Developmental Myelinogenesis and Galanin: *In vivo* and *In vitro*

By

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Contributions of Authors to the Manuscript

The manuscript presented here as part of the Master's thesis has been submitted to a scientific journal for publication and was written by Hanna Lyubetska, the author of this thesis, as a first author of the manuscript. Lin Zhang was also listed as first author and contributed equally to the manuscript. Dr. Jiming Kong is listed as a second author. Dr. Maria Vrontakis is listed as a senior author and is also the corresponding author for this manuscript. This thesis adheres to the guidelines for theses in the 'Manuscript/Grouped Manuscript Style' (As described in the "Report of the Faculty Council of Graduate Studies on Regulation Changes" 2013).

Abstract

Correct myelin formation and maintenance is essential for normal functioning and is affected in the demyelinating disease, Multiple Sclerosis (MS). To better understand this disease and identify important targets in promoting remyelination, the study of developmental myelination is important. Galanin, a 29 amino acid neuropeptide has been identified as a potentially important modulator in early myelin development. In our Galanin transgenic mouse model, myelin basic protein (MBP) levels are highly elevated at postnatal day 10 compared to the wild type. A preliminary investigation of Galanin's behavior at various doses *in vitro*, yielded results that agreed with Galanin's effect *in vivo*. Proteolipid protein (PLP) was highly elevated in the 10nM dose *in vitro* indicating Galanin exerts its effects in a time and dose dependent manner. Overall, this study identifies Galanin as a potentially important modulator of developmental myelination that may become a therapeutic target in future studies of demyelinating diseases.

Chapter 1: Introduction

Myelination is a lengthy process that starts just before birth and can progress well into the third decade of life in humans (Czopka *et al,* 2013). While this important process has been studied for many years, myelination, and the temporal and regulatory mechanisms involved in its initiation and control are still poorly understood. Despite this, many studies continue to try to elucidate the mechanisms by which the events of myelinogenesis proceed. A considerable body of knowledge does exist and continues to expand daily. It is to this body of knowledge that this thesis strives to add with regard to developmental myelination. Considering the large host of diseases that affect the myelin sheath in the central nervous system (CNS), the study of developmental myelination and any potential modulators of the process can contribute to the understanding of why disease may occur and identify any potential targets for therapy. One such potential modulator, the neuropeptide Galanin, is examined here with regard to its effect on developmental myelinogenesis.

Myelin and Disease

Myelin, the specialized lipid membrane surrounding the nerves in both the peripheral and central nervous systems has long been studied for its structure and particularly its function. As any important component in the body, myelin is linked to many diseases that cause neurodegeneration in both a progressive and irreversible fashion. Among these diseases are included a host of hereditary leukodystrophies, most of which have devastating effects early in life that are often lethal (Zuccaro and Arlotta, 2013). Others include diseases that are more familiar and prevalent in the world today such as Multiple

Sclerosis (MS) (Miller, 2012). As the number of diseases affecting the myelin sheath and other cells in the CNS continue to increase in prevalence, there is a need to understand not only the regulatory mechanisms for the repair of damaged areas, but also the normal developmental process of these regions in order to pinpoint when and why disease occurs.

MS in particular is of concern to densely populated northern regions of the world where this disease is the most prevalent (Dutta and Trapp, 2010). This is especially true for Canada, where the country as a whole experiences a higher incidence of this disease (Miller, 2012). MS is characterized as an autoimmune disease resulting in eventual irreversible axonal damage and it is believed that a combination of both genetic and environmental factors contribute to its development (Ascherio and Munger, 2007). Among the neurodegenerative diseases, MS is considered to be one of the few where damage to the white matter of the CNS is the primary pathology of the disease (Hawellek *et al,* 2011). Moreover it is a progressive disease that targets a relatively young age group (20-40yrs), where patients deteriorate in a general sense rather than in one area of functioning (Hawellek *et al,* 2011).

While most cases of MS are initially characterized as relapse remitting multiple sclerosis (RRMS), most eventually progress to secondary progressive multiple sclerosis (SPMS). Others bypass this process completely, and develop primary progressive multiple sclerosis (PPMS), which can be more aggressive (Tullman *et al,* 2004). RRMS is characterized by periods of multifocal inflammation caused by inflammatory cytokines

attacking the myelin sheath of the nervous system, resulting in neurological symptoms (Miller, 2012). Remitting phases follow periods of clinical symptoms in which there can be complete or partial relief of the symptoms (Miller, 2012), for which the length of time is variable (Dutta and Trapp, 2011). Multifocal inflammation is due to the differentiation of autoreactive T cells which recruit a variety of inflammatory cytokines that target the myelin sheath in the CNS resulting in multifocal regions of axonal damage. When this axonal damage progresses far enough it becomes irreversible and the patient progresses into SPMS where there is continuous neurodegeneration (Leray *et al,* 2010; Dutta and Trapp, 2011). This type of disease progression starting with RRMS followed by SPMS occurs in a significant majority of individuals, reportedly as high as approximately 85% (Dutta and Trapp, 2011; Ascherio and Munger, 2007). As mentioned above, a small amount of individuals develop PPMS, in which the clinical symptoms of neurodegeneration and brain atrophy occur without any remitting phases. The onset of this type of MS is typically 40 years later at its beginning as compared to other forms of MS, which may appear as early as twenty years of age (Leray *et al*, 2010; Dutta and Trapp, 2011). At this point in time, there are no treatments available to effectively counter the accumulated damage that occurs in the progressive stages of the disease, nor are they able to effectively prevent the progression of the disease (Leray *et al,* 2010 Miller, 2012; Le Bras *et al,* 2005). As a result it is becoming increasingly important to find long-term treatments that can either effectively ameliorate or repair the damage that has occurred.

Administration of β-interferon, one of the most well known treatments, has been shown to be effective in slowing disease progression for many years now (Bertolotto *et al,* 2003). However, these treatments do not work for all individuals and it may take years to identify whether or not an administered treatment is proving effective (Bertolotto *et al,* 2003). As a result, simply targeting the immune aspect of the disease in hopes of slowing or stopping the progression is not the best treatment option for those suffering in progressive stages. It is becoming increasingly important to find treatments that can either effectively ameliorate or repair the damage that has occurred and that these treatments remain effective in the long term. However, as the exact etiology of MS remains a mystery, in part due to the fact that the process of myelination itself is not yet fully understood, the development of novel treatments to target myelin repair can be difficult (Gibson *et al,* 2014; Dutta and Trapp, 2011; Jones and Coles, 2010). Therefore increasing the knowledge of the important factors and regulators in the process of myelinogenesis, may be especially important in understanding how to treat the progressive stages of demyelinating diseases in which the body's own remyelination attempts fail (Chari, 2007).

Much focus has been devoted to exogenous methods of myelin induction, such as pluripotent stem cell transplantation (Fancy *et al,* 2010; Miron *et al,* 2011). However, many complications and obstacles arise in these types of therapies and alternatives that are more effective and less invasive are being examined (Fancy *et al,* 2010, Metcalfe, 2011). The fact that remyelination does occur on its own following demyelinating events, (Chari, 2007) suggests that the body does have the capability and the machinery to repair

this type of damage. Thus, pursuing studies that attempt to enhance existing endogenous mechanisms of preventing disease progression and remyelinating damaged areas is of interest (Fancy *et al* 2010). Indeed some of the most promising therapies being researched today involve the modulation or enhancement of endogenous mechanisms that act to utilize the organism's own endogenous molecules or machinery to delay or stop the progression of disease (Metcalfe, 2011; Fancy *et al,* 2010). Many of these novel therapies also focus on the immune aspect of the disease and attempt to prevent the inflammatory response that results in demyelination (Metcalfe, 2011). However, in later stages, losing the inflammatory reaction may be partially responsible for remyelination failure. Inflammatory responses may be essential in stimulating the oligodendrocyte precursor cells (OPC's), progenitors of the myelinating cells of the CNS, to migrate, differentiate and initiate remyelination (Miron *et al,* 2011).

While stopping progression of the disease is certainly important in preventing irreversible axon damage, these immune-targeted treatments are only valuable to those individuals in the early stages of the disease. Those who have progressed into later stages, where they have accumulated irreversible axon damage, do not have many treatments available to them. Therefore it would be ideal to find a method of utilizing the hosts own endogenous repair mechanisms to not only prevent further damage, but also to initiate remyelination in order to recover some function. With the recent promising research mentioned above it would seem to follow that other endogenous molecules may have a similar effect. This suggests that a better understanding of the various regulators and factors involved with myelination may help in identifying important players in this

process. It has been noted that developmental myelination is similar to the remyelination of MS lesions that already occurs (Woodruff and Franklin 1999). Thus, it would seem that this is a logical starting point for identifying future therapeutic targets in the treatment of MS.

Myelin, Myelinogenesis and Oligodendrocytes

Regardless of the gaps in knowledge that do exist concerning myelinogenesis, the basic process has been characterized as a primarily postnatal event occurring in the CNS (Bauman and Pham-Dinh, 2001; Zuccaro and Arlotta, 2013). Myelination is generally known as the process in which the axons in the CNS and the peripheral nervous system (PNS) are surrounded with a specialized lipid membrane called the myelin sheath. The sheaths' insulatory function in conjunction with the breaks in the sheath, called nodes of ranvier, permit the rapid transmission of nerve impulses known as saltatory conduction (Jahn *et al,* 2009). This is one of the primary roles of the myelin sheath and the one that is lost in MS.

The process through which axons become ensheathed is highly regulated and proceeds in a sequential fashion. The initial event that occurs in myelination is that the cells that will become the primary myelinating cells in the CNS, the oligodendrocytes (OL's) arise from neural stem cells from the tissue surrounding the developing neural tube (Kamholz, 1996). These neuroectodermal pleuripotent cells will become committed to an oligodendroglial lineage once they differentiate into oligodendrocyte precursor cells. Once differentiated they will begin to diffuse to areas where they will develop into

myelinating OL's and begin the process of axon ensheathment (Kamholz, 1996; Huang *et al*, 2013). Once differentiated, OL's can extend multiple processes to target multiple axons to begin myelination (Jahn *et al*, 2009). In order to get to this point there is a host of regulatory molecules that direct this process and it is this portion of myelinogenesis that is least understood. Among the first regions to be myelinated are the spinal cord and the hindbrain after which more rostral regions will begin myelination (Smith 1973). The process will continue throughout childhood and can continue well into adulthood (Zuccaro and Arlotta*,* 2013; Czopka *et al,*2013).

Myelin biogenesis and the production of the specific components therein is a sequential process requiring the activity of many regulators to signal the synthesis of myelin components at specified times (Aggarwal *et al,* 2010; Zucccaro and Arlotta 2013). In terms of structure, two major components comprise the myelin sheath. The majority of the sheath is made up of lipids, approximately 70-80%, with the rest being protein (Aggarwal *et al*, 2011; Jahn *et al,* 2009). Of the variety of lipids that make up the myelin sheath, the most abundant are glycosphingolipids and plasmalogens, a unique type of glycerophospholipid found in membranes (Aggarwal *et al*, 2011, Braverman and Moser, 2012). As there is with myelin lipids, there is a diverse amount of myelin proteins. However, two proteins predominate while others make up only a small portion of the total protein content in myelin (Wrathall *et al,* 1998). Of the myelin proteins present in the CNS, Proteolipid protein (PLP) and splice variant DM20 are the most abundant comprising approximately 50% of the myelin protein whereas myelin basic protein (MBP) amounts to about 30% of myelin protein (Izawa *et al,* 2010). PLP, which can be

detected early in myelin development in the CNS is also commonly found with its splice variant DM20 and together they are one of many regulators of proper oligodendrocyte maturation and myelin stability (Michalski *et al,* 2011; Timsit *et al*, 1992). PLP is most often expressed by mature myelinating oligodendrocytes, at which stage its expression is highest (Michalski *et al,* 2011) whereas the DM20 variant is expressed in its highest proportion during embryonic development (Michalski *et al,* 2011; Woodruff and Franklin, 1999). PLP has a functional role of assisting with the adhesion of extracellular leaflets to the myelin membrane (Jahn *et al,* 2009; Aggarwal *et al*, 2010) along with MBP, which also plays a structural role that is highly important in the compaction of myelin and in ensuring proper myelin synthesis and assembly (Readhead *et al*, 1987; Aggarwal *et al,* 2011).

As the deposition of myelin is sequential, the study of the temporal aspects of the process has been examined. From what is currently understood, there appears to be a reliable schedule when certain events of myelination occur, particularly in the CNS (Huang *et al,* 2013). However, there is a lack of full understanding about the majority of the regulatory mechanisms that are involved with the regulation and maintenance of the myelination timeline. Other parts of the nervous system's myelination process are better understood, such as the peripheral nervous system (PNS) where the primary activators of myelination are known. In the CNS the molecule(s) that have the analogous function is/are still unknown. Thus making exact estimation of timing of myelination activation hard to pinpoint. There have been some suggestions as to various important molecules

and transcription factors as regulators of initiating and directing myelination however no one such molecule has been identified in the CNS (Aggarwal *et al,* 2011).

With regard to the components that regulate the initiation of myelination, the process can only begin at those neural components that are developmentally ready to be myelinated (Czopka *et al,* 2013). As this is the case, it follows that a primary regulator of myelination may be neuronal in nature. Attempts have been made to find such a regulator, however, it would appear that it is not simply neuronal regulators alone that play a part in initiating and maintaining the myelination process. Rather, there are a variety of extrinsic and intrinsic pathways that appear to be involved with this complex process (Aggarwal *et al,* 2011). In addition to regulatory pathways, temporal factors (as mentioned above) and spatial factors (positioning of OL's) involved with the myelinating cells in the CNS may play an important role, (Aggarwal *et al*, 2011; Glenn and Talbot, 2013; Czopka *et al*, 2013). Therefore, some of the research focus has begun to concentrate on the OL's, OPC's and associated cells involved in reciprocal signaling during myelination. Many regulators can often be endogenous molecules that already exist in the body but function in several other capacities other than regulators of myelination. This is particularly true, as molecules that have long been characterized in one or another region of the body have been found to play a role in regulating oligodendrocytes. For example, leukemia inhibitory factor (LIF) has only recently been linked to myelination (Ishibashi *et al,* 2009; Metcalfe, 2011).

