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A New Axonal Splice Variant of HDGF-related protein 3 Increases Mature Oligodendrocyte Numbers

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Shereen Georges

Committee in charge:

Professor Fred H. Gage, Chair
Professor Nicholas C. Spitzer, Co-chair
Professor Alysson R. Muotri

2015

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The Thesis of Shereen Georges is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2015

DEDICATION

I would like to dedicate this thesis to my family:

To my father, George, and my mother, Sara, for their unending love, encouragement, and support.

To my sister, Nadeen, for knowing exactly how to make me laugh during my times of stress and frustration.

Last but not least, to my loving fiancé, Jason. You are a remarkable man. Thank you for always providing encouragement, love and support through the busiest and hardest of times.

EPIGRAPH

Somewhere, something incredible is waiting to be known.

Carl Sagan

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I would also like to thank Bilal Kerman for his unending patience and time in training me in the techniques necessary to succeed and perform these experiments. Bilal was always there to help me ask the right questions, interpret results, troubleshoot, and design experiments. I will forever be indebted to Bilal for his continuing guidance and support throughout my undergraduate and graduate years.

Section 2.1, in full, is currently in press, as it will appear in *Development*. Kerman, B., Kim, H.J., Padmanabhan, K., Mei, A., Georges, S., Joens, M.S., Fitzpatrick, J., Jappelli, R., Chandross, K., August, P., Gage, F.H. "*In vitro* myelination using embryonic stem cells" (in press). The thesis author was a co-author of this paper.

Section 2.2, in full, is currently being prepared for submission for publication of the material. Genoud, Stéphane *; Kerman, Bilal E.*; Denli, Ahmet M.; Georges, Shereen; Xu, Xiangdong; Ettle, Benjamin; Yeo, Gene W.; Kim, Hyung Joon; Winkler, Jürgen; Gage, Fred H. "A new axonal splice variant of HDGF-related protein 3 regulates the myelinating glia biology." The thesis author was a co-author of this material.

ABSTRACT OF THE THESIS

A New Axonal Splice Variant of HDGF-related protein 3 Increases Mature Oligodendrocyte Numbers

by

Shereen Georges

Master of Science in Biology

University of California, San Diego, 2015

Professor Fred H. Gage, Chair

In the central nervous system, axons are myelinated by oligodendrocytes (OLs). However, though our understanding of OL biology is increasing, the signals that emanate from the axons and modulate myelination are still poorly understood. Previous studies have found that HRP3-II, a newly identified isoform of the hepatoma-derived growth factor (HDGF) family, shows peak expression in the axons of spinal motor neurons

before and during the myelination period. Furthermore, overexpression of HRP3-II increased Schwann cell proliferation and myelination, suggesting its importance for regulating the local pool of Schwann cells. Therefore, our goal is to determine whether HRP3-II also plays a critical role in regulating OLs in the central nervous system (CNS) using a recently developed CNS myelination model. The model is based on co-culturing mouse embryonic stem cell derived cortical neurons and OLs in microfluidic devices. We overexpressed HRP3-II in the neurons using a viral construct in the co-cultures. The average number of Myelin Basic Protein-positive OLs was significantly larger in the HRP3-II overexpressing co-cultures compared to controls; however, a similar increase was not seen on myelination in the HRP3-II overexpressing co-cultures, suggesting its role in the proliferation and/or maturation of OLs.

Chapter 1:

Introduction

1.1 The Importance of Myelin in the Nervous System

In both the Central Nervous System (CNS) and Peripheral Nervous System (PNS), the process of myelination is critical for neuronal survival and proper functioning. In the CNS, glial cells known as oligodendrocytes (OLs) myelinate the axons; and in the PNS, glial cells known as Schwann cells myelinate the axons. Myelin is constituted of proteins and phospholipids that form a whitish, electrically insulating sheath around axons, increasing the speed at which impulses are conducted[1]. Nodes of Ranvier are the gaps on the axon in between the myelin sheaths that are uncoated, and therefore capable of generating electrical activity[2]. Sodium channels are clustered into these nodes during the myelination process, which in turn enables the action potential to jump from one node to the other, a process known as saltatory conduction[2]. An action potential is a short-lasting event in which the electrical membrane potential of a neuron rapidly rises and falls to provide the basis for fast processing of information in a relatively small space[2]. Many processes that are initiated, maintained, or terminated by rapidly-propagated nerve signals could not occur at a sufficient rate without the myelin sheath; and its fundamental importance is highlighted in demyelinating diseases such as multiple sclerosis and various leukodystrophies[1-3]. However, the molecular mechanisms underlying these complex cellular interactions between the specialized glial cells and neurons, culminating in the formation of myelin, are still largely not understood in detail. In other words, how an OL or Schwann cell identifies and wraps neurons has yet to be fully examined.

1.2 Hepatoma-derived growth factor and related proteins

Hepatoma-derived growth factor (HDGF), which was initially purified and identified from the supernatant of human hepatoma cells lines, has been shown to have growth factor activity for cells including: hepatoma cells, fibroblasts, endothelial cells and smooth muscle cells[4]. HDGF has two nuclear localization signals, although expression has also been seen in the cytosol of some cells[5]. Subsequently, five related proteins, which show high identity in their N-terminus, but differ at the C-terminus, have been identified, suggesting similar function; however, aside from their growth factor activity, the functions of these proteins remain largely unknown[6]. Four of these proteins were named Hepatoma-derived growth factor related proteins (HRPs) 1 to 4; and the fifth was termed lens epithelium-derived growth factor (LEDGF). In mice HDGF and HRP2 expression show a broad tissue distribution, while HRP1 and HRP4 mRNAs have shown expression restricted to the testis[7]. HRP3 mRNA expression, on the other hand, has only been examined in human tissues thus far with prominent expression in the brain, and low amounts of mRNA also found in heart, testis, and kidney tissue[8]. Therefore, this evidence points to a particular role of HRP3 in the human nervous system.

Recently, HRP3 has been implicated in neurite outgrowth and neuronal survival[6]. Even though both nuclear and axonal localization of HRP3 has been reported in neurons, neither the precise function nor mechanism of axonal localization of HRP3 has been determined[6,9].

Chapter 2:

Background and Motivation

2.1 A Newly Developed Myelination Assay

Currently, the approaches that have been taken to study the myelination process *in vitro* with isolated primary neuronal cells are restricted by the limited number of cell types, making larger-scaled experiments difficult to perform[3-4]. Therefore, to achieve myelination, members of our lab developed a novel method to differentiate mouse embryonic stem cells (ESCs) into myelinating OLs[10]. First, neural progenitor cells (NPCs) were generated from mouse ESCs. OL precursor cells (OPCs) were then induced from NPCs using Differentiation Medium. At the end of one week (Day 8), about 70% of the population expressed markers for OPCs and/or OLs. In the second week, we switched to Maturation Medium to promote the maturation of OPCs. By Day 15 (D15), mature OLs were generated expressing markers such as O4 and myelin basic protein (MBP) and showing mature OL morphology, i.e., extended membrane sheaths and highly branched processes.

Additionally, an existing protocol was adapted to generate cortical neurons from mouse ESCs[11]. These newly generated myelinating OLs and cortical neurons were co-cultured, and their ability to interact and form myelin was examined[10]. Previous experiments performed in our lab have confirmed functional interaction between neurons and OLs and the existence of compact myelin by immunostaining with markers such as MBP and contactin-associated protein (Caspr), and transmission electron microscopy (TEM) analysis[10].

