

Published in final edited form as:

J Gene Med. 2012 May ; 14(5): 299–315. doi:10.1002/jgm.2626.

Lentiviral vectors encoding shRNAs efficiently transduce and knockdown LINGO-1 but induce an interferon response and cytotoxicity in CNS neurons

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Abstract

Background—Knocking down neuronal LINGO-1 using short hairpin RNAs (shRNAs) might enhance axon regeneration in the CNS. Integration-deficient lentiviral vectors have great potential as a therapeutic delivery system for CNS injuries. However, recent studies have revealed that shRNAs can induce an interferon response resulting in off-target effects and cytotoxicity.

Methods—CNS neurons were transduced with integration-deficient lentiviral vectors *in vitro*. The transcriptional effect of shRNA expression was analysed using qRT-PCR and northern blots were used to assess shRNA production.

Results—Integration-deficient lentiviral vectors efficiently transduced CNS neurons and knocked down LINGO-1 mRNA *in vitro*. However, an increase in cell death was observed when lentiviral vectors encoding an shRNA were applied or when high vector concentrations were used. We demonstrate that high doses of vector or the use of vectors encoding shRNAs can induce an up-regulation of interferon stimulated genes (OAS1 and PKR) and a down-regulation of off-target genes (including p75^{NTR} and NgR1). Furthermore, the northern blot demonstrated that these negative consequences occur even when lentiviral vectors express low levels of shRNAs. Together, these results may explain why neurite outgrowth was not enhanced on an inhibitory substrate after transduction with lentiviral vectors encoding an shRNA targeting LINGO-1.

Conclusions—These findings highlight the importance of including appropriate controls to verify silencing specificity and the requirement to check for an interferon response when conducting RNA interference experiments. However, the potential benefits that RNA interference

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Conflict of Interest Statement

The authors declare no conflict of interest.

and viral vectors offer to gene-based therapies to CNS injuries cannot be overlooked and demand further investigation.

Keywords

RNA interference; neurons; lentiviral vector; interferon response; shRNA; CNS; Integration-deficient

Introduction

Following an injury to the adult central nervous system (CNS), neurons show a very limited regenerative response, which results in their failure to successfully form functional connections with their original targets. This is due to a number of factors including a reduced intrinsic growth state [1–6] and the presence of growth inhibitory molecules in the CNS that form a molecular and physical barrier to regeneration [7–14]. Over the past twenty years there have been steady advancements in molecular techniques that have elevated gene therapy into a promising therapeutic strategy for CNS repair.

Integration-deficient lentiviral vectors are among the most promising therapeutic delivery systems for gene therapy to the CNS. Lentiviral vectors can provide long-term expression by integration into the host cells' genome. Although the specific integration sites are unpredictable it has been demonstrated that lentiviral vectors preferentially integrate into active gene loci [15], which can result in insertional mutagenesis, the activation of proto-oncogenes [16, 17] and the formation of cancers in both mouse models and human clinical trials [18–20]. To circumvent this, integration-deficient lentiviral vectors can be generated by mutating the integrase coding sequence, resulting in the expression of the transgene from episomes [21, 22]. Integration-deficient lentiviral vectors can be produced at high titres and express a transgene at a level comparable to vectors incorporating the wild-type integrase [21]. Robust and stable expression of enhanced green fluorescent protein (eGFP) has been observed for at least nine months in post-mitotic retinal pigment epithelium, and for at least one month in striatal and hippocampal neurons *in vivo* [21]. Both integration proficient and deficient lentiviral vectors have been shown to efficiently transduce CNS neurons *in vitro* [23, 24] and in the brain and spinal cord *in vivo* [21, 25–30]. Furthermore, integration-proficient lentiviral vectors have been shown to successfully mediate RNA interference in neurons *in vitro* [31] and *in vivo* where they have been successfully used to reverse the neurodegenerative and behavioural deficits in animal models of Amyotrophic lateral sclerosis and Alzheimer's disease [32, 33]. We have produced the first integration-deficient lentiviral vectors capable of RNA interference (see also Peluffo et al., manuscript in preparation), and the present study aims to characterise in more detail the cellular response to such vectors.

RNA interference is a process of post-transcriptional gene regulation, which results in the knockdown of a target gene [34, 35]. DNA plasmids can be designed to express short hairpin RNAs (shRNAs), which mimic the structure of endogenous pre-miRNAs and are usually transcribed from Pol III promoters [36, 37]. Once expressed, the shRNAs are processed by the endogenous micro RNA (miRNA) biogenesis pathway resulting in the generation of

functional small interfering RNA (siRNA) that can silence the targeted mRNA [38]. RNA interference has the potential to attenuate the production of proteins that are inhibitory to neurite outgrowth thereby enhancing neurite outgrowth *in vitro* and regeneration *in vivo*. Previous studies have demonstrated that siRNA-mediated knockdown of NgR1, p75^{NTR} and RhoA can enhance the neurite outgrowth of dorsal root ganglion (DRG) neurons and retinal ganglion cells (RGCs) when cultured in the presence of myelin extract *in vitro* [39, 40]. Furthermore RNA silencing of the ubiquitin ligase Cdh1-APC has been shown to increase neurite outgrowth of postnatal cerebellar granule neurons (CGNs) on permissive and inhibitory myelin substrates *in vitro* [41–43].

Over the past decade there have been an increasing number of studies reporting side effects of RNA interference and it has now been extensively demonstrated that shRNAs and siRNAs can induce a cellular innate immune response resulting in the production of interferons (IFNs) that can activate interferon stimulated genes (ISGs), induce a global inhibition in gene expression and result in cell death [40, 44–50]. IFNs are inducible cytokines produced and secreted by multiple cell types including neurons in response to double stranded RNA (dsRNA), a common indicator of viral infection [51–53]. Both viral and non-viral mediated RNA interference has been reported to augment the expression of three key ISGs; 2',5'-oligoadenylate synthase 1 (OAS1) [44–47, 54], protein kinase R (PKR) [45, 49] and myxovirus resistance 1 (Mx1) [40, 49].

The aim of this study was to enhance the neurite outgrowth of postnatal CNS neurons cultured on a growth inhibitory substrate by constructing an integration-deficient lentiviral vector capable of mediating RNA interference. LINGO-1 was selected as the target for knockdown as it has been shown to be an essential component of the neuronally expressed Nogo receptor (NgR) complex [55], which restricts axon growth. Several myelin associated inhibitory proteins, including myelin associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (OMgp) and the Nogo-66 domain of Nogo-A bind and signal through the NgR complex [13, 56–59]. Furthermore inhibition of LINGO-1 using a LINGO-1-Fc fusion protein has previously been shown to enhance neurite outgrowth *in vitro* and sprouting and functional recovery *in vivo* [55, 60]. To date, it was not known whether RNA interference can reduce levels of LINGO-1 in neurons and whether or not this promotes axon growth.

Using integration-deficient lentiviral vectors expressing shRNAs, primary postnatal neurons were efficiently transduced and LINGO-1 expression was significantly knocked down *in vitro*. However, an increase in cell death was observed when lentiviral vectors encoding an shRNA were applied (relative to those that did not encode shRNAs). Moreover, an increase in cell death was observed for all the lentiviral vectors (with or without shRNAs) when high viral concentrations were used. Furthermore, following transduction and LINGO-1 knockdown the neurite outgrowth of CGNs was not enhanced on an inhibitory MAG substrate. Subsequent experiments demonstrated that high concentrations of lentiviral vectors and low level shRNA expression generated an IFN response that may have been responsible for a component of the observed knockdown, cytotoxicity and failure to promote neurite outgrowth.

Materials and Methods

Cell lines

HEK 293T and HeLa cells were maintained in Dulbecco's modified eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen, UK). CHO-MAG and CHO-R2 cells (kind gift from Professor Marie Filbin) and were maintained in a humidified incubator at 37°C, 5% CO₂ in DMEM supplemented with 10% FBS (Invitrogen, UK), 2mM L-glutamine (Sigma, UK), 0.04 mg/ml L-Proline (Sigma, UK), 7.5 µg/ml Glycine (Sigma, UK), 0.73 µg/ml Thymidine (Sigma, UK), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen, UK).

Cerebellar granule neuron (CGN) dissociation

Postnatal day 7-9 (P7-9) Lister hooded rat pups were killed by decapitation, the cerebellum was dissected and the meninges were removed in 3 ml Calcium and Magnesium free media (CMF) (sterile water supplemented with 0.4 mg/ml KCl (Sigma, UK), 0.06 mg/ml KH₂PO₄ (Sigma, UK), 7.65 mg/ml NaCl (Sigma, UK), 0.35 mg/ml NaHCO₃ (Sigma, UK), 0.048 mg/ml Na₂HPO₄ (Sigma, UK), 2.38 mg/ml Hepes (Sigma, UK), pH 7.2). The dissected cerebellum was then finely diced with a razor blade before being incubated in 5 ml 0.05% trypsin EDTA (Invitrogen, UK) in CMF for 15 minutes at 37°C. The trypsin EDTA (Invitrogen, UK) was deactivated using an equal volume of 10% FBS (Invitrogen, UK) in CMF and the cell pellet was transferred to a fresh tube containing 2 ml CMF and 0.5 ml 5 mg/ml DNase I (Sigma, UK). The cell pellet was mechanically triturated 8 times using a 5 ml pipette and 4 times using a 2 ml pipette and left to settle for 5 minutes. 1.5 ml of supernatant was removed and pelleted by centrifugation at 100 x g for 5 minutes and then resuspended in 5 ml SATO media (D-MEM supplemented with 2% FBS (Invitrogen, UK), 1% N2 supplement (Invitrogen, UK) and 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen, UK)). Cells were counted using a haemocytometer.

Plasmid construction

Plasmid Lenti-H1 (Figure 1B) was generated by amplifying the H1 promoter from the pSUPER_ *gfp_neo* plasmid (kind gift from Dr. Thijn R. Brummelkamp, Dr. Rene Bernards and Dr. Reuven Agami) using PCR and cloning it into the pRRLsin_PPT_CMV_GFPpre [61] lentiviral transfer plasmid (Figure 1C). The PCR primers added a unique MCS consisting of four restriction enzyme sites (MluI, SbfI, PstI and XmaI) after the H1 promoter. PCR primer sequences were as follows: H1 cloning Forward 5'-GCG-CGC-GAA-TTC-GAA-CGC-TGA-CGT-CAT-CAA-3' and Reverse 5'-GCG-CGC-GGT-ACC-CCC-GGG-ATA-CCT-GCA-GGA-CGC-GTG-TGG-TCT-CAT-ACA-GAA-CTT-ATA-AGA-3'. The primers were reconstituted to 100 µM using 1xTE (10 mM Tris (Sigma, UK), 1 mM EDTA (Sigma, UK), pH 8.0) and a published set of PCR conditions were used [62]. The PCR product and the pRRLsin_PPT_CMV_GFPpre plasmid were cleaved overnight at 37°C using EcoRI and Acc65I (New England BioLabs, USA). The PCR product was then ligated into the linearised pRRLsin_PPT_CMV_GFPpre plasmid downstream of an existing expression cassette encoding eGFP under the control of a CMV promoter and a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) generating Lenti-H1 plasmid (Figure 1).

shRNA design

Four shRNA constructs (LINGO1-sh1-4) were designed according to the pSUPER protocol (OligoEngine, USA) (Table 1). The antisense sequences targeting LINGO-1 were designed using the Dharmacon siDESIGN[®] centre algorithm with the exception of LINGO1-sh4, which was based on a previously published sequence [63]. Each shRNA was 64 nucleotides in length consisting of a 19 nucleotide sense and antisense sequence separated by a 9 nucleotide non-complementary loop sequence. This was flanked by a 5' mutated MluI restriction site and a 3' RNA polymerase III termination signal followed by a PstI restriction site. The antisense sequences were designed to target the ORF of the rat LINGO-1 gene (GeneID: 315691) and to exclude sequences with a GC content lower than 30% and higher than 64%. Sequences with a predicted high score for successful silencing, low seed frequency, high number of mismatches to sequence records other than the target gene were preferentially selected. A scrambled non-targeting shRNA sequence (Lenti-Scr) was used as a negative control (Table 1). The shRNA sequences were cloned in to the Lenti-H1 plasmid downstream of the H1 promoter (Figure 1A).