A variety of factors are known to modulate the development of myelinating OL's. Several of these have been identified to be important for OPC's and OL's at their various stages of proliferation, differentiation and maturation. Among them are transcription factors, such as the *Olig*1/2 transcripts, growth factors including platelet derived growth factor (PDGF), insulin-like growth factor (IGF) as well as neurotrophic molecules such as ciliary neurotrophic factor (CNTF) (Yang *et al,* 2011; Stankoff *et al,* 2002; Ahrendson *et al,* 2013; Goddard *et al,* 1999). However, how these various factors interact and how they are involved in the regulation and timing of OPC maturation, as well as OL myelination, is still not well understood (Wang *et al,* 1998). Increasingly more factors are being suggested as potential modulators or regulators of the process as a whole. In fact, it is unlikely that these regulators can act on their own to ameliorate symptoms of demyelinating disease or induce remyelination, as was seen in the failed clinical trial with the growth factor IGF (Frank *et al,* 2002; Miron *et al,* 2011). It is likely that an interplay of many signaling molecules is necessary in order to promote myelination.

To this point, although proliferation of OPC's may be increased through the activity of proliferative growth factors, this does not translate to more myelinating mature OL's (Yang *et al,* 2011; Calver *et al,* 1998). It has been suggested that density dependent regulation using the p27 and Rb phosphorylation pathway is employed in order to ensure a uniform final number of myelinating OL's (Yang *et al,* 2011). This type of regulation has been observed in other oligodendrocyte lineage cells, such as astrocytes (Nakatsuji and Miller, 2001). In addition, not all of the factors regulating myelination are stimulatory; in fact some act to inhibit differentiation of OPC's, thus having a 'negative'

regulatory effect on myelination (Huang *et al,* 2013). The presence of such regulators once again points to the fact that timing is important and rigorously maintained in the process of myelination. This suggests that the timing of OPC maturation into myelinating OL also plays a large role in the capacity of OL's to synthesize myelin. It has been demonstrated in zebrafish that myelinating OL's may only be able to do so for a specified period of time (Czopka *et al,* 2013). Thus, this developmental knowledge alone opens a new avenue of understanding the process of myelination.

As it has been pointed out previously, the primary myelinating event in most organisms occurs primarily postnatally (Huang *et al,* 2013) but is not a singular event to occur throughout an organisms' lifetime. As it is known that OPC's and myelination both persist into adulthood, whether these OPC's act as a reservoir for later differentiation into myelinating OL in later stages of life, continues to be of some contention (Czopka *et al,* 2013; Yang *et al,* 2011; Franklin, 2002). Further, though these OPC's may be found in regions of axonal injury, they often do not differentiate to form myelinating OL's capable of repairing the damage that has occurred (Franklin, 2002; Kotter *et al,* 2011;). It would appear that other factors are required to be present in order to initiate efficient and permanent remyelination and that simply increasing OPC amount in these regions is not sufficient to induce greater remyelination (Woodruff *et al,* 2004). What has become abundantly clear is that myelination is exceptionally complex. A wide range of factors influence the development of OPC's, their maturation, the activation of myelinating OL's and the assembly of the various components of the myelin sheath. In disease states most treatments available focus on prevention of demyelination and axonal injury as opposed

to remyelinating efforts. As a result it is essential that regulators capable of inducing myelinating cells to promote regeneration are identified and developed into viable and effective long term treatments for individual suffering from neurodegenerative diseases.

Galanin

Galanin is a neuropeptide that was first characterized in the early 1980's and has been found to be widely distributed throughout the body, where it has been implicated in many physiological roles (Tatemoto *et al,* 1983; Xu *et al,* 2008; Lang *et al,* 2007; Perumal and Vrontakis, 2003). Galanin's many roles include its action as a growth factor, neurotrophic factor, roles in analgesia and anxiety, as well as a neurprotective agent both in the hippocampus and during central nervous system demyelination (Perumal and Vrontakis, 2003; Holmes *et al,* 2005; Polgar *et al,* 2013; Xu *et al,* 2008; Elliot-Hunt *et al,* 2004; Zhang *et al*, 2012). In the rat CNS, Galanin has been detected as early as postnatal day 2-5 through postnatal day 40 by in situ hybridization (Shen,et al 2005, Butzkueven and Gundlach, 2010). Recently, neuropeptides have become the target of more interest in the study of myelination. Galanin and another neuropeptide, neuropeptide Y (NPY), have been examined with regard to their capability of affecting OL's in initiating myelination. This is interesting as both NPY and Galanin expression has been found in neurons and OL's (Hashimoto *et al,* 2011; Shen *et al,* 2005). As they are both expressed in these cells, NPY and Galanin may also be capable of influencing OL particularly at early developmental stages (Hashimoto *et al,* 2011; Ubink *et al,* 2003). Due to the similar behavior of Galanin and NPY, interest was taken in examining whether a potential relationship existed between the two neuropeptides (Merchenthaler *et al,* 2010). With

regard to the effect that these two neuropeptides have in the hypothalamus in both in rats and humans, some evidence has shown that these two neuropeptides are capable of interacting and influencing one another (Horvath *et al,* 1996; Merchenthaler *et al,* 2010).

Galanin has also been noted to act alongside more classical neurotransmitters in the CNS like acetylcholine, dopamine and noradrenaline (Shen *et al,* 2003). As such, Galanin is thought to act through both direct post-synaptic signaling and regulation of presynaptic neurotransmitter release (Shen *et al,* 2003). More recently Galanin has also been implicated in its role as a bidirectional modulator of stress, either in activating stress and anxiety pathways or ameliorating these pathways (Mitsukawa *et al,* 2009). It is also suggested that, Galanin's effect is related to the dose, which contributes to either a positive or negative response (Mitsukawa *et al,* 2009). As for a consensus as to what constitutes a low/moderate/high dose of Galanin, there is no agreement to date, though in the literature it has long been suggested that a high dose of Galanin is often above 5nM (Mitsukawa *et al*, 2009; Ohhashi & Jacobowitz, 1985). Further, in the variety of capacities that Galanin is thought to act, it may act in an indirect fashion, as a neuromodulator, or more directly as a neurotransmitter. Regardless of the finer aspects of Galanin's ability to promote a positive effect, Galanin has an established role as a neruoprotective and neurotrophic molecule in the central nervous system with regard to the myelin sheath and neuronal survival (Shen *et al,* 2005; Zhang *et al,* 2012).

Galanin exerts its effects through three receptors, GalR1, GalR2, and GalR3, which are G-protein coupled receptors (Shen *et al,* 2003; Mitsukawa *et al,* 2009; Lang *et*

al, 2007; Zhang *et al,* 2012). The distribution of these receptors is somewhat varied, with GalR1 and GalR2 being more abundantly distributed in the CNS. The receptors have some homology between them (Jurkowski *et al,* 2013) and yet they show significant differences in their functional coupling (Hobson *et al,* 2008; Lang *et al,* 2007). This may explain why Galanin exhibits a variety of physiological effects. Different pathways are activated by the Galanin receptor subtypes, likely due to the fact that each receptor subtype can couple with different G proteins, which can lead to the activation of a variety of downstream effectors (Hobson *et al,* 2008; Lang *et al,* 2007). Mechanistically, GalR1 activation has been linked to upregulation in MAPK through G_i protein coupling (Kanazawa *et al,* 2007). GalR2 on the other hand has been shown to interact more broadly with G-proteins, such as G_i, $G_{q/11}$, G_o, and $G_{12/13}$ in order to activate phospholipase C and many other downstream effectors including AKT1 (Kanazawa *et al,* 2007; Lang *et al,* 2007). It is through the activation of AKT1 and GalR2 that Galanin has been implicated in having a role in neuronal survival and regenerative neuronal outgrowth in the CNS (Hobson *et al,* 2006; Lang *et al,* 2007). Recent studies suggest even further divergence between GalR1and GalR2 with some evidence pointing to a contradictory effect of Galanin when each of these receptors is stimulated (Jurkowski *et al,* 2013; Zhao *et al*, 2013). GalR3 signaling pathways on the other hand are not fully defined (Lang *et al,* 2007). What is known, suggests that GalR3 acts through $G_{i/o}$ proteins primarily in regions of the peripheral nervous system (Lang *et al,* 2007; Zhao *et al,* 2013).

The distribution of the receptor subtypes is correlated with Galanin expression in a variety of regions in the CNS. Galanin receptors and Galanin expression has been found in the CNS in the cortex, locus coeruleus, hypothalamus, and amygdala in high levels (Lang *et al,* 2007). Cells like neurons and astrocytes have been found to express galanin (Shen *et al,* 2005; Hosli *et al,* 1997; Lang *et al,* 2007). Oligodendrocytes in both adult and developing brains were also found to express Galanin in the corpus callosum, subventricular zone and in the rostral migratory stream (Shen *et al,* 2003, Ubink *et al,* 2003). Interestingly, the subventricular zone is also the region from which OL's are generated in the adult brain, amongst other stem cells (Zuccaro and Arlotta, 2013). In this region, Galanin has also been implicated as a factor capable of stimulating neuronal differentiation (Agasse *et al,* 2013).

It has also been established that Galanin outside the CNS is capable of crossing the blood brain barrier (BBB) along with other neuropeptides and serum proteins (Hashimoto *et al,* 2011; Broadwell and Sofroniew, 1993). There are several methods by which this can occur, including transport mechanisms, as well as certain circulatory pathways that allow the traversing of the BBB (Pan and Kastin, 2004; Begley, 1994; Broadwell and Sofroniew, 1993). Recently it has been noted that exogenous administration of Galanin intravenously has a fast-acting anti-depressant effect suggesting that Galanin is capable of action even after traversing the BBB shortly after its administration (Murck *et al,* 2004). The manuscript in the upcoming chapter discusses the possible methods of Galanin delivery to the CNS from high circulating levels of Galanin in the bloodstream.

Galanin's expression and signaling is well established in the CNS, but it is not limited to this region and is also demonstrated in the peripheral nervous system in the spinal cord, particularly in the dorsal root ganglion (Xu *et al,* 2008; Holmes *et al,* 2000, 2005; Hobson *et al,* 2006). Galanin is also suggested to act as an autocrine or paracrine signaling molecule with regard to developmental regulation of myelination and neuronal development (Ubink *et al,* 2003). Furthermore, Galanin is upregulated following traumatic injury to myelinated regions, largely in the spinal cord where it may also exert some modulatory effect (Zhang *et al,* 1998; Xu *et al,* 2008; Holmes *et al,* 2005; Ubink *et al,* 2003). This modulatory effect is also extended to the CNS where it has been demonstrated in experimental autoimmune encephalomyelitis (EAE), the most common mouse model of demyelinating disease, Galanin overexpression attenuates the disease response and loss of function of Galanin/Galanin receptors results in disease exacerbation (Wraith *et al,* 2009). Moreover, previous studies in this laboratory have confirmed this finding demonstrating that in a cuprizone-induced demyelinating event overexpression of Galanin attenuates the loss of myelin *in vivo* (Zhang *et al,* 2012).

As a result of these observations, it would appear that Galanin could have a role in both developmental and particularly in reparative myelination. In order to be able to effectively treat individuals suffering from diseases related to myelin dysfunction or damage, the developmental process of myelination and the regulators therein must be better understood. As such, the investigation of Galanin's role in developmental

myelination is essential in order to better comprehend the role that it could play as a therapeutic target in remyelination efforts.

Hypothesis and Objectives

The working hypothesis for this project was two-fold; 1) Galanin affects myelination by increasing the rate of myelination and 2) that the relationship between Galanin and myelination is both time and dose dependent. The hypothesis has been tested by examining the role of Galanin in an *in vivo* mouse model with increased circulating levels of Galanin, as well as in an *in vitro* cell culture model where Galanin has been added in two doses. The *in vitro* study has provided a controlled study environment that mimics the CNS *in vivo*, allowing for the more minute study of the cellular events that are occurring in response to Galanin's addition. The manuscript included in chapter two below details the *in vivo* study in its entirety. The results and discussion detailed below for the *in vivo* study (chapter two and five) and the establishment of the *in vitro* study (chapter four and five), demonstrate yet another role that Galanin may play. This is especially important for those individuals in the progressive stages of severe demyelinating diseases where novel targets for therapy need to be identified in order to develop more effective treatments. Galanin may be one such target and better understanding its role in the context of developmental myelination may lead to further applications in the future.

An Elevated Level of Circulating Galanin Promotes Developmental Myelinogenesis in the Mouse Brain*

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Abstract

Myelinogenesis is a scheduled process that is regulated by the intrinsic properties of the cell and extracellular signals. Galanin (GAL) is a bioactive neuropeptide that is widely distributed throughout the nervous system. Chronic increase in GAL promotes and protects the demyelination and remyelination processes. Furthermore, GAL is synthesized in myelin-producing glial cells, such as oligodendrocytes and its expression level is at its highest between postnatal days 10 and 40. In the present study, we use our GAL transgenic mouse model to examine the effects of GAL on postnatal myelinogenesis in the CNS. Although we observed no difference in the proliferation of oligodendrocyte precursor cells, we found that GAL has a strong pro-myelinating effect. The transgenic mice at postnatal day 10 appeared to undergo myelinogenesis at an accelerated rate, as demonstrated by the increase in myelin basic protein (MBP) synthesis. This finding suggests that oligodendrocytes mature more rapidly in these transgenic mice. The immunohistochemical results are consistent with our preliminary findings that suggest that GAL is a regulator of myelination and may be one of the myelination promoters. This finding is especially important for studies focusing on endogenous molecules for treating myelin-related diseases, such as multiple sclerosis and other leukodystrophies.

Introduction

The process of myelination in the central nervous system begins with the presence of oligodendrocyte precursor cells (OPCs), which originate from areas around the ventricles of the developing brain, specifically from the neuroectoderm (Kamholz, 1996). When the OPCs are no longer influenced by inhibitory signals (Aggarwal et al., 2011), they differentiate and promote the synthesis of the major components that make up myelin. Most OPCs begin to differentiate between birth and postnatal day 5 (Huang et al., 2013). These cells then migrate to myelination sites, mature and begin myelin production. The OPC maturation timeline proposed by Ishibashi et al. 2009 suggests that migration occurs at approximately postnatal day 7 and maturation occurs at approximately postnatal day 10 (Ishibashi et al., 2009). In the absence of inhibitory signals, a variety of transcription factors and other regulatory factors are involved in regulating OPCs, their differentiation, their maturation and the synthesis of myelin. Timing appears to be especially important for oligodendrocyte maturation and the onset of myelination. The literature suggests that there is only a brief time frame during which oligodendrocytes are able to myelinate (Glenn and Talbot, 2013).

