
30. Arís, A and Villaverde, A (2003). Engineering nuclear localization signals in modular protein vehicles for gene therapy. *Biochem Biophys Res Commun* **304**: 625–631.
31. Gonzalez, P, Peluffo, H, Acarin, L, Villaverde, A, Gonzalez, B and Castellano, B (2012). Interleukin-10 overexpression does not synergize with the neuroprotective action of RGD-containing vectors after postnatal brain excitotoxicity but modulates the main inflammatory cell responses. *J Neurosci Res* **90**: 143–159.
32. Peluffo, H, Gonzalez, P, Acarin, L, Arís, A, Beyaert, R, Villaverde, A *et al.* (2013). Overexpression of the nuclear factor kappaB inhibitor A20 is neurotoxic after an excitotoxic injury to the immature rat brain. *Neurol Res* **35**: 308–319.
33. Yáñez-Muñoz, RJ, Balaggan, KS, MacNeil, A, Howe, SJ, Schmidt, M, Smith, AJ *et al.* (2006). Effective gene therapy with nonintegrating lentiviral vectors. *Nat Med* **12**: 348–353.
34. DePolo, NJ, Reed, JD, Sheridan, PL, Townsend, K, Sauter, SL, Jolly, DJ *et al.* (2000). VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivated by human serum. *Mol Ther* **2**: 218–222.
35. Gonzalez, P, Peluffo, H, Acarin, L, Villaverde, A, Gonzalez, B and Castellano, B (2012). Interleukin-10 overexpression does not synergize with the neuroprotective action of RGD-containing vectors after postnatal brain excitotoxicity but modulates the main inflammatory cell responses. *J Neurosci Res* **90**: 143–159.
36. d'Avila, JC, Lam, TI, Bingham, D, Shi, J, Won, SJ, Kauppinen, TM *et al.* (2012). Microglial activation induced by brain trauma is suppressed by post-injury treatment with a PARP inhibitor. *J Neuroinflammation* **9**: 31.
37. Hwu, WL, Muramatsu, S, Tseng, SH, Tzen, KY, Lee, NC, Chien, YH *et al.* (2012). Gene therapy for aromatic L-amino acid decarboxylase deficiency. *Sci Transl Med* **4**: 134ra61.
38. LeWitt, PA, Rezai, AR, Leehey, MA, Ojemann, SG, Flaherty, AW, Eskandar, EN *et al.* (2011). AAV2-GAD gene therapy for advanced Parkinson's disease: a double-blind, sham-surgery controlled, randomised trial. *Lancet Neurol* **10**: 309–319.
39. Mittermeyer, G, Christine, CW, Rosenbluth, KH, Baker, SL, Starr, P, Larson, P *et al.* (2012). Long-term evaluation of a phase 1 study of AADC gene therapy for Parkinson's disease. *Hum Gene Ther* **23**: 377–381.
40. Stoessl, AJ (2014). Gene therapy for Parkinson's disease: a step closer? *Lancet* **383**: 1107–1109.
41. Kenny, GD, Bienemann, AS, Tagalakis, AD, Pugh, JA, Welser, K, Campbell, F *et al.* (2013). Multifunctional receptor-targeted nanocomplexes for the delivery of therapeutic nucleic acids to the brain. *Biomaterials* **34**: 9190–9200.
42. Peluffo, H, Arís, A, Acarin, L, González, B, Villaverde, A and Castellano, B (2003). Nonviral gene delivery to the central nervous system based on a novel integrin-targeting multifunctional protein. *Hum Gene Ther* **14**: 1215–1223.
43. Skinner, AM, Chakkaramakkil Verghese, S and Kurre, P (2013). Cell-cell transmission of VSV-G pseudotyped lentivector particles. *PLoS One* **8**: e74925.
44. Croyle, MA, Callahan, SM, Auricchio, A, Schumer, G, Linse, KD, Wilson, JM *et al.* (2004). PEGylation of a vesicular stomatitis virus G pseudotyped lentivirus vector prevents inactivation in serum. *J Virol* **78**: 912–921.
45. Hwang, BY and Schaffer, DV (2013). Engineering a serum-resistant and thermostable vesicular stomatitis virus G glycoprotein for pseudotyping retroviral and lentiviral vectors. *Gene Ther* **20**: 807–815.
46. Lu, B, Sun, YX, Li, YQ, Zhang, XZ and Zhuo, RX (2009). N-Succinyl-chitosan grafted with low molecular weight polyethylenimine as a serum-resistant gene vector. *Mol Biosyst* **5**: 629–637.
47. Minnich, JE, Mann, SL, Stock, M, Stolzenbach, KA, Mortell, BM, Soderstrom, KE *et al.* (2010). Glial cell line-derived neurotrophic factor (GDNF) gene delivery protects cortical neurons from dying following a traumatic brain injury. *Restor Neurol Neurosci* **28**: 293–309.
48. Degeorge, ML, Marlowe, D, Werner, E, Soderstrom, KE, Stock, M, Mueller, A *et al.* (2011). Combining glial cell line-derived neurotrophic factor gene delivery (AdGDNF) with L-arginine decreases contusion size but not behavioral deficits after traumatic brain injury. *Brain Res* **1403**: 45–56.
49. Siddiq, I, Park, E, Liu, E, Spratt, SK, Surosky, R, Lee, G *et al.* (2012). Treatment of traumatic brain injury using zinc-finger protein gene therapy targeting VEGF-A. *J Neurotrauma* **29**: 2647–2659.
50. Hermens, WT, Giger, RJ, Holtmaat, AJ, Dijkhuizen, PA, Houweling, DA and Verhaagen, J (1997). Transient gene transfer to neurons and glia: analysis of adenoviral vector performance in the CNS and PNS. *J Neurosci Methods* **71**: 85–98.
51. Burger, C, Gorbatyuk, OS, Velardo, MJ, Peden, CS, Williams, P, Zolotukhin, S *et al.* (2004). Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Mol Ther* **10**: 302–317.
52. Hermens, WT and Verhaagen, J (1997). Adenoviral vector-mediated gene expression in the nervous system of immunocompetent Wistar and T cell-deficient nude rats: preferential survival of transduced astroglial cells in nude rats. *Hum Gene Ther* **8**: 1049–1063.
53. Delepine, P, Montier, T, Guillaume, C, Vaysse, L, Le Pape, A and Ferec, C (2002). Visualization of the transgene distribution according to the administration route allows prediction of the transfection efficacy and validation of the results obtained. *Gene Ther* **9**: 736–739.
54. Debinski, W and Tatter, SB (2009). Convection-enhanced delivery for the treatment of brain tumors. *Expert Rev Neurother* **9**: 1519–1527.
55. Domingo-Espín, J, Petegnief, V, de Vera, N, Conchillo-Solé, O, Saccardo, P, Unzueta, U *et al.* (2012). RGD-based cell ligands for cell-targeted drug delivery act as potent trophic factors. *Nanomedicine* **8**: 1263–1266.
56. Tenenbaum, L, Chtarto, A, Lehtonen, E, Velu, T, Brotchi, J and Levivier, M (2004). Recombinant AAV-mediated gene delivery to the central nervous system. *J Gene Med* **6** (suppl 1): S212–S222.
57. Gray, SJ, Matagne, V, Bachaboina, L, Yadav, S, Ojeda, SR and Samulski, RJ (2011). Preclinical differences of intravascular AAV9 delivery to neurons and glia: a comparative study of adult mice and nonhuman primates. *Mol Ther* **19**: 1058–1069.