Lentiviral production and titration

Third generation, self-inactivating, integration-deficient lentiviral vectors pseudotyped with the VSV-G envelope glycoprotein were produced as previously described [21, 64]. Briefly, lentiviral vectors were generated by transient transfection of HEK293T cells with the pMD2.VSV-G, pRSV.REV and pMDLg/pRREintD64V packaging plasmids and the relevant transfer plasmid. The harvested HEK293T cell medium was centrifuged at 690 x g for 10 minutes at room temperature and then filtered through a 0.22 µm filter (Nalgene, USA) to remove cell debris. The filtered medium was then harvested and transferred to high speed polyallomer centrifuge tubes (Beckman, USA) and centrifuged at 50 000 x g in a SW32Ti rotor (Beckman, USA) for 2 hours at 4°C. The vector was then resuspended in DMEM, centrifuged at 1400 x g for 10 minutes and incubated with 5 u/ml DNaseI (Promega, USA) and 10 mM MgCl₂ (Sigma, UK) for 30 minutes. The vector was then aliquoted and stored at -80°C. The lentiviral titre was determined by serial dilution and transduction of HeLa cells followed by flow cytometry as previously described [21]. Prior to use, all the lentiviral vectors were titre matched to 1x10⁸ transducing units/ml.

Quantitative RT-PCR analysis of mRNA

Total cellular RNA was isolated using TRIZOL reagent (Invitrogen, UK) and treated with DNase I (Qiagen, UK). For first strand cDNA synthesis, 100 units of Superscript II reverse transcriptase (Invitrogen, UK) was used with 250 ng random primers (Invitrogen, UK), 10 mM dNTP mix (Invitrogen, UK) and 500 ng total RNA. Quantitative RT-PCR (qRT-PCR) was performed using the RotorGene-3000 (Corbett Life Science, Australia). Each PCR reaction contained 20 ng of cDNA, 25 ng/µl of the relevant forward and reverse primers and 4 µl SYBR Green PCR Master Mix (Roche, USA). Forward and reverse primers for LINGO-1 were designed using Primer3, sequences were as follows: Forward 5'-TGG-ACA-TCA-GCG-AGA-ACA-AG-3' and Reverse 5'-ATG-CAA-TCT-GAC-CTC-CAT-CC-3'. Forward and reverse primers for GAPDH, p75^{NTR}, NgR1, OAS1, PKR and Mx1 were designed using NCBI primer-BLAST tool, sequences were as follows: GAPDH forward 5'-

ATG-GGA-AGC-TGG-TCA-TCA-AC-3' and reverse 5'-CCA-CAG-TCT-TCT-GAG-TGG-CA-3'. p75^{NTR} forward 5'-GAG-GTG-GGC-TCG-GGA-CTC-GT-3' and reverse 5'-CGG-GGG-CGT-AGA-CCT-TGG-GA-3'. NgR1 forward 5'-CGG-CTG-CCG-ACA-TGG-GTG-TT-3' and reverse 5'-ATA-GGC-CAG-GCC-CCA-GCT-CC-3'. OAS1 forward 5'-ACA-GCA-ATC-CTG-ATC-CCA-AG-3' and reverse 5'-ACC-AGT-TCC-AAG-ATT-GTC-CG-3'. PKR forward 5'-AAC-AGC-CCT-GGA-AAA-TGA-TG-3' and reverse 5'-TTA-CGG-GTT-GTC-AAT-GCT-TTC-3'. Mx1 forward 5'-AGA-GGA-GCC-ATG-GAG-AGT-CA-3' and reverse 5'-AAA-GCC-AGG-AGA-CAT-CCC-TT-3'. Standard curves were obtained for each of the target genes (LINGO-1, GAPDH, p75^{NTR}, NgR1, OAS1, PKR and Mx1) using three-fold serial dilutions of E15 rat head cDNA. A single peak on the melt curve analysis confirmed the specificity of the PCR primers.

Transduction efficiency and viability assay

24-well assay plates (Nunc, UK) were coated with 0.01% poly-L-lysine (PLL) (Sigma, UK) in PBS (Invitrogen, UK) for 2 hours and washed twice with PBS (Invitrogen, UK). CGNs were dissociated from postnatal day 7-9 rat pups and 5×10^4 cells were plated per well and cultured at 37°C, 5% CO₂ for 24 hours in 300 µl SATO media. Integration-deficient lentiviral vectors were added to the wells at a range of viral concentrations (MOI 1, 5, 10, 20 or 50). For the NVC, un-supplemented D-MEM was added to the wells instead of virus. The plates were then incubated for 72 hours at 37°C, 5% CO₂.

Neurite outgrowth assay

96-well assay plates (Nunc, UK) were pre-coated with 30 µl 0.01% poly-L-Lysine (Sigma, UK) in PBS (Invitrogen, UK) for 2 hours and washed with PBS (Invitrogen, UK). The 96-well plate was then coated with 30 µl 10 µg/ml fibronectin from bovine plasma (Sigma, UK) in DMEM (Invitrogen, UK) for 2 hours at 37°C and washed once with DMEM (Invitrogen, UK). The CHO-MAG and CHO-R2 cells were plated at a density of 4×10^4 cells per well in 100 µl CHO cell media and incubated overnight at 37°C, 5% CO₂. 24 hours later P7-9 CGNs were dissociated and 1×10^4 plated onto the confluent monolayer of CHO-MAG or CHO-R2 cells in 100 µl SATO media. 2 hours after plating, the CGNs were transduced with the integration-deficient lentiviral vectors at an MOI 10. A NVC was included whereby un-supplemented DMEM was added instead of virus. A positive control was also included where NVC cells were given 50 µM of the synthetic ROCK inhibitor Y-27632 (Sigma, UK). After incubating for 24 hours an additional 100µl SATO media was added to the wells, which were then incubated for a further 48 hours.

Immunocytochemistry

CGN cultures were fixed using cold 4% paraformaldehyde in PBS (pH 7) for 20 minutes and then washed once with PBS (Invitrogen, UK). Neurons were stained for beta-III tubulin using a mouse monoclonal antibody (1:2000 Promega, USA) in PBS (Invitrogen, UK) containing 10% normal goat serum (NGS) (Invitrogen, UK) and 0.3% Triton X (Sigma, UK) and incubated on a shaker for 1 hour at room temperature. The cultures were then washed 3 times with PBS (Invitrogen, UK) and incubated with goat anti-mouse Alexa Fluor 546 conjugated secondary antibody (1:1000, Molecular Probes) and DAPI to visualise the nucleus in PBS (Invitrogen, UK) with 0.3% Triton X-100 (Sigma, UK) on a shaker for 45

minutes at room temperature. The cultures were then washed a further 3 times with PBS (Invitrogen, UK) and left in PBS containing 0.02% sodium azide (Sigma, UK).

Northern blot

Northern blotting was used to detect the expression of the LINGO1-sh4 shRNA and siRNA following transduction with Lenti-LINGO1-sh4 or transfection with the LINGO1-sh4 DNA plasmid. The oligonucleotide probe was designed to be complementary to the LINGO1-sh4 antisense siRNA sequence. The probes sequence was: 5'-GAT-CGT-CAT-CCT-GCT-AGA-C-3'. DNA oligonucleotide sequences that were identical to the shRNA and siRNA sequences that should be expressed by LINGO-sh4 were designed and included as positive controls. The sequence of the shRNA positive control was: 5'-GAT-CGT-CAT-CCT-GCT-AGA-CTT-CAA-GAG-AGT-CTA-GCA-GGA-TGA-CGA-TCT-T-3' and the siRNA positive control was: 5'-GTC-TAG-CAG-GAT-GAC-GAT-C-3'. HEK 293T cells were either transduced with Lenti-LINGO1-sh4 at an MOI 10 or transfected with 3 µg of the LINGO1-sh4 plasmid using PEI. 72 hours after transduction/transfection, the total cellular RNA was isolated using TRIZOL reagent (Invitrogen, UK). End labelling of the oligonucleotide probe and ladder was performed using the Decade labelling kit (Ambion, USA). Briefly, 1 µl of 100 pmol oligonucleotide probe or 100 ng Decade ladder, 1 µl 10x forward labelling buffer, 1 µl Diethylpyrocarbonate (DEPC)-pretreated water, 1 µl γ -³²P-ATP 250 µCi (Perkin Elmer, UK) and 1 µl T4 polynucleotide kinase were combined and incubated at 37°C for 60 minutes. The probe and ladder were then purified using G25 Illustra Micropin purification cartridges (GE Healthcare Life Sciences, UK). 20 µg of cellular RNA, 0.5 µl of γ -³²P-labelled ladder and 100 ng of the positive control oligonucleotides in DEPC water and 2x Novex loading buffer (Invitrogen, UK) were separated on a 15% acrylamide gel. The RNA was then transferred to a nitrocellulose membrane (Invitrogen, UK) and UV-crosslinked. The membrane was pre-hybridized with UltraHyb-Oligo (Ambion USA) and then probed with the γ -³²P-labelled probe overnight at 35°C. The membrane was then washed in 2x standard sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) for 5 minutes at 35°C and exposed to Biomax maximum resolution autoradiography film (Sigma, UK) for 21 days. Transcript sizes were determined using the Decade ladder (Ambion, USA).

Imaging and analysis

8 fields of view were taken per well using the IN Cell Analyzer 1000 semi-automated cell imager with a 10x Nikon ApoPlan objective (GE Healthcare Life Sciences, UK). Cell counts were obtained using the semi-automated IN Cell Developer Toolbox software (GE Healthcare Life Sciences, UK) and custom-written algorithms. Transduction efficiency was determined by counting the number of eGFP and beta-III tubulin positive cells. Neuronal viability was assessed by counting the total number of beta-III tubulin immunoreactive cells. Percentage transduction was then calculated by dividing the number of transduced neurons by the total number of neurons multiplied by one hundred. The data represents 3 independent experiments using 1 well per condition, ($n = 3$ /group). The relative levels of LINGO-1 mRNA were determined by cross-referencing with the standard curve and the fold change was determined by normalising to GAPDH: the knockdown experiment using an MOI of 10 was carried out once using three wells per condition ($n = 3$ /group), while the knockdown experiment using an MOI of 50 was carried out once using four wells per

condition ($n = 4/\text{group}$). The relative levels of p75^{NTR} and NgR1 were determined by cross-referencing with a standard curve and the fold change by normalising to GAPDH. Both experiments (MOI 10 and 50) were carried out once using four wells per condition ($n = 4/\text{group}$). The mean neurite length per neuron was determined by measuring the neurite length of beta-III tubulin positive cells divided by the total number of neurons: this experiment was carried out once using 8 wells per condition, ($n = 8/\text{group}$). The relative levels of OAS1, PKR and Mx1 mRNA were determined by cross-referencing with a standard curve and the fold change was determined by normalising to GAPDH. The ISG qRT-PCR experiments at an MOI 10 was carried out once using three wells per condition ($n = 3/\text{group}$) and the ISG qRT-PCR experiments using an MOI 50 were carried out once using four wells per condition ($n = 4/\text{group}$).