Growing this newly developed co-culture system on conventional cell culture dishes made quantification and monitoring of myelination difficult due to the random distribution of neurites and myelin within the plate. Therefore, to overcome these

limitations, this co-culture system was combined with microfluidic technology. The compartmentalization provided by microfluidic platforms allowed us to concentrate myelin formation into a defined region, which we named the myelination compartment. Neurons were plated in such a way that only the axons are able to project to the opposing compartment where the OLs are plated (Figure 2.1.1 a-b). Similar to conventional co-cultures, myelin was observed in the microfluidic devices (Figure 2.1.1 c-d).

Counting the number of smooth, tubular-looking segments that extend from the cell body of the OL membrane, or manually tracing these segments are two of the main strategies currently employed to quantify myelination[12-13]; however, these approaches are often prohibitive when trying to compare several conditions and/or to analyze large experimental areas due to the extensive time and manpower requirements. With the ability to observe the entire experimental area containing almost only the axons and OLs, our lab then developed a custom computer-assisted method; Computer-assisted Evaluation Myelination (CEM) to quantify myelination. We imaged and quantified the entire myelination compartment using CEM, in order to show that it is feasible to identify and quantify myelin in a large area with minimal human intervention (Figure 2.1.2).

Section 2.1, in full, is currently in press, as it will appear in *Development*. Kerman, B., Kim, H.J., Padmanabhan, K., Mei, A., Georges, S., Joens, M.S., Fitzpatrick, J., Jappelli, R., Chandross, K., August, P., Gage, F.H. "*In vitro* myelination using embryonic stem cells" (in press). The thesis author was a co-author of this paper.

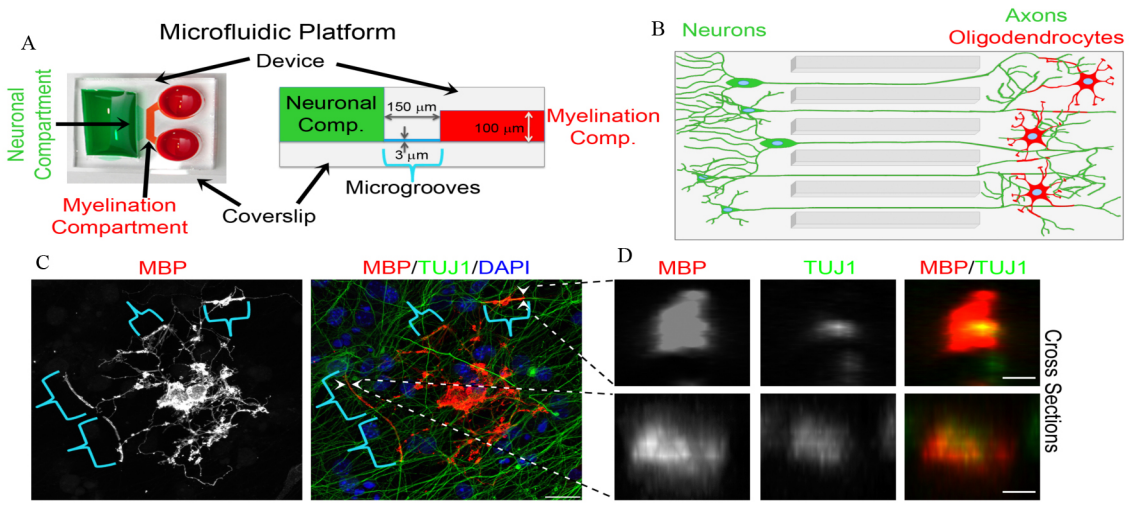

Figure 2.1.1. Microfluidic device used for myelination assay. (**A**) Left top view, right schematic cross section. Neurons were plated into the open Neuronal Compartment. OLs were plated into the closed Myelination Compartment. (**B**) A representation of myelination in devices. (**C**) Left panel OLs (MBP) only, right panel neurites (TUJ1) and OLs. Brackets mark regions indicating myelin. (**D**) Optical cross sections

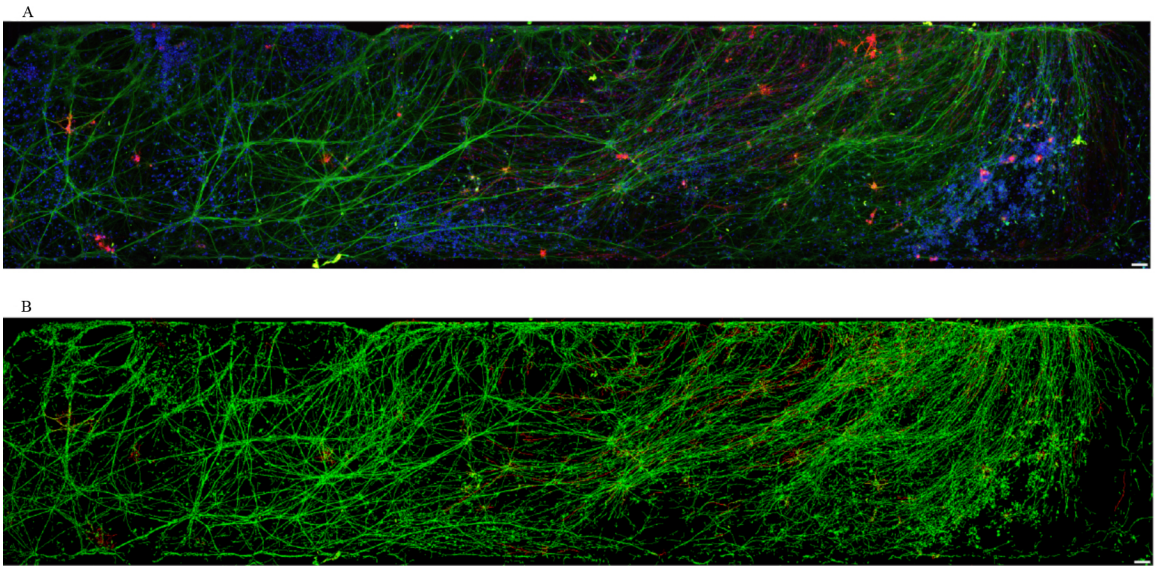

Figure 2.1.2. Images of the entire myelination compartment of microfluidic devices before and after processing. (A) A representative image of the entire myelination compartment at 2 weeks of co-culture. The image is a mosaic of about 100 overlapping tiles stitched on a computer showing neurons (TUJ1) and OLs (MBP). Scale bar is 100 μm. (**B**) A representative image of the entire myelination compartment shown in (A) after processing.

2.2 Identification of a novel splice variant of HDGF-related protein 3 as a myelin modulator

In order to identify new core components of the myelination process within the neurons, gene expression profiling studies during the myelinogenic phase of development were previously performed in our lab[14]. A microarray analysis approach, combined with laser capture microdissection of spinal motor neurons (MNs), identified strong expression of HRP3 at the myelinating regions at the onset of myelination, with significant neuronal body and axonal expression in the developing white matter tract of spinal cord (Figure 2.2.1 E, 2.2.1 I). Upon comparing HRP3 with myelin gene expression, HRP3 appeared one to two days earlier than well established myelination markers (Figure 2.2.1 F, 2.2.1 L), then decreased after P21 through adulthood (Figure 2.2.1 G). These results were also confirmed via Western Blot analysis (Figure 2.2.1 A). Lastly, HRP3 was detected in the axons of myelinated and unmyelinated neurons, but not in the dendrites, in the early postnatal spinal cord (P3-P7) and in cultures of spinal motor neurons (Figure 2.2.1 L-N). However, when myelination had not yet started in earlier stages of spinal cord development (E18), HRP3 expression was restricted to the nuclei of motor neurons (Figure 2.2.1 C), suggesting that HRP3 is essentially nuclear but can also be targeted to the axonal compartment of motor neurons during myelination.

