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Lentiviral vector mediated knockdown of the NG2 proteoglycan or expression of Neurotrophin 3 promotes neurite outgrowth in a cell culture model of the glial scar.

Abbreviated title: Effect of LV vectors on neurite outgrowth in an inhibitory environment

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Abstract

Background; Following spinal cord injury, a highly inhibitory environment for axonal regeneration develops. One of the main sources of this inhibition is the glial scar which is formed after injury by reactive astrocytes. The inhibitory environment is mainly due to chondroitin sulphate proteoglycans (CSPGs). Neuroglycan 2 (NG2), one of the main inhibitory CSPGs, is upregulated following spinal cord injury.

Methods; siRNA was designed to target NG2 and this shRNA was cloned into a lentiviral vector. The neurotrophic factor Neurotrophin-3 (NT-3) promotes the growth and survival of developing neurites and has also been shown to aid regeneration. NT-3 was also cloned into a lentiviral vector. *In vitro* assessment of these vectors using a co-culture system of dorsal root ganglia (DRG) neurons and Neu7 astrocytes was carried out. The Neu7 cell line is a rat astrocyte cell line which over expresses NG2, thereby mimicking the inhibitory environment following spinal cord injury.

Results/Discussion; These experiments showed that both the knockdown of NG2 via shRNA and over expression of NT-3 can significantly increase neurite growth, but that a combination of both vectors did not confer any additional benefit over the vectors used individually. These lentiviral vectors show promising potential for growth and survival of neurites in injured CNS tissue

Key Words: Glial Scar, NG2, Neurotrophin-3, Lentiviral Vector, Spinal cord injury, Neu7 astrocytes

Introduction

When the spinal cord suffers an injury it responds by commencing wound repair and scar formation. It is widely accepted that the glial scar, which is formed after spinal cord injury (SCI), creates an environment which is highly inhibitory to axonal regeneration through the injured tissue (1, 2) . Though initially, at the time of injury this inhibition serves to protect the surviving neurons from entering the highly toxic environment of the glial scar, in the long term it is this inhibitory environment which prevents recovery from SCI (3).

The inhibitory environment of the glial scar is due to a complex cellular and molecular response. The reactive astrocytes, oligodendrocyte precursor cells and

meningeal cells that are present in the glial scar have been shown to express a number of CSPGs including aggrecan, brevican, neurocan, versican, phosphacan and NG2. Studies have shown that the digestion of CSPG GAG side chains by the enzyme chondroitinase ABC (chABC) can increase axonal regeneration and functional recovery *in vivo* (4).

NG2 has been shown to be up regulated following SCI (5) and is observed as early as 2 days post injury (6), with peak expression at 7 days (7). NG2 has been shown to induce growth cone collapse, suggesting that it is similar to other axon guidance molecules that collapse and repel growth cones (8). Targeting NG2 by the use of blocking antibodies following SCI has been observed to promote regeneration (9). In *in vitro* studies neurons are unable to attach to tissue culture surfaces which have been coated with NG2, unless further adhesion molecules are provided (10) and axons have also been shown to avoid NG2 rich areas in culture (11). Knockdown of NG2 by shRNA is a rational approach to reducing the inhibitory environment for neurite outgrowth, in particular, using lentiviral vector expression as a delivery vector for shRNA provides long term expression. Whilst reducing the capacity of CSPGs to inhibit regeneration, providing a parallel growth factor stimulus may also be needed.

An important process in the development of the nervous system is axon guidance and pathfinding. NT-3 is involved in axon pathfinding by a process mediated by its chemoattractive properties (12). NT-3 promotes growth of axons through the inhibitory dorsal root entry zone and into the spinal cord (13). Viral vector delivery of NT-3 has been described using adenoviral and lentiviral vectors (14, 15) and has also been expressed from a variety of modified transplanted cells such as olfactory ensheathing cells, fibroblasts and bone marrow derived cells (16-18). Primary DRG cultures are a commonly used model for assessing neurite growth *in vitro* (19). DRG cultures have been utilized to assess the effectiveness of viral vectors expressing neurotrophic factors on neurite growth (20, 21). NT-3 is the first chemotropic molecule to be seen to promote reinnervation into the correct brainstem target *in vivo* and the formation of possible functional synapses (15).

Lentiviral vectors are a well established vector system for gene delivery to the CNS, due to their long term stable transduction, and their ability to transduce non-dividing cells (22). Therefore the ideal viral vector for CNS would potentially give long term stable integration, reducing the need for repeat surgical delivery of viral vector. Obviously

lentiviral vectors ability to transduce non-dividing cells makes them a very advantageous vector for gene therapy to the non dividing cells of the CNS. Interestingly the NT-3 study by Alto *et al* 2009 used lentiviral vector to deliver NT-3 and to create a gradient to coax axons to reinnervate the correct natural brain stem target. Hence they are a useful tool to attempt to modify the inhibitory environment of the glial scar via genetic modification of the tissue.

Our hypothesis in this study is that through the use of shRNA in a lentiviral vector system we can sufficiently reduce levels of NG2 to promote neuronal growth in an inhibitory environment. We also proposed to increase the growth and survival of neurons through over expression of NT-3 via lentiviral vectors. Lentiviral vectors offer the advantage of long term stable expression, which will give long term knockdown of NG2 via shRNA, over the transient knockdown obtained with enzymatic or antibody methods.

Methods

siRNA sequence Design

Four siRNA oligonucleotide sequences to the sequence of NG2 were designed, by Miss Elizabeth Geurts using NCBI BLAST to check homology. A random scrambled sequence was also designed, as a control. These siRNA sequences were assessed for efficient NG2 knockdown, as described below. Following selection of the most effective siRNA sequence, the sequence was used to construct a shRNA to be cloned into the Lentiviral expression vector pLVTHM, to give pLVTHM shNG2.

Electroporation of siRNA NG2

Neu7 astrocytes were seeded in a 6 well plate at a density of 1×10^5 cells per well. 24 hours after seeding the cells were trypsinised. 3×10^5 cells were spun at 200g for 6 minutes and resuspended in 300 μ l of siPORT electroporation buffer (Ambion). 10 μ l of siRNA was added to the cells and cells were transferred to Bio-Rad 4mm electroporation cuvettes. Cuvettes were placed in the Bio-Rad GenePulser Xcell electroporator. The cells were electroporated under the following conditions: 4 pulses of 1200V for 90 μ s, 5 sec apart. Cells were allowed to recover in the cuvettes for 10 minutes in a cell culture incubator. The cells were then reseeded into a 6 well plate. 24 hours post electroporation RNA was extracted from the cells using the QuiagenTM RNeasy spin kit as per manufacturers protocol. The previously prepared RNA was used to produce cDNA for use in quantitative real time PCR. cDNA was synthesized from the RNA using the ImProm-IITM reverse transcription system (Promega). PCR was then carried out on the resultant RNA for NG2 and Lamin as a control. As mentioned about the most effective siRNA was used to construct the shRNA. The scramble control had no effect on NG2 levels.

