Spinal cord injury

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2019

document version
Publisher's PDF, also known as Version of record

Summary

Spinal cord injuries are most often irreversibly debilitating. So far, an effective treatment to promote spinal cord regeneration in humans remains elusive. Rodent models of spinal cord injury allow for the investigation of the physiological roadblocks to axonal regeneration following injury and the possibility of therapeutic intervention to overcome these roadblocks. Furthermore, insights gained from studying regeneration in the peripheral nervous system (PNS) and the spinal cord could be applied to the treatment of brain injuries or degeneration.

Axotomy of peripheral neurons (sensory neurons and motor neurons) triggers an intrinsic cell body response (Lieberman, 1971) resulting in the simultaneous activation and repression of hundreds of genes in the neuron, these genes that are activated by axotomy in the injured neurons are usually referred to as regeneration-associated genes (RAGs) (Tetzlaff et al., 1991; Smith and Skene, 1997; Kiryu-Seo and Kiyama, 2011; van Kesteren et al., 2011). The absence of a coordinated RAG response in central nervous system (CNS) neurons is a major reason for the failure of axon regeneration in the CNS. Transcription factors (TFs) jointly regulate the expression of multiple genes and may therefore be central to the regulation of the RAG program. In this thesis, I test the hypothesis that overexpression of a combination of certain regeneration-associated transcription factors in dorsal root ganglion (DRG) neurons could promote RAG expression and regeneration and/or functional recovery following a lesion of their axonal projections in the dorsal column (DC) of the spinal cord. We tested two sets of TFs, chosen either based on findings in the literature (**Chapter 2**) or selected based on a computational analysis of RAG gene promoters followed by *in vitro* screening for their effects on neurite outgrowth (**Chapter 3**). TFs were delivered to DRG neurons via direct injection of adeno-associated viral vectors (AAVs) harbouring the TFs of interest into the DRG of animals which subsequently received a DC lesion, followed by functional testing of recovery and finally histological analysis of axonal tracts (**Chapters 2 and 4**). Finally, we utilised RNA sequencing (RNA-seq) to probe the mechanisms underlying our *in vivo* findings (**Chapter 5**).

In **Chapter 1,** I discuss the DC lesion model, where the centrally projecting axons of the DRG are injured, and the unique properties of this model that make it relevant for the study of axonal regeneration, especially the neuron-intrinsic component (Attwell et al., 2018). The DRG neurons relay sensory information including pain, pressure and proprioception from their peripherally-projecting axonal branches, and along their CNS-projecting axon branches to the spinal cord or to the brain stem after ascending in the DC. The DRG can successfully regenerate its peripheral axons following injury but not its central branch axons. Remarkably, it was discovered that a so-called conditioning lesion (CL) of the peripheral axons (i.e., the sciatic nerve) prior to a central branch lesion drives axonal regeneration that can penetrate the DC lesion site. Gene expression profiling studies of the DRG neurons following peripheral

injury have revealed hundreds of RAGs. The discovery of the RAG program and the observation that the PNS extrinsic cellular environment is much more conducive to regeneration than the CNS environment, led to the distinction of neuron-intrinsic and neuron-extrinsic barriers to regeneration. The DC lesion model is well-suited to study both. Interventions to enhance neuron-intrinsic regeneration have utilized gene therapy for overexpression of RAGs in the DRG, with small effects on axonal outgrowth into the lesion but limited or no effect on functional recovery. Efforts to render the extrinsic environment more permissive to regeneration have improved the observed regeneration that can be promoted following a CL.