To further explore the potential role of HRP3 in myelination, its expression in the sciatic nerve and in various brain areas was examined (Figure 2.2.2). The tissues were examined during the most active period of myelin development: P1-P10 for the sciatic nerve (Figure 2.2.2 A-D), and P10-P30 for the corpus callosum (Figure 2.2.2 E-H). It is shown that HRP3 expression in defined CNS and PNS white matter fiber tracts

consistently peaks with their period of myelination. Furthermore, HRP3 expression was examined using an experimental demyelination model. Cuprizone treatment induces myelin loss in large areas of mouse brain; and in a matter of weeks following injury, complete remyelination occurs[15]. Using this model, after cuprizone treatment, HRP3 expression in the corpus callosum began to increase immediately when remyelination was occurring, and declined after complete remyelination to levels observed prior to injury (Figure 2.2.3). Together, this data illustrates that HRP3 expression is temporally controlled and regulated in neurons to be myelinated, and thus may play a role in modulation of myelination.

To further examine the potential role of HRP3 on myelination, HRP3 was overexpressed via fusion to GFP; however, unlike endogenous HRP3, this version failed to be expressed in the axons of dorsal root ganglia and motor neuron cultures. Sequence analysis of this version of HRP3 showed that it contains a nuclear localization signal (NLS), but no nuclear export signal (NES). A search through NCBI database of expressed sequence tags revealed an alternatively spliced isoform of HRP3 with a functional NES motif (Figure 2.2.4 A-B). This new isoform of HRP3, named HRP3-II, is a novel, highly conserved splice variant that differs from HRP3-I by four extra amino acids at its C-terminal end[14]. Peak expression of this isoform was found to coincide with the period of myelination in the white matter fiber tracts during developmental myelination and during re-myelination after injury. Unlike HRP3-1, which remains in the cell nuclei of neurons (Figure 2.2.4 C), HRP3-II is exported out of the cell bodies and into the axons of neurons (Figure 2.2.4 D), but was not found in dendrites or non-

neuronal cells, such as astrocytes (Figure 2.2.4 E-F). Expression of this protein was also highest before and during the myelination period by Schwann cells in the PNS[14].

In myelinating co-cultures, increased levels of HRP3-II, but not HRP3-I, resulted in increased Schwann cell proliferation and myelination, suggesting its importance for regulating the local pool of Schwann cells (Figure 2.2.5). However, it cannot be distinguished if the increased myelination was due to an indirect effect of increased Schwann cell numbers or if HRP3-II also directly modulates myelination by Schwann cells

In all, this data suggests that HRP3-II may play a critical role in OL maturation and proliferation in the CNS as well. Therefore, based on these findings, the goal of this thesis is to determine whether HRP3-II is also involved in the proliferation, maturation and/or myelination of neurons by OLs in the CNS.

Section 2.2, in full, is currently being prepared for submission for publication of the material. Genoud, Stéphane *; Kerman, Bilal E.*; Denli, Ahmet M.; Georges, Shereen; Xu, Xiangdong; Ettle, Benjamin; Yeo, Gene W.; Kim, Hyung Joon; Winkler, Jürgen; Gage, Fred H. "A new axonal splice variant of HDGF-related protein 3 regulates the myelinating glia biology." The thesis author was a co-author of this material.

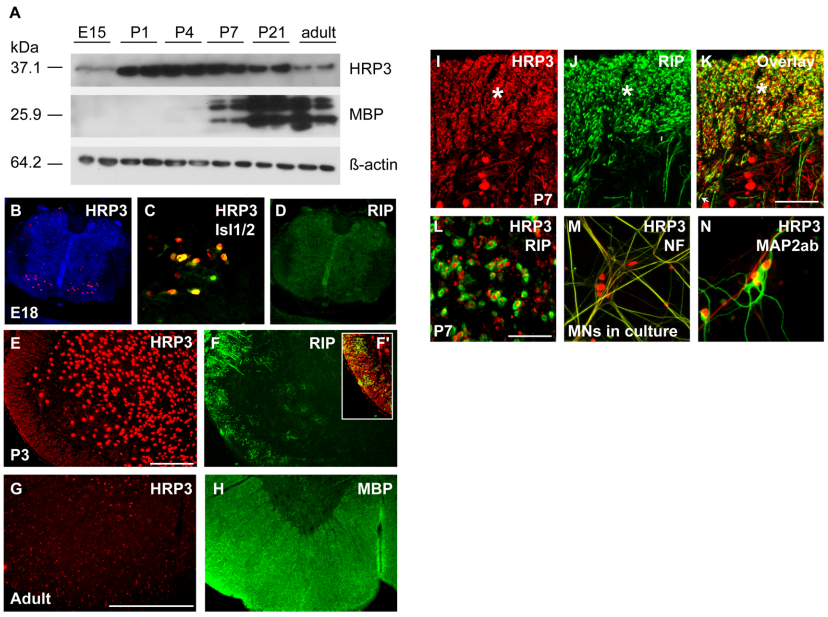

Figure 2.2.1. HRP3 is upregulated at the onset of myelination, with significant neuronal body and axonal expression in the developing white matter tract of spinal cord. (A) Western blot showing peak expression of HRP3 at onset of myelination. **(B-N)**. Immunohistochemistry with antibodies to HRP3 in the rat spinal cord at E18 (no myelination yet), P3-P7 (period of active myelination) and adult.

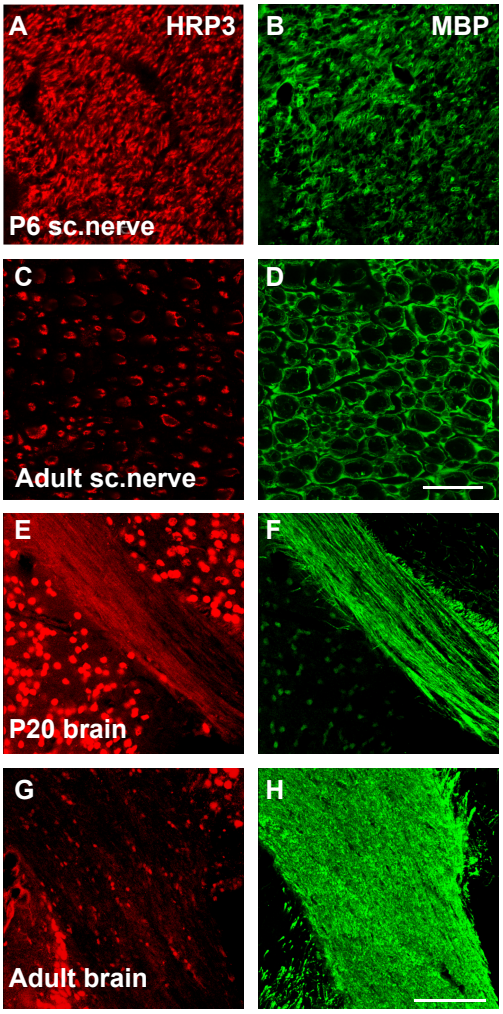

Figure 2.2.2. HRP3 expression in defined CNS and PNS white matter fiber tracts peaks with their period of myelination. (A-D). Immunofluorescence analysis of HRP3 expression pattern in rat sciatic nerves at P8 (period of active myelination) and adult shows transient upregulation of HRP3 protein. **(E-H).** HRP3 is also upregulated in the developing corpus callosum during myelination (p20).

