Final Narrative Report

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1. Original aims of the project

We proposed to test the consequences of adeno-associated virus-mediated L1 expression in a mouse spinal cord regeneration paradigm. Adeno-associated virus carrying either L1 (AAV-L1) or GFP (AAV-GFP) under the control of the mouse CMV promoter will be injected into the lesion. The expression level of L1 will firstly be tested by Western blot and immunohistochemical analyses. To assure that the exogenously applied L1 is not only overexpressed, but also functionally active, the downstream targets in the L1 signaling pathways such as phosphorylared ERK and CREB will be measured. Immunohistochemistry of serotonergic axons, anterograde labeling of the corticospinal tract, and a newly developed quantitative videotaping system for locomotor evaluation will be used to evaluate the axonal regeneration and functional recovery after the spinal cord injury. We planned to investigate if the functional recovery and axonal regrowth will be enhanced more by introducing the AAV-L1 in both the lesion site and the motor cortex, where the neuronal cell bodies of the corticospinal axons locate. By doing this, not only the cellular components in the lesion site, but also the axons will express exogenous L1. We expected that the homophlic binding between the axonal L1 and local L1 could further improve the axonal regeneration and functional recovery.

2. Project successes

Adeno-associated virus (AAV) mediated expression of target genes in lesioned adult mouse spinal cord

We first studied whether AAV-5 was capable of stably transducing neural cells in injured spinal cord by introducing AAV-5 encoding GFP into the lesion. Strong GFP signal was found in spinal cord 5 weeks after the operation (Fig. 1A-C). GFP fluorescence was found in the spinal cord at distances of 10mm away from the center of the lesion site. Thus, the AAV-5 vector is capable of stably transducing neural cells in the injured spinal cord for at least 5 weeks. The transduction of AAV-L1 was comparable to AAV-GFP (Fig. 1D-G), with the knowledge that L1 expression is weaker than GFP expression because AAV-GFP contains the WPRE which stabilizes mRNA, resulting in a 2 - 10 fold higher level of protein expression in comparison to L1 expression. Western blot analysis confirmed the immunohistochemical results in exogenous L1 expression time course (Fig. 1Q, R).

We observed transgenic expression in AAV-GFP transduced mice mainly in cells which co-localized with the neuronal marker NeuN (~50% of all transduced cells) and the astrocytic marker GFAP (~30% of all transduced cells). A few transduced cells also co-localized with the oligodendrocyte marker

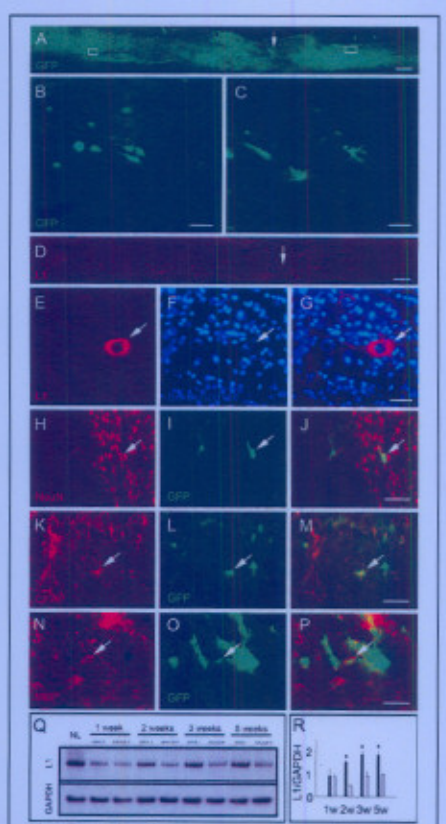

Fig1. Expression of transgene products after spinal cord compression and injection of adeno-associated viral vectors encoding green fluorescent protein (AAV-GFP) or full-length mouse L1 (AAV-L1).

MBP (~10% of all transduced cells).