Galanin (GAL) is a bioactive neuropeptide that is 29 amino acids long and widely distributed throughout the rat, mouse and human nervous systems. GAL has diverse neuromodulatory effects and acts as a "classical neurotransmitter" (Vrontakis, 2002, Lang et al., 2007). Furthermore, GAL has neurotrophic effects in the developing and adult brain (Shen et al., 2005). It has also been suggested that GAL plays a role in neurogenesis, specifically in the proliferation and differentiation of neural stem cells (Agasse et al., 2013) and that GAL acts as a growth and survival factor (Holmes et al.,

2000, O'Meara et al., 2000, Elliott-Hunt et al., 2004, Butzkueven and Gundlach, 2010) for various neurons (Shen et al., 2003) and oligodendrocytes (Habert-Ortoli et al., 1994, Ubink et al., 2003, Zhang et al., 2012). GAL acts through the three receptor subtypes GalR1 GalR2 and GalR3 (Habert-Ortoli et al., 1994, Fathi et al., 1997, Howard et al., 1997, Wang et al., 1997, Lang et al., 2007). Although all three of these receptors are members of the G-protein-coupled receptor family, they demonstrate differences in their functional coupling, which might explain the variety of physiological effects exhibited by GAL (Lundstrom et al., 2005a, Lundstrom et al., 2005b, Lang et al., 2007). GAL and its receptors have been found in the corpus callosum, where GAL may play a role in regulating oligodendrocyte precursor cells and, thus, myelination (Shen et al., 2005, Lang et al., 2007). Additionally, in situ hybridization has shown that GAL expression can be detected in the corpus callosum region starting at postnatal day 10. The expression level peaks at postnatal day 20 and is barely detectable at postnatal day 40 (Shen et al., 2005). Furthermore, a study on the rat brain showed that some non-neuronal cells express high levels of GAL and that there is a temporally restricted GAL mRNA expression pattern in the corpus callosum (Butzkueven and Gundlach, 2010). Recently, we demonstrated that GAL has pronounced neuroprotective effects on demyelination and remyelination in a multiple sclerosis animal model (Zhang et al., 2012). In the present study, GAL is assessed as a potential regulator of oligodendrocyte maturation and as a potential factor that promotes myelinogenesis.

We investigated the effects of GAL by comparing the myelin development in GAL transgenic (TG) mice and wild-type mice (WT) with the same genetic background. Tests for detecting the expression of biomarkers for myelin synthesis (such as MBP protein)

were conducted at postnatal days 10, 15 and 30 (P10, P15 and P30), and the results were used to monitor the rate of myelination.

Materials and Methods

Experimental animals

All mice, including the wild-type (WT; C57BL/6) and homozygous transgenic mice (TG) maintained on a C57BL/6 background, were housed in the University of Manitoba animal facility in a temperature-controlled environment (at 20°C under a 12 h light/dark cycle). Food and drinking water were available ad libitum. WT mice were obtained from the University of Manitoba Genetic Modeling of Disease Centre; the TG mice were generated using a method described in previous studies (Perumal and Vrontakis, 2003). The transgenic animals were first created by fusing a 320bp fragment of the rat GH promoter to the full length preprogalanin cDNA clone, then purifying the new construct and injecting it into mouse embryo's (Perumal and Vrontakis 2003). In these transgenic animals Galanin was over expressed in the pituitary and over secreted in the circulation. Thus, the construction of these transgenic mice allow for the study of the effect of Galanin in a variety of regions in its many roles. Before the experiment began, homozygous TG mice were backcrossed to WT; C57BL/6 from Charles River and bred again to homozygosity. The WT and TG mice were collected at P10, P15, and P30 and sorted into three groups per genotype. Each group consisted of 4–6 mice, and experiments were repeated twice. To confirm the results of Day 10, 6 more male mice were added to the control group and 4 more transgenic male mice were added to the experimental group. All of the procedures were conducted in accordance with the Animal

Protocol Review Board of the University of Manitoba, which approved this study under protocol #10-013/1/2.

Tissue collection and preparation.

Mice were anesthetized and intracardially perfused with 0.1M phosphate buffer saline (PBS;pH 7.4) containing 50U/ml heparin sodium, followed by 4% paraforlmadehyde prepared in 0.1M PBS. Whole brains were carefully harvested after decapitation. To exclude the regional differences in the myelination rate (Smith, 1973), the cerebellums were not included in this study. The dissected brains were cut along the longitudinal fissure to generate two hemispheres of equal size. Half of the hemispheres were collected and stored at -80°C until protein extraction for western blot analysis, and the remaining half of the hemispheres were incubated in RNAlater solution (Cat. # AM7020, Life Technologies^{TM}) at 4°C overnight and then stored at -80°C until RNA extraction for real-time PCR. Animals intended for the histological studies were perfused and fixed with 4% paraformaldehyde. The fixed brains were cryoprotected in PBS containing 30% sucrose. The brains were then snap frozen on dry ice and stored at -80°C until the samples were sectioned.

Western blot analysis

The protocol used in this study is a modified version of a protocol provided by Abcam. The frozen tissues were homogenized in RIPA lysis buffer containing a protease inhibitor cocktail (CKT, Cat. # P8340-1ML, Sigma-Aldrich). The protein concentration was quantified using a BCA Protein Assay Kit (Product # 23225, Pierce, Thermo Scientific).

Protein samples were denatured and reduced following the manufacturer's protocol and were then stored in aliquots at -80°C.

Samples of 25 μg protein and a protein marker (EZ-Run Prestained REC Protein Ladder, Cat. # BP3603-500, Fisher BioReagents) were separated on 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) using a Mini Protean 3 cell system at room temperature (Cat. # 165-3301, BIO-RAD). Membranes were blocked using a solution of 5% milk in TBST buffer for 1 h and then incubated in the primary antibodies (Table 1) overnight at 4°C. The next day, the membranes were incubated in the HRP-conjugated secondary antibodies (Table 1) for 1 h at room temperature on a rocking shaker. Finally, the bands were visualized using the Western Lightning® Plus-ECL kit (Cat. # NEL104001EA, PerkinElmer) and autoradiography films.

Histology staining.

Serial 25 μm coronal sections were cut from the frozen brains. The sections used for staining were in the 185 to 195 level according to the High Resolution Mouse Brain Atlas by Sidman et al. (http://www.hms.harvard.edu/research/brain/atlas.html). MBP staining was performed using the previously described avidin-biotin-peroxidase complex technique (Zhang et al., 2012).

RNA preparation

All of the instruments and laboratory supplies were nuclease-free. The stored samples in RNAlater solution were thawed on ice. The total RNA was extracted from each homogenized tissue sample using the manufacturer's phenol/chloroform extraction

protocol (Cat. # 15596-026, TRIzol Reagent, Invitrogen, Life Technologies™). The total RNA was purified using the RNeasy Mini Kit (Cat. # 74104, QIAGEN) according to the manufacturer's instructions; a DNA-decontamination treatment was also used. The purified RNA samples were dissolved in RNase-free water and stored at -80°C until needed.

Real-time reverse transcription polymerase chain reaction (real-time RT-PCR) Real-time RT-PCR was performed using a two-step reaction that consisted of first-strand synthesis (reverse transcription, RT) and real-time PCR.

1. Reverse transcription (RT)

One microgram of total RNA was used to generate single-stranded cDNA. The samples were denatured at 70°C for 10 min, and reverse transcription was then performed following the manufacturer's protocol (Cat. # 18064014, SuperScript® II Reverse Transcriptase, Invitrogen). The following reaction conditions were used: 25°C for 10 min, 42°C for 50 min and 72°C for 15 min with a 0°C hold at the end. The generated cDNA was diluted in 50 µl RNase-free water.

2. Real-time PCR.

The primer pairs were designed and generated by SABiosciencesTM, QIAGEN; the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the internal quantitative control (Table 2). Each PCR consisted of 12.5 µl RT^2 SYBR Green/ROX qPCR MasterMix (Cat. # PA-012-24, SABiosciences, QIAGEN), 6.5 µl RNase-free water and 1 µl Primer Mix. PCRs for each gene of interest were run in

triplicate using the StepOneTM Real-Time PCR System (Applied BiosystemsTM). The following PCR cycling program was used: 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The following melt curve program was used: 95°C for 15 s, 60°C for 1 min, 65°C to 95°C at 2°C/min and 95°C for 12 s. To confirm the accuracy of the primers, a default melting program was run after each PCR cycling program to ensure that the dissociation curves for each pair of primers contained a single peak and that the agarose gels of the amplified product produced a single band that corresponded with the predicted amplicon length. To determine the amplification efficiency, a calibration curve was performed prior to beginning the experiments.

Results

Data collection and processing

IHC staining slides were digitally captured using an OLYMPUS BH-2 light microscope equipped with an OLYMPUS Q-color5 digital camera. The software program used for the microscope was ImagePro Plus 5.0 version (MediaCybernetics). Quantitative image analysis with densitometry for immunohistochemistry was applied using ImageJ 1.48 (NIH Image). Densitometry was first conducted for the half brain sections (figure 3) and an optical density calibration was set according to the developer's instruction for all the densitometric analyses (http://imagej.nih.gov/ij/,1997-2014). The MBP-immunostaining revealed many fine fibers, at the knee region of the corpus callosum (figure 4), which ran parallel to the cortex, condensed starting in the area of caudoputamen and external capsule of corpus callosum and projected to the adjacent cortex layer 6a (http://mouse.brain-map.org). We measured the optical density of the MBP-

immunostaining according to our previous study (Zhang et al., 2012), and relative optical density values were calculated.

Images of the western blot films were captured using a scanner, and the signal intensity of each band was measured using the Quantity One 1-D Analysis Software (basic mode, version 4.6.5, Bio-Rad) following the manufacturer's protocol. The processed results were expressed as the relative quantity (RQ) to the WT control group.

The real-time RT-PCR results were calculated using the StepOne^{TM} software version 2.1 and the comparative threshold cycle quantitation method. The final results were expressed as the fold-change in the target gene expression level, i.e., the expression level relative to the WT control samples.

Statistical Analysis:

The statistical calculations and graphs were made using GraphPad Prism® version 5. All comparative data from Western-blot assays and real-time PCR tests were analyzed using two-factor mix-measures analysis of variance (two-factor mix- measures ANOVA) assuming that genotype was the factor for independent grouping and that time elapsed was the other factor for repeated measures. All two-way ANOVA tests were accompanied by Bonferroni's multiple comparison tests. When considering variances caused by either factor one-factor ANOVA tests accompanied by Tukey's multiple comparison tests were applied. A p-value less than 0.05 (difference/effect) was considered to be significant.

Results:

1. Myelin basic protein (MBP) expression level was higher in the GAL-over-expressing TG mice.

The MBP was isolated using SDS-PAGE gel electrophoresis, and the amount of protein present in the mouse brain was determined by the signal intensity on the western blot film. The samples collected from mice of different ages and genotypes were measured from the same membrane, as shown in the representative western blot image in Figure 1 (A). Five and four more animals were added to each day 10 group (WT and TG respectively)

The results show that the total amount of MBP in the WT and TG mice quickly accumulated within 15 to 30 days after birth (P15, P30), and the quantitative analysis revealed that there was no significant difference in the expression level between the WT and TG mice at these two stages (Figure 1 (B)). At postnatal day 10 (P10), the MBP expression level in the WT group was almost undetectable, but there was a clear signal indicating MBP expression on the films for the TG group. A densitometric analysis on the western blot images showed that there was a 6.44±1.44 (mean ± SD)-fold increase in the MBP expression level in the TG group ($p<0.05$) compared with the WT group. On the second experiment (Figure 2) the difference was even more significant ($p< 0.001$). The real-time RT-PCR (quantitative PCR, qPCR) assays revealed a similar pattern in the MBP gene and protein expression levels from P10 to P30 (Figure 1 (C)). In the WT and TG groups, the MBP gene expression level increased significantly until 15 days after birth. The qPCR comparative analysis showed that there was a 2.85±0.35-fold higher gene expression level in the P10 TG mouse brain compared with the P10 WT mouse brain ($p<0.01$) and that there was no significant difference in the gene expression level

between the two genotypes at P15 and P30.

Immunohistochemistry (IHC) analysis was conducted on P10 WT and TG mice to further investigate the MBP protein expression pattern because the western blot and qPCR results showed that the MBP gene and protein expression levels were significantly different between these groups at this age. In the WT and TG mice, the early MBP expression was mainly localized in the corpus callosum and striatum (Figure 3 and Figure 4), and there were fine MBP-positive fibers scattered throughout the adjacent cortex. Although the MBP IHC staining revealed that myelinogenesis (stain patterns) was occurring in similar locations in the mouse brains of the two different genotypes at P10, the staining intensity in the TG mouse brain was significantly higher ($p<0.001$), which indicates that the protein expression level was higher in the TG mouse brain (Figure 4). Similar to the MBP expression pattern, the PLP expression level increased from P10 to P30 in both mouse groups. Despite the higher MBP expression level in the TG mice at P10, the western blot analysis showed that there was no significant difference in the PLP expression level between the WT and TG mice at P10 (Figure 5 (A) (C)).

To determine whether the higher MBP expression level was a result of an increase in the number of myelinating cells, we have further examined the expression level of the cell marker for oligodendrocyte precursor cells, PDGFR-α. PDGFR-α was highly expressed in the WT and TG mice at P10, and the expression level decreased from P10 to P30 as the CNS matured. We did not observe any significant difference in the PDGFR-α expression level between the WT and TG mice at any of the developmental stages analyzed (Figure 5 (B) (D)).

Discussion

The process of oligodendrocyte maturation and subsequent myelination is a highly regulated and time-specific process. The presence of GAL in the corpus callosum indicates that it may be involved in regulating the scheduled process of myelination. The purpose of this study was to determine whether there was a difference in myelination, oligodendrocyte maturation or the myelination assembly rate between WT and TG mice (postnatal days 10, 15 and 30). Because this study was a preliminary investigation to determine whether differences between these groups of mice existed and could be observed, indirect analyses were used. Western blot analysis was used to determine the expression levels of markers for oligodendrocyte proliferation, maturation, and mature myelin products. Additionally, IHC staining was used to detect changes in the spatial expression patterns in the brain, where abundant myelination is expected to occur. Taken together, these analyses were considered sufficient for determining whether there are observable differences between WT and galanin TG mice. Furthermore, the results from these analyses were considered sufficient for determining whether future studies using more direct methods, such as using HPLC (high-performance liquid chromatography) analysis to investigate the lipid profile of myelination, are necessary.

