NeuroImmune Modulation of Multiple Sclerosis via the Dorsal Root Ganglia

by

Maria Melanson M.D.

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University of Manitoba
Winnipeg

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Abstract

Background: Multiple sclerosis (MS) is a chronic, neurological disease characterized by targeted destruction on central nervous system (CNS) myelin. The autoimmune theory is the most widely accepted explanation of disease pathology. Circulating Th-1 cells become activated by exposure to CNS-specific antigens such as myelin basic protein. The activated Th-1 cells secrete inflammatory cytokines, which are pivotal for inflammatory responses. We hypothesize that enhanced production of inflammatory cytokines triggers cellular events within the dorsal root ganglia (DRG) and/or spinal cord, facilitating the development of neuropathic pain (NPP) in MS. NPP, the second worst disease-induced symptom suffered by patients with MS, is normally regulated by DRG and/or spinal cord.

Objective: To determine gene and protein expression levels of tumor necrosis factor-alpha (TNF α) within DRG and/or spinal cord in an animal model of MS.

Methods: Experimental autoimmune encephalomyelitis (EAE) was induced in adolescent female Lewis rats. Animals were sacrificed every 3 days post-disease induction. DRG and spinal cords were harvested for protein and gene expression analysis.

Results: We show significant increases in TNFα expression in the DRG and of EAE animals at peak disease stage, as assessed by clinical symptoms.

Conclusion: Antigen-induced production of inflammatory cytokines such as TNFα within the DRG identifies a potential noel mechanism for MS-induced NPP.

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ABBREVIATIONS

Abbreviation	Meaning
aEAE	active EAE
APCs	antigen presenting cells
ATF-3	activating transcription factor three
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
CA	carbonic anhydrase
CGRP	Calcitonin gene related peptide
CNS	central nervous system
dpi	days post induction
DRG	dorsal root Ganglia
EAE	experimental autoimmune encephalitis
FA	Freund's incomplete adjuvant
Gd-DTPA	Gadopentetic acid
GDNF	glial cell line-derived neurotrophic factor
Hsp	heat shock protein
i.p.	intraperitoneal
ICAM-1	intracellular adhesion molecules
IFNγ	interferon gamma
IHC	immunohistochemical
IL-12	interleukin-12
LFA-1	leukocyte functional antigen
LPS	lipopolysaccharide
MAG	myelin associated glycoprotein
MAPKs	mitogen-activated protein kinases
MBP	myelin basic protein
MMP	matrix metalloproteases
MOG	myelin oligodendrocytic glycoprotein
MRI	magnetic resonance imaging
MRI	magnetic resonance image
MS	Multiple Sclerosis
MT	*Mycobacterium Tuberculosis*
NAA	[N-Acetyl Aspartate
NAWM	normal appearing white matter
NGF	nerve growth factor
NPP	neuropathic pain
NT-3	neurotrophin-3
NT-4	neurotrophin-4
OL	oligodendrocyte

p75NTR	p75 receptor
PBS	phosphate buffered saline
PBS-T	PBS-Tween
PLP	proteolipid protein
PSGL-1	P-selectin glycoprotein ligand-1
PT	pertussis toxin
qRT-PCR	quantitative real time reverse-transcription polymerase chain reaction
RNA	ribonucleic acid
RRMS	relapsing-remitting MS
sc	subcutaneous
SPMS	secondary progressive MS
TCR	T-cell receptor
TGFβ	transforming growth factor-β
TNFR1	p55 TNF receptor type 1
TNFR2	p75 TNFα receptor 2
TNFα	tumor necrosis factor alpha
Treg	T regulatory cells
VCAM-1	vascular cell adhesion molecules
VLA-4	very late antigen

BACKGROUND

Multiple Sclerosis

Multiple Sclerosis **(MS**) was first described by Dr. F. M. Charcot in 1868. Despite considerable research advancements, the cause of this devastating white matter disease remains to be fully elucidated. MS is a common neurological disease, primarily affecting young adults between the ages of 15 and 30. MS can affect up to 0.2% of people in high prevalence areas (Klein, Rose et al. 1994; Weinshenker 1996). It is one of the leading causes of disability among young adults of Caucasian origin (Kurtzke, Page et al. 1992; Warren, Warren et al. 2003). Canadians have one of the highest rates of MS in the world (100-300/100, 000 population). There are currently 55,000-75,000 MS patients in Canada, with three more people being diagnosed with MS daily. MS is also one of the most expensive diseases for the Canadian health care system with health care costs ranging from *$10,598 to $51,698 per patient per year,* depending on the severity of illness [1]. As a result, any therapy that can delay disease progression or any diagnostic procedure that can definitively diagnose MS earlier will greatly reduce the heavy pharmacoeconomic burden imposed on the health care system.