The RAGs contain a number of TFs, some of which have been functionally linked with regeneration. In **Chapter 2,** we sought to utilise existing knowledge (reviewed in Verhaagen et al 2012 and Attwell et al 2018) as well as our own laboratories observations (Stam et al., 2007; MacGillavry et al., 2009, 2011) to inform the selection of a number of TFs that are induced by nerve injury and had a high probability of physically or functionally interacting, therefore having a greater chance of driving RAG expression together than on their own. The TFs we selected were ATF3, c-Jun, Smad-1 and STAT3. The effects of these TFs were studied *in vivo* in a dorsal root (DR) and DC lesion model of axonal injury. We succeeded in delivering multiple TFs via dual promoter AAVs to the DRG. Our dual promoter AAV vector was designed to express the TF, alongside farnesylated GFP (eGFPf), which served as an effective axonal tracer in the DC lesion. In the DR injury model, we observed a faster rate of axonal outgrowth until the dorsal root entry zone (DREZ) of the spinal cord with the co-overexpression of ATF3, c-Jun, STAT3 and Smad1 and with the ATF3-only group. After 20 days, the eGFPf control group's axons had regenerated as far as the TF groups. Neither of the TF-treated groups showed sensory recovery following DR lesion and there was no effect of either group on regeneration following a DC lesion. We concluded that while RAG TF overexpression could transiently enhance the speed of regeneration until the DREZ after DR lesion, the TFs we selected to express in combination worked no better than ATF3 alone. This suggested that ATF3, c-Jun, STAT3 and Smad1 are not an adequate combination of TFs to drive RAG expression and promote regeneration of injured neurons to overcome the barrier of the DREZ to the spinal cord, nor the DC lesion.

In **Chapter 3**, we sought to identify RAG-associated TFs based on transcription factor binding sites (TFBSs) within the promoter regions of known RAGs and I performed subsequent cellular screening for their effect on neurite outgrowth. To identify TFs that directly interact with the promoter sequences of RAGs, a TFBS overrepresentation analysis on the promoter regions of RAGs in a facial motor neuron (FMN) injury RAG dataset was performed (Mason et. al., manuscript in preparation). Similar to DRG neurons, FMNs are known to be able to successfully regenerate their axons after sustaining a lesion. This analysis identified nine TFs with TFBS that are overrepresented in RAG promoters, which are thus potentially directly

involved in RAG expression. These were ATF3, CEBPδ, c-Jun, KLF7, MEF2C, SMAD1, SOX11, STAT3 and SRF. Seven were described in the context of regeneration previously and two were novel (MEF2C and SRF). I systematically screened these TFs *in vitro* to evaluate their effect on total neurite outgrowth (an *in vitro* proxy of axon-regeneration) in a DRG-like cell line. The TFs were overexpressed by themselves, in all possible pairs and then selectively in higher number combinations. From this medium-throughput screen, I identified two combinations from these nine TFs, a pair (KLF7/MEF2C) and a triple combination (ATF3/KLF7/MEF2C) that increased total neurite outgrowth significantly (pairs compared to single TFs, triples compared to pairs of TFs, etc.). These data suggest that there are combinations of TFs that are capable of promoting neurite outgrowth more effectively together than when expressed alone.

The single TFs KLF7, MEF2C, and the TF combinations KLF7/MEF2C and ATF3/KLF7/MEF2C that were discovered in **Chapter 3** were studied *in vivo* in **Chapter 4**. The L4 and L5 DRGs of rats were injected with AAV5 vectors that each express a TF and eGFPf. These animals subsequently received cervical spinal (C4) lesions of the DC, as described in **Chapter 1 and 6,** and performed in **Chapter 2**. Animals in this experiment were evaluated for sensorimotor functional recovery for 11 weeks. At 11 weeks, a transganglionic tracer was injected in the sciatic nerve to label regenerating axons. Animals were sacrificed 1 week later. The TF combination KLF7/MEF2C significantly increased axonal sprouting, prevented axonal retraction, and in two independent ladder tasks showed significant functional recovery over 11 weeks compared to controls that received AAV- eGFPf injections only. The triple TF combination ATF3/KLF7/MEF2C, surprisingly, did not result in better regenerative outcomes than the TF singles, double, or the controls, in contrast to *in vitro* results of **Chapter 3**. The promising improvements in axonal sprouting and functional recovery caused by KLF7/MEF2C indicate that we are one step closer to finding an optimal group of TFs, which, when overexpressed in the DRG neurons may be able to promote regeneration and recovery following injury of the DC of the spinal cord.