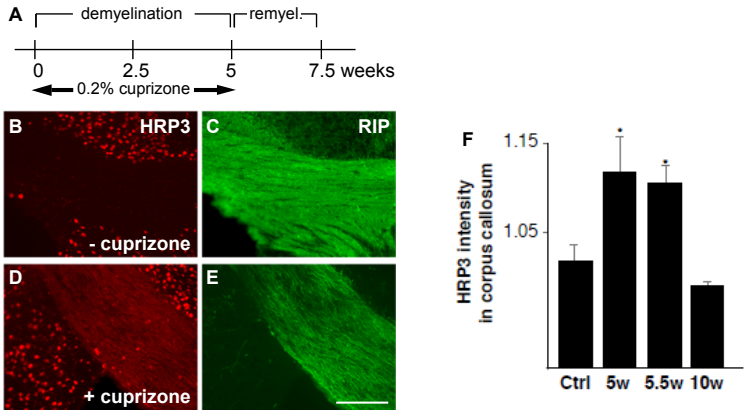

Figure 2.2.3. HRP3 is transiently upregulated in white matter fiber tracts during remyelination. (A) Experimental design schematic. **(B-E).** Coronal sections through the forebrain region of adult animals treated with or without cuprizone. **(F)** Quantification of immunohistochemistry revealed increased HRP3 protein expression in the corpus callosum of cuprizone treated mice during the active remyelination phase.

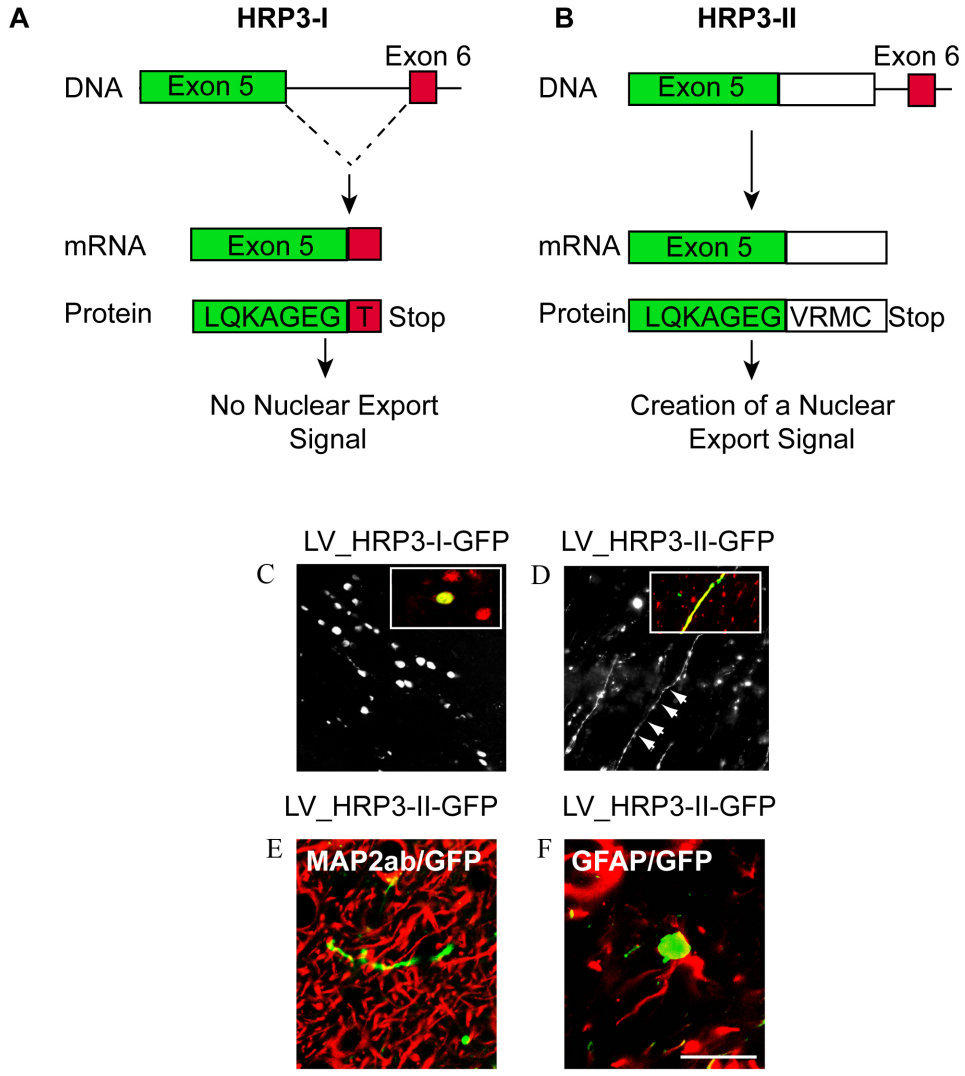

Figure 2.2.4. Identification of the axonal variant of HRP3 (HRP3-II). (A-B). Schematic representation of the genomic organization of the rat HRP3-I nuclear variant AAK72965 and the axonal variant HRP3-II. **(C-F)**. Immunofluorescence analysis on brain sections immunolabelled with GFP (green), NeuN (C), neurofilament (D), MAP2ab (marker of dendrites; E) and GFAP (marker of astrocytes; F).

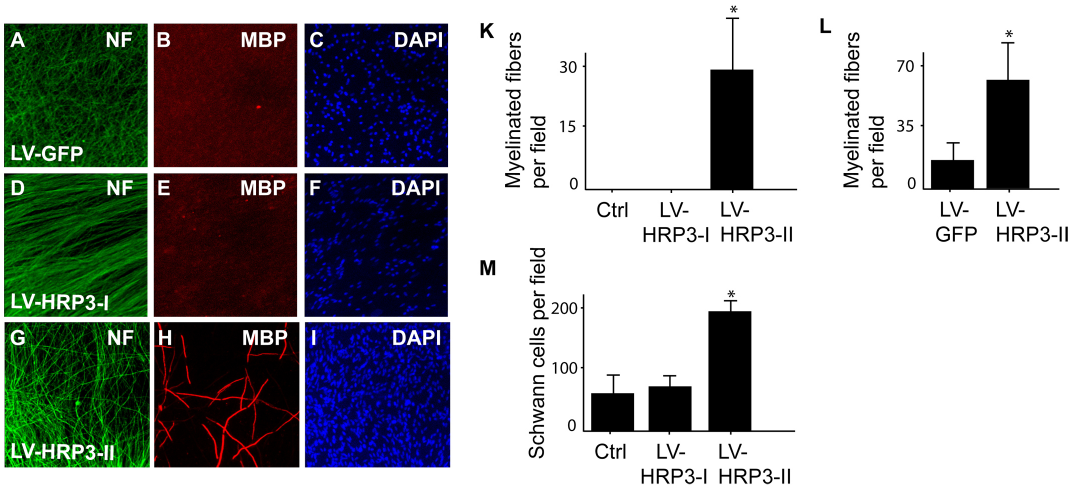

Figure 2.2.5. HRP3-II, but not HRP3-I, stimulates Schwann cell proliferation and myelination. (A-I) Lentiviral-mediated overexpression of GFP, HRP3-I nuclear variant and HRP3-II axonal variant in DRG neurons co-cultured with primary Schwann cells. **(K-L).** Overexpression of HRP3-II in neurons stimulated myelination, whereas HRP3-I had no significant effect, compared with GFP infected cultures at two different seeding densities. **(M)** The number of Schwann cells found in K was counted