The TG mice used in this study were generated in our lab to study the functional roles of GAL, and these mice have a circulating GAL level that is ten-fold higher than the level found in WT mice (Perumal and Vrontakis, 2003). The increased level of circulating GAL provides a unique environment for studying the neuromodulatory roles of GAL. The blood-brain barrier (BBB) was once believed to be an impermeable peptide barrier that prevented peptides from entering the parenchyma of the brain. Recent findings,

however, indicate that peptides are capable of crossing the BBB using both non-saturable and saturable transport mechanisms and in quantities that are sufficient to affect the activity of the CNS (Pan and Kastin, 2004, Banks, 2008). Several potential mechanisms may be responsible for allowing the circulating GAL to enter the brain. First, the circumventricular organs may provide a broad passage way (Broadwell and Sofroniew, 1993). Additionally, active transport mechanisms that also protect the substances from rapid degradation have been described (Begley, 1994). Furthermore, GAL can cross the BBB, and the intravenous administration of GAL has a quick-acting anti-depressant effect and affects sleep EEG recordings (Murck et al., 2004). The neurophysiological events induced immediately after extraneous GAL is introduced suggest that circulating GAL can permeate or be transported into the brain and regulate its functions. One limitation of the study is that in our Galanin TG mouse, besides chronic increase of serum Gal there is small increase of prolactin (PRL) and growth hormone (GH)(Perumal and Vrontakis, 2003). Both hormones have been described to have an effect on myelination (Gregg C 2009, Aberg B, 2010), but this effect is mainly through the proliferation of OPCs. In our TG mice OPC proliferation is not different from WT mouse, since PDGFR-α expression levels are not different in TG and WT mice.

MBP is one of the structural proteins that makes up the myelin sheath, and it is believed to be the only myelin component, along with cholesterol, that can be directly linked to the myelination rate because it is a vitally important functional protein for myelin assembly and maintenance (Popko et al., 1987, Readhead et al., 1987, Saher et al., 2005). This positively charged “sticky” protein interacts with the negatively charged cytoplasmic membrane and produces the dynamic wrapping action of the myelin sheath

during the myelination process (Simons and Trotter, 2007). Because of its unique physiological and structural functions, MBP has been widely accepted as a marker for myelinogenesis (Strait et al., 1997, Chekhonin et al., 2000, Givogri et al., 2001). Therefore, the increase in MBP protein and mRNA expression observed in the current study strongly indicates that there is an increase in myelinogenesis in the GAL TG mouse brain. PLP is another major protein that is abundant in mammalian CNS myelin. In contrast to MBP, PLP is more closely associated with the myelin lipids, including the phospholipids and cholesterol. PLP is considered one of the early expressed myelin genes, and its expression has been detected as early as postnatal day 2 (Timsit et al., 1992, Le Bras et al., 2005). It has been shown, however, that myelin assembly is not significantly affected when PLP is completely absent, which indicates that PLP does not play an important rate-limiting role during myelin development (Le Bras et al., 2005). In the present study, we did not find a significant difference in the PLP expression level between the WT and TG mouse brains at the observed developmental stages. Potential explanations for these findings are that early changes in the PLP expression level occur prior to the earliest developmental stage we analyzed in this study or that there is no correlation between the rate of PLP expression and the rate of MBP expression or myelin development.

The findings of this study suggest that there is no difference in the number of oligodendrocytes between TG and WT mice. This conclusion is based on a lack of difference observed in the expression level of the marker PDGFR-α and PLP. However, there do appear to be changes in the OPC maturation rate, based on the earlier increase of MBP levels (characteristic of mature OL's) in the day 10 transgenic mice. Previous

studies have shown that the final number of mature oligodendrocytes will remain the same even when the number of oligodendrocyte precursor cells increases (Yang et al., 2011). This behavior may be the result of several different regulatory mechanisms in the brain, including apoptotic pathways triggered by a high OPC density, the inhibition of OPC proliferation when a critical number of cells has been reached or even competition between early and late OPCs that results in the survival of one group over the other (Yang et al., 2011). These potential mechanisms suggest that changes in OPC proliferation may not be observable even when other changes are observed in this mouse model. As such, it would seem that OPC maturation and molecules that promote OPC maturation are of more importance to myelination than the amount of OPC's that are produced during myelinogenesis.

In terms of the regulation of the processes discussed above, GAL may act as a neuro- signaling molecule in addition to its trophic effects described in the introduction. In this regard, GAL may be similar to other factors that stimulate myelinogenesis, such as insulin-like growth factor-I (IGF-I), ciliary neurotrophic factor (CNTF) and the more recently identified neuropeptide Y (NPY) (Hashimoto et al., 2011, Ahrendsen and Macklin, 2013). CNTF has been shown to promote the final maturation of oligodendrocytes, and through this role, it promotes myelination. Thus, the final stage of maturation is one step in myelinogenesis that these factors may act on to influence myelination (Hashimoto et al., Goddard et al., 1999, Stankoff et al., 2002).

The results from the western blot analysis on the PDGFR-α expression level exclude the possibility that a difference in OPC concentration could cause these changes in myelin synthesis. The MBP results, however, indicate that the OPC maturation rate may have

increased. The effects of GAL on the regulation of oligodendrocyte maturation may be the result of several different mechanisms. The effect may be direct, which means that the GAL expressed in the oligodendrocytes may trigger the increases in the maturation and myelination rates. Additionally, GAL may act indirectly as a neuro-signaling molecule that acts on the oligodendrocytes to increase myelination. Future studies investigating the precise regulatory mechanism are needed.

In summary, we provide new evidence suggesting that galanin may play a key role in regulating and stimulating myelinogenesis. These findings may be particularly important for the study and treatment of demyelinating and dysmyelinating diseases, such as multiple sclerosis and leukodystrophies. These diseases have remitting phases in which remyelination may occur, and understanding the factors and mechanisms that regulate this process may contribute substantially to the development of viable treatments.

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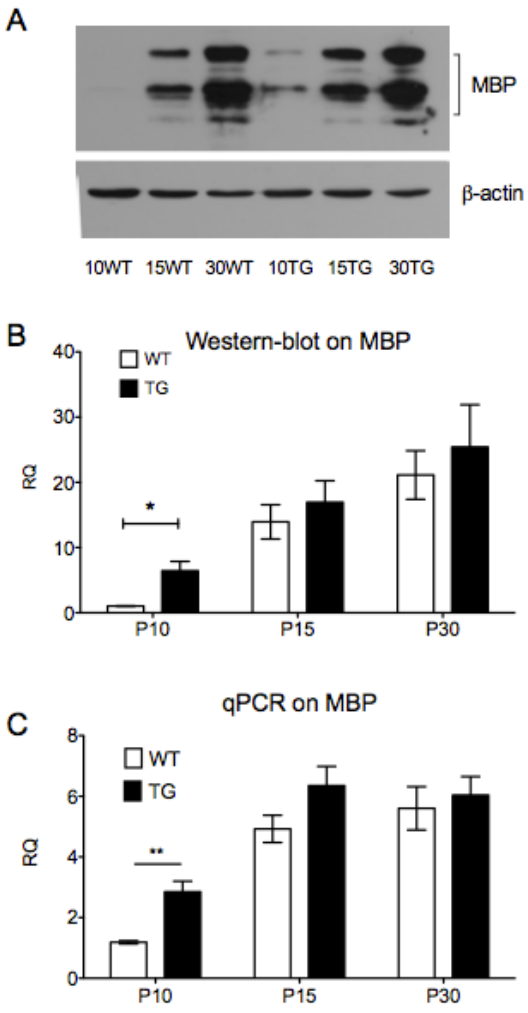


Figure 1. MBP expression level was higher in galanin-over-expressing TG mice. The 10, 15 and 30 were determined by western blot analysis and real-time RT-PCR, respectively. WT and TG samples from all of the analyzed developmental stages were separated on the same gel as shown in the representative image (A). Densitometric analyses were performed using the Quantity One 1-D© Analysis Software. The bar chart (B) was calculated based on the results from the films that included WT and TG samples at all of the developmental stages analyzed (A). The bar chart (C) shows the real-time RT-PCR results for MBP gene expression. Data are expressed as mean ± s.e.m values (n=4-6 per group. $p<0.05$, $p<0.01$).

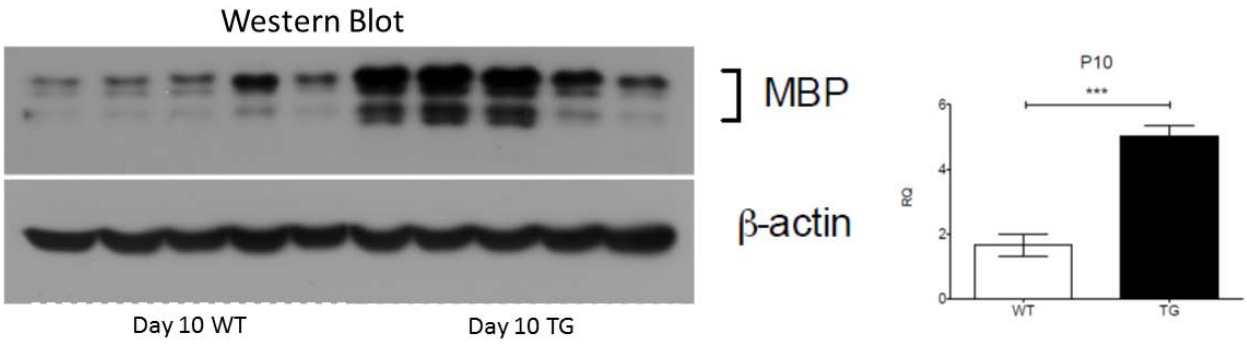


Figure 2. The amount of MBP protein level at P10 was significantly higher in galanin TGs. The MBP was isolated using SDS-gel electrophoresis, and the amount of protein present in the mouse brain was determined by the signal intensity on the western blot film. In the central nervous system there are three different forms of the protein made by alternate transcription from a single gene, which have molecular weights of 21.5, 18.5, and 17.2 kDa. Since the two lower molecular weight forms are very close in molecular size, MBP antibodies typically show two bands on Western blots, one at about 22kDa and another at about 18kDa. Densitometric analyses were performed using the Quantity One© 1-D Analysis Software. Data are expressed as mean ± s.e.m values (n=5 per group) $p<0.0001$

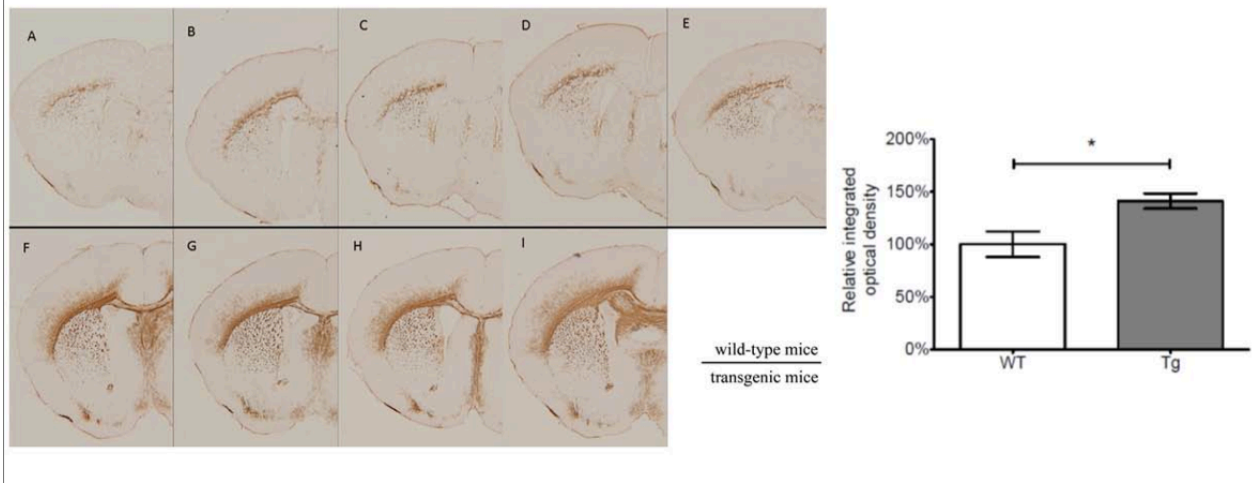


Figure 3. MBP expression levels were higher at P10 in half brain sections, whereas the spatial expression patterns remained the same. MBP expression was detected using IHC staining and an MBP antiserum at a dilution 1:1000. The staining was visualized using DAB color development. Representative images (A-E) show P10 WT mouse brain sections (10WT), and (F-I) show P10 TG mouse brain sections (10TG). Although the MBP IHC staining revealed that myelinogenesis (stain patterns) was occurring in similar locations in the mouse brains of the two different genotypes at P10, the staining intensity in the TG mouse brain was significantly higher. Half brain section densitometry data are expressed as mean ± s.e.m values (n=5 per group). $p<0.05$

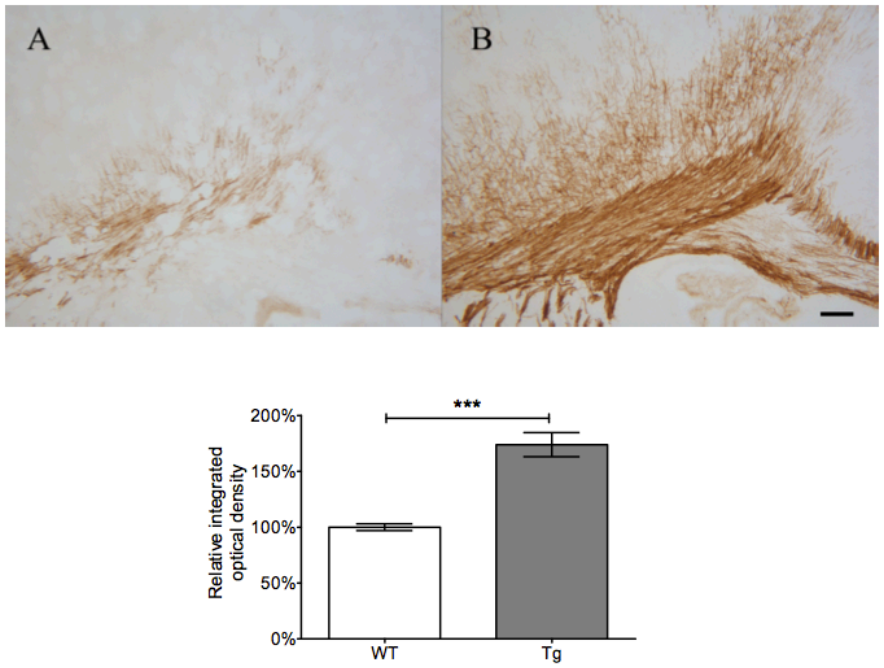


Figure 4. In the WT and TG mice, the early MBP expression was mainly localized in the corpus callosum. There were fine MBP-positive fibers scattered throughout the adjacent cortex. The bar graph represents the measurement of optical density of MBP IHC staining in the knee region of corpus callosum. Data are expressed as mean ± s.e.m values (n=5 per group). $p<0.0001$