In **Chapter 5** RNA-seq experiments were performed to compare the changes in gene expression induced by overexpression of the TFs studied in **Chapters 3 and 4** with the RAG program. To this end, a strategy was devised of simultaneously expressing each TF together with a unique fluorophore in DRG neurons, with the aim of identifying neurons which had been transduced by multiple TFs (and therefore expressed multiple coloured fluorophores) in DRG tissue sections (**Chapter 5**). After overexpressing individual TFs and TF combinations in DRG neurons, we successfully selectively laser capture micro-dissected (LCMd) transduced (and co-transduced) DRG neurons that were collected for RNA-seq analysis. We compared the gene expression changes induced by the TFs to a RAG dataset generated from selectively LCMd, large-diameter DRG neurons that had undergone peripheral nerve injury at two time-points prior to LCM. Initial comparisons of significant differentially expressed genes induced

by TFs in comparison to RAGs did not reveal a marked overlap in expression changes except for KLF7. In a cluster analysis of significantly expressed genes, it was interesting to note that the TF groups, ATF3 alone, and ATF3/ KLF7/ MEF2C, induced very similar gene expression changes, indicating a dominant effect of ATF3. This is in some ways similar to the observations of Chapter 2, where the phenotype induced by 4 factors including ATF3 was the same as that induced by ATF3 only.

Rather than looking at significant gene expression changes on an individual gene level, we next plotted RAG expression changes induced by TF overexpression against the changes induced by axotomy. We found that in all TF groups except MEF2C, there was a strong correlation of TF induced fold-changes with axotomy-induced fold-changes. This indicates TF overexpression-induced changes in expression are partially overlapping with the changes induced by axotomy.

This analysis was followed by Weighted Gene Co-expression Network Analysis (WGCNA), which considers the whole input of all gene expression to derive co-regulated gene clusters. WGCNA indicated that all TF groups except MEF2C alone were regulating clusters of genes with well-established links to regeneration such as the STAT and MAPK axotomy-induced signalling pathways, integrin signalling, cytoskeletal processes, and cell motility. With regards to the hypothesis posed earlier that RAGs are primarily coordinated under control of TF regulated gene expression, the RNA-seq analysis revealed that forty percent of genes (155/392) classified as strongly up-regulated by axotomy were regulated by all TF groups, with the exception of MEF2C alone. This supports the hypothesis that certain combinations of TFs can be key regulators of the RAG response to axotomy.

Interestingly, WGCNA revealed a class of 666 genes weakly regulated by axotomy as well as specifically by the KLF7/ MEF2C group (and not the other TF groups). Gene ontology (GO) analysis showed that this class of 666 genes contained genes involved in myofibril and muscle contraction, consisting of myosins and actinins. These components of the contractile apparatus may be important for axon extension. These genes, regulated by KLF7/ MEF2C and not the other TF groups, will be important to characterize further to understand the *in vivo* recovery demonstrated by this group in Chapter 4.

Chapter 6 describes our functional testing optimisation (that preceded the functional testing of Chapter 4) in which we evaluated the functional deficits caused by DC lesions at either the cervical or thoracic level. The functional deficits were measured in five sensory or sensorimotor functional tests, including one novel test, the inclined rolling ladder. Cervical lesions caused longer-lived functional deficits than thoracic lesions, detectable up to 7 weeks following lesion, and whole time-course comparisons revealed significant deficits in most tests.

The main findings of this thesis can be summarized as follows:

- *ATF3 promotes axon regeneration following a dorsal root lesion but not after a dorsal column lesion (**Chapter 2**)*

- *The combination ATF3, c-Jun, STAT3 and Smad1 also promotes regeneration following a dorsal root lesion but there is no synergistic effect of the four factors (**Chapter 2**)*

- *The combination of the TFs KLF7 and MEF2C increased axonal sprouting, prevented retraction, and improved functional recovery in two sensorimotor ladder tasks following dorsal column lesion (**Chapter 4**)*

- *Adding ATF3 to the dual combination KLF7/MEF2C abolishes the effect on axonal regeneration (**Chapter 4**)*

- *Gene expression profiling of TF-overexpressing neurons coupled with WGCNA analysis reveals that four of the five TF groups derived from TFs identified in **Chapter 3** regulate forty percent of the genes that are strongly up-regulated by axotomy, and finally, identifies a large cluster of genes weakly regulated by both axotomy and KLF7/ MEF2C which merits further investigation (**Chapter 5**)*