MS is characterized by targeted destruction of central nervous system (**CNS**) myelin (Prineas 1975; Martino and Hartung 1999; O'Connor, Bar-Or et al. 2001; O'Connor 2002). Destruction of CNS myelin ultimately leads to the disruption of the propagation of electrical impulses along nerve axons. Damage or destruction of myelin along axons results in the formation of “lesions” or

“plaques” that characteristically cluster around the ventricles and other areas within the brain as detected by magnetic resonance imaging (**MRI**) (Raine, Scheinberg et al. 1981; Lyman, Roth et al. 1988; Raine, Moore et al. 1988). Sensory abnormalities have been reported to be the first symptom described by many adult MS patients *prior to diagnosis* (Portenoy, Yang et al. 1988; Namaka, Turcotte et al. 2008).

Myelin

Myelination is critical for normal mammalian nervous function. An intact myelin system facilitates efficient saltatory conductance of nerve impulses through the CNS. Saltatory conduction (from the Latin *saltare*, to hop or leap) is the propagation of action potentials along the axon, from one node of Ranvier to the next (Huxley and Stampfli 1949). The axonal cytoplasm is electrically conductive.However, the axonal membrane contains ion channels that result in the leakage of charge across the membrane. The myelin sheath prevents charge leaking through the axonal membrane, ensuring that membrane depolarization occurs only at the nodes of Ranvier. Depolarization at one node of Ranvier elevates the voltage at a neighboring node to the threshold for action potential initiation. Thus the action potentials effectively "hop" along the axon, traveling faster than they would if the electrical charge were propagated in the form of a wave (Huxley and Stampfli 1949).

Oligodendrocytes (**OL**) are the myelinating cells of the CNS (Bunge 1968; Baumann and Pham-Dinh 2001). They produce extensive sheets of lipid rich

membrane that wrap around the axon to form a multi-layered sheath (Bunge 1968). The myelin membrane is composed of about 80% lipid and about 20% protein (Dyer 2002). Some of the principle proteins that make up myelin are myelin basic protein (**MBP**), proteolipid protein (**PLP**), and myelin associated glycoprotein (**MAG**) (Campagnoni 1988; Baumann and Pham-Dinh 2001). The importance of these proteins to myelin function has been demonstrated using transgenic mouse models. Specifically, MBP ablation results in a dysmyelinated mouse called the *Shiverer* because of its shivering (Palma, Owh et al. 1997). PLP ablation, or overexpression, results in a mouse with severe hypomyelination analogous to the human disorder Perlizaeus Merzbacher disease (Griffiths, Klugmann et al. 1998; Karim, Barrie et al. 2007), and MAG knockout mice show a phenotype representative of the human disorder Schizophrenia (Sokolov 2007). Myelin also contains high levels of cholesterol and galactosylceramide, which play significant roles in the structure and function of myelin (Jahn, Tenzer et al. 2009). Each OL can interact with up to 50 different neurons and myelinates each axon in 1-2 mm lengths known as internodes. Saltatory conductance along the axon is accelerated by the clustering of sodium channels at the nodes of Ranvier (Kaplan, Meyer-Franke et al. 1997; Salzer 2003; Nie, Ma et al. 2006). Myelin loss results in severely impeded or weakened nerve impulse conduction (Rasminsky and Sears 1972).