Statistical analysis

Statistical analysis was carried out using SPSS 17.0 (SPSS Inc, USA). A threshold level of significance (α) of 0.05 was selected. Graphs show means \pm standard errors of the mean. The Kolmogorov-Smirnov and Levene's tests were used to test for normality and the equality of variances. To statistically analyse the transduction efficiency and viability a one-way ANOVA was used to test for differences among the groups followed by Tukey post-hoc testing to analyse the effects between groups. LINGO-1 knockdown was analysed using a one-way ANOVA to test for differences among the groups followed by Tukey post-hoc testing to analyse the effects between groups. To analyse the expression level of p75^{NTR} and NgR1 a one-way ANOVA was used to test for differences among the groups followed by Dunnett's post hoc tests to compare the groups to the no virus control. To analyse the expression of ISGs at an MOI of 10 a one-way ANOVA was used to test for differences among the groups followed by Tukey post-hoc tests to analyse the effects between groups. The data for an MOI of 50 was not normally distributed so a Kruskal-Wallis was performed to test for differences among the groups followed by Mann-Whitney post-hoc tests to analyse the effects between groups. We report exact P values for the Mann-Whitney comparisons: these were not adjusted for multiple comparisons. To analyse the neurite outgrowth a one-way ANOVA was used to test for differences among the groups followed by Dunnett's post hoc tests to compare groups to the negative control.

Results

The generation of shRNA encoding integration-deficient lentiviral vectors

The H1-RNA promoter (H1 promoter) was cloned into a lentiviral transfer plasmid downstream of an eGFP expression cassette as described in the methods, generating Lenti-H1, which also contains cloning sites for shRNAs downstream of the H1 promoter. The H1 promoter has previously been demonstrated to efficiently drive the expression of the shRNAs [36]. We then generated integration-deficient lentiviral vectors expressing shRNAs targeting LINGO-1 (Lenti-LINGO1-sh1, Lenti-LINGO1-sh2, Lenti-LINGO1-sh3, Lenti-LINGO1-sh4), a vector expressing a scrambled non-targeting shRNA that had been microarray-tested by Dharmacon to have minimal targeting of known genes in the rat genome (Lenti-Scr) and a vector containing the H1 promoter and multiple cloning site

(MCS) but not expressing an shRNA (Lenti-H1). A schematic of the different lentiviral vectors can be seen in Figure 1.

Integration-deficient lentiviral vectors efficiently transduce postnatal CGNs in vitro

P7-9 rat CGNs were transduced in 96 well plates using a range of viral doses (multiplicity of infection (MOI) 1, 5, 10, 20 or 50). Importantly, a no virus control (NVC) was also included where the cells were left untreated. Cultures were fixed after seventy two hours and immunolabelled for beta-III tubulin. An automated image capture and analysis system was used for unbiased measurement of transduction efficiency and cell survival. Lentiviral vectors transduced neurons with high efficiency at all MOIs (Figure 2). Transduction efficiency was similar for all the lentiviral vectors tested. Using Lenti-LINGO1-sh4 as an example, the number of transduced neurons was affected by MOI ($df = 5(12)$, $F = 167$, $P < 0.001$, one-way ANOVA, $n = 3/\text{group}$) (Figure 2A). The number of transduced neurons increased between an MOI 1 and 10, before decreasing at higher viral concentrations. Tukey post-hoc tests indicated a difference in the number of neurons transduced between all the concentrations used and the NVC (P values < 0.001). Significantly more neurons were transduced when using an MOI 10 compared to using an MOI 1 ($P < 0.01$) and MOI 50 ($P < 0.01$) but not an MOI 5 and 20 ($P > 0.05$). The percentage of neurons that were transduced, however, generally did not change with viral concentrations higher than MOI 10 where the transduction efficiency was already above 90 % (Figure 2B, D). MOI 10 was therefore optimum for CGN transduction.

CGN viability is reduced by both lentiviral concentration and shRNA expression in vitro

We investigated whether lentiviral vector concentration affected neuronal viability. The pattern of neuronal viability was similar for all the shRNA expressing lentiviral vectors. For Lenti-LINGO1-sh4 a difference in neuronal viability was detected between the different viral concentrations, indicating that MOI contributes to cell death ($df = 5(12)$, $F = 12.40$, $P < 0.001$, one-way ANOVA, $n = 3/\text{group}$) (Figure 2C). Tukey post-hoc tests indicated that there was a difference in neuronal viability between all the MOI's used and the NVC (P values < 0.05). There was also a significant reduction in neuronal viability between MOI 10 and MOI 50 ($P < 0.05$). A different pattern of viability was observed when the lentiviral vectors did not express an shRNA. Using Lenti-H1, neuronal viability was affected by viral concentrations ($df = 5(12)$, $F = 6.41$, $P < 0.01$, one-way ANOVA, $n = 3/\text{group}$) (Figure 2C). However, unlike lentiviral vectors that encode an shRNA, Tukey post-hoc tests indicated that there was no difference in neuronal viability between MOI 1-20 and the NVC (P values > 0.05). Only at high viral concentrations was a statistically significant difference in viability detected: Using MOI 50 significantly reduced viability compared to MOI 1-10 ($P < 0.05$) or the NVC ($P < 0.01$). Thus neuronal viability appears to be affected by both viral concentration and the expression of an shRNA. As there was a significant reduction in viability between MOI 10 and 50 using Lenti-LINGO1-sh4 and MOI 10 achieved almost complete transduction, we conclude that MOI 10 is the optimal concentration for CGN transduction.

Lenti-LINGO1-sh4 significantly knocks down LINGO-1 mRNA in vitro at an MOI 10 and 50, although knockdown is also observed with the control vectors

Using an MOI of 10 or 50, P7-9 CGNs were transduced with either Lenti-LINGO1-sh1, Lenti-LINGO1-sh2, Lenti-LINGO1-sh3, Lenti-LINGO1-sh4, Lenti-Scr or Lenti-H1. A NVC was also included. Seventy two hours after transduction, knockdown of LINGO-1 mRNA was assessed using qRT-PCR. LINGO-1 mRNA levels were significantly affected by the different lentiviral vectors at an MOI of 50 ($df = 6(21)$, $F = 29.0$, $P < 0.001$, one-way ANOVA, $n = 4/\text{group}$) (Figure 3A). Tukey post-hoc tests revealed that Lenti-LINGO1-sh4 significantly knocked down LINGO-1 expression 2.1 fold, 2.2 fold and 4.8 fold respectively compared to Lenti-Scr ($P < 0.05$), Lenti-H1 ($P < 0.05$) and NVC ($P < 0.001$). Lenti-LINGO1-sh1, Lenti-LINGO1-sh2 and Lenti-LINGO1-sh3 did not significantly knockdown the level of LINGO-1 mRNA compared to the Lenti-Scr and Lenti-H1 controls (P values > 0.05). However, unexpectedly all the lentiviral vectors including the Lenti-Scr and Lenti-H1 controls significantly knocked down the level of LINGO-1 mRNA compared to the NVC ($P < 0.001$). Due to concerns with cytotoxicity at high viral concentrations we investigated whether using an MOI 10 would also significantly knockdown LINGO-1. A statistically significant difference in LINGO-1 mRNA levels was detected between the different lentiviral vectors ($df = 6(14)$, $F = 24.1$, $P < 0.001$, one-way ANOVA, $n = 3/\text{group}$) (Figure 3B). Tukey post-hoc tests revealed that Lenti-LINGO1-sh4 significantly knocked down LINGO-1 expression 2.2 fold, 2.3 fold and 4.6 fold respectively compared to Lenti-Scr ($P < 0.05$), Lenti-H1 ($P < 0.05$) and NVC ($P < 0.001$). Lenti-LINGO1-sh1, Lenti-LINGO1-sh2 and Lenti-LINGO1-sh3 did not significantly knockdown the level of LINGO-1 mRNA compared to the Lenti-Scr and Lenti-H1 controls (P values > 0.05). Again surprisingly, all the lentiviral vectors including the Lenti-Scr and Lenti-H1 controls significantly knocked down the level of LINGO-1 mRNA compared to the NVC ($P < 0.001$).

Transduction of CGNs with Lenti-LINGO1-sh4 at an MOI of 10 does not enhance neurite outgrowth on an inhibitory MAG substrate

CGNs were plated onto either a confluent layer of MAG expressing CHO cells (CHO-MAG) or CHO cells that express the reverse peptide sequence of MAG (CHO-R2) as a control. The cells were subsequently transduced with integration-deficient lentiviral vectors expressing either Lenti-LINGO1-sh4, Lenti-Scr or Lenti-H1 at an MOI 10. A NVC was included as a negative control and 50 μM Y-27632, a synthetic Rho-kinase inhibitor was added to other NVCs as a positive control. Seventy two hours after transduction the CGNs were fixed, stained and the mean neurite length per transduced neuron was measured. Statistical analysis revealed a significant difference between the groups ($df = 5(18)$, $F = 33.61$, $P < 0.001$, one-way ANOVA, $n = 4/\text{group}$) (Figure 4A). Dunnett's post-hoc test indicated that CGNs plated on the CHO-R2 cells had significantly longer neurites compared to the CGNs plated on the CHO-MAG cells (CHO-R2, $148 \pm 5 \mu\text{m}$; CHO-MAG, $78.3 \pm 3.6 \mu\text{m}$; values represent mean \pm SEM, $n = 4/\text{group}$, *** $P < 0.001$) (Figure 4A and B). Addition of 50 μM Y-27632 to NVC CGNs cultured on the CHO-MAG cells resulted in a significant increase in the neurite outgrowth (Y-27632, $122.7 \pm 10.9 \mu\text{m}$; CHO-MAG, $78.3 \pm 3.6 \mu\text{m}$; values represent mean \pm SEM, $n = 4/\text{group}$, *** $P < 0.001$) (Figure 4A, D). In contrast, there was no significant difference in the neurite outgrowth of CGNs transduced with lentiviral vectors expressing an shRNA targeting LINGO-1 compared to CGNs transduced with any of the control lentiviral

vectors or the NVC (Lenti-LINGO1-sh4, $71 \pm 3.6 \mu\text{m}$; Lenti-Scr, $71.9 \pm 2.4 \mu\text{m}$; Lenti-H1, $69.5 \pm 4.9 \mu\text{m}$; NVC, $78.3 \pm 3.6 \mu\text{m}$; values represent mean \pm SEM, $n = 4/\text{group}$, $P > 0.05$) (Figure 4A, C). Thus despite substantial knockdown of LINGO-1 mRNA by lentiviral vectors in general, and by Lenti-LINGO1-sh4 in particular (Figure 3), this did not promote neurite growth *in vitro*.