Lentiviral vector production

Lentiviral vectors (LV) were produced as previously described (22). A siRNA was designed against NG2 and this was the cloned into the pLVTHM lentiviral expression

plasmid under the control of the H1 promoter. An siRNA scramble, was also designed as a control. The siRNA sequence that was used to construct the shRNA is as follows; sense- 5'CGAGUGAGGUACCUGAGUAUU 3' anti-sense- 5' UACACAGGUACCUCACUCGAA 3'. The shRNA was cloned into the pSUPER vector, from this the shRNA was cloned into pLVTHM at the BamH1 and Mlu1 sites. (pLVTHM shNG2), which also contains GFP as a marker gene. The pWPT expression plasmid was modified by replacing the GFP gene with the human NT-3 gene. Lentiviral vectors were produced by standard techniques (22), using Jet PEI (Star Labs) according to manufacturers protocol. Virus was harvested 48 and 72h post transfection and concentrated by ultracentrifugation at 27,000g for 2.5 hrs. Virus was aliquoted and stored at -80°C. Fixed volumes of the virus were used to transduce HeLa cells and quantitative PCR was performed on the HeLa DNA for the viral GAG gene to determine viral titre. The following primers for GAG were used; forward 5' GGAGCTAGAACGATTCGCAGTTA 3', reverse GGT 5' TGTAGCTGTCCCAGTATTTGTA 3' . All quantitative real time PCR was performed on the ABI onestep machine, on the standard programme, using beta actin as a control gene.

Lentiviral vector validation

pLVTHM shNG2

1 X 10⁵ Neu7 cells were transduced with LVshNG2 at MOI 100 and 72 h post-transduction, RNA was extracted from the cells using Qiagen RNeasy spin kit, and cDNA was generated by reverse transcription, using the Promega ImProm-II Reverse Transcription Kit. Quantitative PCR was performed on the cDNA for NG2 to assess the effectiveness of NG2 knockdown. Lamin was used as a control gene. NG2 primers forward 5' AGCAGGCCAAACAGATCATCT 3', reverse 5' AGTCACTCAGCACCGTGTCTG 3'. Lamin primers forward 5' AATGACCGTCTGGCCGTGTA 3', reverse 5' TCGGCTGACCACCTCTTC 3'.

LV NT-3

1×10^5 Neu7 and primary rat astrocytes were transduced with LV NT-3 at MOI 100. Medium was removed at 72 and 120h after transduction and levels of NT-3 expression determined using ELISA (R&D Systems).

Recombinant (rh) NT-3 treatment of DRG cultures

DRG's were seeded in 8 well chamber slides. rhNT-3 (R&D Systems) was placed in triplicate into each well at the following concentrations: 1000, 750, 500, 250, 100, 50, 10 pg/ml. Control wells contained media without rhNT-3. After one day *in vitro* the DRG cells were fixed and immunostained with β -tubulin to detect levels of neurite outgrowth (see immunocytochemistry protocol below). Images were taken using a Olympus IX81 microscope using Volocity software. Stereology was performed to assess levels of neurite growth (see stereology protocol below).

Astrocyte culture

Rat cortical cultures were prepared from P1-P2 Sprague Dawley pups. Pups were anaesthetised on ice and decapitated. Cortex was dissected out in HBSS and chopped using a scalpel blade. Tissue was triturated and expelled through a 70 μ m Nitex Mesh filter (Falcon) into a 50ml tube. The cells were spun at 1000rpm to pellet the cells. Cells were resuspended in astrocyte medium, i.e. DMEM/F12 (Sigma) containing 10% FBS (Sigma), 1% Pen/Strep (Sigma), 1% L-Glutamine (Sigma), and seeded into tissue culture flasks at the equivalent of 2 brains per flask. Cells were fed every 2-3 days. Approximately 10 days after initial culture, cells are purified to obtain pure astrocyte cultures. Purification involved shaking the flasks at 190rpm for 1hr on a shaker platform inside a 37°C incubator in a horizontal position. This shaking removes the microglia. Medium containing microglia was removed and replaced with fresh medium. Cells were returned to the shaker in the incubator and allowed to shake at 250rpm for 15-18 hours to separate the oligodendrocytes from the astrocytes. The suspended cells were removed, leaving a pure population of astrocytes adherent in the flask. The purity of each culture was assessed at this point by light microscopy (~95%).

Lentiviral transduction of astrocytes co-cultured with DRGs

Neu7 and primary rat astrocytes were seeded in Falcon 12 well companion plates at 4.2×10^4 per well. 24 hrs post-seeding, the cells were transduced with either LV pLVTHM shNG2, LVNT-3 or both, each virus being used at MOI 100. 24 hrs post transduction the medium on the cells was changed with fresh medium. At 48 hrs post transduction the inserts containing the acclimatize DRGs were placed on top of the transduced cells and allowed to grow in co-culture for 1, 2 and 3 days. A control group was also completed using LV GFP to transduce both astrocytes cells.

DRG co-culture

Female Sprague Dawley rats were mated with males of the same strain and females were allowed to gestate to day 16, at which time both the mother and foetuses were euthanized and the dorsal root ganglia (DRG) were harvested into ice cold Hanks Solution. DRG were spun at 500g for 5min to pellet the cells. The cell pellet was then resuspended in Gibco alpha MEM, 15% FBS, 0.7% L-glutamine, 1% Pen/Strep, 3% 20% w/v glucose, 10ng/ml NGF, 0.02M FrdU and 0.8M uridine. Undissociated DRG were seeded into Falcon cell culture inserts 1.0 μm pore size, and allowed to acclimatize for 24 hrs before being grown with the viral transduced astrocytes for 1, 2 or 3 days. n=3 for viral treated groups, n=2 for controls.

Immunocytochemistry and image analysis

DRGs were fixed with 4% PFA for 10 min and then washed 3 times with 1 X PBS. DRG's were then stained with mouse anti tubulin, beta III isoform monoclonal antibody (Millipore) 1:200 dilution, anti mouse FITC secondary. Five images were taken for each insert using an Olympus IX81 microscope using Volocity software. Image J software was used to analyse the images by converting the image to an 8-bit image and measuring the percentage area comprised by the DRG neurites. Briefly this was done by automatically adjusting the thresholds of the 8-bit image. The mean +/- standard error for each sample was calculated. Analysis of variance was carried out using Minitab software. Tuckey's post hoc test was used to determine whether statistical differences existed between samples ($P \leq 0.05$).

Stereology

Random fields of view (n=5) were captured from each sample. Images were converted to grayscale and inverted using Adobe Photoshop software in order to observe fine detail of the neurites. Each image captured was analysed using stereology to estimate the length per unit area of each sample. The total length per unit area of neurite outgrowth from the DRG cells was measured by placing a grid over the images. The distance between each point on the grid was measured (T). The number of neurites crossing a line on the grid (I) was counted. The total length of neurite outgrowth per unit area was estimated using the formula:

$$\text{Total Length/Unit Area} = \pi/2 \cdot I/2 \cdot T$$

The mean \pm standard deviation for each sample was calculated. Analysis of variance was carried out using Minitab software. Tuckey's post hoc test was used to determine whether statistical differences existed between samples ($P \leq 0.05$).