Etiology of MS

At present, the autoimmune theory represents the most plausible and widely accepted explanation of disease pathology (Martino and Hartung 1999; O'Connor, Bar-Or et al. 2001; O'Connor 2002). According to this theory, circulating Th1-cells in the blood become activated upon exposure to specific CNS antigenic determinants such as MBP, PLP and/or myelin oligodendrocytic glycoprotein (**MOG**). Once activated in the blood, Th1-cells are able to adhere to and cross the blood brain barrier (**BBB**) (Martino and Hartung 1999; O'Connor 2002; O'Connor and Group 2002). Activated Th1-cells secrete pro-inflammatory cytokines such as interleukin-12 (**IL-12**), interferon gamma (**IFNγ**) and tumor necrosis factor alpha (**TNFα**) (Zeis, Graumann et al. 2008). IFNγ and TNFα secreted by activated Th1-cells leads to up-regulation of adhesion molecules, such as vascular lectins (e.g. ICAM-1, VCAM-1), on the surface of endothelial cells of the BBB. These adhesion molecules mediate the initial attachment of activated circulating Th1-cells to the endothelium. As more Th-cells dock at the BBB surface, they continue to release chemo-attractant cytokines, which promotes additional T-cell recruitment and aggregation. The subsequent release of matrix metalloproteases 3 and 9 (**MMP-3**, **MMP-9**) loosens the tight-junctions between the endothelial cells of the BBB, which facilitates the passage of Th1-cells directly into the CNS (Dhib-Jalbut, Jiang et al. 1996). BBB disruption is a hallmark of MS. However, it is not known whether BBB disruption is the cause or consequence of Th1-cell infiltration into the CNS.

Th1-cells are re-activated following entry into the CNS and continue to promote inflammation by the sustained production of inflammatory cytokines. It is known that elevated levels of TNFα in the CNS correlate well with MS disease activity and BBB damage (Merrill 1992). In particular, TNFα and complement are known to cause OL death (Kornek and Lassmann 2003; Nakazawa, Nakazawa et al. 2006). These cytokines orchestrate a pathogenic immune response directed against CNS myelin. The subsequent myelin damage interferes with effective propagation of nerve impulse transmission to effector targets. The reduction or total loss of nervous transmission to effector targets can lead to a variety of symptoms such as weakness, fatigue, cognitive dysfunction and sensory abnormalities such as neuropathic pain (**NPP**) (Pollmann and Feneberg 2008).

Several theories have been proposed to explain the source of the antigens that activate the Th-cells. These include epitope spreading, superantigenic activation, and molecular mimicry (Namaka, Kapoor et al. 2010). Regardless of the antigenic source, once activated, the Th-cells are able to attach to the endothelial cells of the BBB, cross it and mediate and inflammatory response with cytotoxic damage specifically directed toward myelin and/or myelin-producing cells of the CNS.

Th-cells

The immune system response is orchestrated by three main types of T helper cells which include Th1, Th2, and Th17. In addition, another subset of T-cells called T regulatory cells (**Treg**) is also involved. The Th1 subtype is responsible for mediating inflammation, while the Th2 subtype is responsible for the protective or anti-inflammatory activity. At present the role of Th17 cells in the immune response and the mechanisms underlying their entry into the CNS is still unresolved, However, there is increasing evidence that Th17 cells may play a significant role in the immune mediated events that occur during an MS attack (El-Behi, Rostami et al. 2010). T helper cell proliferation is regulated by Treg cells and Treg cells are inhibited by cytokines released by Th17 cells (O'Connor, Taams et al. 2010). The specific activity of Th-cells is regulated by cytokines that are expressed by other Th-cells (O'Connor, Taams et al. 2010). Organ-specific autoimmune disease develops when the dynamic balance between Th1 and Th2 is disturbed. Further, Th1 and Th17 cells regulate the behavior of each other. Specifically, Th17 cells secrete IL-17, which inhibits the activity of Th1 cells (El-Behi, Rostami et al. 2010). Consequently, autoimmune diseases like MS may be largely driven by a Th1 inflammatory response, which far outweighs the protective activity of Th2 mediated response as well as the failure of Treg cells to suppress Th cell activity (Kumar and Sercarz 1998; Martino and Hartung 1999; Hohlfeld and Wekerle 2001; Ozenci, Kouwenhoven et al. 2002; O'Connor, Taams et al. 2010).