Interferon stimulated genes OAS1 and PKR but not Mx1 are up-regulated following transduction with lentiviral vectors encoding shRNAs

Due to the non-specific knockdown of LINGO-1 and the cytotoxicity observed after transduction with high concentrations of lentiviral vector, the expression of three typical ISGs (OAS1, PKR and Mx1) was examined seventy two hours after transduction using qRT-PCR. OAS1 expression was significantly affected after transduction with lentiviral vectors at an MOI of 10 ($df = 6(14)$, $F = 5.83$, $P < 0.01$, one-way ANOVA, $n = 3/\text{group}$) (Figure 5A). Tukey post-hoc tests indicated that there was a significant increase in OAS1 expression between Lenti-LINGO1-sh1 ($P < 0.05$), Lenti-LINGO1-sh2 ($P < 0.01$), Lenti-LINGO1-sh4 ($P < 0.05$) and the NVC. There was a trend for increased OAS1 expression between Lenti-Scr and NVC ($P = 0.06$). However, there was no significant difference in OAS1 expression between either Lenti-LINGO1-sh3 or Lenti-H1 and the NVC ($P \text{ values} > 0.05$). CGNs were also transduced with lentiviral vectors at an MOI 50, which also led to a significant difference in OAS1 expression between the vectors ($df = 6$, Chi-square = 13.31, $P < 0.05$, Kruskal-Wallis, $n = 4/\text{group}$) (Figure 5B). Mann-Whitney post-hoc tests revealed that all lentiviral vectors significantly increased OAS1 expression compared to the NVC ($P \text{ values} = 0.03$). There was no significant difference in OAS1 expression between the shRNA expressing viruses and the non-shRNA expressing Lenti-H1 ($P \text{ values} > 0.05$). There was also no significant difference in OAS1 expression between the two different MOI used ($df = 1(35)$, $F = 1.04$, $P > 0.05$, two-way ANOVA, $n = 3/\text{group}$).

Any apparent differences in PKR expression did not reach statistical significance following transduction of CGNs with lentiviral vectors at an MOI 10 ($df = 6(14)$, $F = 1.934$, $P > 0.05$, one-way ANOVA) (Figure 5C). In contrast, at an MOI of 50 a statistically significant difference in PKR expression was observed between the groups ($df = 6$, Chi-square = 16.94, $P < 0.05$, Kruskal-Wallis, $n = 4/\text{group}$) (Figure 5D). Mann-Whitney post-hoc tests revealed that PKR expression was significantly increased in CGNs transduced with any of the shRNA expressing lentiviral vectors compared to the NVC ($P = 0.03$) but no significant difference in PKR expression was detected between the non-shRNA expressing Lenti-H1 and the NVC ($P = 0.49$). There was also a significant increase in PKR expression between CGNs transduced with Lenti-LINGO1-sh1 ($P \text{ values} = 0.03$) and Lenti-H1 and a trend for Lenti-LINGO1-sh2 and Lenti-LINGO1-sh3 ($P = 0.06$). However, there was no significant difference in PKR expression between Lenti-LINGO1-sh4 and Lenti-H1 ($P = 0.11$) or Lenti-Scr and Lenti-H1 ($P = 0.34$). There was also a significant increase in PKR expression between CGNs transduced with lentiviral vectors at an MOI 10 and 50 ($df = 1(35)$, $F = 7.47$, $P < 0.01$, two-way ANOVA, $n = 3/\text{group}$).

Mx1 expression was not significantly affected following transduction by any of the viral vectors at an MOI of 10 ($df = 6(14)$, $F = 0.936$, $P > 0.05$, one-way ANOVA) (Figure 5E) or

at an MOI of 50 ($df = 6$, Chi-square = 2.68, $P > 0.05$, Kruskal-Wallis) (Figure 5F). There was also no significant difference in Mx1 expression between the two different viral concentrations used ($df = 1(35)$, $F = 0.48$, $P > 0.05$, two-way ANOVA, $n = 3/\text{group}$).

The expression of off-target genes p75^{NTR} and NgR1 are reduced following lentiviral vector transduction

To determine whether lentiviral vector transduction reduces the expression of off-target genes, we compared the expression of two other genes (p75^{NTR} and NgR1) in transduced and untransduced CGNs using qRT-PCR. p75^{NTR} expression was significantly affected after transduction with lentiviral vectors at an MOI 10 ($df = 6(21)$, $F = 3.33$, $P < 0.05$, one-way ANOVA, $n = 4/\text{group}$) (Figure 6A). Dunnett's post-hoc tests demonstrated that p75^{NTR} expression was significantly attenuated in CGNs transduced with Lenti-LINGO1-sh2 ($P < 0.05$), Lenti-LINGO1-sh3 ($P < 0.05$) or Lenti-Scr ($P < 0.05$) compared to the NVC, but no differences in p75^{NTR} expression were detected between the NVC and Lenti-LINGO1-sh1, Lenti-LINGO1-sh4 or Lenti-H1 (P values > 0.05). Transduction using an MOI 50 also resulted in significant differences in p75^{NTR} expression between the groups ($df = 6(21)$, $F = 3.13$, $P < 0.05$, one-way ANOVA, $n = 4/\text{group}$) (Figure 6B). Dunnett's post-hoc tests indicated that compared to the NVC p75^{NTR} expression was significantly reduced in CGNs transduced with Lenti-LINGO1-sh2 ($P < 0.05$), Lenti-LINGO1-sh3 ($P < 0.01$), Lenti-LINGO1-sh4 ($P < 0.05$) and Lenti-H1 ($P < 0.05$). Lenti-LINGO1-sh1 and Lenti-Scr did not affect p75^{NTR} expression compared to the NVC (P values > 0.05).

NgR1 expression was also assessed. NgR1 mRNA was significantly affected when CGNs were transduced using an MOI of 10 ($df = 6(21)$, $F = 2.91$, $P < 0.05$, one-way ANOVA, $n = 4/\text{group}$) (Figure 6C). Dunnett's post-hoc tests revealed that NgR1 expression was significantly decreased in CGNs transduced with Lenti-LINGO1-sh2 ($P < 0.05$), Lenti-LINGO1-sh3 ($P < 0.05$), Lenti-LINGO1-sh4 ($P < 0.05$) or Lenti-Scr ($P < 0.05$) compared to the NVC. However no difference in NgR1 expression was detected between Lenti-LINGO1-sh1 or Lenti-H1 and the NVC (P values > 0.05). Transduction of CGNs using an MOI of 50 resulted in significant differences between the groups ($df = 6(21)$, $F = 4.04$, $P < 0.01$, one-way ANOVA, $n = 4/\text{group}$) (Figure 6D). Dunnett's post-hoc tests demonstrated that all the lentiviral vectors significantly attenuated NgR1 expression compared to the NVC (P values < 0.05).

Northern blots detect low level expression of shRNA after plasmid transfection but do not confirm the expression of the shRNA or siRNA following lentiviral transduction

Cellular RNA was extracted from HEK cells that had either been transfected with the DNA plasmid expressing LINGO1-sh4 using polyethylenimine (PEI) or transduced with integration-deficient lentiviral vectors expressing Lenti-LINGO1-sh4 at an MOI 10. Both viral and non-viral methods of transfection were effective, giving high transfection and transduction efficiencies that were visually assessed using eGFP expression (Figure 7A and B). RNA samples were loaded onto a polyacrylamide gel, separated using electrophoresis and stained using ethidium bromide (thereby confirming good quality RNA; Figure 7C) prior to transferring onto a membrane for hybridisation. Expression of the shRNA and siRNA from LINGO1-sh4 was examined using a radioactively labelled DNA probe

complementary to the siRNA antisense sequence. In addition, two DNA oligonucleotides were designed; one identical to the LINGO-1-sh4 shRNA and the other identical to the LINGO-1-sh4 siRNA. These were used as positive controls to confirm the northern blot had worked. These probes were loaded into gel lanes in parallel with the samples of RNA. The northern blot did not display bands for the LINGO1-sh4 shRNA or the siRNA in RNA extracted from the viral transduced HEK cells (Figure 7D). In the lane containing RNA from the DNA transfected HEK cells, a faint band representing the shRNA was detected. However, no band was present for the siRNA (Figure 7D). Bands representing the positive control shRNA and siRNA were present, confirming that the probe was correctly designed and labelled and that the northern blot had been carried out correctly (Figure 7D, white arrowhead = shRNA, black arrowhead = siRNA).

Discussion

The aim of this study was to enhance the neurite outgrowth of postnatal CNS neurons by attenuating their response to myelin associated proteins. To achieve this, an integration-deficient lentiviral vector capable of mediating RNA interference were designed and generated to knockdown the expression of LINGO-1, an essential component of the NgR that has previously been shown to be involved in the inhibition of neurite outgrowth [55]. However, while conducting these experiments we unexpectedly detected several negative side effects of transduction with integration-deficient lentiviral vectors encoding shRNAs.

LINGO-1 mRNA was significantly knocked down in CGNs by a lentiviral vector expressing LINGO1-sh4 compared to the control lentiviral vectors. This is consistent with a previous study that demonstrated knockdown of the LINGO-1 mRNA level in oligodendrocytes following transduction with lentiviral vectors expressing an identical siRNA sequence [63]. The three other shRNA sequences targeting LINGO-1 did not mediate significant mRNA knockdown compared to the control lentiviral vectors, demonstrating the requirement to test multiple shRNA sequences to determine the optimal shRNA. Unexpectedly, LINGO-1 mRNA was significantly reduced following transduction with any of the lentiviral vectors when compared to the NVC. This included the control lentiviral vectors, one of which encoded the H1 promoter without an shRNA and the other a scrambled non-targeting shRNA. It is possible that the lentiviral vector encoding just the H1 promoter did lead to expression of some downstream RNA sequence because this vector lacked an RNA polymerase III termination sequence immediately downstream of the H1 promoter. This data suggests that transduction with lentiviral vectors that do not encode an shRNA targeting LINGO-1 can knockdown the LINGO-1 transcript level via a non-specific mechanism. This may be due to the induction of an IFN response, leading to the production of IFNs that activate ISGs and result in a non-specific global inhibition in gene expression and a subsequent reduction in LINGO-1. Since this effect was observed using an MOI 10 it raises concerns that even relatively standard concentrations of lentiviral vector can generate non-specific knockdown. The time scale and cellular system under study may also be of relevance. We have also shown reduced protein levels of the channel transient receptor potential cation channel subfamily V member 1 (TRPV1) in cultured DRG neurons, using an integrating and non-integrating lentiviral vector of similar architecture to the one in the current study (Peluffo et al., manuscript in preparation). In this study the control H1

lentiviral vector lacking an shRNA did not reduce TRPV1 protein levels after five days compared to the NVC, even though the study was carried out at MOI 50 (Peluffo et al., manuscript in preparation). However, in a separate study investigating knockdown of the voltage gated calcium channel subunit $\alpha_2\delta_1$ in DRGs, two western blots demonstrated a reduction in $\alpha_2\delta_1$ protein level using the control H1 lentiviral vector compared to the NVC after 8 days in culture (Supplementary figure 1). Taken together these studies highlight the importance of using appropriate controls (*e.g.*, no virus and scrambled controls) and monitoring off-target effects, including the interferon response, in studies involving lentiviral vectors and RNAi.