Chapter 3:

Results

In order to determine whether HRP3-II levels have an effect on OL maturation and/or myelination ability, we utilized the myelination assay described above. ESC-derived cortical neurons were infected either with an HRP3-II overexpressing (GFP IRES HRP3-II) or a control virus after being plated into the microfluidic devices. The OLs were plated into the myelination compartment of the devices. For myelin quantification, the devices were stained with TUJ1 (a neuronal tubulin marker) to identify the neurons and MBP, which identifies mature, myelinating OLs (Illustration 3.1). The myelination ratios were compared to determine the effect of HRP3-II isoform on maturation, and/or myelination ability of OLs *in vitro*. HRP3-II overexpression caused a significant increase in the number of MBP-expressing OLs; however, no effect was seen on overall myelination. Furthermore, we hypothesize that close proximity of neurons to OLs was required by HRP3-II to modulate OL biology, based on conditioned medium experiments that we performed.

3.1 HRP3-II overexpression increased the number of MBP-expressing OLs

In order to determine whether or not HRP3-II similarly modulates OL biology as previously seen in Schwann cells, we overexpressed HRP3-II in ESC-derived cortical neurons in the myelination assay. Because HRP3-II was expressed from a construct that also expresses GFP via an IRES sequence, we overexpressed GFP as a control. The devices were fixed and stained for MBP and TUJ1 before imaging the entire myelination compartment Figure 3.1.2 A-B and 3.2.1. A).

First, we asked if HRP3-II modulates number of mature OLs. The number of MBP expressing mature OLs (MBP^+ cells) was counted in each device to obtain and

compare the number of MBP^+ cells between the control and overexpression conditions (Figure 3.1.1). A total of nine GFP control devices and seven HRP3-II overexpression devices in three independent experiments were analyzed. The number of MBP^+ cells for each device was normalized to the average number of MBP^+ cells of all GFP control devices for each experiment. HRP3-II overexpression resulted in an overall 1.5 fold increase of mature OLs (p value= 0.0372; Figure 3.1.1 C-D). Thus, our results parallel the effect seen on Schwann cells, since increased levels of HRP3-II in a portion of the axons increases the number of Schwann cells.

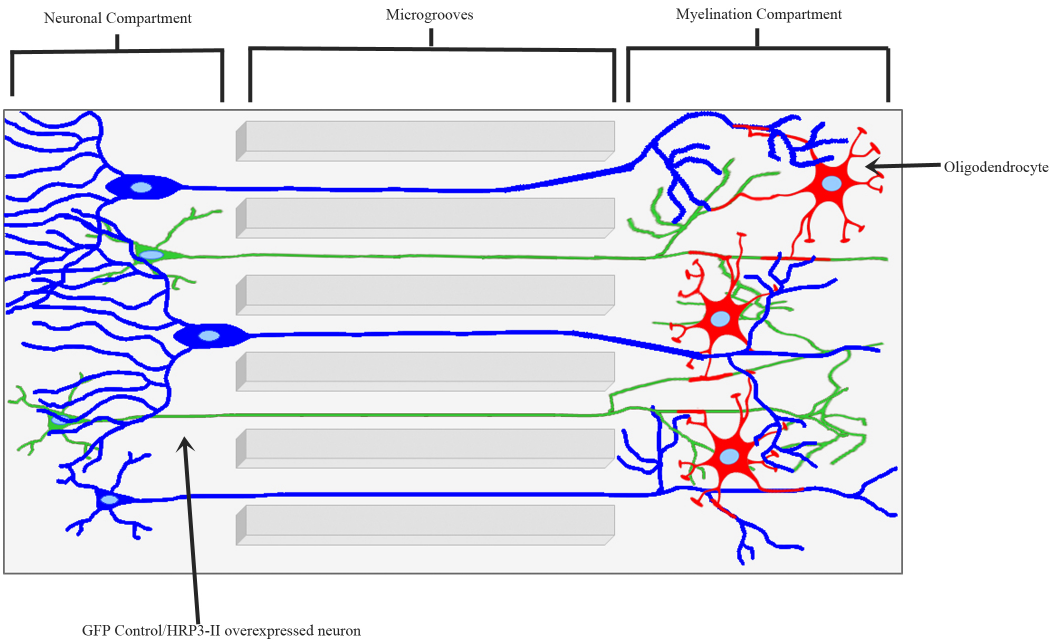

Illustration 3.1.1. Experimental scheme of microfluidic devices used and myelination in devices. Neurons were plated into the Neuronal Compartment. GFP infected neurons are depicted in green while non-infected neurons are depicted in blue. OLs were plated into the Myelination Compartment. Axons extended through microgrooves into the Myelination Compartment, where OLs ensheathed them.