Cytokines

The Th1 subtype is often identified by the presence of CD4 on its surface. The Th1 subtype secretes inflammatory cytokines such as IL-12, IFNγ, IL-6, IL-2 and TNFα, while the Th2 subtype secretes anti-inflammatory cytokines such as IL-4, IL-6, IL-10 and TGFβ (Kumar and Sercarz 1998; Martino and Hartung 1999; Hohlfeld and Wekerle 2001; Ozenci, Kouwenhoven et al. 2002). Cytokines are soluble molecules that can mediate both the pro-and anti-inflammatory responses of the immune system. Despite the wide variety of inflammatory cytokines, TNFα is thought to play a key role in induction of the pathophysiological steps underlying the development of MS by its contributions to immune-mediated demyelination (Brosnan, Selmaj et al. 1988). TNFα is believed to mediate myelin damage by causing the up-regulation of MHC Class II expression on astrocytes. This increased antigen presentation leads to activation of Th-cells and subsequent Th-cell-induced myelin destruction. TNFα also contributes to cell- mediated myelin damage through both its induction of cytokine (IL-1 and IL-6) and its possible ability to work synergistically with other cytokines such as IFNγ (Cannella and Raine 1989; Zoja, Wang et al. 1991; Vassalli 1992). Specifically, TNFα was also found to stimulate production of IL-12, which in turn induces IFNγ production. This creates a positive feedback cycle for additional Th1 activation and recruitment that facilitates inflammation (Martino and Hartung 1999; Hohlfeld and Wekerle 2001; Ozenci, Kouwenhoven et al. 2002). IFNγ is involved in antiviral immune response, macrophage activation as well as up-regulation of MHCII expression on the antigen presenting

cells (**APCs**) (Cannella and Raine 1989; Trinchieri 1995; Mosmann and Sad 1996; Cannella, Hoban et al. 1998; Elenkov, Wilder et al. 2001). In patients with MS, increased levels of IFNγ may increase incidence of exacerbations (Panitch, Hirsch et al. 1987). It can also damage OL progenitors, which are responsible for the formation of new myelin in the CNS (Selmaj and Raine 1988). TNFα can also damage endothelial cells leading to further breakdown of BBB through up regulation of intracellular adhesion molecules (**ICAM-1**) and vascular cell adhesion molecules (**VCAM-1**) (Barten and Ruddle 1994; Navikas and Link 1996).

The only cytokine secreted by both Th1 and Th2 cell subtypes is IL-6, which has both pro-inflammatory and anti-inflammatory activity (Naugler and Karin 2008). IL-12 secretion by Th1 cells is central to promoting the preferential differentiation of T-lymphocytes in the blood into the inflammatory Th1 subtype, as described above. Thus, IL-6, IL-12, IFNγ and other inflammatory cytokines including TNFα are thought to be key factors in the underlying pathogenesis of MS (Traugott and Lebon 1988; Olsson, Zhi et al. 1990; Frei, Fredrikson et al. 1991; Sun, Olsson et al. 1991; Maimone, Reder et al. 1993; Woodroofe and Cuzner 1993; Link, Soderstrom et al. 1994; Windhagen, Newcombe et al. 1995; Navikas and Link 1996).

T-cell activation

It is important to note that Th-cells are unresponsive until they become activated. Thus, T-cell activation is a crucial component of disease pathology. In

order to become activated, a circulating antigen has to be detected by the APCs of the immune system, such as macrophages and microglia. The function of these APCs is to "present" the detected antigen to the specific Th-cells that recognize the specific amino acid sequence displayed by the presented antigen. The presented antigen will then bind to the T-cell receptor (**TCR**) found on the surface of the T-cell, which starts the initial activation process. This initial interaction with the TCR facilitates the up-regulation of the B-family proteins [B7-1 (CD80) and B7-2 (CD86)] on the antigen-presenting cell. The up-regulated B7 proteins then interact with a CD28 receptor on the T-cell to produce the "co-stimulatory" effect required to complete T-cell inflammatory activation (Kumar and Sercarz 1998; Hohlfeld and Wekerle 2001; Ozenci, Kouwenhoven et al. 2002; Hohlfeld, Meinl et al. 2008). This T-cell activation usually occurs in the peripheral blood and is essential for eventual adhesion to and migration across the endothelial cells of the BBB. In addition to peripheral activation, T-cell activation can also occur within the CNS, as discussed above (Kumar and Sercarz 1998; Martino and Hartung 1999; Hohlfeld and Wekerle 2001; Ozenci, Kouwenhoven et al. 2002).