AAV-L1 improves recovery of motor functions

To evaluate the motor functions after spinal cord injury, we adopted the Basso Mouse Scale (BMS) (Engesser-Cesar et al., 2005), as well as a newly developed motion analysis approach (Apostolova et al., 2006). Spinal cord compression caused severe disabilities in both AAV-L1 and AAV-GFP treated mice as indicated by the BMS at 1 week after injury. Between 1 and 5 weeks after injury, the mean score values improved more in AAV-L1 treated than in AAV-GFP treated mice at 5 weeks. The time course of BMS recovery is shown in Fig. 2A using recovery indices. Analysis of the foot-stepping angle also revealed that enhanced recovery in AAV-L1 compared to AAV-GFP treated mice at 3 and 5 weeks (Fig. 2A).

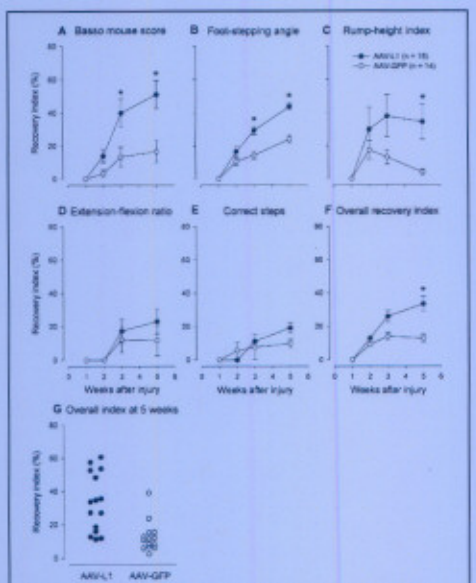

Fig2. Time course and degree of functional recovery after spinal cord compression in mice treated with AAV-L1 or AAV-GFP. Shown are mean values ± SEM of recovery indices for open field locomotion scores (Basso mouse score, BMS)

We analyzed the rump-height index, a measure of the ability to support body weight during ground locomotion. AAV-L1 treated mice recovered significantly, compared both to zero and to the AAV-GFP group, within the 5-week observation period (Fig. 2C). The animals' ability to perform voluntary movements without body weight support, estimated by the extension-flexion ratio, was not significantly affected by the kind of treatment (Fig. 2D). The same conclusion was reached for numbers of correct steps made by the animals during inclined ladder climbing (Fig. 2E). We calculated overall recovery indices for each animal (Fig. 2G) and group mean value (Fig. 2F). This analysis revealed an overall better outcome in mice treated with AAV-L1 compared to AAV-GFP application.

AAV-L1 promotes serotonergic fiber regrowth and prevents corticospinal tract axons from degenerative retraction 5 weeks after injury

We found robust 5-HT fibers growing into the lesion site in AAV-L1 mice. In contrast, in mice having received AAV-GFP, the 5-HT fibers could only occasionally be observed in the lesion site and caudal to the lesion site.

We also anterogradely labeled corticospinal tract axons with rhodamine-conjugated dextran Fluoro-ruby. The distance between the tips of the Fluoro-ruby labeled axons and the rostral border of the lesion site demarcated by GFAP immunostaining was measured and used as an indicator of the extent of retrograde axonal degeneration. This distance was significantly shorter in AAV-L1 infected spinal cords ($282.46 \pm 33.07\mu m$) compared to AAV-GFP infected spinal cords ($1077.19 \pm 111.04\mu m$, $P < 0.01$), indicating that overexpression of L1 protects the corticospinal tract axons from retraction in a hostile environment.

To investigate if the functional recovery and axonal regrowth will be enhanced more by introducing the AAV-L1 in both the lesion site and the motor cortex, where the

neuronal cell bodies of the corticospinal axons locate, we injected the AAV-L1 or AAV-GFP into the sensorimotor cortex as well as into the spinal cord lesion site. We found that although the corticospinal tract axons were successfully labeled by AAV transduction, the corticospinal axons retraction was not significantly different from the ones in the group which only received AAV-L1 treatment.