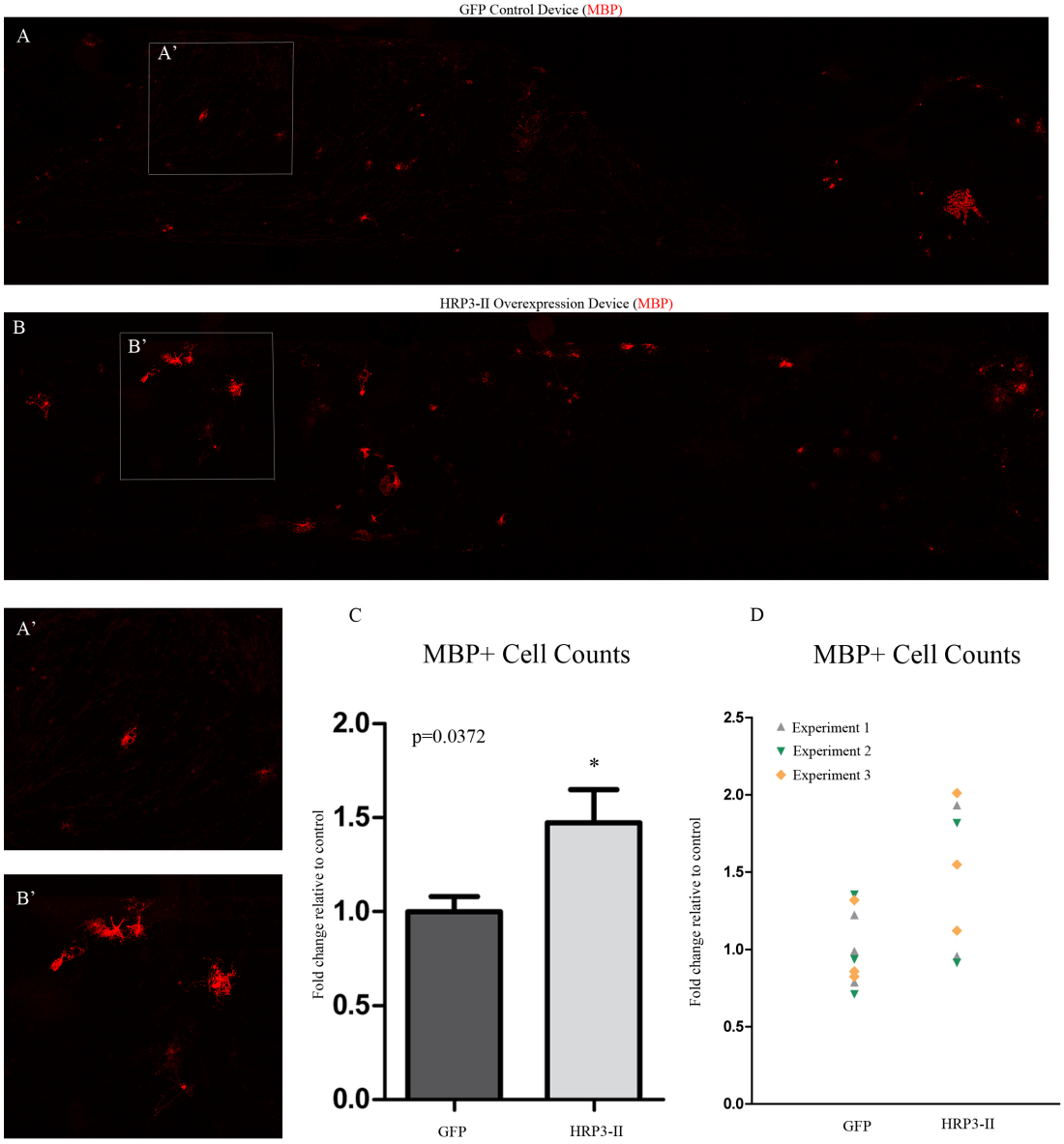

Figure 3.1.1. HRP3-II overexpression significantly increased the number of MBP+ cells. (**A-B**). Control (**A**) and HRP3-II overexpression (**B**) devices were stained, imaged, and analyzed. (**C**). The normalized number of MBP^+ cells was 1.5 fold higher in HRP3-II overexpression devices. (**D**) Number of MBP^+ cells for each device.

3.2 HRP3-II overexpression did not increase overall myelination

Next, we sought to determine whether this increased number of MBP^+ cells was also accompanied by an increased amount of myelination when HRP3-II was overexpressed in neurons in the myelination assay. As all of the neurons were not infected, GFP was used to identify axons with increased levels if HRP3-II in the experimental devices. Axons that were positive for both TUJ1 and GFP indicated that these neurons did contain the construct, and thus expressed HRP3-II either at higher levels (in the experimental devices) or at normal levels (control devices). Whereas axons that were positive for only TUJ1 indicated that they were not infected and expressed HRP3-II at normal levels in both control and experimental devices (Illustration 3.1.1).

In total, the myelination compartments of nine control devices and seven HRP3-II overexpression devices were analyzed. Upon imaging the entire myelination compartment of all devices (Figure 3.2.1 A), the myelin quotient (MQ) was calculated using CEM as was previously described[10]. Briefly, the total amount of myelin was calculated by counting overlapping pixels of MBP+ processes and TUJ1 processes (Figure 3.2.1 A'). This value was then divided by the total pixel count of neurons to establish each device's "myelination ratio". To further clarify the comparison and eliminate variability within and among the three experiments, a MQ was calculated by normalizing the myelination ratios of each device within an experiment to the average control myelination ratio for that experiment (Figure 3.2.1 B-C). Averaging the three normalized MQ's for the three independent experiments resulted in a HRP3-II overexpression MQ of 1.02. Therefore, there appears to be no trend of increased overall myelination in the overexpression condition compared to control.

It is unclear why an increase in number of MBP^+ OLs did not also result in increased myelin. One possibility was that, though HRP3-II increased the local pool of mature OLs, it did not modulate the pro-myelination signals on axons. Thus, the number of axons that could be myelinated stayed the same in both control and experimental devices. Another possibility was that the myelination promoting factors such as thyroid hormone in the medium might have masked the effect of HRP3-II. Furthermore, overexpressing HRP3-II may not be increasing the later stages of the myelination process (i.e. myelin sheath formation); but rather, is increasing the maturation of OLs, which may eventually lead to myelin. However, our myelin quantification does not measure this hypothesis, directly. Regardless, these results do not contradict our previous observation with Schwann cells and DRG neurons as the baseline myelination in those co-cultures were very low. It is also possible that HRP3-II's affect in PNS was different than in CNS.

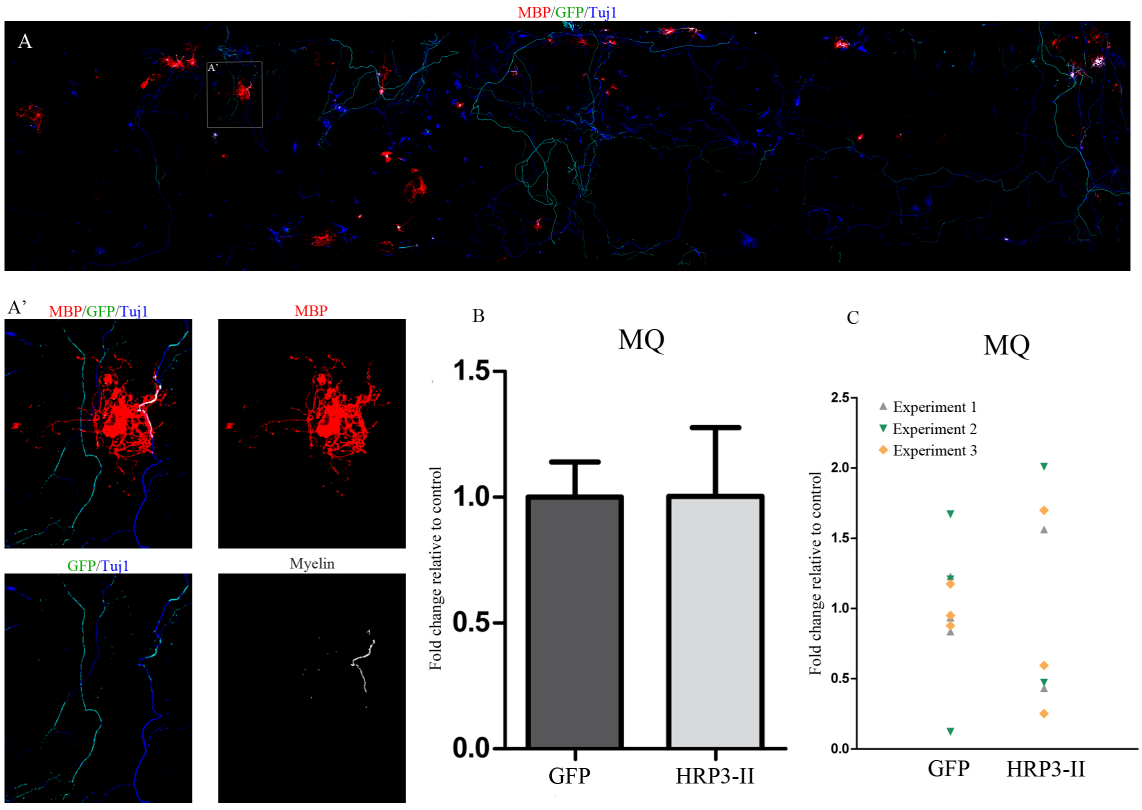

Figure 3.2.1. HRP3-II overexpression did not affect total myelin. (**A**) The entire myelination compartment of an HRP3-II overexpression device. (**A'**) A close-up of the boxed region in A showing CEM identified myelin. (**B**) HRP3-II overexpression did not affect MQ. (**C**) MQ for each device.