Entry into the CNS:

The transmigration of Th-cells into the CNS is a multi-step process (Figure 1). During the initial phase, the adhesion molecule subclass, known as selectins (P-selectin and E-selectin), on the endothelial cells mediate transient contact with lymphocytes via low affinity lymphocyte ligands known as P-selectin glycoprotein

ligand-1 (**PSGL-1**). PSGL-1 is capable of binding to P, E and L selectins, if glycosylated in the correct way. The expression of these adhesion molecules by endothelial cells is up-regulated by the secretion of the pro-inflammatory cytokines, IL-12, IFNγ and TNFα produced by activated Th-cells. During the interaction with the endothelium, the lymphocytes encounter endothelial chemokines. The endothelial chemokines activate the chemokine receptors on the surface of the lymphocytes. This leads to the induction of G protein-linked intracellular signaling, which in turn results in the activation of integrins on the surface of the lymphocytes (Prendergast and Anderton 2009). Specifically, lymphocyte adhesion molecules called Leukocyte functional antigen (**LFA-1**), also known as αLβ2 integrin, and very late antigen (**VLA-4**), also known as $\alpha_4\beta_1$ integrin, are upregulated on the surface of activated Th-cells. LFA-1 and VLA-4 facilitate the interaction between T-cells and endothelial cells via their counter receptors ICAM-1 and VCAM respectively (**Figure 1**) (Kumar and Sercarz 1998; Martino and Hartung 1999; Hohlfeld and Wekerle 2001; Ozenci, Kouwenhoven et al. 2002; Prendergast and Anderton 2009). The interaction between the lymphocyte integrin and its corresponding endothelial counter receptor results in firm adhesion of the lymphocyte to the endothelium surface of the BBB. The combined up-regulation of inflammatory cytokines and adhesion molecule expression on the BBB results in a positive feedback cycle essential to the aggregation of additional activated Th-cells to the BBB. These aggregated Th-cells secrete MMP-3 and MMP-9, which disrupts the basement membrane underlying the endothelium. The basement membrane is the final barrier to T-

cell entry into the CNS (Kumar and Sercarz 1998; Hohlfeld and Wekerle 2001; Ozenci, Kouwenhoven et al. 2002; Hohlfeld, Meinl et al. 2008). Following T-cell migration/ aggregation on the BBB, the activated T-cells begin to pass between the endothelial cells across the basement membrane into the CNS. Not only do MMPs affect the BBB, but they have also been shown to damage the myelin sheath. In several models of white matter disease, MMPs play a significant role in myelin destruction (Walker and Rosenberg 2009). MMPs have been shown to degrade MBP *in vitro* (Walker and Rosenberg 2009). It is important to note that as part of ongoing immune-surveillance, naïve Th-cells are also able to cross the BBB. (Mondino, Khoruts et al. 1996). During a sustained period of chronic inflammation, tight junctions of the BBB are significantly disrupted and may remain permeable to cellular infiltration after the initial inflammatory response (Mondino, Khoruts et al. 1996).

Cytokine Release

Once these activated Th-cells cross into the CNS they release inflammatory cytokines (IL-12, IL-2, IL-6, TNFα, and IFNγ) and begin their indirect destruction of myelin and OL. Further, the production of IFNγ is critical for macrophage and/or microglia recruitment to the site of inflammation resulting in direct damage to OL and myelin segments. Researchers have confirmed the damaging effects of IFNγ using *in vitro* assays. The addition of exogenous IFNγ to OL in culture made them susceptible to Fas ligand-mediated apoptosis by inducing Fas expression on OL cell surface (Trapp, Bo et al. 1999; Pouly, Becher et al. 2000;

Buntinx, Gielen et al. 2004). The damaging effects of other inflammatory cytokines, such as TNFα also became apparent on examination of brain lesions of MS patients. Pathological studies have shown that TNFα levels are significantly elevated in active demyelinating lesions as compared to inactive or remyelinating lesions (Oka, Akiguchi et al. 1998; Bitsch, Schuchardt et al. 2000). In addition to TNFα, IFNγ and other cytokines, such as IL-10, IL-12, p40 and IL-6 have also been found in the plaques of active MS lesions (Merrill 1992; Maimone, Reder et al. 1993; Issazadeh, Ljungdahl et al. 1995; Toulmond, Parnet et al. 1996; Hesselgesser and Horuk 1999; Glabinski, O'Bryant et al. 2000; Hohlfeld and Wekerle 2001). Ongoing research is exploring the functional significance of the presence of these cytokines in active MS lesions of the brain.