L1 affects signal transduction mechanisms

We studied whether L1 overexpression would activate endogenously expressed MAPK, PI3K and PKA pathways *in vivo*. Spinal cord homogenates were analysed five weeks after injury by Western blot using antibodies recognizing the phosphorylated and non-phosphorylated forms of the extracellular-regulated kinases ERK1 and ERK2, and PI3K and PKA. We found that AAV-L1 enhanced phosphorylation of ERK1/2 when compared with the AAV-GFP group (Fig. 3A), while the total protein form of ERK1/2 was not affected. In contrast, phosphorylation of PKA was similar in the two groups. However, the total level of PKA expression was elevated in the AAV-L1 group compared with the AAV-GFP group, causing a decrease in the ratio of the phosphorylated/total value in the AAV-L1 group (Fig. 3B). Total PI3K levels were also elevated in the AAV-L1 group compared with the AAV-GFP group (Fig. 3C). We did not detect phosphorylated PI3K in the AAV-L1 or AAV-GFP groups.

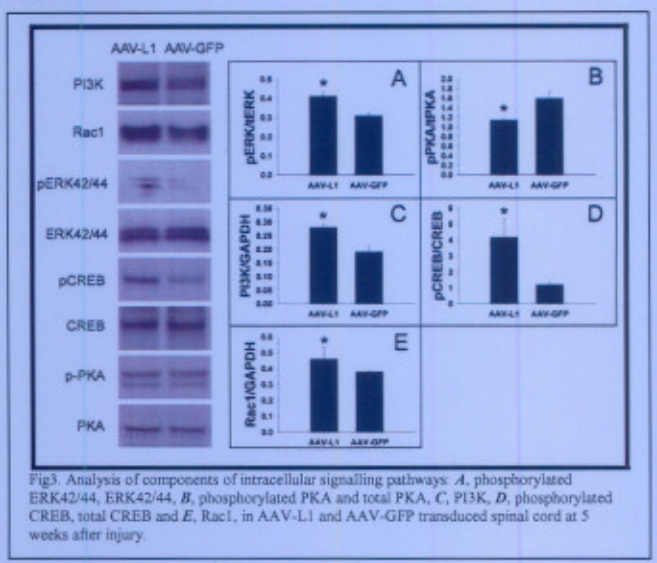

Fig3. Analysis of components of intracellular signalling pathways: *A*, phosphorylated ERK42/44, ERK42/44, *B*, phosphorylated PKA and total PKA, *C*, PI3K, *D*, phosphorylated CREB, total CREB and *E*, Rac1, in AAV-L1 and AAV-GFP transduced spinal cord at 5 weeks after injury.

We analysed spinal cord homogenates by Western blotting using antibodies against phospho-CREB and total-CREB. The ratio of phosphorylated/total levels of CREB was significantly higher in the AAV-L1 group than in the AAV-GFP group (Fig. 3D) indicating that exogenous L1 expression increases CREB activation.

L1-triggered activation of the ERK1/2 pathway has been shown to induce cell motility-associated gene products, among them the small GTPases Rac-1 (Silletti et al., 2004), which also stimulates neurite outgrowth *in vivo* (Yip et al., 1998; Schmid et al., 2000; Causeret et al., 2004). We observed that Rac1 expression levels were considerably higher in AAV-L1 transduced spinal cords versus AAV-GFP transduced spinal cords (Fig. 3E).

The small GTPase RhoA is another regulator of the actin cytoskeleton in neurites and its activation results in growth cone collapse,

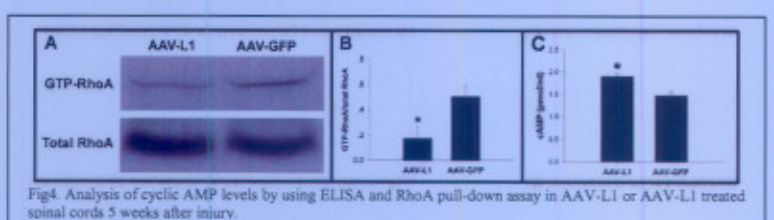

Fig4. Analysis of cyclic AMP levels by using ELISA and RhoA pull-down assay in AAV-L1 or AAV-L1 treated spinal cords 5 weeks after injury.

neurite retraction, and neurite growth inhibition (Lehmann et al., 1999; Wahl et al., 2000). Given that chondroitin sulfate proteoglycans inhibit neurite extension via the Rho pathway in the glial scar (Monnier et al., 2003), we investigated whether decreased astrocytic gliosis accompanied by lower NG2 expression levels in the AAV-L1 transduced spinal cords would correlate with a lower activation level of the Rho pathway (Fig. 4A). GTP-RhoA was pulled down with the Rhotekin Rho binding domain, and the ratio of GTP-RhoA/total RhoA was calculated. This ratio was significantly lower in the AAV-L1 treated spinal cords compared with the AAV-GFP treated ones (Fig. 4B), suggesting that RhoA activation is decreased by overexpression of L1.