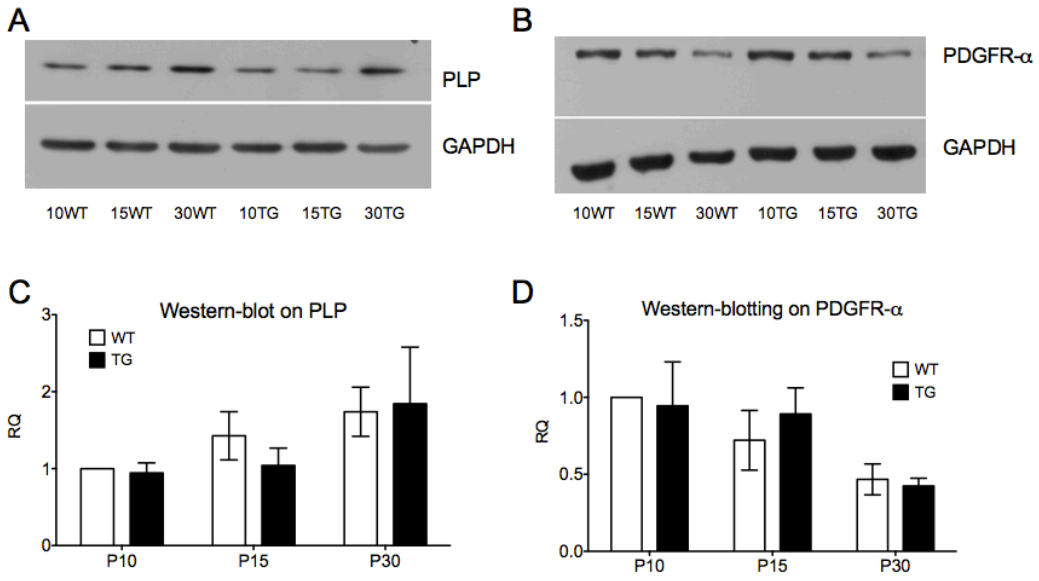


Figure 5 No significant difference was found in the PLP and PDGFR expression levels between the two genotypes. The expression levels of myelin proteolipid protein (PLP), a marker for early myelination, and Alpha-type platelet-derived growth factor receptor (PDGFR-a), a cell marker for oligodendrocyte precursor cells, were determined using western blot analysis. The PLP expression level gradually increased from P10 to

P30 in the WT and TG mice, but there was no significant difference in the expression levels between these developmental stages (A). The bar chart (C) represents the quantitative analysis of the PLP expression level. The PDGFR-a expression level decreased from P10 to P30, which coincides with the maturation of the central nervous system, (B). The chart (D) shows the results of the quantitative analysis of the PDGFR-a expression level. Data are expressed as mean ± s.e.m values. There was a significant decrease ($p<0.05$) between P15 and P30 PDGFR-a levels in the TG animals.

Table 1 Primary antiserum list

	Target	Dilution Ratio	Order #	Supplier
Anti-MBP	Myelin	1:3000 1:1000*	SC-13914	Santa Cruz
Anti-PLP	Myelin	1:3000	AB105784	Abcam
Anti-PDGFR-α	OPC	1: 3000	SC-338	Santa Cruz
Anti-β-actin	Loading control	1:3000	Sc-69879	Santa Cruz
Anti-GAPDH	Loading control	1:3000	Sc-32233	Santa Cruz

* The higher concentration of antibody was used for IHC staining.
Abbreviations: MBP, myelin basic protein; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; PLP, myelin proteolipid protein; PDGFR-α, Alpha-type platelet-derived growth factor receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table 2 Primer pairs provided by SABiosciences™, QIAGEN

Symbol	Description	Band size	Order #
MBP	Myelin basic protein	129 bp	PPM04745F
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	140 bp	PPM02946E

Chapter 3: *In vitro* Cell Culture

The cell culture was initiated in order to study Galanin's effects on myelination in a controlled environment that mimicked that of the CNS. This type of culture allows for the close monitoring of the processes and cells involved therein.

Methods and Materials for Cell Culture

The method of creating the spinal cord cell culture was modeled after that cited in the literature (Thomson *et al*, 2008). The final development of the cell culture is a modified version of that described in Thomson *et al.* (2008). The difficulty of culturing pure CNS tissue in order to study myelination has been noted particularly with regard to generating abundant myelinating axons. To remedy this, CNS and PNS tissue has been co-cultured in order to stabilize the culture growth, however, this does not effectively generate an accurate myelinating culture that mimics what is seen in the CNS (Thomson *et al*, 2008). The value of the mixed spinal cord cell culture that is described and utilized here is that it is an accurate method of generating abundant myelinating axons that allows for both biochemical analysis and easy visualization and monitoring of the development of the axons. Further, as a mixed cell culture this type of culture retains the interactions between cells that occur *in vivo* necessary for the proper timing and development of myelin (Bauman and Pham-Dinh, 2001). Myelination *in vitro* is seen around day 15 and the culture is sustainable to approximately 4-5 weeks (Thomson *et al,* 2008). Variability of the onset of myelination and its sustenance is dependent on what embryonic day the tissue is cultured (ex: E13.5 vs. E15.5). Regardless of this fact, this time frame allows for the monitoring of the onset of myelination and the events therein.

Experimental Design

The cell culture was carried out on three groups, the control (no Gal), the 1nM (low dose) and 10nM (high dose). A previous iteration of this cell culture had been established in our laboratory as a trial of Galanin doses, with 10nM being utilized as the low dose and 100nM as the high dose. The procedure for both cultures was the same, as follows. Four 24-well plates with autoclaved glass coverslips were used for the immunocytochemistry. Twelve 35mm dishes were used for western blot analysis. Each group was collected at four time points, 1 week, 2 week, 3 week, and 4 week. The addition of Galanin was not done until day 5 of the cell culture as this was the time point at which none of the plating medium (see below) remained in the cell culture.

Experimental Animals

One Sprague-Dawley time pregnant female with pups at 15.5 embryonic days (E15.5) was used as this was found to be the optimal time for use of the spinal cords (Thomson et al. 2008). The female was housed at the University of Manitoba animal facility in a temperature-controlled environment (20 °C under a 12 h light/dark cycle) with food and water available *ad libitum*. The amount of pups per female varies somewhat but is generally found to be between 12-16, our female yielded 12 embryos. All procedures were in accordance with the Animal Review Board of the University of Manitoba, which approved this study under the protocol #10-013.

Tissue culture media and reagents

*Hanks Balanced Salt Solution w/ Ca^{2+}, Mg^{2+} (HBSS)**

*all ingredients obtained from Sigma-Aldrich© and combined sterilely, the solution was filtered and transferred into a sterile bottle and stored at 4-8°C.

Plating Medium (PM)

The plating medium was composed of 50% full DMEM (D6429, Sigma-Aldrich ©), 25% Fetal Bovine Serum (Cat # 10439-016, Life Technologies™), and 25% HBSS with calcium and magnesium.

Differentiating Medium (Dfm)

All reagents are listed in their final volumes and concentrations. The differentiating medium is composed of full DMEM, 0.5% Hormone Mix, 10ng/mL Biotin (B4501-100MG, Sigma-Aldrich©), 50nM hydrocortisone (H0888-1G, Sigma-Aldrich©), 10 ug/mL insulin (I9278-5ML, Cat. # SLBC3574, Sigma-Aldrich©; only added for the first 2 weeks), Glutamax 100x (Cat. #35050-061 Life Technologies™) and Penicillin-Streptomycin-Neomycin (PSN) (Cat. # 15640-055, Life Technologies™).

Hormone Mix

These ingredients were mixed into HBSS 1mg/mL apo-transferrin (T1428-50MG, sigma-Aldrich©), 20mM putrescine (P5780-5G, Sigma-Aldrich©), 4uM progesterone (P8783-1G, Sigma-Aldrich©), 6uM sodium-selenite (S5261-10G, Sigma-Aldrich©).

Galanin Peptide

Galanin peptide (1-29) for addition to cell culture was prepared into a stock solution by dissolving galanin powder (Abcam©1mg; Ab141153) into fresh sterile HBSS. It was then aliquoted at several dilutions, 10μM, 50μM, and 100μM and stored at -80°C until ready for use where it was further diluted to 1μM and the appropriate volume of Galanin added to each cell culture group.

Preparation and Tissue Collection

The day prior to tissue collection, four 24 well plates had autoclaved glass coverslips placed inside the wells using autoclaved fine forceps under the laminar hood. The coverslips were then coated with poly-D-lysine (P6407, Sigma-Aldrich©) in order to create an adherent surface for the cells. Twelve 35 mm dishes were also coated with poly-D-lysine. Both the plates and dishes were left under the laminar hood overnight in order for the surface of the dishes and coverslips to become adherent to the cells. The poly-D-lysine was removed from the plates and dishes the morning of the tissue collection and washed with sterile autoclaved ddH_2O and allowed to dry for 2.5 hours during the dissection in the laminar hood under UV light.

The pregnant female was sacrificed on the day when her pups would be embryonic day 15.5 (E15.5). The sacrifice was performed by Dr. Teng Guan and the collection of the embryo's from the uterus was done by Wenyen Li. There were 12 spinal cords (without meninges) dissected from the pups under a dissecting microscope in HBSS with calcium and magnesium using fine forceps. The spinal cords were collected by both myself, Hanna Lyubetska, and Lin Zhang. The procedure was completed within

2.5 hours on ice blocks as the tissue would have been less viable for culture after this time. The tissue was then transferred into the laminar hood and washed four times with cold sterile HBSS without calcium and magnesium. The HBSS was then removed very carefully and the spinal cords were minced into small pieces using a sterile surgical blade. The minced tissues were then dissociated by the addition of accutase (A6964, Sigma-Aldrich©). The tissues in accutase were then transferred to a 15mL tube and incubated in a water bath at 37°C for approximately 20 minutes, the tissues were agitated every five minutes during this incubation. The digestion was then quenched by adding an equal amount of fetal bovine serum (FBS) to the tissue/accutase mixture and the tissue was then further dissociated by pipetting the mixture no more than 30 times. The tube was then spun down at 1200Xrpm in a centrifuge for 10 minutes. The supernatant was discarded and the erythrocyte layer removed carefully from the pellet. The pellet was then resuspended with approximately 3 mL of plating medium and the cell suspension was further dissociated through pipetting. The cells were allowed to rest at the bottom of the 15 mL tube so that only single cells remained in the suspension. The cell suspension was then transferred to a 50 mL tube through a 70 um cell strainer. Another 2-3mL of plating medium was added to the remaining cells in the 15mL tube and the dissociation and transfer of the suspension through the 70um mesh basket was repeated twice. Following this the cell strainer was washed with another 3mL of plating medium. The cell counting was then completed by the addition of 15uL of the cell suspension to a plastic slide and the cells counted by the TC20^{TM} automated cell counter (Cat #145-0102, Bio-Rad). The cell count was: found to be $3.36x10^6$. The cell suspension was then diluted by 5mL of plating medium in order to obtain a cell density of $2.5x10^6$. In previous

iterations of this cell culture we found that a cell density between 1-2.5x10^6 was appropriate in order develop a viable myelinating culture.

Cell Seeding

The cells were seeded on both 24-well plates as well as 35mm petri dishes. Initial seeding consisted of 100uL of cell suspension being seeded in the middle of each of the coverslips in the 24-well plates and 500uL of cell suspension being seeded into the middle of the 35mm dishes. The cells were allowed to attach for 3 hours followed by the addition of 400uL of plating medium plus an additional 500uL of differentiating medium (Dfm) to bring the final volume to 1mL/well in the 24 well plate. In the dishes, another 500uL of plating medium was added in conjunction with 1mL of Dfm for a final volume of 2mL.

Addition of Galanin and Media change

Galanin peptide, as listed above, was added to the culture in amounts that gave final concentration doses of 1nM and 10nM. In the 24 well plate in order to obtain a dose of 1nM the addition of 1uL of 1uM of Galanin was added to a media volume of 1mL. For the 10nM dose 10uL of 1uM Galanin was added to a media volume of 1mL. In the 24 well plate a 2uL of 1uM Galanin was added for a dose of 1nM and 20uL of 1uM galanin was added for the 10nM dose. Galanin peptide was added to the all cell culture starting on the fourth day *in vitro* in order to ensure the plating medium containing FBS was no longer in either of the dishes. Galanin was added daily in the amounts listed above and the medium was changed every second day for the duration of the culture.

Culture Collection and Analysis

The cells were collected from each group at four time points; Week 1, 2, 3, and 4. The cells were collected at the same time each week under the same conditions.

1.Western Blot

The protocol used here is a modified from that indicated by Abcam©. The cells were collected from the 35mm dishes for western blot analysis. Before the cells were removed, pictures were taken of the dishes for each of the groups with the Nikon eclipse TE2000-E microscope. Following this, the medium was removed from each dish and the cells were washed with DPBS and homogenized with RIPA lysis buffer containing protease inhibitors (CKT, Cat. # P8340-1ML, Sigma-Aldrich©). A sterile scraper was used to scrape up the cells and then transferred into a sterile 1.5mL collection tube. The samples were then denatured and reduced following the manufacturer's protocol and were then stored in aliquots at -80°C until ready for use.