Results

Si/shRNA validation

The designed siRNA and scramble control were used to transduce Neu7 cells, by electroporation. The most effective siRNA sequence was chosen to construct a shRNA in a lentiviral vector construct. The scramble control had no effect on NG2 expression (Figure 1)

Lentiviral Vector validation

LVpLVTHM shNG2 was used to transduce Neu7 cells, which over express NG2. Using qPCR a 76% knockdown of NG2 was obtained at MOI 100 (Figure 2 A), when normalised against untransduced Neu7 cells.

Both Neu7 cells and primary astrocytes were transduced with LVNT-3 at MOI 100, the medium was removed at various time points and assessed for NT-3 levels by ELISA. At both 72 and 120 hrs both cells types produced over 1000 pg/ml (figure 2 B). The transduced primary astrocytes produced less NT-3 then the Neu7 cells, this is due to Neu7 cells being more readily transduced then primary cells.

Treatment of DRGs with rhNT-3

To ensure that the levels of NT-3 expressed from the lentiviral vector transduced cells achieved a concentration that would promote neurite outgrowth we first tested the effect of rhNT-3 on DRGs. The effect of treating DRGs with increasing concentrations of rhNT-3 was determined after a 24 hr incubation period. Length of neurites was calculated by placing a grid over photomicrographs of β -tubulin stained DRGs within each sample, as shown in Figure 3(A). Calculation of neurite length shows that at 100pg/ml rhNT-3 and above, a significant increase in length of neurites was observed (Figure 3 B).

Co Culture of astrocytes and DRGs

LVpLVTHM shNG2 was used to transduce both Neu7 cells and primary astrocytes. Figure 3 A shows that the LVpLVTHM shNG2 gives a statistically significant increase in neurite area of DRGs grown with transduced Neu7 cells vs. those grown with untreated Neu7 cells at all three time points. Treatment of the Neu7 cells with LVpLVTHM shNG2 appears to improve the growth of the DRGs over those grown in the inhibitory environment (Figure 4 B: A & C). LVpLVTHM shNG2 has minimal effect on growth of DRG grown with primary astrocytes, as these astrocytes produce no significant quantity of NG2

The over expression of NT-3 by the transduction of Neu7 cells with LVNT-3 significantly increased neurite area of DRGs grown with transduced Neu7 cells vs. those grown on untreated Neu7 cells at all three time points. Also at day 3 a significant increase in neurite area was seen in the primary astrocytes. Transduction of Neu7 cells with LVNT-3 also increased the neurite area in the primary astrocytes by day 3 *in vitro*. A morphological improvement was observed in all DRGs grown in the LVNT-3 transduced cultures, where neurites appeared to grow more parallel to each other in more concise patterns than those grown in the absence of NT-3 (Figure 5 B).

As both vectors gave positive results alone in the inhibitory Neu7 cells, a combination of the vectors was used to transduce the inhibitory and control astrocytes to see if an accumulative effect could be observed. There appears to be no increase in DRG growth using both vectors together vs. using them alone, (Figure 6) though a trend of improvement is observed. This experiment was carried out using MOI 100 of each vector,

i.e. a total MOI of 200, a preliminary experiment was also carried out using MOI 50 of each vector, and similar results were observed (data not shown).

Figure 7 shows the LVGFP has no effect on neurite growth and that any change in neurite growth in other groups can be attributed to the transgene and not the vector itself.

As no accumulative effect was observed with both LV pLVTHM shNG2 and LV NT-3, the level of NT-3 produced by the transduced Neu7 cells was observed by ELISA for NT-3 on the culture medium. No decrease in NT-3 levels was seen in the combination treatment, showing the LV pLVTHM shNG2 does not interfere with NT-3 production from LV NT-3 (Figure 8).

Discussion

Our work showed that the knockdown of NG2 by shRNA via lentiviral vector delivery can overcome the inhibitory environment created by the Neu7 cell line, at all three time points analysed (Figure 4). The effect of the inhibitory environment created by the Neu7 cells can be clearly seen in figure 4 B: A vs.C, where the increase in the density of neurites from the DRGs in the treated group over the poorly formed neurites in the untreated groups is clearly visible. This inhibition of DRG growth was as reported by Fok-Seang *et al* (1995). The use of blocking antibodies for NG2 by Fidler *et al* (1999) also showed that a reduction in the level of NG2 resulted in an increase DRG growth. The use of shRNA for NG2, via lentiviral vector, offers a more stable and long term knockdown of NG2 levels. This observation was confirmed by the use of Image J analysis (Figure 4 A). The images were also analysed by stereology, to assess increase in total neurite length per unit area. This analysis showed the same trend as the Image J method, but no statistical difference was observed (data not shown). Dou and Levine (1994), observed that neurons grown on laminin coated surfaces grew to greater lengths than those which were grown in the presence of NG2 and NG2 which has been subjected to chABC digestion (34). Our work has shown that the use of lentiviral vectors can effectively deliver shRNA for NG2 and cause sufficient knockdown to allow an increase in DRG growth. Potentially the use of lentiviral vectors should provide long term stable knockdown of NG2, via shRNA, over the passive knockdown by chABC. This work confirms the potential of NT-3 as a suitable growth factor to aid axonal regeneration through the lesion site of injured spinal cord, as has been shown in other work

(12, 13, 15, 31). The results of this study also showed that NT-3 has the ability to promote neurite growth in an inhibitory environment *in vitro* (Figure 5). An improvement in the organization of the neurites in the presence of NT-3 can be clearly seen in figure 5 B: A vs.C. NT-3 had not previously been used to overcome the inhibitory effect of NG2 in Neu7 cells. Showing that NT-3 alone has the ability to overcome this inhibitory environment, increases the attractiveness of NT-3 as a potential therapeutic in SCI. Weight is added to this by the results of Alto *et al* (2009) who showed that NT-3 can guide axons through the site of injury to the correct site and form potentially functioning synapses. NT-3 has also been shown to promote regeneration of the corticospinal tract (CST), which is mainly responsible for motor axon activity. NT-3 increases synaptic plasticity in the absence of inhibitory molecules following SCI. This was demonstrated when the CST was hemisectioned at the level of the pyramids. NT-3 was then administered to the lumbar spinal cord via adenoviral vectors and this induced sprouting of neurons from the contralateral uninjured CST (Zhou *et al.*, 2003).