AAV-L1 alters cellular responses to injury

To evaluate the influence of AAV-L1 on reactive astrogliosis and expression of NG2, a member of the CSPG family produced by polydendrocytes and other cell types (Nishiyama, 2007), we analysed homogenates from 500 µm-long spinal cord segments containing the lesion site by Western blot analysis using antibodies against GFAP and NG2. As opposed to L1 expression, which was elevated in the AAV-L1 treated mice compared with the AAV-GFP treated animals (Fig. 5A), we observed a marked decrease in GFAP (Fig. 5B) and NG2 expression levels (Fig. 5C). This indicates that L1 overexpression limits astrogliosis and downregulates expression of growth-inhibitory molecules.

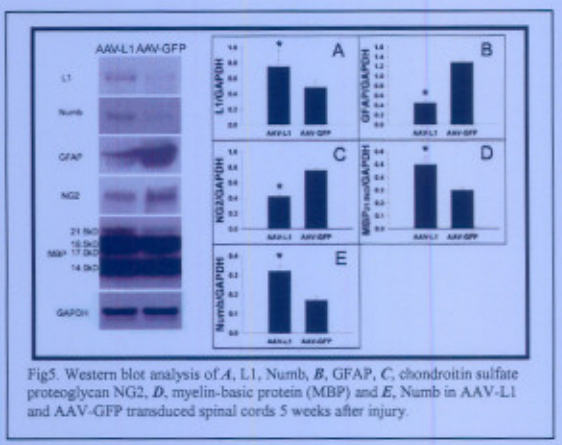

Fig5. Western blot analysis of *A*, L1, Numb, *B*, GFAP, *C*, chondroitin sulfate proteoglycan NG2, *D*, myelin-basic protein (MBP) and *E*, Numb in AAV-L1 and AAV-GFP transduced spinal cords 5 weeks after injury.

We also studied the expression of myelin basic protein (MBP) to monitor treatment-related alterations in de-/remyelination in the injured spinal cord. We found that one of the MBP isoforms, the larger 21.5 kD isomer, was significantly upregulated in the AAV-L1 group (Fig. 5D). No apparent differences between the groups were found for the other MBP isomers (18.5 kD, 17.0 kD and 14.0 kD, left panel in Fig. 5), Since only 3% of the oligodendrocytes were infected by the AAV constructs, the observed significant upregulation of the 21.5 kD isoform of MBP was unexpected. The 21.5 kD isoform is the earliest to become expressed during myelination and is re-expressed in remyelinating lesions in multiple sclerosis (Capello et al., 1997). Upregulation of this 21.5 kD isoform may reflect the ability of L1 to initiate remyelination after spinal cord injury.

To determine whether L1 expression alters the microglial reaction after spinal cord injury, we used the microglial marker Iba-1 by immunohistochemistry and Western blot analysis. We found, in contrast to the other molecular markers, no differences between AAV-L1 and AAV-GFP treated mice (data not shown).

Numb is a cell fate determinant which regulates neurogenesis by antagonizing the activity of the Notch receptor and was found to improve neurite outgrowth by promoting L1 endocytosis at growth cones (Nishimura et al., 2003). This observation suggests that the L1 and Numb signalling pathways may affect each other in regulating neurite outgrowth. To investigate whether L1 overexpression alters Numb expression, we

measured levels of Numb expression and found that the Numb expression was higher in AAV-L1 treated compared with AAV-GFP transduced spinal cords (Fig. 5E).