Samples of 10uL and a protein ladder (EZ-Run Prestained REC Protein Ladder, Cat. # BP3603-500, Fisher BioReagents) were separated via a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) using a Mini Protean 3 cell system at room temperature (Cat. # 165-3301, Bio-Rad). The membranes were blocked for 1 h using a 5% milk in Tris Buffered Saline with 0.05% Tween (TBST) and then incubated in the primary antibodies (Table 3) overnight at 4°C on a rocking shaker. The next day, the membranes were incubated in the HRP-conjugated secondary antibodies for 1 h at room temperature on a rocking shaker. The bands were visualized using the Western

Lightning® Plus-ECL kit (Cat. # NEL104001EA, PerkinElmer) and autoradiography films.

2. Immunocytochemistry (ICC)

The protocol used is a modified version of the protocol indicated for use by Abcam ©. Pictures were taken of the three wells for each group before the collection of the coverslips from the 24-well plate. The glass coverslips were removed carefully from the 24-well plate using autoclaved fine forceps and a sterile needle and transferred to another clean 24-well plate. They were then washed with cold sterile Phosphate Buffered Saline (PBS) and the slides were fixed with 4% paraformaldehyde (PFA) for approximately 20 min. They were washed again after the fixation and held in DPBS in 4-8°C until they were ready to be stained.

The cells were permeabilized using a solution of 0.25% Triton X-100 Tris Buffered Saline (TBS-TritonX) for 10 min followed by washing with PBS three times for 5 min each. The coverslips were then blocked in 1% Bovine Serum Albumin (BSA) in PBST for 1 h. The coverslips were incubated overnight at 4°C in a mix of primary antibodies (Table 3). The next morning the cells were washed with PBS three times for 5 mins. This was followed by incubation with fluorescent secondary antibody (Table 4) in 1% BSA in PBST for 1 h at room temperature in the dark. The coverslips were then washed twice for 10 min with PBS in the dark. The cells were counterstained with Hoechst for 5 min and rinsed with PBS afterwards. The coverslips were mounted onto slides with one drop of mounting fluorescent mounting medium (S3023, Dako). The slides were stored in the 4-8°C fridge in the dark until used for imaging.

Analysis of Western Blot Films

Images of the western blot films were captured using a scanner, and the signal intensity of each band was measured using the Quantity One 1-D Analysis Software (basic mode, version 4.6.5, Bio-Rad) following the manufacturer's protocol. The processed results were expressed as the relative quantity (RQ) to the control group.

Imaging of ICC Slides

The slides were imaged using the Nikon eclipse T2000-E using the X-Cite^{TM} 120 fluorescence illumination system. Images were taken at 10x and 20x magnifications.

Statistical Analysis

As the results presented here demonstrate only two iterations of the cell culture at varying doses, the analysis done here is simplified and carried out to provide some context to the results obtained. The dose identified as most effective, 10nM, was analyzed using standard deviation of the two samples from each iteration of the cell culture which was then used to calculate the standard error, denoted by error bars on figures 10 and 11 below. The results on the graphs are represented as averages of repeats of the western blotting for each sample.

Chapter 4: *In vitro* Cell Culture Results

The *in vitro* cell culture results are displayed as preliminary results demonstrating the valuable nature of this type of cell culture particularly with regard to its relevance in the study of developmental myelination. The time frame encompassed in this study allowed for the monitoring of the cellular events taking place during the process of myelination. Further, this cell culture allowed for the visualization of the developing myelin sheath and subsequent biochemical analysis of the proteins as they appeared sequentially.

The preliminary results, depicted below are in accordance with what has been found *in vivo*, as it was seen that myelination rate was also increased *in vitro*, and demonstrates promise for the continuation of this work. From the two cell culture iterations that were conducted, 10nM was identified as a moderate dose that was most successful in inducing a sustained increase in myelination, between doses of 1nM and 100nM. In addition to indicating that Galanin's effects are likely dose dependent, these initial results give further credence to the potential role that Galanin plays as one of the factors that is important in modulating the process of myelination.

The targets for immunocytochemistry were selected in order to visualize the process of myelinogenesis, from the development of the neurofilaments and the synthesis of myelin sheath. Therefore in order to visualize the developing myelin sheath and axons the targets anti-PLP and anti-pNFH were chosen, respectively. PLP has been noted as one of the major myelin proteins that is most abundantly distributed and is expressed in

earlier stages than other major myelin proteins, thus it was deemed to be well suited for the visualization of the developing myelin sheath (see chapter one). The marker for axons, p-NFH, is a major structural protein in axons (Singh *et al,* 2011) and thus deemed to be well suited for axon visualization.

1) *Immunocytochemistry result*

In figure 6 below, it is seen initially that there are no differences between the control group and the experimental groups in week one, as expected. However, by week two some subtle differences can be observed. This is in accordance with the onset of myelination, which is expected to be around day 15 *in vitro*. In both the 1nM and 10nM doses there is increased expression of PLP observed as compared to the control group. At week three the amount of PLP signal intensity indicates that the expression of PLP is even further upregulated. It can be seen that there are more myelinated axons in the 10nM dose as compared to both the 1nM dose and the control. The most pronounced difference however, was observed at the final time point, week four. Here it is clearly seen that the myelin deposition is much more abundant with more axons being fully myelinated in the 10nM dose. The signal intensity of PLP is greatly increased and localized heavily at the axons. As a result of the amount of PLP present, the signal intensity of the axons is scarcely visible. This staining pattern indicates that myelin deposition has occurred at a greater rate in the 10nM dose as compared to the other two groups of 1nM Galanin and the control group. Considering that the 1nM dose, while also appearing to be more myelinated than the control group, still has visibly unmyelinated regions of axons, suggesting this dose may not be as effective at increasing myelination rate as the 10nM

dose. Upon examination of the control group, the differences are even more apparent as there are entire axons that remain unmyelinated. This series of images also shows the progression of the cell culture from immature to myelinating, demonstrating the capacity of this cell culture to mimic the myelinating environment in the CNS.

Figure 7 demonstrates the high magnification images of the myelinating OL's at week three which has been observed as the time where OL's are capable of myelination (Thomson *et al,* 2008). What is seen here is that the control group contains more unmyelinated axons and actively myelinating OL's as compared to the 1nM and 10nM dose where the axons are almost completely myelinated indicating an accelerated timeline of myelination.

Overall the results show that of the doses compared, the 10nM dose appears superior to the 1nM dose and the control group. In both the 10x magnification and in the 20x magnification it appears that myelination is completed at a greater rate in the 10nM dose, followed by the 1nM dose and finally the control. Western blot analysis below provides further insight into these findings.

Figure 6. **Increased myelination of axons is seen by week 3 in the 10nM dose**. Immunocytochemistry of the control, 1nM Galanin and 10nM Galanin cell culture at four time points, 1 week, 2 week, 3 week, and 4 week is seen below. Proteolipid protein (PLP) denoting myelin was stained green, phosphorylated neurofilament heavy chain (p-NFH) representing axons was stained red. Hoechst stain, blue, was used to identify the nuclei. Here it is observed at week 2 that there is increased PLP expression is both the1nM and 10nM dose with the pronounced myelination being observed particularly in week 3 and 4 in the 10nM dose as compared to the control and 1nM groups. Overall, myelination rate is increased in the 10nM dose. Hoechst, p-NFH, PLP

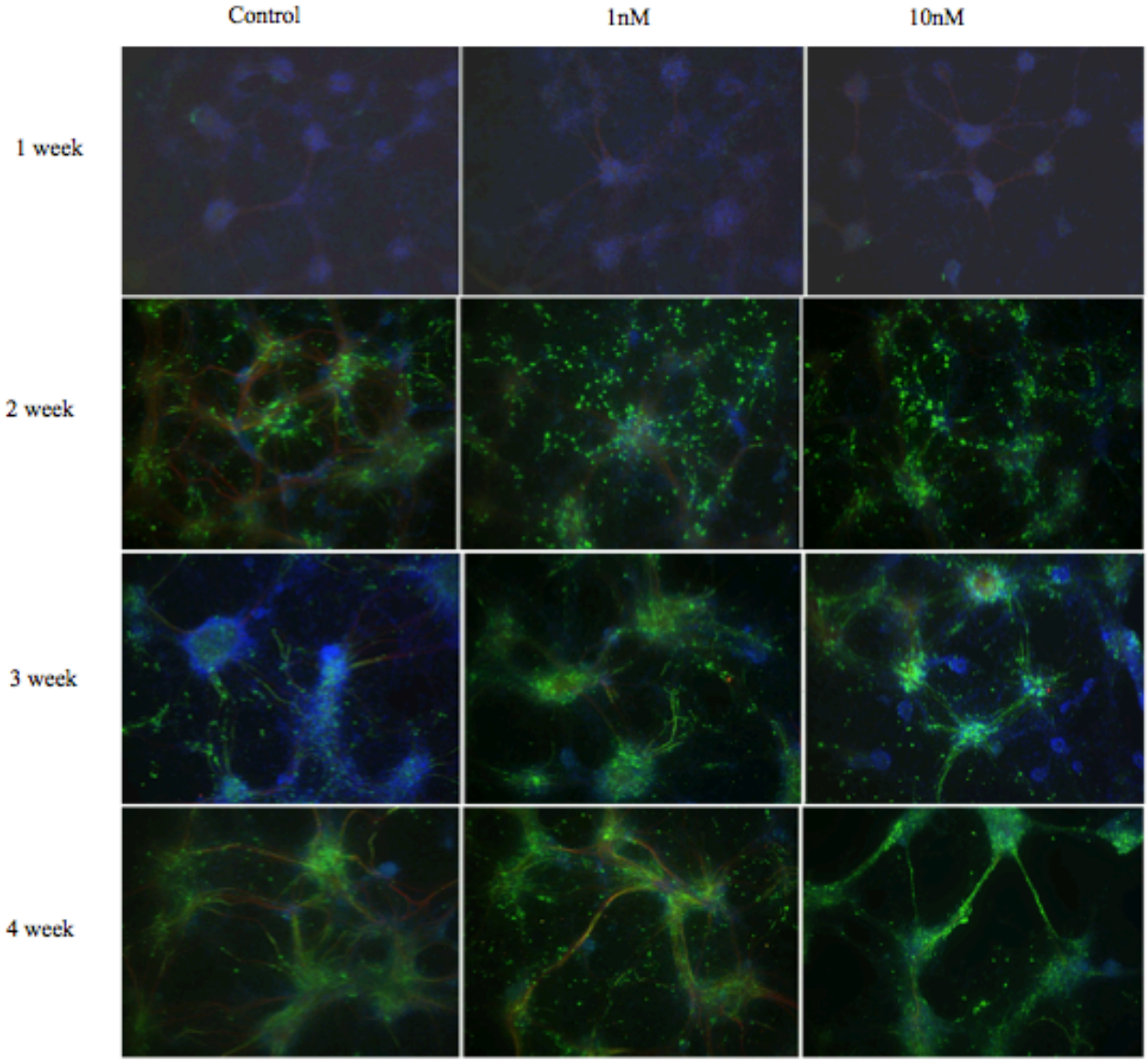
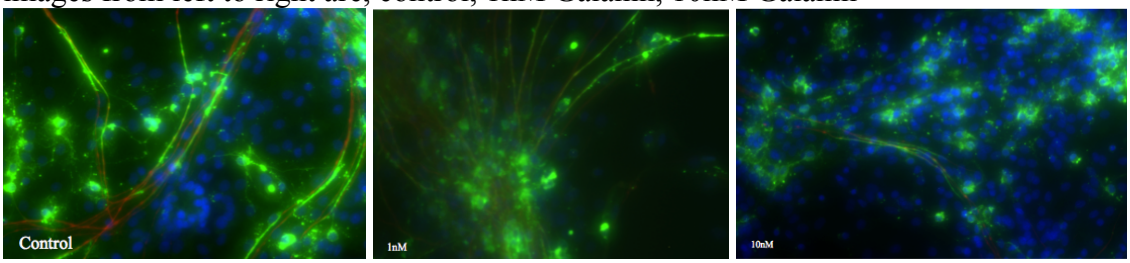


Figure 7. High magnification of myelinating oligodendrocytes in the control, 1nM and 10nM doses, respectively at week 3. Green staining indicates PLP expression, denoting the myelinating oligodendrocytes and the axons that are being ensheathed. The images from left to right are, control, 1nM Galanin, 10nM Galanin



2) *Western Blot result*

The preliminary western blot shows some interesting results that require further investigation. The two major myelin proteins were probed, anti-PLP and anti-MBP, in order to ascertain the amount of myelin that was being produced at each time point. The expression of PLP was found to be upregulated in the 10nM dose in particular, starting at week two and peaking at week three and the expression remained increased at week four in the 10nM dose as compared to the 1nM dose and the control (figure 8). This expression pattern matches that which was observed in the immunocytochemistry results (figure 6 above). The second major myelin protein, MBP was also probed, however, it would appear as though there were no significant differences between the expressions of the control and 1nM doses at all time points (figure 9 and 11). In the western blot, MBP appears in two bands, close in molecular weight due to alternative transcripts that are produced (Givogri et al 2001). In figures 8 and 9 the average of two western blot results are depicted as relative changes in fold from the most recent iteration of the cell culture.

MBP and PLP were also examined between the control and 10nM dose (the 10nM dose was identified as a moderate dose at which Galanin can exert promyelinating

effects) in the two samples from both iterations of the cell culture. For clarity, these results are depicted in figures 10 and 11 below. These results are consistent with the ICC indicating that the 10nM dose is superior to the control in terms of PLP and, while MBP continues to show no differences between the control and experimental group.

Two other targets were probed only in the most recent iteration of the cell culture to see if any differences of expression between the experimental and control groups. As PDGRFα, demonstrated no differences *in vivo,* it was of interest to examine if there was any increase in the differentiation of OPC's. Therefore, Olig2 was probed in order to examine if this was a possibility. The western blotting indicates that Olig2 was upregulated at week one and two in the 1nM and 10nM doses then falling at weeks three and four (figure 12). This result suggests that differentiation of the OPC's into maturing oligodendrocytes is possibly occurring at a greater rate in the experimental groups than in the control group. As this result shows promise, it will be further investigated in future repeats of this cell culture.

Figure 8. Increased expression of PLP in the 1nM and 10nM Galanin dose, peaking at week three. Western Blot analysis of Proteolipid Protein (PLP), a myelin structural protein found in abundance. The graph indicates the relative fold changes as compared to the control group.