Transduction with Lenti-LINGO1-sh4 did not significantly enhance the neurite outgrowth of CGNs on a growth inhibitory MAG substrate. This was inconsistent with previous studies that have demonstrated that inhibiting the function of LINGO-1 using DN-LINGO-1 or LINGO-1-Fc in CGNs attenuated RhoA activation and the inhibitory effects of myelin inhibitors resulting in enhanced neurite outgrowth *in vitro* [55] and functional recovery *in vivo* [60]. There are several possible explanations for this disparity. Firstly, the neurite outgrowth assay used in this experiment may not be sensitive enough to detect the enhanced neurite outgrowth, although this seems unlikely as enhanced CGN neurite outgrowth was detected with Y-27632 (a synthetic ROCK inhibitor), which was used as a positive control. Secondly, the residual expression of LINGO-1 left after knockdown may be enough to maintain the neuron's responsiveness to myelin associated inhibitors, although the majority of LINGO-1 mRNA was reduced by this vector (relative to the NVC). Thirdly, *de novo* gene expression has been shown to be necessary for some phases of axon growth [65] and the induction of ISGs and an IFN response, which was shown to accompany transduction with these lentiviral vectors, may initiate a cellular shift away from a potential neurite growth state via the widespread down-regulation of gene expression, thereby masking any growth promoting effects of LINGO-1 knockdown. Fourthly, the lentiviral vectors may not be expressing the shRNA at sufficiently high levels, which would mean the previously detected LINGO-1 knockdown was non-specific and solely the result of an IFN response.

To address the latter possibility, northern blots were used to evaluate expression of the shRNA and siRNA from the lentiviral vectors and the DNA transfer plasmid. To our surprise, the northern blots did not detect expression of the shRNA or siRNA from the lentiviral vector in HEK cells. However, after non-viral transfection of the LINGO1-sh4 plasmid in to HEK cells, a low intensity band was detected, which represented the shRNA. However, no subsequent siRNA band was detected and the RNA in this lane had smeared during separation potentially making the shRNA band a false positive. The failure to detect the shRNA or siRNA was not a consequence of poor transduction/transfection as the HEK cells demonstrated high transfection and transduction efficiency (assessed *via* eGFP fluorescence). In addition, the positive controls built into the experiment were detected, confirming that the northern blot was executed successfully. However, a large amount of siRNA positive control oligonucleotide was loaded into the gel and yet only a faint band was observed even with a long exposure, potentially suggesting that the blot was not sensitive enough to detect the siRNA and that this assay requires further optimisation. The result indicates that the shLENTImax plasmid expresses only low levels of shRNA and undetectable levels of siRNA. However, our other experiments do provide multiple lines of

indirect evidence for expression of the shRNAs by the lentiviral vectors: namely, increased cell death, knockdown of LINGO-1 and the up-regulation of ISGs in cells transduced with lentiviral vectors encoding shRNAs relative to the lentiviral vectors that did not encode an shRNA. The failure to express shRNAs at high levels could be due to the relative positioning of the promoter to the shRNA, which has been demonstrated to be important for the production of a functional shRNA [66, 67]. Alternatively, it may be a result of promoter interference between the two heterologous promoters: The Pol II CMV promoter driving expression of eGFP may be interfering with the expression of the shRNA by the Pol III H1 promoter. Promoter interference between the CMV promoter and elongation factor-1 α (EF1 α) promoter in late generation lentiviral constructs has previously been demonstrated [68].

We assessed the expression of three well characterised ISGs (OAS1, PKR and Mx1) following transduction of CGNs. OAS1 expression is induced by IFNs and once expressed OAS1 is activated by double stranded RNA. This leads to the synthesis of 2',5'-oligoadenylates, which bind and activate the endoribonuclease RNase-L. RNase-L non-specifically degrades cellular and viral RNA, resulting in a global non-specific inhibition in gene expression and cell death [51]. There have been multiple studies showing OAS1 up-regulation following lentiviral vector mediated expression of shRNAs [44, 46, 47, 54] or transfection with siRNAs [45]. Following transduction of CGNs using an MOI 10, OAS1 expression was only up-regulated in CGNs transduced with lentiviral vectors encoding an shRNA. However, following transduction using an MOI 50, OAS1 expression was up-regulated in CGNs transduced with any of the lentiviral vectors, including the lentiviral vector that did not encode an shRNA. This indicates that at lower vector concentrations low level shRNA expression is required to stimulate up-regulation of OAS1 expression but at higher viral concentrations lentiviral transduction alone is sufficient to induce OAS1 expression. PKR is an IFN inducible protein that is autophosphorylated and activated by dsRNA. Following activation, PKR can phosphorylate and activate eukaryotic protein synthesis initiation factor 2 (eIF-2) that results in a global inhibition of mRNA translation. PKR also induces the further production of IFNs leading to enhanced expression of ISGs and induces cellular apoptosis through eIF-2 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappa B) mediated signalling [48, 51, 69]. PKR has previously been shown to be up-regulated following addition of siRNAs [45, 49]. In the present study PKR expression was not significantly up-regulated following transduction with any of the lentiviral vectors at an MOI 10. However, at an MOI 50, PKR expression had increased in CGNs following transduction with any of the lentiviral vectors encoding shRNAs. Mx1 is also an IFN-induced gene and is part of the Mx GTPase family of proteins that inhibits RNA synthesis in response to viral infection and double stranded RNA [51, 70]. Several studies have shown that siRNAs increase the expression of Mx1 in a dose dependent manner in CNS neurons [40, 49]. However, in the present study Mx1 expression was not affected by any of the lentiviral vectors at either concentration. This suggests that Mx1 is not involved in the lentiviral vector induced cell death or the non-specific knockdown observed in the present study. These results confirm the findings from previous studies which demonstrated that lentiviral vectors and shRNAs can induce an IFN response. This data emphasises that it is

crucial to assess ISG levels relative to cells that have not been transduced or transfected at all.

Due to the observed interferon response we investigated whether transduction with lentiviral vectors results in a reduction in the expression of off-target genes. We measured the expression of p75^{NTR} and NgR1 because these genes are expressed by CGNs [55], can bind to LINGO-1 and form a functional NgR complex [55] but are not targeted for knockdown by the LINGO-1 shRNAs. Using an MOI of 10, p75^{NTR} and NgR1 expression were only attenuated in CGNs transduced with lentiviral vectors expressing an shRNA. However after transduction at an MOI of 50 both p75^{NTR} and NgR1 expression levels were significantly reduced by both lentiviral vectors encoding an shRNA as well as those not encoding an shRNA. This data suggests that at low viral vector concentrations, shRNA expression stimulates an interferon response leading to a non-specific down-regulation of both target and off-target gene expression. However at higher viral concentrations lentiviral transduction alone is able to stimulate an interferon response, resulting in the non-specific down-regulation of both target and off-target genes and conceivably induce an even more widespread reduction in gene expression.

Both CGN transduction and viability were affected by viral concentration and shRNA expression. This is consistent with previous studies showing that shRNA expression by Pol III promoters can result in cytotoxicity *in vitro* and fatality *in vivo* [40, 44–47, 49, 71, 72]. These studies have extensively demonstrated that high levels of shRNA expression can activate an IFN response leading to global changes in gene expression and cell death. In contrast, the results from the present study suggest that even at very low levels of expression, shRNAs can induce an IFN response leading to an increased incidence of cell death. Lentiviral vectors have generally been demonstrated to be safe and well tolerated in the CNS [23, 29]. However, our data also suggests that the integration-deficient lentiviral vectors themselves may contribute to the induction of an IFN response, as has been previously reported for integrating lentiviral vectors. Lentiviral vectors have previously been shown to rapidly induce a type I IFN response in hepatocytes, which interfered with cell transduction and viability [73, 74]. In addition, a recent study by Bauer et al showed that transduction of cortical neurons with integration-proficient lentiviral vectors resulted in elevated levels of cleaved caspase-3 protein and increased expression of the ISG OAS1, which was then further increased by shRNA expression [54]. Our data is in agreement with these studies and demonstrates that at viral concentrations above MOI 10, integration-deficient lentiviral vectors themselves can induce the expression of OAS1 and an IFN response in CNS neurons resulting in a down-regulation of off-target genes and decreased viability. Furthermore, when the integration-deficient lentiviral vectors encode an shRNA we saw additional effects on cytotoxicity and ISG expression. These results strongly suggest that the viral concentration should be titrated to determine the lowest MOI that can be used to produce effective knockdown and the required functional effect.

Our data emphasises the need for appropriate controls to confirm sequence-specific RNA silencing and assess the level of cytotoxicity. What constitutes an appropriate control has recently come under scrutiny [75] as each siRNA sequence has a unique immunostimulatory response, and it must be ensured that the control siRNA has a similar immunostimulatory

response to the therapeutic siRNA sequence. This problem was recently highlighted by a set of studies that had used an siRNA to induce the silencing of vascular endothelial growth factor (VEGF) to generate a therapeutic effect, which was not observed when using a commonly used negative control siRNA that targeted eGFP [75]. However, it was later discovered that the control siRNA had an unusually low immunostimulatory response and that the observed therapeutic effect was actually due to the induction of an IFN response by the VEGF siRNA [50, 76]. These studies raise concerns over how many other RNA interference induced therapeutic effects are the result of an induced IFN response rather than the sequence-specific silencing of the targeted gene. Measures can be taken when designing an siRNA to minimise its IFN response [77]. Specific immunostimulatory sequence motifs such as GU-rich sequences and 5'-UGU-3' have been identified that should be avoided [78], it has also shown that the U6 promoter produces a larger IFN response compared to the H1 promoter [79]. It has been reported that specific modifications to the siRNA, such as the "Stealth" modification [40] or the transfer of the shRNA into a shRNA-miR structure can reduce the induction of ISGs and the IFN response [54, 80].

In conclusion, this study has demonstrated that although integration-deficient lentiviral vectors can efficiently transduce postnatal CGNs *in vitro*, transduction with lentiviral vectors at low concentrations when encoding shRNAs or at high concentrations without an shRNA can induce the up-regulation of ISGs that result in an IFN response leading to the non-specific down-regulation of both target and off-target genes and ultimately cell death. In addition, it was demonstrated via northern blot that the lentiviral transfer plasmid only expressed low levels of shRNA, which may be a result of promoter interference. Together, these results may explain why CGN neurite outgrowth was not enhanced on an inhibitory MAG substrate following transduction with lentiviral vectors expressing a shRNA targeting LINGO-1 despite substantial knockdown.

While there are still concerns with using lentiviral vectors and shRNAs for RNA interference, they still present a powerful tool that can be effective as long as the appropriate design criteria, modifications and controls are used. Alternatively, AAV vectors have been reported to display negligible cytotoxicity and immunogenicity and provide efficient CNS transduction [81–85]; however cytotoxicity and lethality associated with shRNA expression has still been reported for AAV vectors [86–90]. shRNA-miRs that mimic the structure of endogenous miRNAs seem to be capable of bypassing the majority of the silencing side effects and may therefore represent more promising therapeutic mediators of RNA interference [54, 79, 91].

Acknowledgements

This study was supported by a Research Councils UK Academic Fellowship (L.M.), the British Pharmacological Society's Integrative Pharmacology Fund (L.M), Friends of Guy's Hospital Research Grants (L.M. and R.J.Y.-M.), a Biotechnology and Biological Sciences Research Council's Doctoral Training Grant (T.H.), financial support from the 7th EU Framework Programme (PERSIST project, grant agreement n°222878, R.J.Y.-M.) and a grant from Genoma España (R.J.Y.-M.). We would also like to thank Dr. Thijn R. Brummelkamp, Dr. Rene Bernards and Dr. Reuven Agami for the pSUPER plasmid and Professor Marie Filbin for the CHO-MAG and CHO-R2 cells.