3. Project challenges

Lack of permissive molecules and abundance of inhibitory molecules in the local lesion environment after spinal cord injury prevent the axons from successful regeneration and therefore, contribute to the failure to satisfactory functional recovery. Corticospinal tract axons are the most vulnerable axons and their regeneration is very limited. In this study, we found that the degenerative retraction of corticospinal axons were prevented by the AAV-L1 application. However, no significant regeneration was observed. To exclude the possibility that the sprouting axons are too few to be observed by traditional anterograde labeling method, we used a transgenic mouse strain whose corticospinal axons are YFP positive. However, we did not observe significant axonal regeneration in this mouse strain either. To investigate if the corticospinal axonal regrowth will be enhanced more by introducing the AAV-L1 in both the lesion site and the motor cortex, where the neuronal cell bodies of the corticospinal axons locate, we injected the AAV-L1 or AAV-GFP into the sensorimotor cortex as well as into the spinal cord lesion site. We found that although the corticospinal tract axons were successfully labeled by AAV transduction, the corticospinal axons regeneration was not significantly different from the ones in the group which only received AAV-L1 treatment in the spinal cord lesion site. This finding suggests that AAV-L1 is not sufficient to overcome the inhibitory environment in the lesion site and stimulate the corticospinal tract regeneration. Combinatory therapies, such as Chondroitinase ABC or stem cell transplantation, might improve the corticospinal tract regeneration more.

4. Implications for future research and/or clinical treatment

Here we show that an adeno-associated viral construct can be used as an efficient vector to introduce a beneficial adhesion molecule into the injured spinal cord. By transducing the spinal cord with the cell adhesion molecule L1, we observed improved motor functional recovery, enhanced axonal regeneration and amelioration of the local microenvironment. We attribute these effects to the ability of L1 to activate multiple signalling pathways in regrowing neurites and/or surrounding tissue, to limit reactive astrogliosis, and to influence intraspinal circuitries. Thus, L1 transduction using an adeno-associated viral vector is likely to provide incentives to a therapeutic venue to spinal cord injury in adult mammals, including humans. To study the feasibility of using AAV-L1 in humans, we will first study if similar beneficial effects will be observed when AAV-L1 is administered in primate, such as monkey. We will construct human L1 plasmid and package it into adeno-associated viral vector. The human AAV-L1 will be applied in a monkey contusion spinal cord injury model, which has been established in our lab. We will use a novel video-taping system to analyze the locomotor behavior recovery in monkey.

5. Plans to continue this research, including applications submitted to other sources for ongoing su

1. Apply AAV-L1 in chronic spinal cord injury

Since AAV-L1 has been proved to be efficient to improve functional and morphological recovery in the acute mouse spinal cord injury model. It is hence plausible to apply this treatment into the chronic mouse spinal cord injury model. Compared to the acute model, the chronic model is more clinic-oriented, since most spinal cord injured patients are admitted to the hospital weeks after the primary injury. The chronic spinal cord injury is more difficult to be treated since the neuropathological environment is more hostile than the acute one. We expect that the L1 overexpression mediated by AAV will provide similar beneficial functions in the chronic model as in the acute model, hence stimulates the axonal systems regeneration, ameliorates the inhibitory glial scar, and improves functional recovery in the chronic model.

2. Combine AAV-L1 with other treatments, such as chondroitinase ABC, to achieve better functional and morphological recovery

Lack of regeneration in the mature central nervous system (CNS) of mammals is attributed to prevalence of neurite outgrowth inhibitory over conducive molecules. Combinatory treatments might be more efficient to overcome the hostile lesion environment and improve functional recovery in the acute and chronic spinal cord injury model. Astrocytes express axon growth inhibitors such as CSPGs which potently restrain neurite outgrowth both *in vitro* and *in vivo*. Enzymatic degradation of the chondroitin sulfate moiety of CSPGs with chondroitinase ABC improves axon regeneration of the nigrostriatal and dorsal column tracts after spinal cord injury in adult rats, indicating a prominent role of these ECM constitutes in preventing axonal regeneration. We plan to combine AAV-L1 with chondroitinase ABC in the acute and chronic mouse spinal cord model. We expect that the L1 and chondroitinase ABC will function with separated mechanisms and hence work synergetically to treat the spinal cord injury.