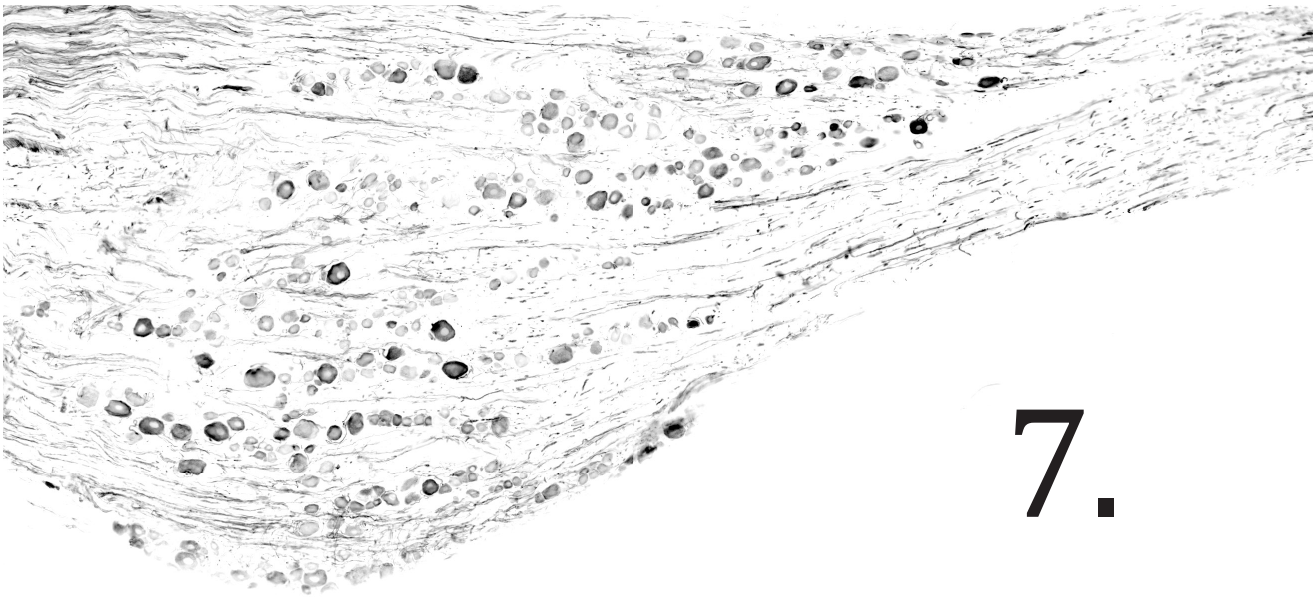
Adeno-associated viral vector-mediated delivery of transcription factors to promote axon regeneration

Fagoe, N.D.

2014



7.

Summary and general discussion

Nitish D. Fagoe [1], Matthew R.J. Mason [1], Joost Verhaagen [1, 2]

[1] Laboratory for Neuroregeneration, Netherlands Institute for Neuroscience, an Institute of the Royal Academy of Arts and Sciences, Amsterdam, The Netherlands

[2] Center for Neurogenomics and Cognition Research, Neuroscience Campus Amsterdam, Vrije Universiteit Amsterdam, The Netherlands

1. Summary

Injury to the brain or spinal cord leads to severe and permanent disability. In contrast, injury to the peripheral nervous system (PNS) is followed by axonal regeneration and usually results in some degree of functional recovery. A major reason for the failure of neurons in the central nervous system (CNS) to regenerate an axon is the poor neuron-intrinsic response upon axotomy. In the PNS a strong response occurs consisting of the expression of several hundred regeneration-associated genes (RAGs) and includes a number of regeneration-associated transcription factors (TFs). Regeneration-associated TFs are potential key regulators of the RAG program and may act in a combinatorial manner to drive the expression of the full RAG program. In this thesis we aimed to deliver a combination of TFs to dorsal root ganglion (DRG) neurons and study their effects on regeneration of their central axons. First we developed methods to deliver and express multiple transgenes simultaneously in DRG neurons. In the second part of this thesis we tested combinations of TFs in neurite outgrowth screens and performed *in vivo* experiments using the methods developed in the first part of this thesis to assess the effect of a combination of regeneration-associated TFs on the regeneration of the axons of injured DRG neurons.

In **chapter 1** we review the consequences of spinal cord injury and studies that focus on enhancing axon regeneration by manipulating the RAG-response, and propose that adeno-associated viral vector (AAV)-mediated gene delivery of combinations of regeneration-associated TFs could be a powerful strategy to activate the RAG program in injured CNS neurons and achieve long distance axon regeneration.

AAV vectors are efficient molecular tools to deliver genes to the nervous system *in vivo*. In **chapter 2** we describe two techniques to transduce DRG neurons *in vivo* using AAV: the direct injection technique and an intrathecal delivery method. Both methods are efficient at transducing DRG neurons *in vivo*. Each method has its advantages and disadvantages. The main advantages of direct injection are that specific DRGs can be targeted with high transduction rates with low amounts (µl) of a viral vector stock, however the procedure is complex, invasive and time consuming. Intrathecal injection has the advantage of being a fast and simple method to transduce multiple DRG bilaterally, and involves no surgical manipulation of the DRG. However, intrathecal delivery does require much larger amounts of viral stock (10 to 20µl), and has the disadvantage that viral particles will leak from the cerebrospinal fluid to the spinal cord and/or peripheral tissues.