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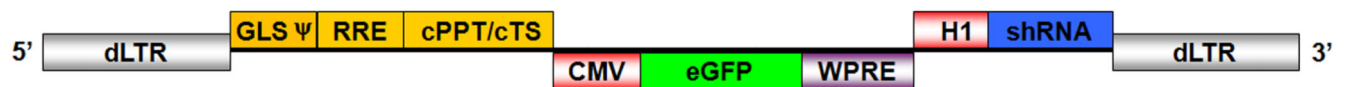
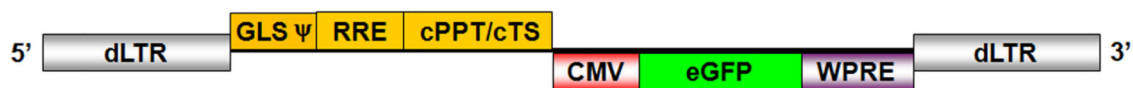
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(A) Lenti-Lingo1-sh1-4 / Lenti-Scr**(B) Lenti-H1****(C) pRRLsin_PPT_CMV_GFPpre****Figure 1.**

Schematic representation of the integration-deficient lentiviral vectors. Schematic depicts linear form of double stranded DNA after completion of reverse transcription. All vectors contain an eGFP expression cassette under the control of a CMV promoter and including a WPRE. The cassettes are flanked by the self-inactivating 5' deleted LTR. (A) Lenti-LINGO1-sh1-4/Lenti-Scr contain an shRNA expression cassette driven by the H1 promoter placed downstream of the eGFP expression cassette. (B) Lenti-H1 contains the H1 promoter but no shRNA or Pol III termination sequence. dLTR, deleted long terminal repeat; GLS, gag leader sequence incorporating the packaging element (ψ); RRE, rev response element; cPPT/cTS, central polypurine tract and central termination sequence; CMV, cytomegalovirus immediate early promoter; eGFP, enhancer green fluorescent protein; H1, H1-RNA promoter; shRNA, short hairpin RNA; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

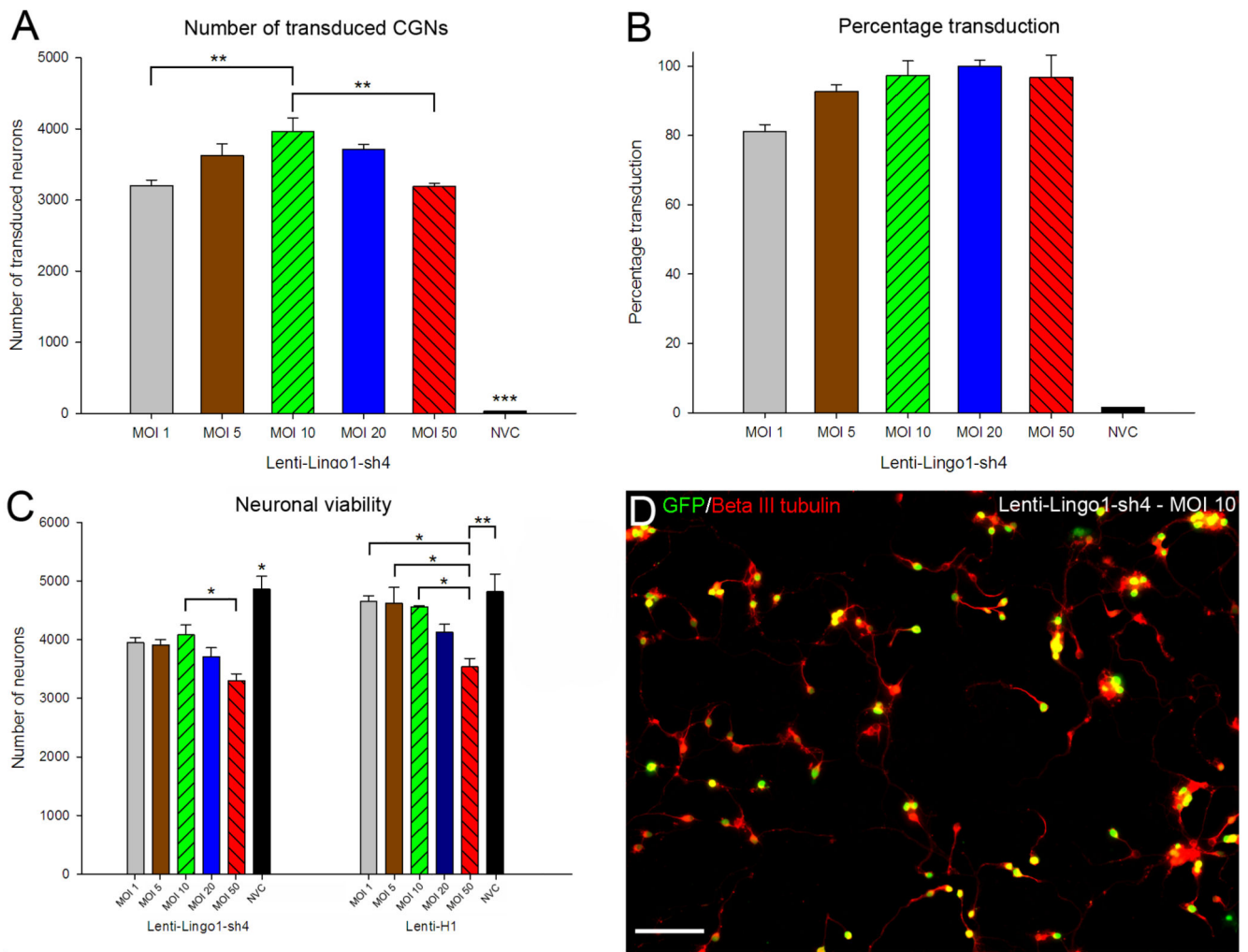


Figure 2.

CGNs are efficiently transduced in vitro, although viability was affected by shRNAs and viral concentration. (A) Quantification of the mean number of transduced CGNs using Lenti-LINGO1-sh4. The number of eGFP positive CGNs was counted and the mean number of transduced CGNs plotted for each MOI. There were significantly more CGNs transduced using an MOI 10 compared to MOI 1 or 50 (** $P < 0.01$). Values represent mean and SEM; analysis was performed using one way ANOVA with Tukey post-hoc tests, $n = 3$. (B) Quantification of the percentage transduction efficiency, which was calculated by dividing the number of transduced CGNs by the total number CGNs multiplied by one hundred. Percentage transduction did not change with viral concentrations higher than MOI 10 where the transduction efficiency was already above 90%. Values represent mean and SEM, $n = 3$. (C) Quantification of the mean number of CGNs after transduction with Lenti-LINGO1-sh4 or Lenti-H1. The number of CGNs was counted and the mean number plotted for each MOI. Neuronal viability was affected by MOI and there was a decrease in neuronal viability at higher viral concentrations. Neuronal viability was also differentially affected by the viral vectors. There were different patterns of neuronal viability between the lentiviral vectors that encoded shRNAs and the viral vectors that did not encode an shRNA. Values represent mean

and SEM, analysis was performed using one way ANOVA with Tukey post-hoc tests, $n = 3/$ group. (D) CGNs are efficiently transduced by Lenti LINGO1-sh4 at an MOI of 10. Transduced CGNs expressing eGFP (green) and stained for beta-III tubulin (red) appear yellow. Scale bar: 100 μm .

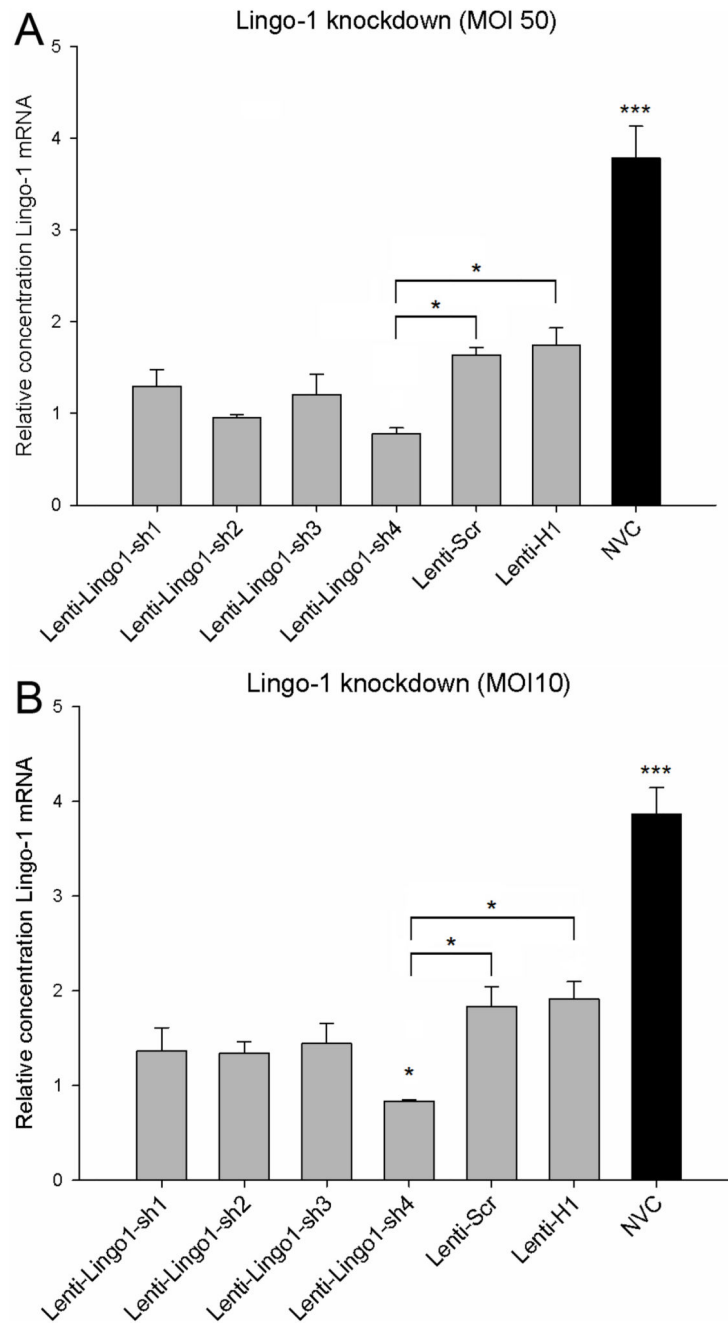


Figure 3.

Lenti-LINGO1-sh4 knocks down LINGO-1 mRNA, although non-specific silencing was also observed with the control vectors. (A) Quantification of the relative level of LINGO-1 mRNA in CGNs transduced with lentiviral vectors at an MOI 50. Lenti-LINGO1-sh4 significantly knocked down LINGO-1 expression 2.1 fold and 2.2 fold respectively compared to Lenti-Scr and Lenti-H1 (* $P < 0.05$). In addition, significantly lower levels of LINGO-1 were detected in CGNs transduced with any of the lentiviral vectors compared to the NVC (***) $P < 0.001$). Values represent mean and SEM, analysis was performed using

one way ANOVA with Tukey post-hoc tests, $n = 4/\text{group}$. (B) Quantification of the relative level of LINGO-1 mRNA in CGNs after transduction at an MOI 10. Lenti-LINGO1-sh4 significantly knocked down LINGO-1 expression 2.2 fold and 2.3 fold respectively compared to Lenti-Scr, Lenti-H1 (* $P < 0.05$). However, significantly lower levels of LINGO-1 were detected in CGNs transduced with any of the lentiviral vectors compared to the NVC (***) $P < 0.001$). Values represent mean and SEM, analysis was performed using one way ANOVA with Tukey post-hoc tests, $n = 3/\text{group}$.