As previously mentioned, IL-6 is a unique cytokine that is secreted by Th1 and Th2 subtypes and possesses both inflammatory and anti-inflammatory properties (Merrill 1992; Mondino, Khoruts et al. 1996; Romagnani 1999; Cousins, Lee et al. 2002; Naugler and Karin 2008). IL-6 is known to play a very important role in the underlying pathogenesis of MS by its ability to promote both B-cell activation and subsequent myelin specific antibody production by differentiated plasma cells (Rotteveel and Lucas 1990; Rodriguez 1992; Comi, Leone et al. 2000; Hauser and Oksenberg 2006). However, it is thought that the B-cell conversion to myelin specific antibodies is a delayed and direct response that follows shortly after indirect Th1 insult (Lindert, Haase et al. 1999; Martino and Hartung 1999; Hohlfeld, Meinl et al. 2008). Following the initial inflammatory

phase of the disease, Th2 cell induction and Th2-cytokine secretion attempts to suppress the inflammatory response that was initially driven by Th1 cells. In order to accomplish this, Th2 subtype secretes anti-inflammatory cytokines such as IL-10, which are known to suppress IL-2, IL-12, TNFα and IFNγ that are driving disease progression (Issazadeh, Ljungdahl et al. 1995; Romagnani 1999; Cousins, Lee et al. 2002).

The role of CNS Antigens in MS

Several CNS-derived antigenic proteins have been identified as possible immune system triggers for activating the disease pathology of MS. The three most common include MBP, PLP, and MOG. These antigens are released into the peripheral blood either via unknown mechanisms or as foreign proteins that mimic the amino acid sequence of CNS antigens (Namaka, Kapoor et al. 2010). Once these CNS-derived antigenic proteins are released into the peripheral blood, they are detected by the APCs of the immune system. The APCs in the circulating blood preferentially bind to these antigenic proteins via the MHCII (animals) or HLA-Class II (humans), which then bind to specific receptors on circulating Th-cells. This results in T-cell activation (Lublin 1984; Rodriguez and Miller 1994; Huitinga, Ruuls et al. 1995; Lassmann and Ransohoff 2004; Melanson, Miao et al. 2009; Zhu, Frost et al. 2010).

PLP is the most abundant myelin protein in the CNS representing 20% of the entire myelin protein content (Mobius, Patzig et al. 2008; Jahn, Tenzer et al.

2009). PLP-reactive Th-cells have been obtained from both cerebrospinal fluid and peripheral blood of patients with MS (Kumar and Sercarz 1998; Martino and Hartung 1999; Hohlfeld and Wekerle 2001; Ozenci, Kouwenhoven et al. 2002). However, numbers of PLP-reactive Th-cells are not significantly increased over PLP-specific Th-cells found in normal healthy individuals without MS (Kumar and Sercarz 1998; Martino and Hartung 1999; Hohlfeld and Wekerle 2001; Ozenci, Kouwenhoven et al. 2002). This raises the question of why or what causes some individuals to go on to develop the disease while others do not? PLP-specific Th-cells are mostly $CD4^+$ and display preferential binding for HLA-Class II-DR found on circulating APCs.

Another CNS-antigenic protein thought to be implicated in MS pathology is MOG. MOG is a member of the immunoglobulin superfamily and is expressed only by OL (Scolding, Frith et al. 1989; Solly, Thomas et al. 1996; Slavin, Johns et al. 1997). MOG is a minor myelin protein representing approximately 0.05% of the total myelin protein (Solly, Thomas et al. 1996; Mobius, Patzig et al. 2008; Jahn, Tenzer et al. 2009). Interestingly, significantly increased numbers of MOG-reactive Th-cells have been found in serum samples from MS patients as compared to healthy, age-matched controls (Kerlero de Rosbo, Milo et al. 1993; Diaz-Villoslada, Shih et al. 1999; Martino and Hartung 1999; Mobius, Patzig et al. 2008; Jahn, Tenzer et al. 2009). Th-cells reactive to other CNS antigens, such as PLP and MAG are not significantly increased in MS patients. Further, there are almost no MBP reactive Th-cells in either serum of MS patients or healthy

age-matched controls (Kerlero de Rosbo, Milo et al. 1993). The use of MOG to induce experimental autoimmune encephalitis (**EAE**) has been a widely published animal model for MS (Mendel, Kerlero de Rosbo et al. 1995; Menon, Piddlesden et al. 1997; Costa, Divoux et al. 2003). Alternatively, EAE animal models for MS have also been successfully developed using MBP and PLP (Yasuda, Tsumita et al. 1975; Zamvil, Nelson et al. 1985; Kawamura, Yamamura et al. 2000; Fuller, Olson et al. 2004).