Although we showed that both the knockdown of NG2 and the over-expression of NT-3 alone can have a positive effect on neurite growth, the combination treatment did not appear to have an additional beneficial effect. The levels of NT-3 were checked to ensure that the shRNA was not having an adverse effect on the expression of NT-3 (Figure 8). The lack of effectiveness of the combination treatment, over the individual treatments, may be due to the shRNA interfering with NT-3 receptors in some way. There is also the possibility that the effect seen with the individual vectors is the maximal improvement achievable with this model, and that any benefit of the combined use of the vectors may not be observed. Though the combination treatment of shNG2 and NT-3 showed no additional benefit over the individual treatments, it is possible that the combination treatment may have greater benefit in the more complex *in vivo* setting of SCI. In an *in vivo* setting, there may be the potential to use both chABC in combination with the dual treatment. Hendriks *et al* (2007) suggested that pre-digestion of the glial scar with chABC, may not only aid in removing the initial inhibitory GAG-side chains, it may also aid breaking down the complex physical barrier of the interphotoreceptor matrix, allowing more efficient lentiviral transduction.

The successful use of lentiviral vectors in this study to deliver shNG2 offers a more specific *in vivo* treatment compared to the delivery of chABC. Additional advantages of this approach would include stable integration and long term expression. These benefits also apply to lentiviral delivery of NT-3. Combining the use of lentiviral vectors to deliver neurotrophic factors *in vivo* with the use of a controllable system would be advantageous, as excess neurotrophins may result in axons making incorrect connections, which may result in adverse effects such as pain. This would also aid in the guidance of axons to the correct target, as different systems could be used if multiple targets were required.

With lentiviral vectors, as with all other vectors, there are safety issues to be considered. The use of third generation, self inactivating, replication incompetent virus, would be advantageous, though a fourth generation of lentiviral vectors exists, its production titres are low, and third generation is the favoured vector.

Some non-primate lentiviral vectors have some very attractive properties in regards to their use in the CNS. The equine infectious anemia viral vector (EIAV) is one such vector. The key, and most exciting aspect of this vector is its ability to be retrogradely transported from muscle to spinal motor neurons, thus possibly removing the need for invasive surgery generally associated with viral vector delivery to the CNS. EIAV has been used successfully in a rat model of Parkinson's disease (PD) to deliver GDNF. Protection of complex motor function was observed (Dowd, Monville et al. 2005).

In this study both the knockdown of NG2 and the over expression of NT-3 have been shown here to have a beneficial effect on the growth of DRGs in an inhibitory environment. Though no additional benefit was observed from the combination treatment, it may be possible that in a complex *in vivo* setting a benefit may occur. The use of lentiviral vectors to deliver the transgenes offers stable long term expression, which in the setting of SCI is very advantageous, as it minimizes the need for multiple administration and invasive surgeries.

References

1. Fawcett JW, Asher RA. The glial scar and central nervous system repair. *Brain Res Bull.* 1999 Aug;49(6):377-91.
2. Yiu G, He Z. Glial inhibition of CNS axon regeneration. *Nat Rev Neurosci.* 2006 Aug;7(8):617-27.
3. Rolls A, Shechter R, Schwartz M. The bright side of the glial scar in CNS repair. *Nat Rev Neurosci.* 2009 Mar;10(3):235-41.
4. Bradbury EJ, Moon LD, Popat RJ, King VR, Bennett GS, Patel PN, et al. Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature.* 2002 Apr 11;416(6881):636-40.
5. Asher RA, Morgenstern DA, Moon LD, Fawcett JW. Chondroitin sulphate proteoglycans: inhibitory components of the glial scar. *Prog Brain Res.* 2001;132:611-9.
6. Massey JM, Amps J, Viapiano MS, Matthews RT, Wagoner MR, Whitaker CM, et al. Increased chondroitin sulfate proteoglycan expression in denervated brainstem targets following spinal cord injury creates a barrier to axonal regeneration overcome by chondroitinase ABC and neurotrophin-3. *Exp Neurol.* 2008 Feb;209(2):426-45.
7. Jones LL, Yamaguchi Y, Stallcup WB, Tuszynski MH. NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. *J Neurosci.* 2002 Apr 1;22(7):2792-803.
8. Ughrin YM, Chen ZJ, Levine JM. Multiple regions of the NG2 proteoglycan inhibit neurite growth and induce growth cone collapse. *J Neurosci.* 2003 Jan 1;23(1):175-86.
9. Tan AM, Colletti M, Rorai AT, Skene JH, Levine JM. Antibodies against the NG2 proteoglycan promote the regeneration of sensory axons within the dorsal columns of the spinal cord. *J Neurosci.* 2006 May 3;26(18):4729-39.
10. Tan AM, Zhang W, Levine JM. NG2: a component of the glial scar that inhibits axon growth. *J Anat.* 2005 Dec;207(6):717-25.
11. Chen ZJ, Negra M, Levine A, Ughrin Y, Levine JM. Oligodendrocyte precursor cells: reactive cells that inhibit axon growth and regeneration. *J Neurocytol.* 2002 Jul-Aug;31(6-7):481-95.
12. Hari A, Djohar B, Skutella T, Montazeri S. Neurotrophins and extracellular matrix molecules modulate sensory axon outgrowth. *Int J Dev Neurosci.* 2004 Apr;22(2):113-7.
13. Ramer MS, Bishop T, Dockery P, Mobarak MS, O'Leary D, Fraher JP, et al. Neurotrophin-3-mediated regeneration and recovery of proprioception following dorsal rhizotomy. *Mol Cell Neurosci.* 2002 Feb;19(2):239-49.
14. Zhou L, Shine HD. Neurotrophic factors expressed in both cortex and spinal cord induce axonal plasticity after spinal cord injury. *J Neurosci Res.* 2003 Oct 15;74(2):221-6.
15. Alto LT, Havton LA, Conner JM, Hollis Ii ER, Blesch A, Tuszynski MH. Chemotropic guidance facilitates axonal regeneration and synapse formation after spinal cord injury. *Nat Neurosci.* 2009 Sep;12(9):1106-13.
16. Lipson AC, Widenfalk J, Lindqvist E, Ebendal T, Olson L. Neurotrophic properties of olfactory ensheathing glia. *Exp Neurol.* 2003 Apr;180(2):167-71.