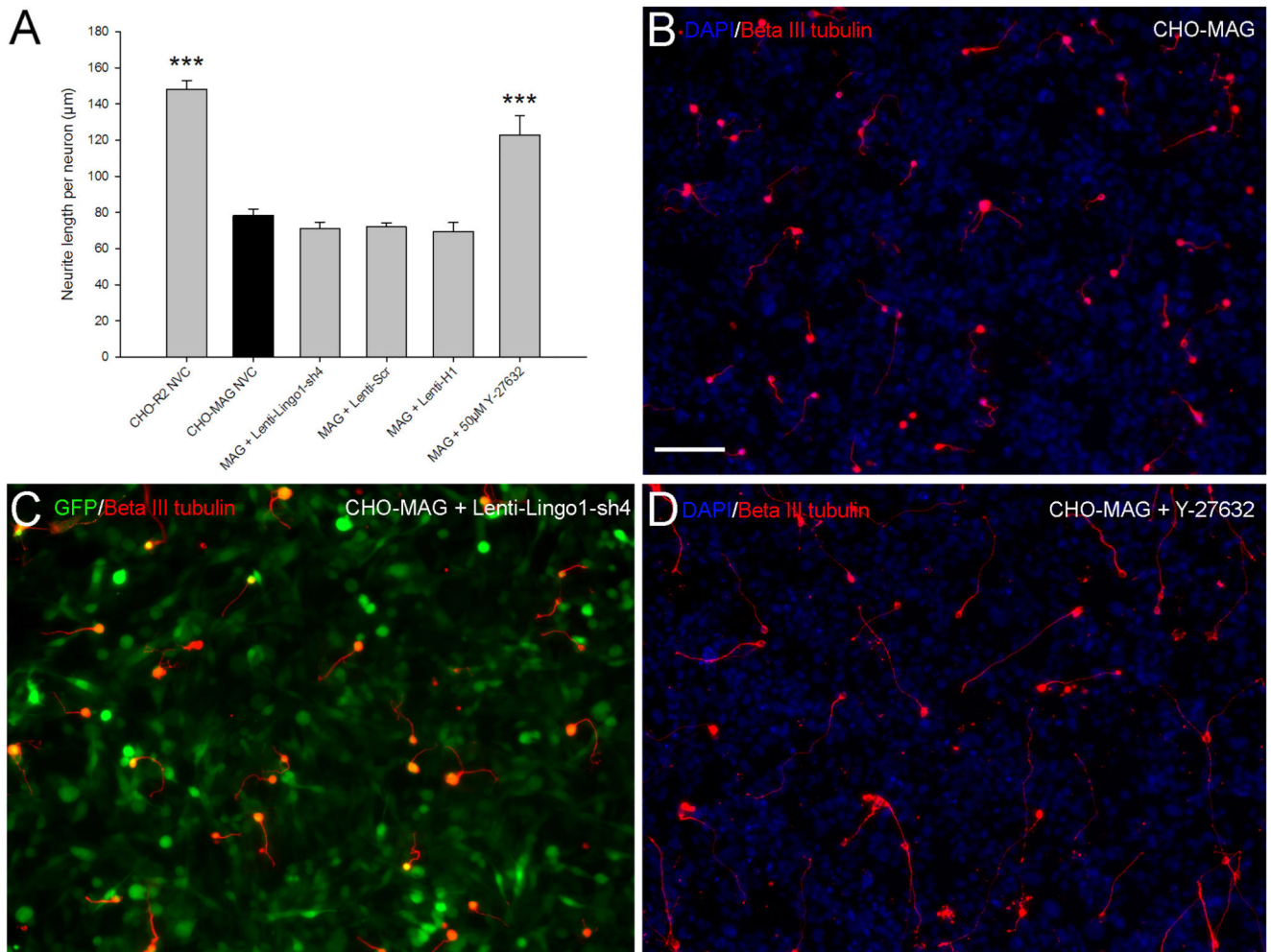


Figure 4.

Integration-deficient lentiviral vectors encoding an shRNA targeting LINGO-1 do not enhance neurite outgrowth. (A) The neurite length of beta-III tubulin positive CGNs was measured, divided by the total number of neurons and the mean neurite length per neuron plotted. CGNs plated on the CHO-R2 cells had significantly longer neurites compared to CGNs plated on the inhibitory CHO-MAG cells (***) $P < 0.001$. CHO-MAG inhibition could be partially reversed with using 50 μM Y 27632 (***) $P < 0.001$. Lenti-LINGO1-sh4 did not significantly increase neurite outgrowth of CGNs plated on an inhibitory MAG substrate compared to the control lentiviral vectors or the NVC ($P > 0.05$). Values represent mean and SEM, analysis was performed using one way ANOVA, with Dunnett's post-hoc tests comparing to CHO-MAG cells, $P < 0.001$, $n = 8/\text{group}$. (B) CGN neurite outgrowth is inhibited by CHO-MAG cells. (C) Transduction with Lenti-LINGO1-sh4 does not alleviate the MAG inhibition and enhance CGN neurite outgrowth. (D) The ROCK inhibitor Y-27632 partially reverses the MAG inhibition and promotes neurite outgrowth. Scale bar: 100 μm .

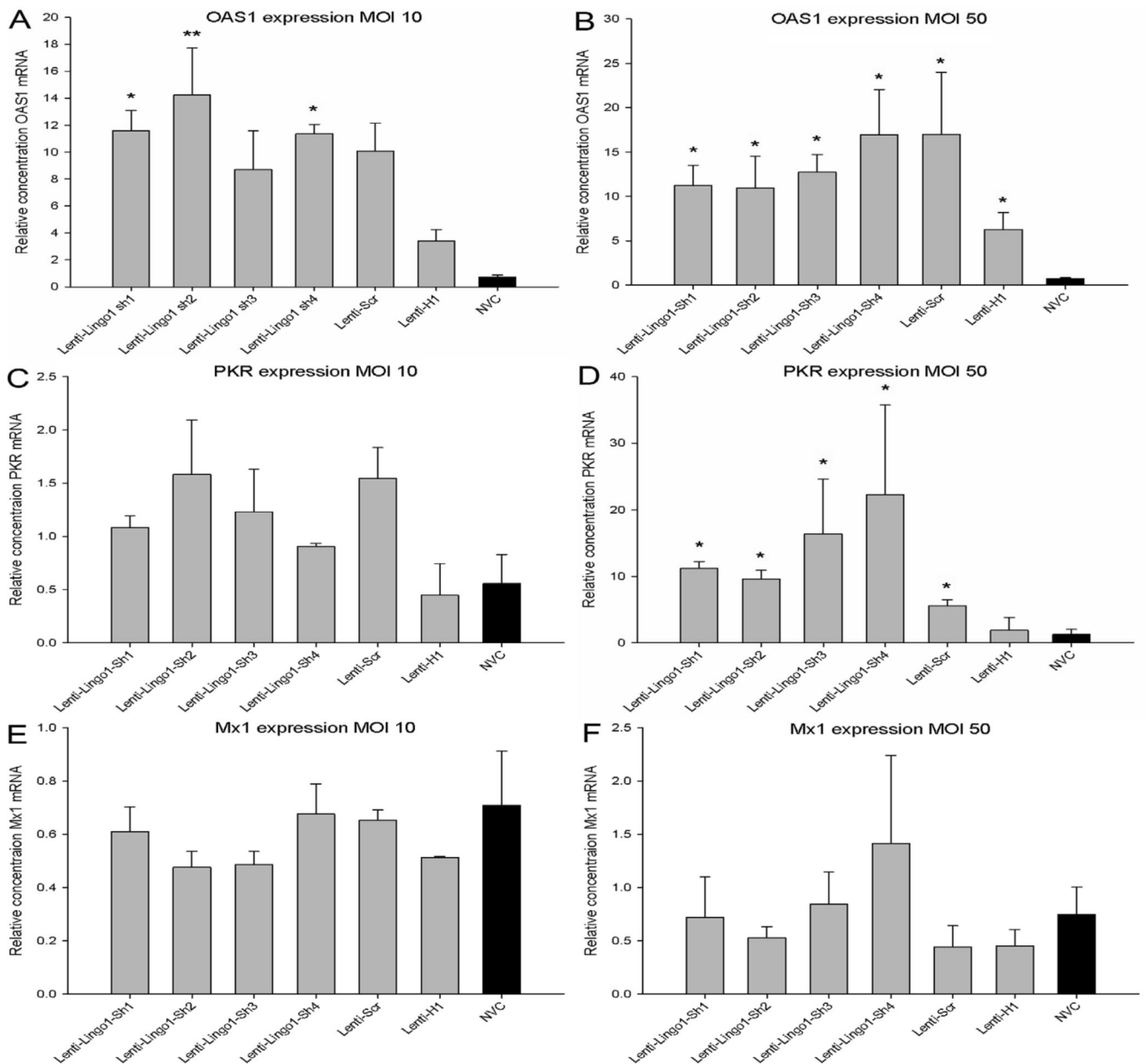
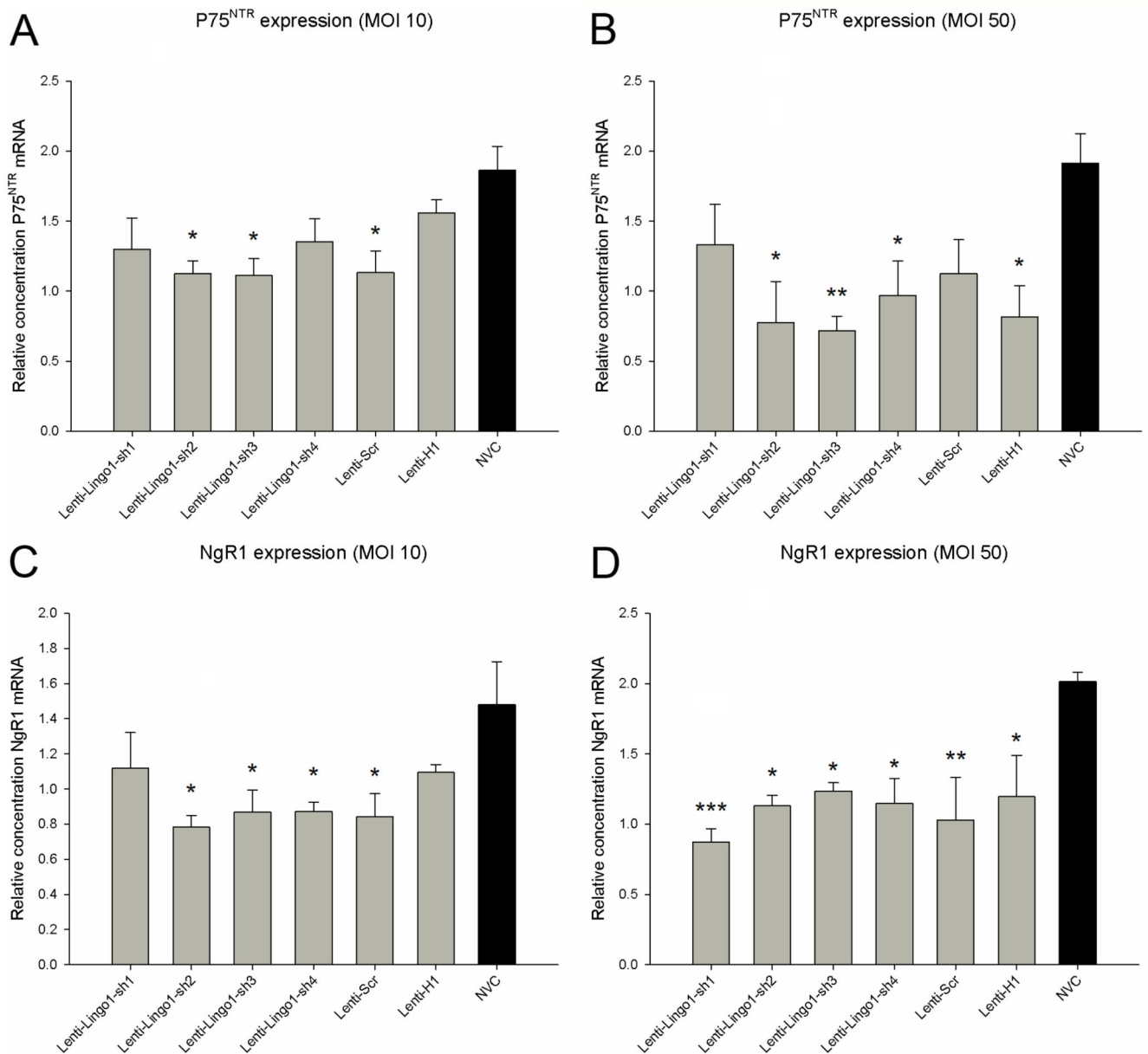