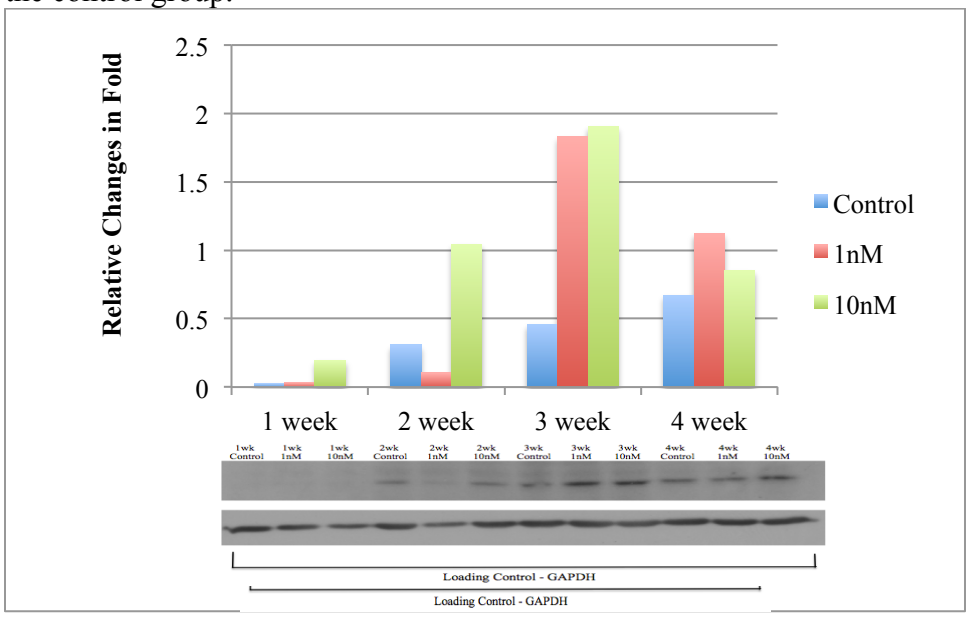


Figure 9. **MBP expression appears relatively similar with expression in the control group being slightly elevated at week two.** Western Blot Analysis of myelin basic protein (MBP), an essential structural myelin protein, deposited at the final stages of myelination.

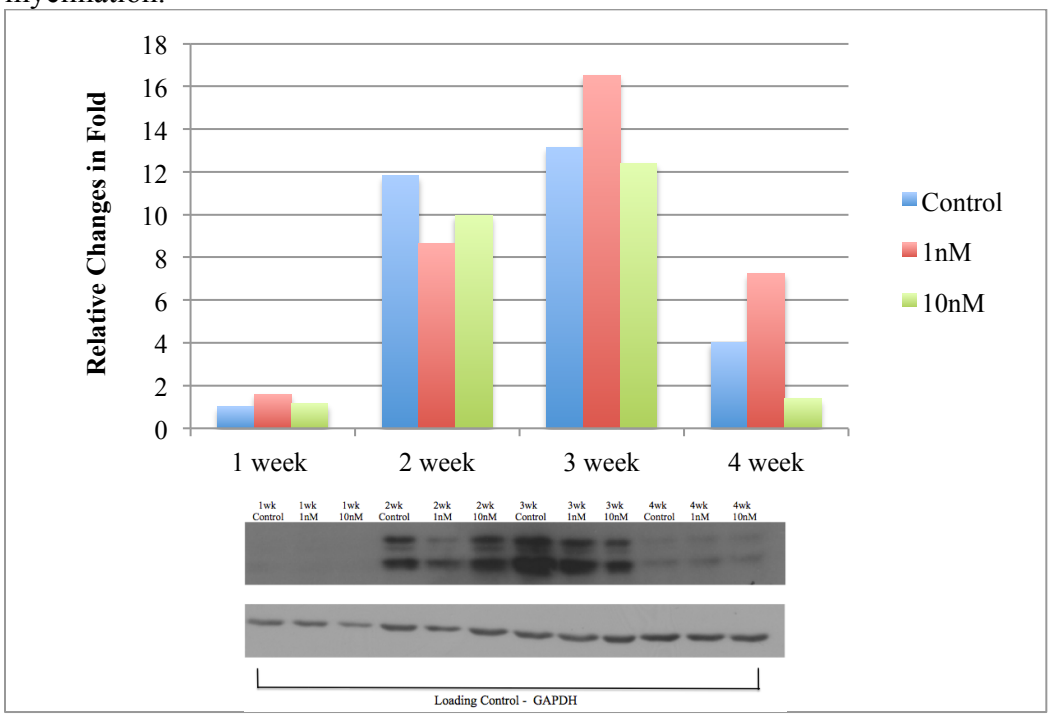


Figure 10. PLP is elevated in the 10nM Galanin dose, compared to the control. Western Blot analysis of Proteolipid Protein (PLP), a myelin structural protein found in abundance. The graph indicates the relative fold changes of the average of the two samples from two iterations of the cell culture for both groups. The error bars represent the standard error.

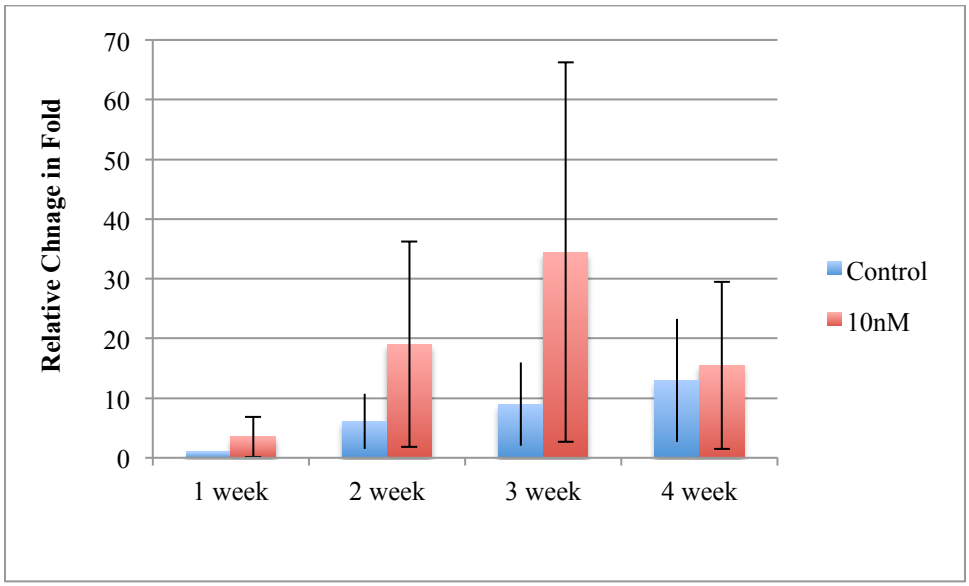


Figure 11. MBP expression remains similar in both control and 10nM dose. Western blot analysis represents the MBP expression in the control and 10nM dose of Galanin, between samples from the two iterations of the cell culture. The expression appears to be relatively similar to one another. The error bars represent the standard error.

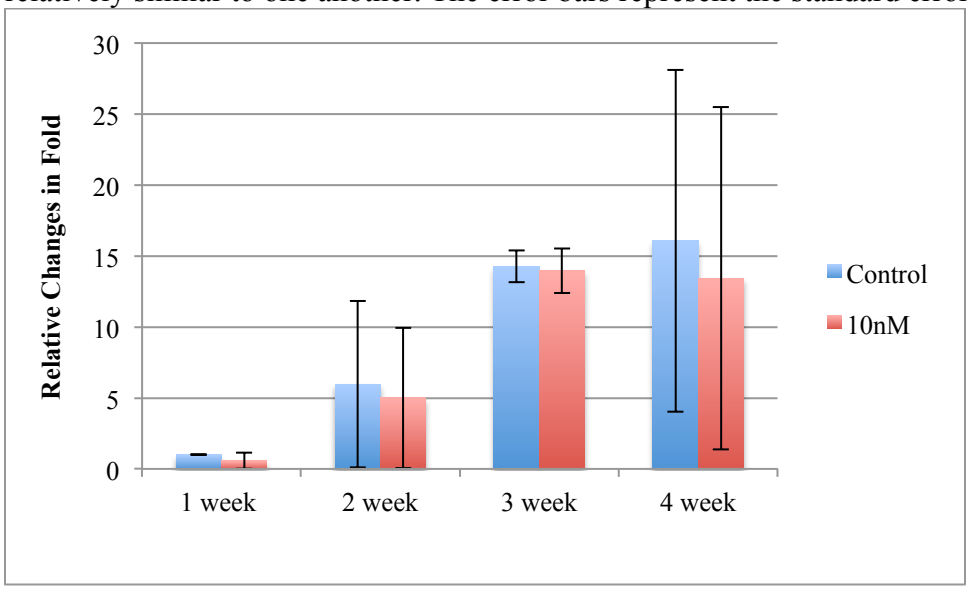


Figure 12. **Olig2 increased in 1nM and 10nM Galanin doses at weeks one, two and three.** Western blot analysis of Olig2, a transcription factor necessary for the differentiation of immature oligodendrocytes into mature myelinating oligodendrocytes. Here the relative changes in fold clearly show increased expression of Olig2 at weeks one, two and three, indicating differentiation of oligodendrocytes is earlier in both 1nM and 10nM doses of Galanin.

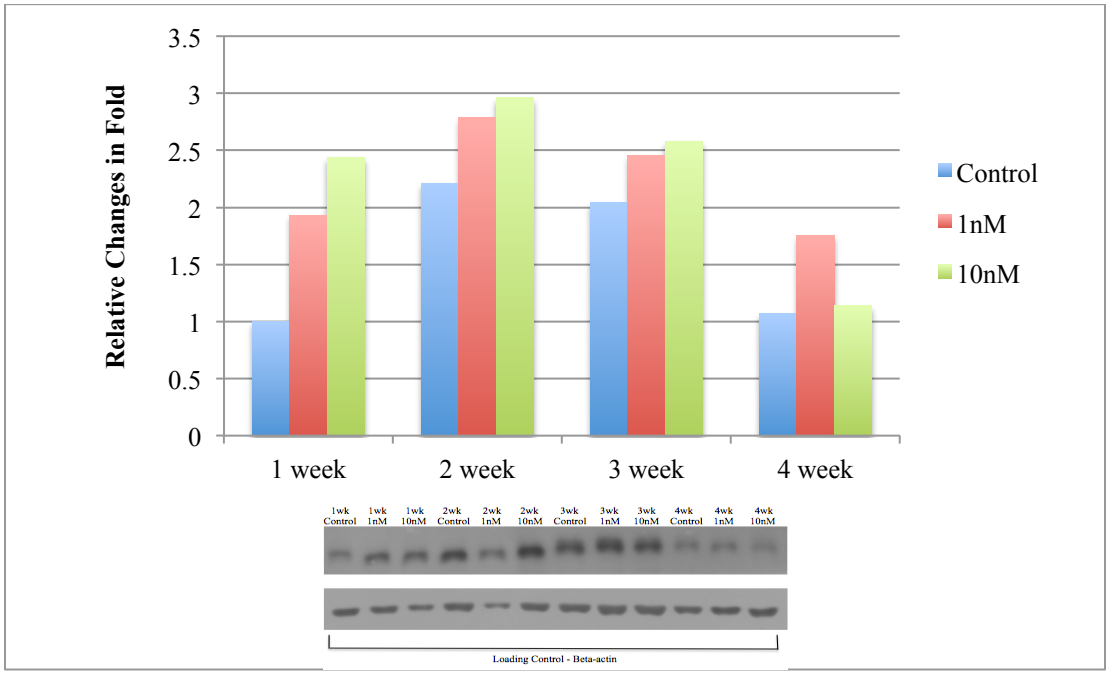


Table 3. Primary antibodies utilized in western blot and immunocytochemistry staining

	Target	Dilution Ratio	Order Number	Supplier
Anti-MBP	Myelin	1:3000	sc13914	Santa Cruz
Anti-PLP	Myelin	1:3000	sc23570	Santa Cruz
		1:200*		
Anti-AKT1	Galanin	1:3000	ab81283	Abcam
Anti-Olig2	OPC	1:3000	AB9610	Millipore
Anti-pNFH	Axons	1:200*	sc32780	Santa Cruz
Anti-β-actin	Loading control	1:5000	sc69879	Santa Cruz
Anti-GAPDH	Loading control	1:5000	sc32233	Santa Cruz

*the higher concentration of antibody was used for immunocytochemistry
Abbreviations: MBP, myelin basic protein; PLP, Proteolipid protein; pNFH, phosphorylated neurofilament heavy chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Table 4. Fluorescent secondary antibody list

	Dilution Ratio	Order Number	Supplier
Alexa Fluor© Anti-Ms IgG	1:1000	A11062	Invitrogen
Alexa Fluor© Anti-Gt IgG	1:1000	A11078	Invitrogen

Chapter 5: Discussion, Conclusions and Future Directions

Developmental myelination, one of the most important processes during early development is still not well understood (Gibson *et al,* 2014). There is a complex interaction of a variety of signaling molecules that regulate the interplay of oligodendrocytes, axons, and other extracellular factors that affect the initiation and maintenance of myelination. In addition to the variety of signaling pathways that initiate and maintain correct myelin formation, it would appear that this process is time sensitive (Czopka *et al,* 2013; Butzkueven and Gundlach, 2010). As mentioned in chapter one, the study of myelination in the context of disease is becoming increasingly important and it has been noted that remyelination capacity in individuals suffering from demyelinating diseases is not as effective as would be desired (Patel *et al,* 2010; Chari, 2007). Galanin, present in many parts of the CNS, can also be detected in the corpus callosum and the subventricular zone of the normal brain (Agasse *et al,* 2013; Shen *et al,* 2003). These are regions where OPC's proliferate and migrate from following injury from MS insults (Patel *et al,* 2010) indicating that Galanin may be important in the process of remyelination as well.

As mentioned previously, it was hypothesized that Galanin would 1) increase the rate of myelination and 2) that the relationship between Galanin and myelination is both time and dose dependent. Therefore, the use of the TG mouse model to study Galanin's effects on developmental myelination seemed appropriate in order to provide some clues into Galanin's role in CNS myelination. The purpose of the development of both models was to examine if, in the presence of Galanin (either at high circulating levels or at either

a high or low dose added to culture), myelination rate is increased, thus implicating Galanin as a potentially important molecule in myelination. The second objective was to determine if Galanin exerts its effects in a time and dose related fashion.