17. Mitsui T, Fischer I, Shumsky JS, Murray M. Transplants of fibroblasts expressing BDNF and NT-3 promote recovery of bladder and hindlimb function following spinal contusion injury in rats. *Exp Neurol*. 2005 Aug;194(2):410-31.
18. Padovan CS, Jahn K, Birnbaum T, Reich P, Sostak P, Strupp M, et al. Expression of neuronal markers in differentiated marrow stromal cells and CD133+ stem-like cells. *Cell Transplant*. 2003;12(8):839-48.
19. Quach TT, Duchemin AM, Rogemond V, Aguera M, Honnorat J, Belin MF, et al. Involvement of collapsin response mediator proteins in the neurite extension induced by neurotrophins in dorsal root ganglion neurons. *Mol Cell Neurosci*. 2004 Mar;25(3):433-43.
20. Blits B, Oudega M, Boer GJ, Bartlett Bunge M, Verhaagen J. Adeno-associated viral vector-mediated neurotrophin gene transfer in the injured adult rat spinal cord improves hind-limb function. *Neuroscience*. 2003;118(1):271-81.
21. Ruitenberg MJ, Levison DB, Lee SV, Verhaagen J, Harvey AR, Plant GW. NT-3 expression from engineered olfactory ensheathing glia promotes spinal sparing and regeneration. *Brain*. 2005 Apr;128(Pt 4):839-53.
22. Naldini L. Lentiviruses as gene transfer agents for delivery to non-dividing cells. *Curr Opin Biotechnol*. 1998 Oct;9(5):457-63.
23. Cui W, Allen ND, Skynner M, Gusterson B, Clark AJ. Inducible ablation of astrocytes shows that these cells are required for neuronal survival in the adult brain. *Glia*. 2001 Jun;34(4):272-82.
24. do Carmo Cunha J, de Freitas Azevedo Levy B, de Luca BA, de Andrade MS, Gomide VC, Chadi G. Responses of reactive astrocytes containing S100beta protein and fibroblast growth factor-2 in the border and in the adjacent preserved tissue after a contusion injury of the spinal cord in rats: implications for wound repair and neuroregeneration. *Wound Repair Regen*. 2007 Jan-Feb;15(1):134-46.
25. Hendriks WT, Eggers R, Verhaagen J, Boer GJ. Gene transfer to the spinal cord neural scar with lentiviral vectors: predominant transgene expression in astrocytes but not in meningeal cells. *J Neurosci Res*. 2007 Nov 1;85(14):3041-52.
26. Fouad K, Dietz V, Schwab ME. Improving axonal growth and functional recovery after experimental spinal cord injury by neutralizing myelin associated inhibitors. *Brain Res Brain Res Rev*. 2001 Oct;36(2-3):204-12.
27. Blomer U, Naldini L, Kafri T, Trono D, Verma IM, Gage FH. Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J Virol*. 1997 Sep;71(9):6641-9.
28. Scherr M, Battmer K, Eder M, Schule S, Hohenberg H, Ganser A, et al. Efficient gene transfer into the CNS by lentiviral vectors purified by anion exchange chromatography. *Gene Ther*. 2002 Dec;9(24):1708-14.
29. Abdellatif AA, Pelt JL, Benton RL, Howard RM, Tsoulfas P, Ping P, et al. Gene delivery to the spinal cord: comparison between lentiviral, adenoviral, and retroviral vector delivery systems. *J Neurosci Res*. 2006 Aug 15;84(3):553-67.
30. Azzouz M, Kingsman SM, Mazarakis ND. Lentiviral vectors for treating and modeling human CNS disorders. *J Gene Med*. 2004 Sep;6(9):951-62.
31. Taylor L, Jones L, Tuszynski MH, Blesch A. Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. *J Neurosci*. 2006 Sep 20;26(38):9713-21.

32. Fok-Seang J, Smith-Thomas LC, Meiners S, Muir E, Du JS, Housden E, et al. An analysis of astrocytic cell lines with different abilities to promote axon growth. *Brain Res.* 1995 Aug 21;689(2):207-23.
33. Fidler PS, Schuette K, Asher RA, Dobbertin A, Thornton SR, Calle-Patino Y, et al. Comparing astrocytic cell lines that are inhibitory or permissive for axon growth: the major axon-inhibitory proteoglycan is NG2. *J Neurosci.* 1999 Oct 15;19(20):8778-88.
34. Dou CL, Levine JM. Inhibition of neurite growth by the NG2 chondroitin sulfate proteoglycan. *J Neurosci.* 1994 Dec;14(12):7616-28.
35. Espejo M, Cutillas B, Arenas TE, Ambrosio S. Increased survival of dopaminergic neurons in striatal grafts of fetal ventral mesencephalic cells exposed to neurotrophin-3 or glial cell line-derived neurotrophic factor. *Cell Transplant.* 2000 Jan-Feb;9(1):45-53.
36. Bradbury EJ, Khemani S, Von R, King, Priestley JV, McMahon SB. NT-3 promotes growth of lesioned adult rat sensory axons ascending in the dorsal columns of the spinal cord. *Eur J Neurosci.* 1999 Nov;11(11):3873-83.

Figures

Figure 1; Agarose gel electrophoresis of reverse transcription products of Neu7 cells. Lane 1; ladder , lane 2-5; candidate siRNAs to NG2, lane 6; scramble siRNA , lane 7-8; untreated. All samples were also subject to PCR for the housekeeping gene Laminin for normalisation (not shown). n=2

Figure 2;A: Knockdown for NG2 in Neu7 cells 72 hrs post transduction with LVpLVTHM shNG2 at MOI 50 & 100, by qPCR for NG2 . NG2 levels in Neu7 cells transduced with LVpLVTHM MOI 100 corresponds to ~76% knockdown. n=3 B: NT-3 over expression from Neu7 and rat primary astrocytes transduced with LVNT-3 MOI 100, n=3. Mean \pm SEM