3.3 Close proximity of neurons to OL was required by HRP3-II to modulate OL biology

Based on the results obtained thus far, we then sought to determine the mechanism by which HRP3-II increased MBP^+ cell numbers. We hypothesized that if HRP3-II, or some other factor regulated by HRP3-II was secreted into the culture medium by the neurons and affected the OLs, then exposing OLs to the culture medium of HRP3-II overexpressing neurons should result in the same increase in MBP^+ cell number. Two experiments were performed using slightly different conditions. In the first experiment, neurons were either infected with control GFP or the HRP3-II overexpression construct, or not infected at all. The medium was collected and plated onto OL-precursor cells (OPCs) at their 2-week differentiation time point after either spinning down or filtering the conditioned medium. This was done to eliminate potential debris or other contaminants that may have been present in the medium, and to determine whether or not either of these actions would affect the outcome on the OLs. The OLs were stained for KI67 (a proliferation marker) and SOX10 (an OPC marker), or with MBP (a mature OL marker) after 1-week and 2-week time points post-conditioned medium (Illustration 3.3.1). After imaging the cells and blinding the images, the cells that were positive for each marker were counted. There was no significant difference between conditions; however, little to no MBP^+ cells were present at both time points (data not shown). This suggested that the growth factors and nourishment in the medium was depleted by neurons resulting in starving OLs. Therefore, the same protocol was followed in the second experiment with one exception: the OLs received half conditioned medium and half fresh OL medium. Upon imaging and blindly counting the images, the data

again was insignificant; however, more cells were present, suggesting that this protocol may have been healthier for cell survival (Figure 3.3.1). Based on these results, we concluded that the effect caused by HRP3-II on OLs might require close proximity, if not completely contact-dependent; or the interaction might be so transient or labial that its effect was lost during transfer from neurons to OLs.

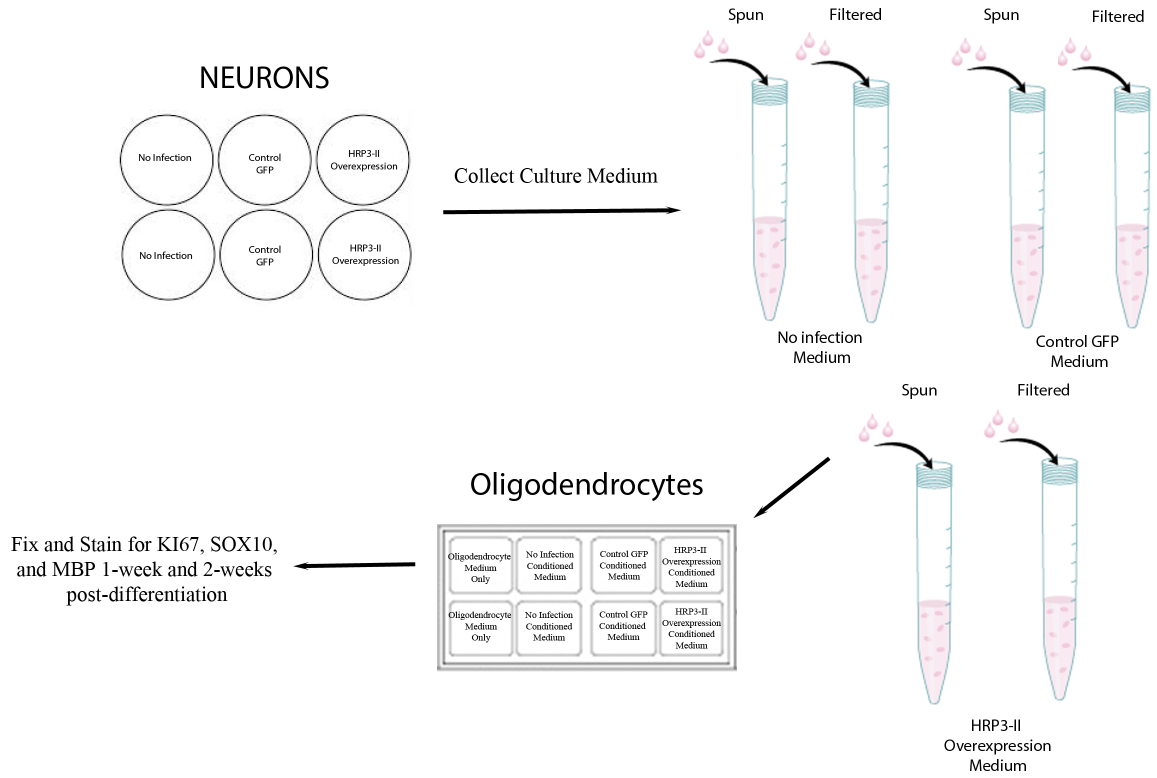

Illustration 3.3.1. Experimental schematic of conditioned medium experiments. Neurons were either infected with LV-GFP or HRP3-II IRES, or not infected at all. The medium was collected and fed to OPCs after either spinning down or filtering. The OLs were analyzed for proliferation and maturation after 1-week and 2-week time points post-conditioned medium.

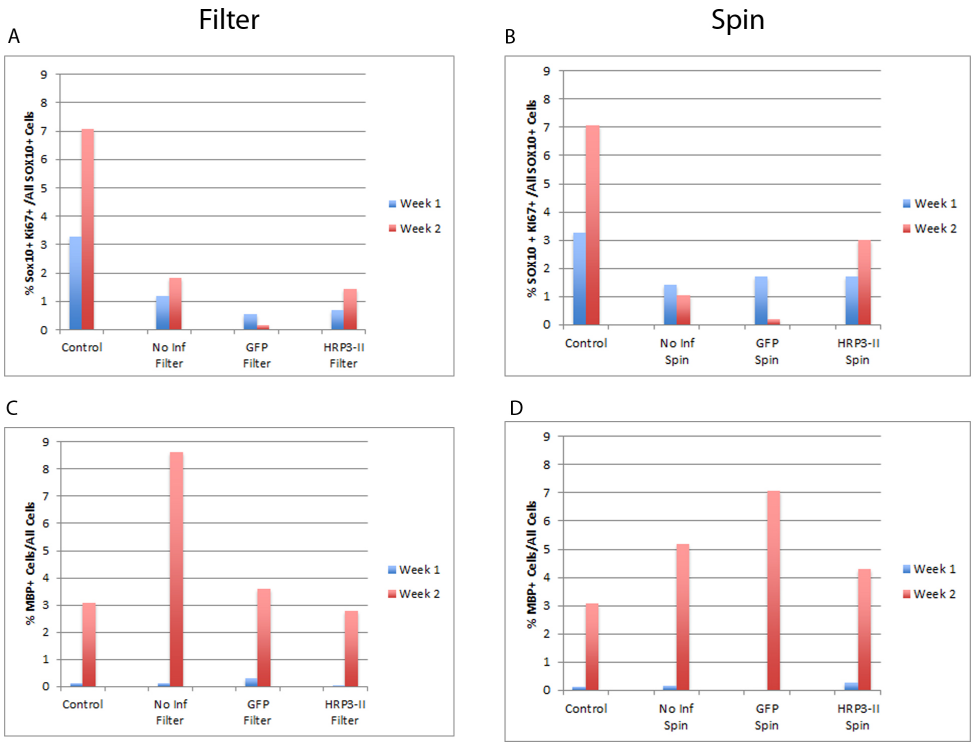

Figure 3.3.1. Cell counts obtained from second conditioned medium experiment showed no significant effect of HRP3-II conditioned medium on OLs. Twelve photos were taken at random, per condition at two time points. (**A-B**). The percent of proliferating ($KI67^+$) OLs (SOX^+) cells, (**C-D**). The percent of mature OLs (MBP^+) cells relative to total number of cells ($DAPI^+$).