Advantages of studying Galanin and myelination both in *in vivo* and *in vitro* allows for the examination of Galanin's effect on myelination in a normal physiological environment as well as a in a controlled environment. The use of both study models provided a more comprehensive understanding of Galanin's role within the process of myelination. While the *in vivo* model provides the capability of examining myelination at many stages of development, the *in vitro* model is limited to approximately four-five weeks of growth as the culture is not as viable both in terms of cell survival and generation of myelin sheath (Thomson *et al,* 2008). Therefore, it is possible that some molecules expressed later in myelination may be underrepresented, particularly with regard to those proteins, like MBP, which is expressed at the final stages of myelin maturation (discussed below). Nonetheless, both models are versatile and in conjunction may be utilized to probe Galanin's effects on myelinogenesis.

Though valuable as a tool to study developmental myelination, the *in vitro* model may be modified in order to study Galanin in the context of demyelinating disease and to examine its potential role on remyelination. In addition, the *in vitro* culture provides a means to study Galanin's mode of action and the pathways involved therein. Overall, the *in vivo* model allowed for the identification of Galanin as having the predicted effect on the rates of myelination in the TG mice as compared to the WT. This further allowed the

development and design of a stable myelinating mixed spinal cord cell culture, which can be utilized to further understand the role Galanin plays in myelination developmentally and in an induced disease state.

As outlined in the manuscript in chapter two, the Galanin TG mouse model demonstrated significant differences, particularly at postnatal day 10 in myelination (figures 1, 2, 3 and 4). The rate of myelination *in vivo* was enhanced in the Galanin TG mice as evidenced by the immunohistochemical staining illustrated in figure 3 from the densitometry of the half brain sections at P10. Differences observed from these images, while significant ($p<0.05$) between the TG and WT, were affected by the large area over which they were analyzed. Therefore images were taken at the corpus callosum where the MBP-immunostaining was concentrated in the TG and WT. This subsequent densitometry at the knee region of the corpus callosum (figure 4) yielded much more significant differences between the TG and WT ($p<0.0001$), as expected. The TG mice at P10 expressed MBP in fine filaments that extended into the cortex whereas the control did not demonstrate this staining pattern (figure 4).

As discussed in chapter two, in conjunction with the qPCR results that also demonstrated significantly increased MBP expression (figure 1 and 2), myelination rate has increased in the TG mice at postnatal day 10. This same effect, increased myelination rate, is seen in the preliminary results of the *in vitro* model. Here, increased myelination and increased levels of the major myelin protein PLP is seen in both in the immunocytochemistry staining of the culture as well as in the western blotting results

(figures 6 and 8). However, while both are major structural proteins of CNS myelin, only one was upregulated either *in vivo* or *in vitro*. The reason for this variation is yet to be determined and is of interest for future study.

The results from the *in vivo* model (P10) and the preliminary results from the *in vitro* model (10nM dose) coincide, in the sense that both corroborate the fact that myelination rate is increased in the presence of increased Galanin levels. As has been stated above, the specific distribution of these major myelin proteins does not agree in either study. In the *in vivo* study, levels of MBP surpass those of the control group and demonstrate significant differences between the TG and WT mice (figure 3 and 4) and show no significant differences in PLP expression (figure 5). The cell culture on the other hand preliminarily demonstrated large differences in PLP expression, to the order of an approximate 30-fold increase (figure 6 and figure 10) as compared to the control. MBP expression showed no increase and in fact levels were either the same or slightly lower than that seen in the control group (figure 9 and figure 11). The variation seen between the *in vivo* and *in vitro* results, while requiring further investigation, may be explained in several ways. Firstly, *in vivo* normal developmental myelination is occurring in the CNS, where it has been established that mature myelin (thus mature oligodendrocytes) can be denoted by the amount of MBP that is being expressed (Bodhireddy *et al,* 1994). MBP's importance has been well established with knockout experiments showing poor survival in animals with MBP-knockout (Readhead *et al,* 1987). Therefore it is important to note that the MBP results came from a whole brain source of a surviving animal as compared to embryonic spinal cord tissue. As such it is important to also note that the distribution

of protein content may change *in vitro.* Interestingly, in a study of spinal cord injury, while MBP expression in the spinal cord is exhibited in similar regions to PLP expression, normal levels of MBP remain lower as compared to levels of PLP (Wrathall *et al,* 1998). In addition to this point it is also known that in neural embryonic tissue that later differentiates into OPC's, PLP and its splice variant DM20 are heavily expressed (Michalski *et al,* 2011). In fact, PLP/DM20 is found to be highly expressed in the spinal cord during embryonic development where the tissue used for the mixed cell culture was isolated (Michalski *et al,* 2011; Woodruff and Franklin, 1999; Harlow *et al,* 2014). Therefore the tissue origin may play a role in affecting the results. However, future studies will need to be employed in order to confirm this possibility.

Secondly, it should also be noted the PLP/DM20 increased expression has been found in the population of OPC's which are migratory and which will eventually differentiate into mature myelinating oligodendrocytes (Harlow et al 2014). While mice that lack PLP are still able to survive and myelinate, OPC's are affected in their capacity to extend processes, suggesting that PLP may play a role in responding to external signaling causing the stimulation of OPC process outgrowth as well as stabilizing OPC's (Harlow *et al,* 2014). From what is seen in the results in chapter 4, the increase in PLP expression may be a response to external signaling stimulating OPC process outgrowth. Thus the highly elevated PLP, in addition to being elevated due to myelin deposition, may also be responding to external stimulus. These results, in conjunction with what has been found in the literature regarding Galanin's capacity to act as an extracellular effector

(see chapter one), suggests that Galanin may be acting as a signaling molecule in a fashion to enhance oligodendrocyte myelinating capacity.

Finally, a third possibility exists which can be attributed to the viability of the cell culture not being more than four-five weeks. As discussed above, myelination and the components produced therein are highly regulated in order to ensure the deposition of the correct proteins at the desired time. Therefore, maximal MBP expression would not be reached until such a time that the myelin was mature. If Galanin were acting not only to increase myelination rate, but also amount of myelin being deposited, it is possible that the deposition of MBP was incomplete at the time of the termination of the cell culture. Further, the quality and survival of the myelinating cells may have decreased as the cell culture came closer to 4-5 weeks in age. As mentioned previously and in the literature, the culture's viability decreases after four weeks (Thompson *et al,* 2008) thus providing a potential possibility for the decreased MBP expression observed *in vitro*.

Examination of the later postnatal days 15 and 30 in the *in vivo* study showed no significant differences at these time points between the TG and the WT (see chapter two). As is well known, OPC's follow strict regulation in terms of differentiation, migration and myelination capacity (Ishibashi *et al,* 2009). The lack of significant increase in myelination at the later postnatal days may be attributed to the recently demonstrated characteristic that oligodendrocytes only have a short window of time where they are capable of initiating myelination (Czopka *et al,* 2013; Glenn and Talbot 2013). Further, as the rate of myelination appears increased at P10 (figure 3 and 4), this could suggest

that by P15, the amount of myelin required at this stage had already been achieved, and the regulatory mechanisms in place to prevent excess myelin deposition prevented further myelin deposition in the TG animals. These observations suggest that the early stages of myelination are particularly important with regard to the ability of Galanin to have an effect on oligodendrocytes within the short window of time provided to increase the rate of myelination.

As there were differences in myelination rate it became apparent that differences in the OPC population had to be examined, both *in vivo* and *in vitro*. An increase in OPC's may have suggested an increasing amount of myelinating OL's, a marker for OPC's was probed in the *in vivo* model. Elevated platelet derived growth factor receptor alpha (PDGFRα), a marker for OPC proliferation, would indicate that the observed increased rate of myelin deposition could be attributed to the elevated levels of OPC's differentiating into more myelinating oligodendrocytes. This however, was not the case, as the expression of PDGFRα was not elevated in the TG as compared to the WT (figure 5). In conjunction with this data, several studies indicate that there are regulatory mechanisms that ensure the amount of myelinating oligodendrocytes is independent of the amount of OPC's that are produced early in development (Yang *et al,* 2011). As a result, there is a very small likelihood that the increased myelination rate is due to an increase in the amount of myelinating cells being generated in the Galanin TG mice.

Following this logic, in the *in vitro* model the Olig2 (indicating immature OPC's soon to undergo differentiation) expression pattern was examined to ascertain if there

were any differences at this point between the control and Galanin dosed groups. Increased expression of Olig2 may indicate increased rate of OPC differentiation potentially causing myelination rate to also be elevated. At week one, the expression of Olig2 was increased more than two fold for the 10nM dose and by week two the expression level was found to be elevated more than two-fold for both the 1nM and 10nM doses (see figure 12). The expression remained elevated in the 1nM and 10nM doses until week three. Olig2 is one of the essential transcription factors necessary to stimulate the differentiation of OPC's into mature myelinating oligodendrocytes (Mie *et al,* 2012). In Olig2 knockout experiments it has been found that oligodendrocytes are not detected, and when Olig2 is forcibly expressed, oligodendrocyte differentiation is induced (Mie *et al,* 2014). This suggests an essential role for Olig2 in OL development and in conjunction with the *in vivo* results indicates an interesting potential mechanism by which Galanin may act. While these results are preliminary findings, they point to the idea that Galanin may be influencing the differentiation of OPC's into myelinating OL's that are then capable of increased myelination both in rate and amount. As the PLP results *in vitro* at the 10nM dose (figure 10), and MBP results *in vivo* in the Galanin TG mice (figure 3 and 4) demonstrate increased myelin deposition in rate and amount, the possibility of Galanin promoting Olig2 will be further investigated in the future.

While the *in vivo* results are not conclusive and do not point to a direct effect of Galanin on the OL's, the results do indicate that Galanin is having an effect on the process as a whole. It has been noted both in chapter two and in the literature that Galanin can act both directly within the OL's which express the neuropeptide, or indirectly as a

signaling molecule in the CNS in order to promote myelination (Merchenthaler *et al,* 2010; Ubink *et al,* 2003). The *in vitro* results demonstrate promise regardless of their preliminary nature and require further study in order to accurately predict Galanin's role in this process, especially in identifying the mechanism by which Galanin exerts its effects. Nonetheless, these results demonstrate yet again the valuable nature of the *in vitro* myelinating culture and potentially yet another role for Galanin in the CNS.

Future iterations of the *in vitro* study will be employed to ensure PLP and MBP are indeed elevated and also to assist in understanding why myelination rate is increased both *in vivo* and in those cells treated with the 10nM dose (in particular) of Galanin.

Conclusion

Galanin is not a novel neuropeptide in the CNS and has been well characterized during the past 30 years since its discovery in the early 1980's. However, novel functions are often being discovered, not only for Galanin, but for neuropeptides like NPY (chapter one) and various other well known endogenous molecules, like LIF (chapter one). Galanin remains an important neuropeptide in many neurobiological functions. In this application we have shown that yet another important role may be attributed to this multifaceted neuropeptide. Galanin, if capable of regulating myelination rate in the fashion described above, would certainly be able to fill one of the gaps in knowledge that surround the process of developmental myelination and remyelination following injury or damage from disease. As was mentioned in the introductory chapter, well-established endogenous molecules are becoming increasingly important in the study of demyelinating

diseases like MS. Many of these novel applications focus primarily on the prevention of the progression of the disease. This of course is a major priority as many individuals continue to suffer in the early stages of the disease. However, many individuals also struggle with progressive variations of MS where immune suppression and progression prevention are not the most useful therapies. A better understanding of the developmental processes that are affected by this disease, namely myelination, and the regulators that control this complicated process would be immensely helpful in identifying the etiology of this disease. Thus the identification of Galanin as an important component of myelin modulation may lead to it being used as a potential therapeutic target in the future.

In summary, this study has demonstrated that Galanin does exert an effect on CNS myelination, namely increasing the rate of myelination *in vivo.* We have also established a protocol for examining Galanin's effects *in vitro* that is highly versatile and valuable. This study also aided in identifying a dose at which Galanin may optimally exert its effects. The *in vivo* and *in vitro* models have also demonstrated that Galanin's effects are both time and dose dependent as many of the significant results were noted at early developmental days.

Overall, both the *in vivo* and *in vitro* models have contributed to the growing knowledge regarding a novel role for the well-characterized molecule Galanin. They have demonstrated Galanin's capacity to act in a key role as a promoter of myelinogenesis. These studies have also suggested a variety of new directions in which Galanin can be examined in the role of a modulator of myelination. This may be especially important in

both the understanding and development of novel treatments for demyelinating and dysmyelinating diseases, and specifically MS. The continued investigation of Galanin in the context of myelination holds promise in the future and may eventually be an important therapeutic target in the treatment of neurodegenerative disease.

Future Directions

While the preliminary results described in chapter 2 and chapter 4 show promise with regard to Galanin as an important promoter of myelination, further analysis, especially with regard to the *in vitro* study does need to be done. In particular, further iterations of the mixed spinal cord cell culture will be initiated in order to ensure the efficacy of the 10nM dose, which we have identified as most optimal to date. Withdraw of Galanin will also be examined in the future and fully analyzed alongside the group to which Galanin will be added. This will allow for the confirmation of the results described in chapter 4 and allow for a clearer understanding of the events of myelination as they occur and Galanin's role therein. Examination of upstream regulators, such as enzymes regulating the production of the components of the myelin sheath, and down-stream regulators activated by Galanin can also be examined to see where and how Galanin may be exerting its effects during myelinogenesis. Real time PCR will also be employed to look at Galanin receptor expression patterns in order to ascertain what changes are occurring (if any), which could give some insight into the pathways being activated in response to Galanin addition.

As the electron microscope (EM) study protocol has recently been standardized in our laboratory with regard to this type of culture, future iterations of the cell culture, will examine sheath thickness using EM as an added confirmation of Galanin's promyelinating effect.

Future studies may also include the examination of Galanin's effect *in vitro* through the use of a demyelinating model to which Galanin is added. As it has been noted above, Galanin has been seen to attenuate disease state in an *in vivo* model of EAE (Wraith *et al,* 2009). Due to the versatility of this cell culture model, the development of a disease state and the subsequent addition of Galanin *in vitro* may shed more light on Galanin's effect on the myelination process and its potential ability to induce reparative functions in the CNS during disease states. This may be particularly essential for those diseases both hereditary and acquired as mentioned in the introduction.

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