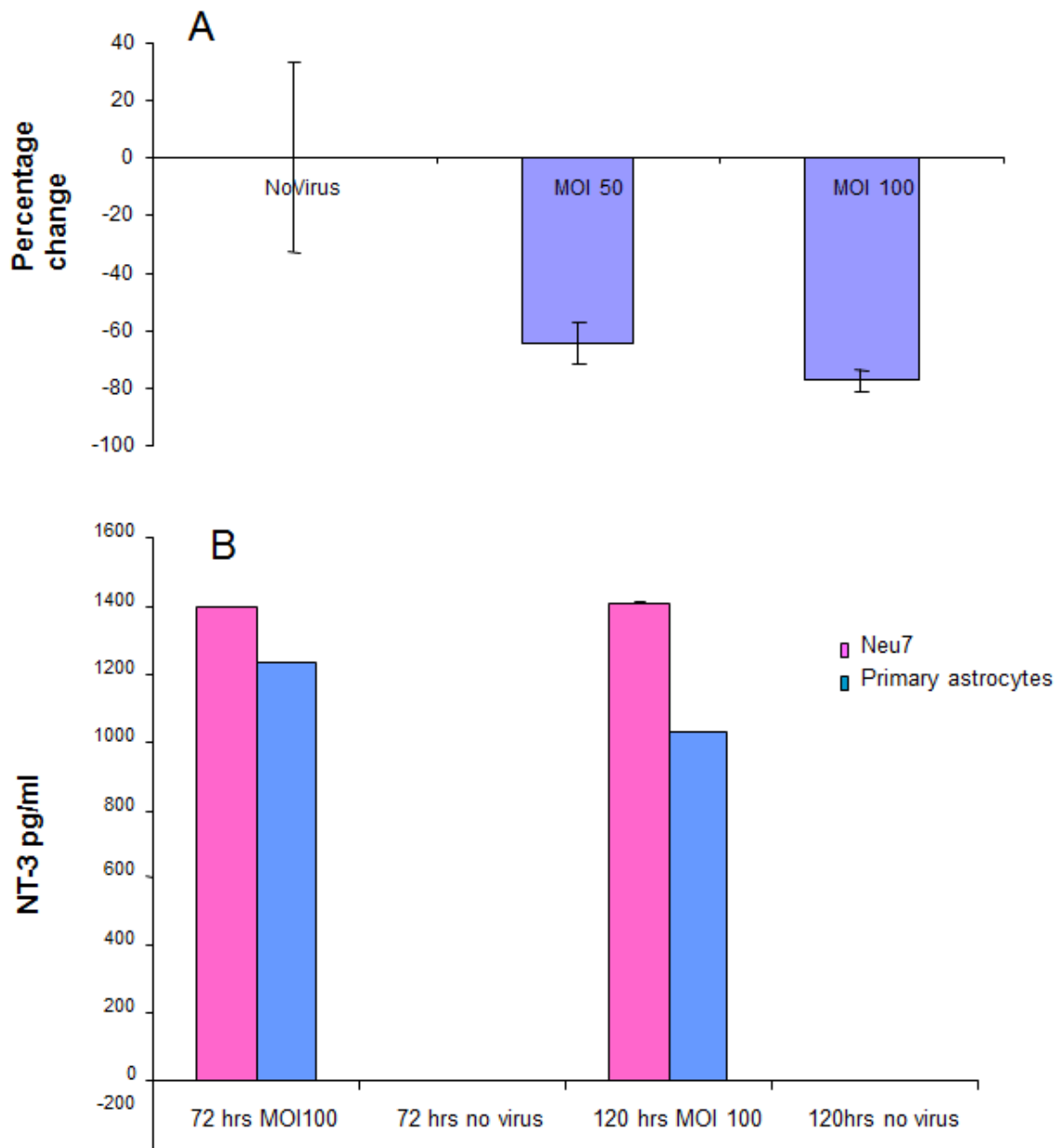


Figure 3, Effect of RhNT-3 on DRG growth. 2A shows the stereology method. 2B; level of neurite growth when treated with rhNT-3, n=3. Mean \pm SEM

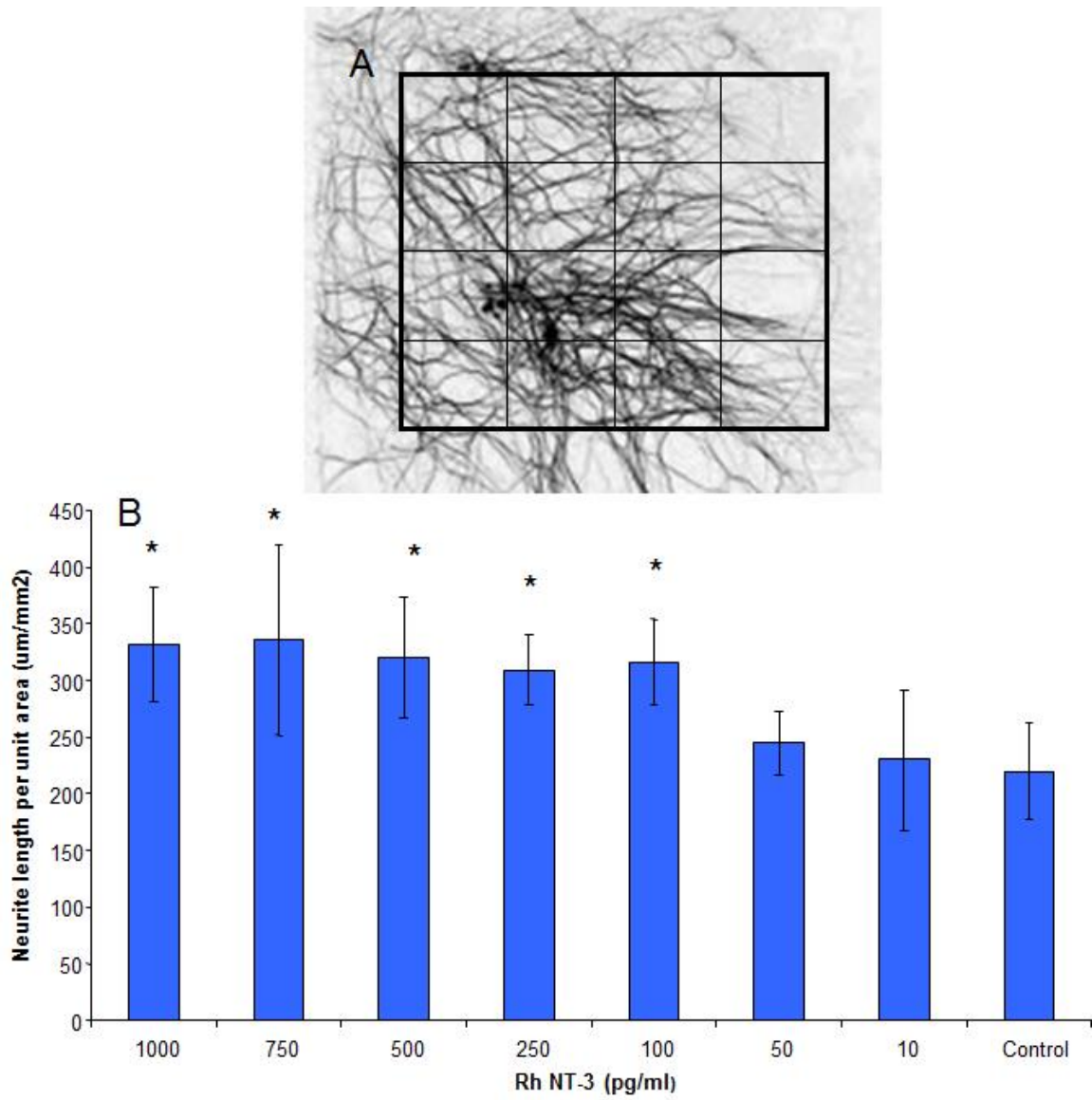


Figure 4; A: DRG neurite growth assessed by image J software in an inhibitory co-culture environment treated with LVpLVTHM shNG2. The knockdown of NG2 in Neu7 cells by LVpLVTHM shNG2 can increase neurite growth over untreated Neu7 cells at all time points. $*=p < 0.05$ Tukeys post hoc test, DRG's 3 days of coculture, stained for β -Tubulin. B: DRG grown with Neu7 cells transduced with LVpLVTHM shNG2. C: DRG grown with primary astrocytes transduced with LVpLVTHM shNG2. D: DRG grown with untransduced Neu7 cells. E: DRG grown with untransduced primary astrocytes. Virally transduced groups n=3, control groups n=3. Mean \pm SEM