Figure 5.

OAS1 and PKR induction following transduction with integration-deficient lentiviral vectors encoding shRNAs. (A) Quantification of the relative level of OAS1 mRNA in CGNs transduced using an MOI 10. There was a significant increase in OAS1 expression with Lenti-LINGO1-sh1, Lenti-LINGO1-sh2 and Lenti-LINGO1-sh4 compared to the NVC. Values represent mean and SEM, analysis was performed using one way ANOVA with Tukey post-hoc tests, * $P < 0.05$, ** $P < 0.01$, $n = 3/\text{group}$. (B) Quantification of the relative level of OAS1 mRNA in CGNs transduced using an MOI 50. All the lentiviral vectors significantly increased OAS1 expression compared to the NVC. Values represent mean and SEM, analysis was performed using Kruskal-Wallis with Mann-Whitney post-hoc tests, * P

< 0.05, n = 4/group. (C) Quantification of the relative level of PKR mRNA in CGNs transduced using an MOI 10. PKR expression was not significantly affected compared to the NVC. Values represent mean and SEM, analysis was performed using one way ANOVA, $P > 0.05$, n = 3/group. (D) Quantification of the relative level of PKR mRNA in CGNs transduced using an MOI 50. PKR expression was significantly increased in CGNs transduced with any of the shRNA expressing lentiviral vectors compared to the NVC. Values represent mean and SEM, analysis was performed using Kruskal-Wallis with Mann Whitney post-hoc tests, * $P < 0.05$, n = 4/group. (E) Quantification of the relative level of Mx1 mRNA in CGNs transduced at an MOI 10. No significant difference in Mx1 expression was observed compared to the NVC. Values represent mean and SEM, analysis was performed using one way ANOVA, $P > 0.05$, n = 3/group. (F) Quantification of the relative level of Mx1 mRNA in CGNs transduced using an MOI 50. Mx1 expression was not significantly affected compared to the NVC. Values represent mean and SEM, analysis was performed using Kruskal-Wallis, $P > 0.05$, n = 4/group.

**Figure 6.**

Off-target genes p75NTR and NgR1 are reduced following lentiviral vector transduction. (A) Quantification of the relative level of p75NTR mRNA in CGNs transduced using an MOI 10. There was a significant decrease in p75NTR expression with Lenti-LINGO1-sh2, Lenti-LINGO1-sh3 and Lenti-Scr compared to the NVC. Values represent mean and SEM, analysis was performed using one way ANOVA with Dunnett's post-hoc tests comparing to the NVC, * $P < 0.05$, $n = 4/\text{group}$. (B) Quantification of the relative level of p75NTR mRNA in CGNs transduced using an MOI 50. p75NTR expression was significantly reduced in CGNs transduced with Lenti-LINGO1-sh2, Lenti-LINGO1-sh3, Lenti-LINGO1-sh4 and Lenti-H1 compared to the NVC. Values represent mean and SEM, analysis was performed using one way ANOVA with Dunnett's post-hoc tests comparing to the NVC, * $P < 0.05$, **

$P < 0.01$ $n = 4/\text{group}$. (C) Quantification of the relative level of NgR1 mRNA in CGNs transduced using an MOI 10. NgR1 expression was significantly decreased in CGNs transduced with Lenti-LINGO1-sh2, Lenti-LINGO1-sh3, Lenti-LINGO1-sh4 and Lenti-Scr compared to the NVC. Values represent mean and SEM, analysis was performed using one way ANOVA with Dunnett's post-hoc tests comparing to the NVC, * $P < 0.05$, $n = 4/\text{group}$ (D) Quantification of the relative level of NgR1 mRNA in CGNs transduced using an MOI 50. There was a significant decrease in NgR1 expression with any of the lentiviral vectors compared to the NVC. Values represent mean and SEM, analysis was performed using one way ANOVA with Dunnett's post-hoc tests comparing to the NVC, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 4/\text{group}$.

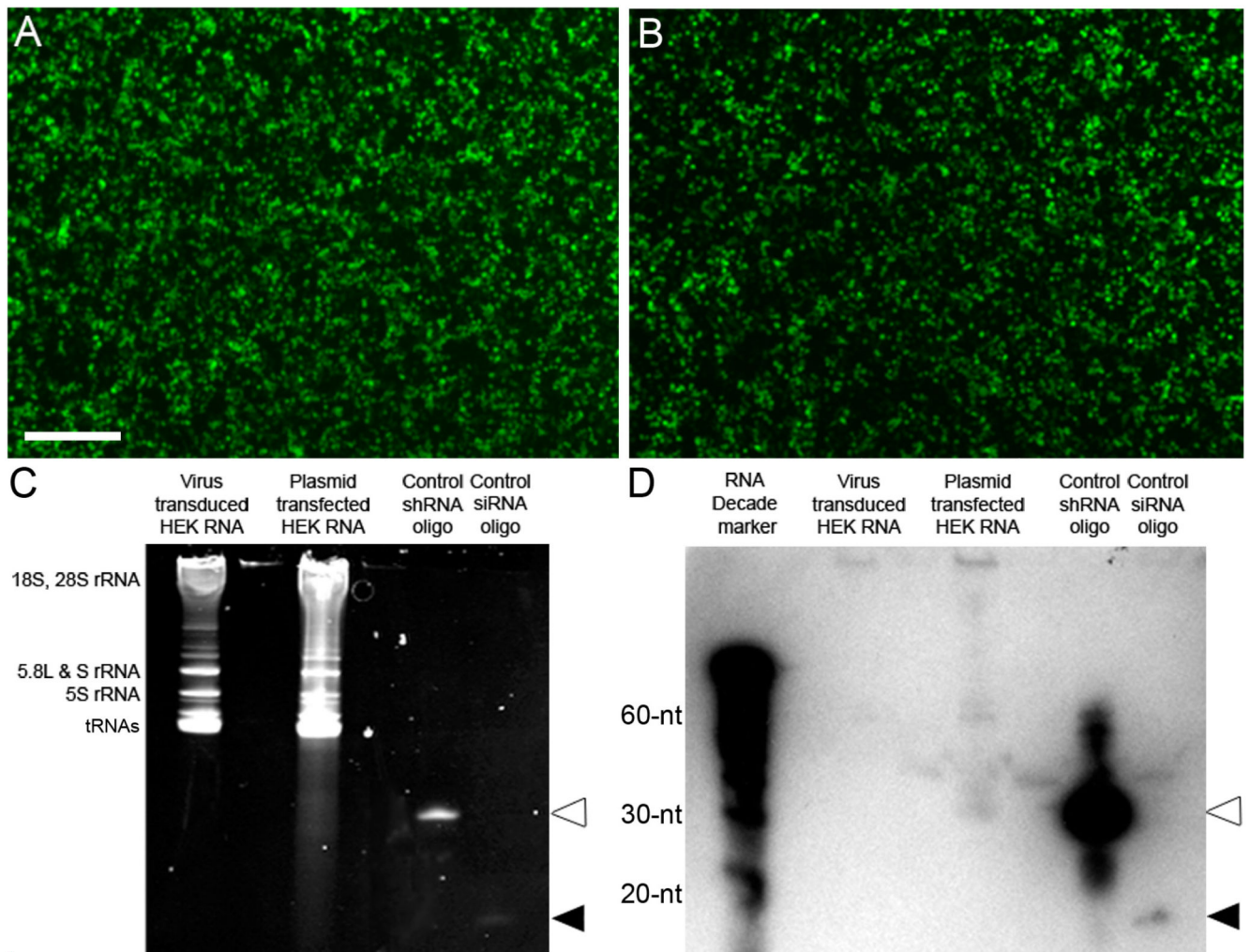


Figure 7. Northern blots do not detect a high level of shRNA expression. (A) Image of eGFP positive HEK cells that were efficiently transduced using Lenti-LINGO1-sh4 at an MOI 10. Scale bar: 500 μ m. (B) Image of eGFP positive HEK cells that were efficiently transfected with the LINGO1-sh4 plasmid using PEI. (C) Image of the resolved RNA gel, the bands correspond to the different ribosomal RNA (rRNA) species or transfer RNAs (tRNAs) as labelled on the left side. The RNA resolved from the plasmid transfected HEK cells shows some smearing. The shRNA (white arrowhead) and siRNA (black arrowhead) positive controls can be seen, confirming the design and sensitivity of the probe (D) Image of the small transcript northern blot. Neither the shRNA nor siRNA was detected from the virus transduced HEK cell RNA. A faint band representing the shRNA was detected from the plasmid transfected HEK cell RNA but a band for the siRNA was not detected. Bands representing the shRNA (white arrowhead) and siRNA (black arrowhead) positive controls were detected.

shRNA sequences.

Table 1

Name	Sense	Loop	Antisense
LINGO1-sh1	CTACATGTTCCAAGACCTA	TTCAAGAGA	TAGGTCTTGGAACATGTAG
LINGO1-sh2	GCTCAATGTTTCTGGCAAC	TTCAAGAGA	GTTGCCAGAAACATTGAGC
LINGO1-sh3	ACAAAGCACAACATCGAAA	TTCAAGAGA	TTTCGATGTTGTGCTTTGT
LINGO1-sh4	GATCGTCATCCTGCTAGAC	TTCAAGAGA	GTCTAGCAGGATGACGATC
Lenti-Scr	TGGTTTACATGTCGACTAA	TTCAAGAGA	TTAGTCGACATGTAAACCA