For regeneration studies, where axon growth is usually assessed distant from the transduced neuronal cell bodies, it is desirable to simultaneously express a gene of interest and a fluorescent marker protein to label the axons of transduced neurons. In **chapter 3** we developed an AAV vector containing the CMV promoter and sCAG promoter in a compact back-to-back configuration with a shared enhancer. We show robust expression of two transgenes simultaneously using two fluorescent proteins, eGFP and mCherry, both by direct injection into the DRG

and via intrathecal delivery (**chapter 2**). Quantification of co-expression shows that when considering all eGFP-positive neurons 77 to 85 percent of these also express mCherry. We demonstrate how this vector is useful for studies on axon regeneration by expressing an actively transported form of eGFP, farnesylated eGFP (eGFPf), which results in superior long distance labelling of axons of DRG neurons compared to normal eGFP. Taken together, this novel molecular tool enables specific tracing and quantification of distant axons from transduced neurons after axonal injury.

In **chapter 4** we performed *in vitro* medium-throughput cellular screens ("Cellomics") to test combinations of TFs of two individual 'modules' (module 1: ATF3, c-Jun, Smad1 and STAT3; module 2: KLF7, Sox11, MEF2 and SRF) on neurite outgrowth using the dual promoter construct co-expressing a TF and eGFPf (**chapter 3**) in the F11 DRG-like cell line. The two combinations were identified based on the analysis of RAG-promoters using an advanced TF binding site overrepresentation algorithm and on data available in the literature. We show relatively small effects in module 1 by overexpression of ATF3 only, several combinations of two and three TFs and the full module containing all four TFs, although this was not significantly better than overexpression of ATF3 alone. The second module gives strong synergistic effects on neurite outgrowth with several combinations. The module 2 TFs are currently followed up in a parallel research line by Callan Attwell.

Although the effects of overexpressing TFs of module 1 (ATF3, c-Jun, STAT3, and Smad1) in F11 cells were limited, we continued to study these TFs in a parallel *in vivo* experiment for a number of reasons. Firstly, the screen in F11 cells does not take the real context of regeneration into account, such as the complex environment and interplay of neurons with other types of cells. Furthermore, F11 cells already express ATF3, c-Jun, Smad1 and STAT3 endogenously, which could explain why the effects of overexpression of these TFs were limited. Secondly, evidence from the literature has linked each of these four TFs to successful regeneration and thirdly, interactions between ATF3, c-Jun, STAT3, and Smad1 are described in the literature, which supports the idea that combining these four TFs could result in synergistic effects on axon regeneration. In **chapter 5** we injected dual promoter AAV vectors developed in **chapter 3** expressing eGFPf-only, ATF3 and eGFPf or ATF3/Smad1/STAT3/c-Jun combined and eGFPf into the L4 and L5 DRG and assessed axon regeneration after dorsal root injury and dorsal column injury. We show clear co-expression of TFs in eGFPf-positive DRG neurons and performed histological analysis at several locations to quantify regenerating dorsal root axons. We show for the first time that ATF3 enhances axon regeneration after dorsal root injury. Combinatorial overexpression of ATF3/Smad1/STAT3/c-Jun also resulted in faster regeneration of injured dorsal root axons, however this was not more effective than expressing ATF3 alone. No functional effects were found using sensory tests based on heat or electrical stimuli, however significant more animals that received ATF3 only showed autotomy behaviour, and a trend was

seen for the TF combination group. We found no effect on axon regeneration of TF overexpression in a dorsal column lesion model.

The primary sensory DRG neurons are a commonly used model for regeneration studies. However, a lesion to the central axons in the dorsal column spares other sensory spinal pathways which could possibly compensate for the loss of function, leading to recovery of function within weeks. In **chapter 6** we therefore compared a number of behavioural tests to reliably measure recovery of function after dorsal column injury. We used the adhesive tape removal test, rope-crossing test, CatWalk™ gait analysis, horizontal ladder test, and developed a new test in which animals climb an inclined ladder with rolling bars. We performed dorsal column injuries or sham injuries at C4 or T7 level and followed the animals for the duration of 8 weeks. We found that in the majority of tests animals show spontaneous recovery within two weeks after injury. In some of the tests, however, including the new inclined rolling ladder test we were able to measure more long-lasting dysfunction over time. This indicates that the inclined rolling ladder may be a suitable functional test for regeneration experiments following lesions of the dorsal column.

2. Discussion

The aim of the work described in this thesis was to deliver a combination of regeneration-associated TFs to DRG neurons and to study their effects on the regeneration of injured dorsal root axons. In this section we will first discuss the challenges for co-delivery of multiple transgenes to DRG neurons *in vivo*. In the second part we discuss our efforts to enhance axon regeneration by overexpression of multiple regeneration-associated TFs simultaneously.

2.1 Overexpression of multiple genes in DRG neurons *in vivo*

The challenges of simultaneous overexpression of multiple transgenes *in vivo*

Many biological processes, including axon regeneration, involve the regulation of large numbers of genes (**chapter 1**). It would therefore be advantageous to be able to express multiple genes simultaneously in a single neuron to gain insight into complex gene expression programs, such as the RAG program. Additionally, for many studies it would be useful to co-express a fluorescent marker to label axons because this would allow the analysis of axon regeneration of the transduced neurons. The simplest method to do this is to mix vectors and deliver them together. However, mixing vectors could lead to possible technical difficulties, such as lower viral titres of individual vectors for an experimental group that receives a mix of multiple vectors when matching total viral titre to a group that receives