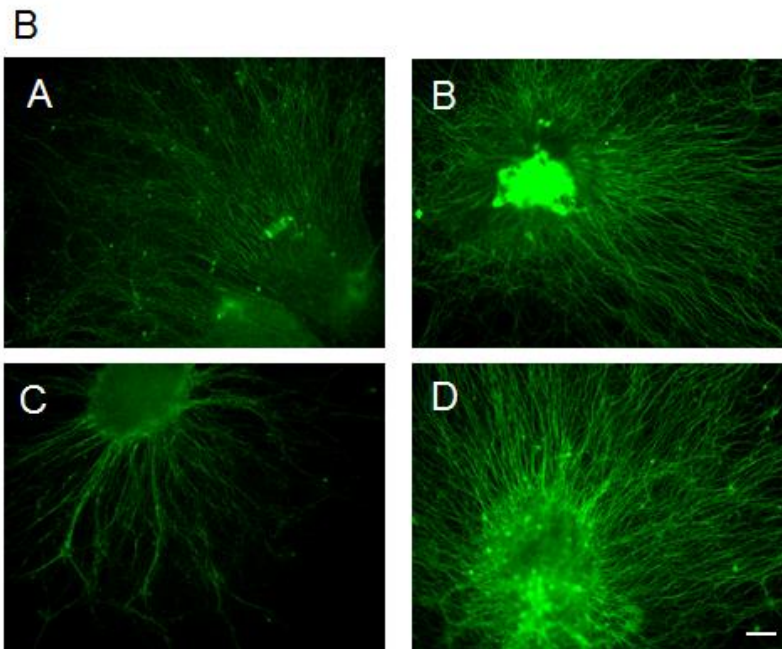
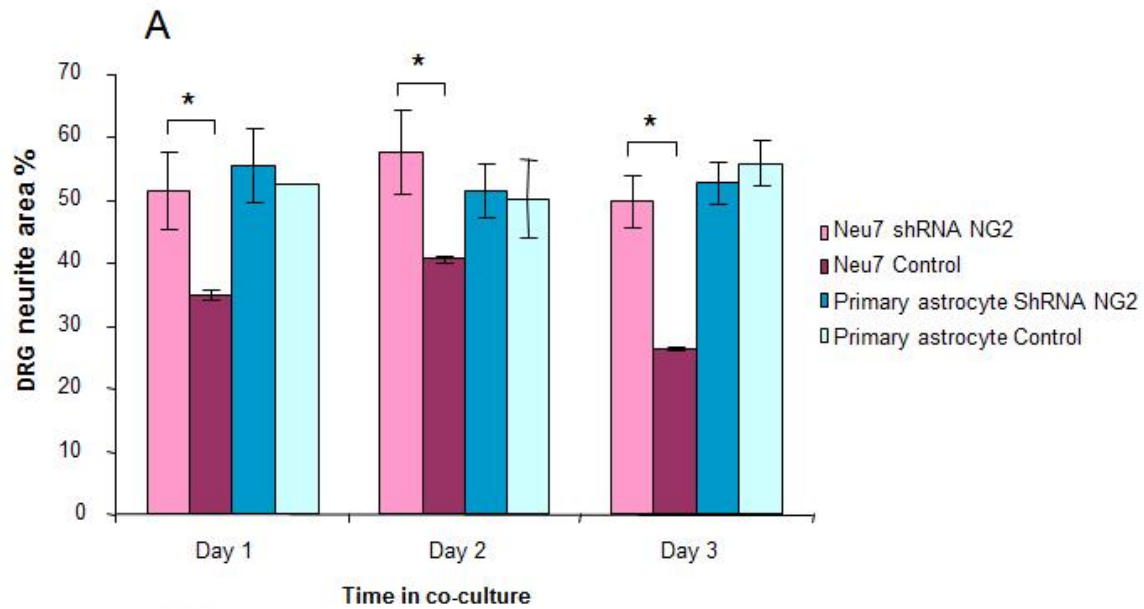


Figure 5; A: DRG neurite growth in an inhibitory co-culture environment treated with LVNT-3. Neurite growth assessed by Image J software. Over expression of NT-3 can increase neurite growth of DRGs grown in culture with Neu7 cells over those grown with untreated Neu7 cells at all time points. Overexpression of NT-3 can also increase neurite growth of DRGs grown with primary astrocytes $*=p < 0.05$, tukeys post hoc test. DRG's 3 days of coculture, stained for β -Tubulin. B: DRG grown with Neu7 cells transduced with LVNT-3. C: DRG grown with primary astrocytes transduced with LVNT-3. D: DRG grown with untransduced Neu7 cells. E: DRG grown with untransduced primary astrocytes. . Virally transduced groups n=3, control groups n=3. Mean \pm SEM

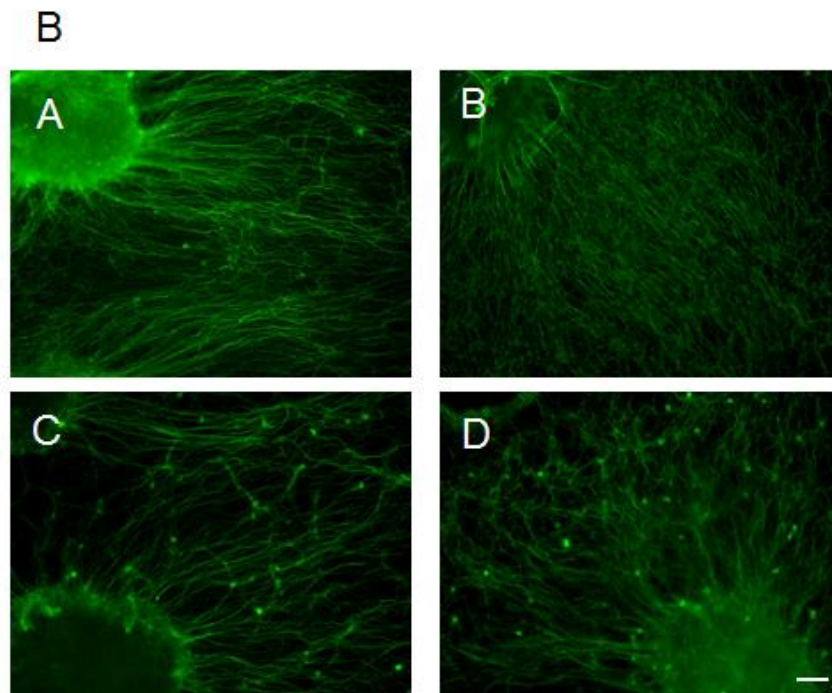
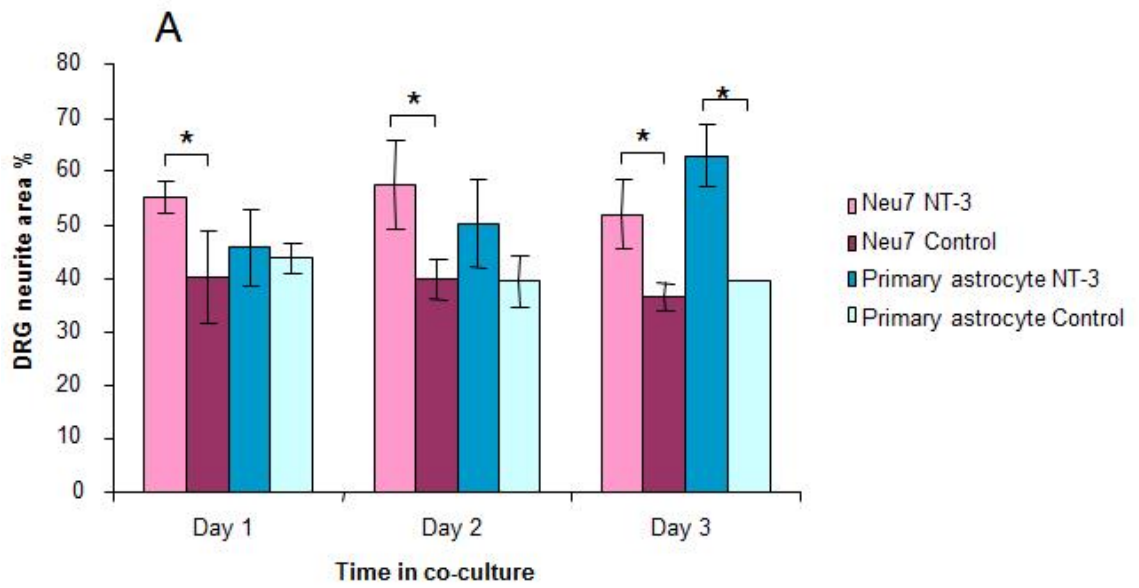