Chapter 4:

Discussion

So far, little is known about the biological functions of members of the HRP protein family. However, in contrast to other HRPs, which are expressed in various tissues, HRP3 expression is restricted to a small number of tissues with the highest expression being in neurons, suggesting rather specific functions in these cells[8-9.] Previous research done in our lab suggested that a specific splice-variant of HRP3, HRP3-II, was involved in modulating myelinating glia. Thus, we overexpressed HRP3-II using a viral construct in neurons and compared the number of mature OLs and the amount of myelination in these experimental devices to control devices, containing neurons infected only with GFP. Overall, a significant fold increase in the number of MBP^+ OLs was observed in the HRP3-II overexpression experimental devices compared to controls. Yet, there did not appear to be a trend of increased myelination by OLs in the presence of HRP3-II overexpression.

Previous work performed in our lab did show an increase in the number of Schwann cells, which was further accompanied by an increase in myelination when HRP3-II was overexpressed in the DRG neurons. The observed difference may be due to dissimilarities of Schwann cells to OLs and/or DRG neurons to ESC-derived cortical neurons. For example, unlike Schwann cells, however, which each myelinate a single axon, OLs can extend their processes to multiple axons. Therefore, it is possible that each OL extended fewer processes and myelinated fewer axons under our experimental conditions. It is also possible that the amount of axons that can be myelinated in our myelination assay is saturated. The saturation may stem from the growth factors added to the myelination medium such as T3 or may be due to HRP3-II's failure to modulate axonal surface to allow myelination. Therefore, there may be more OLs in the HRP3-II

overexpression devices competing for the same amount of axons, which is why no effect is seen. Furthermore, comparing the average total amount of MBP in the control devices versus the HRP3-II overexpression devices shows that the MBP^+ cells in the HRP3-II overexpression devices were larger than those in the control devices, suggesting that the absent myelination effect is not due to the OLs being smaller when HRP3-II is over-expressed.

When taken together our data suggest that HRP3-II may play a role in the survival and/or maturation of OLs. In order to determine the mechanism behind this positive effect on OLs, experiments using conditioned medium were performed to examine the role of a potential secreted factor that may be involved. However, in two independent experiments, the difference between the conditions was insignificant, suggesting that HRP3-II's effect on neurons required close proximity in our experimental design. That may mean the effect may be dependent on contact between the OLs and the neurons themselves. Alternatively, if HRP3-II, or some other factor regulated by HRP3-II, is secreted, the effect may be transient.

Overall, by combining both stem cell and microfluidic technologies with a recently developed ESC-based *in vitro* myelination assay and automated quantification method, we were able to test the effects of HRP3-II on OL regulation. Our results paralleled with previous data obtained on the effects of this novel isoform on Schwann cells. By helping regulate both Schwann cell and OL numbers, this data suggests that HRP3-II is playing an important role in the proliferation, maturation, and/or survival of these myelinating cells in the nervous system. We believe that this project as a whole

will further our understanding of the process and mechanism of myelination, which is currently not fully understood.

Chapter 5:

Materials and Methods

Myelinating Co-Cultures

Neurons were differentiated following a previously published protocol that enriches for cortical neurons[11]. OLs were differentiated and myelinating co-cultures were set-up using protocols generated in our lab[10]. Briefly, on day 12 of the neuronal differentiation protocol, neurons were plated in the neuronal compartment of microfluidic devices. Neurons were grown in Neuron Medium for another one to two weeks to allow for neurite growth. Neuron Medium consisted of 1:1 DDM Medium:Neurobasal/B27 Medium[11] supplemented with 20 ng/ml Glial cell derived neurotrophic factor (GDNF; R&D Systems), 500 μg/ml cAMP (Sigma), and 0.2 μM ascorbic acid (Sigma). Four to five days prior to OL, neurons were infected either with control virus or with HRP3 overexpression virus. 80,000 (per device) OLs at D15 of the OL differentiation protocol were plated in the myelination compartment. Every other day, co-cultures were fed with Neuron Medium supplemented with 40ng/mL T3 (Sigma-Aldrich).

Immunostaining

Cells were fixed in 4% paraformaldehyde-PBS for 15 min, blocked and permeabilized with horse serum (10%) and Triton X-100 (0.1%) in PBS and were incubated overnight with a combination of the following primary antibodies: 1:900 mouse anti-TUJ1 (Covance), 1:40 rat anti-MBP (Serotec), and 1:500 rabbit anti-GFP (Invitrogen). DAPI (Sigma) was used to visualize nuclei. Images were acquired on Zeiss LSM 710 or 780 confocal microscopes at 1 μm intervals and were processed on Zen (Zeiss), and ImageJ (NIH) software.

Constructs

Lentiviral reporter plasmids were derived from pCSC-SP-PW (Marr et al., 2004). Lentiviral constructs were used to express GFP in the neurons, to serve as a control against the HRP3-II overexpression condition. To overexpress HRP3-II in the neurons, a GFP- inner ribosomal entry site (IRES)-HRP3-II viral construct was used.

MBP$^+$ Cell Quantification

After images of the entire myelination compartment of all devices were reconstructed, the images were then blinded. Two individuals counted the number of MBP$^+$ cells in each device, and the values obtained for each device were averaged. The number of MBP$^+$ cells for each device within an experiment was normalized to the average control MBP$^+$ cell count for that experiment.

Myelin quantification

Reconstructed mosaic images of confocal scans of the devices were quantified using CEM. Briefly, each optical Z-section was transformed to a binary image. Then the myelin was identified as the overlapping pixels of neurites and OLs. The amount of myelination was then assessed as the ratio of overlapping pixels to the total amount of neurites. The myelin quotient (MQ) was calculated by normalizing the myelination ratios of each device within an experiment to the average control myelination ratio for that experiment.

Conditioned Medium

ESC-derived cortical neurons were either infected with Control GFP, an HRP3-II overexpression construct, or not infected at all. The medium was collected and either spun down or filtered. Half of the conditioned medium was mixed with half fresh OL medium before being plated onto OL-precursor cells (OPCs) at their 2-week differentiation time point. The OLs were stained for KI67 (a proliferation marker) and SOX10 (an OPC marker), or with MBP (a mature OL marker) after 1 week and 2-week time points post-differentiation. Images were taken and blinded before being counted for further analysis.

Statistical Analysis

Error bars in all figures depict standard error. GraphPad Prism Software (GraphPad) was utilized to construct all graphs. P-values for myelin quantification were calculated using Mann-Whitney U-Test.

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