Figure 6; DRG neurite growth in an inhibitory co-culture environment treated with LVpLVTHM shNG2 and LVNT-3. Neurite growth assessed by Image J software. . Virally transduced groups n=3, control groups n=3. Mean \pm SEM

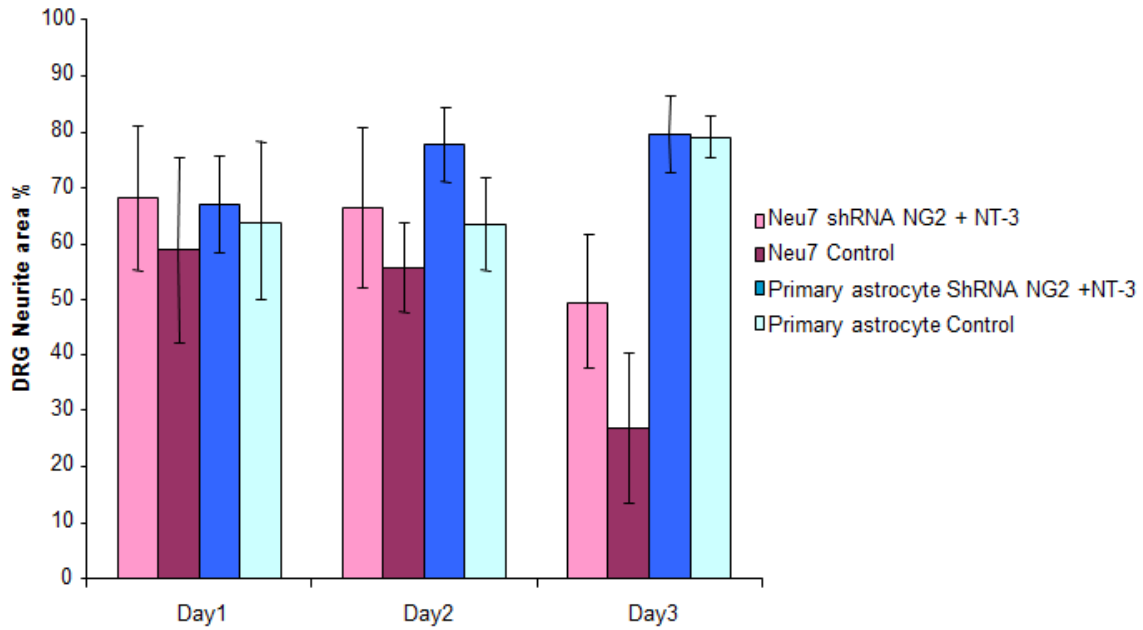


Figure 7; DRG neurite growth in an inhibitory co-culture environment treated with LVpLVTHM. Neurite growth assessed by Image J software. Virally transduced groups n=3, control groups n=3. Mean \pm SEM

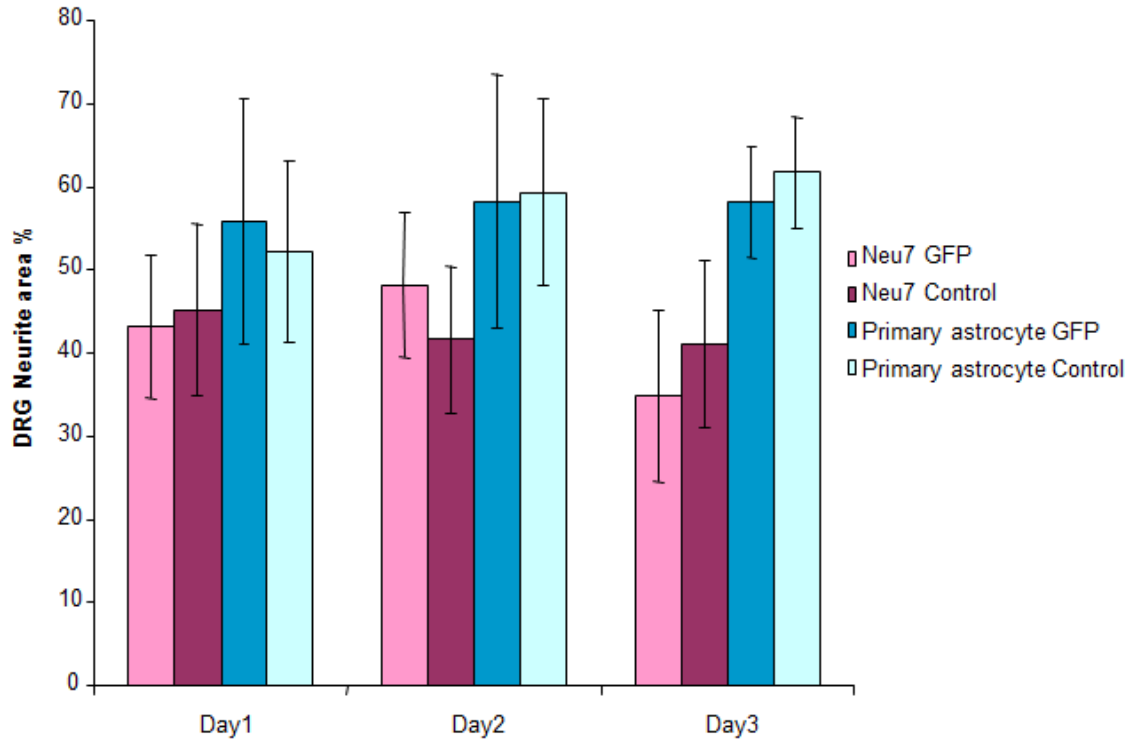


Figure 8; Expression level of NT-3 in Neu7 cells from LV NT-3 and from the combination of LVNT-3 and LV pLVTHM shNG2. Mean \pm SEM