Non-CNS Antigens

Recent studies also indicate that reactivity against non-myelin proteins may also play a role in the pathogenesis of MS. Non-myelin proteins analyzed so far are heat shock protein (**Hsp**), transaldolase and the calcium-binding protein, S-100 (Kumar and Sercarz 1998; Martino and Hartung 1999; Hohlfeld and Wekerle 2001; Ozenci, Kouwenhoven et al. 2002).

Th-cells exposed to certain bacteria and/or viruses that share a similar amino acid sequence to traditional CNS-derived antigens can also activate T-cell receptors. This is known as “molecular mimicry” (Libbey, McCoy et al. 2007; Namaka, Kapoor et al. 2010). Molecular mimicry is the phenomenon by which structural similarities between viral and/or bacterial protein sequences result in the activation of specific T-cell epitopes. Although these disease-induced activated Th-cells are produced to destroy the causative organism, they are also capable of crossing the BBB and entering the CNS for a targeted attack on those myelin components displaying the same or similar amino acid sequencing via the

mechanisms discussed above (Ffrench-Constant 1994; Lucchinetti, Bruck et al. 1996).

MS and Genetics

A variety of factors has been associated with the predisposition and/or development of MS. For example, there is a gender preference (70-75%) for females between the ages of 15-29 years old with an average age of onset of 30 years (Confavreux, Aimard et al. 1980; Kurtzke, Page et al. 1992; Evangelou, Jackson et al. 1999; Jacobs, Wende et al. 1999; Coyle 2005). In addition, genetic factors have also been associated with the development of MS. Familial occurrence is not uncommon with a 20-40 fold-increased risk for first degree relatives (Warren, Cockerill et al. 1991; Kurtzke, Page et al. 1992; Compston 1999; Warren, Warren et al. 2003). Environmental factors also play a role with an increased prevalence north of the equator (Kurtzke 2000; Warren, Warren et al. 2003). Despite extensive research into the underlying pathophysiology of MS, the exact cause of MS remains elusive. However, it is currently believed to be a T-cell mediated autoimmune response that is influenced by genetic and environmental factors (Hauser and Oksenberg 2006; Ludwin 2006; Pleasure, Soulika et al. 2006; Rudick, Stuart et al. 2006; Boissy and Fox 2007).

MS Diagnosis

At the present, the diagnosis of MS relies on clinical features consisting of documented clinical attacks supported by additional laboratory and radiological tests such as MRI's (Poser, Paty et al. 1983; Paty 1997; McDonald, Compston et al. 2001). Despite the apparent diagnostic benefit of the MRI, a positive MRI alone is insufficient to confirm a diagnosis of MS. Accurate diagnosis of MS requires a clinical examination to confirm neurological deficits. Patients with a clinically definite diagnosis of MS must have a positive clinical disease history with at least two attacks that are disseminated in time and space. According to the McDonald diagnostic criteria for MS, each attack must last more than 24 hours and be separated from each other by at least one month (Lublin and Reingold 1996; Paty 1997; McDonald, Compston et al. 2001). There is no direct correlation between the numbers of lesions identified by the MRI and the clinical deficits displayed by the patient. MRI is, at best, a measure of disease activity (Miller, Albert et al. 1996; Weinshenker 2006).

The majority (80%) of MS patients suffer from the relapsing-remitting form of the disease (Poser, Paty et al. 1983; Paty 1997; Poser 1997; Namaka, Gramlich et al. 2004; Miao, Madec et al. 2008; Namaka, Crook et al. 2008). The use of immunomodulatory agents such as interferon β 1a or 1b products, or the use of amino acid polymers such as glatiramer acetate (Copaxone®) are administered to slow the overall disease progression and minimize the frequency and severity of MS attacks (Lucchinetti, Bruck et al. 2001; Pauly, Broll et al. 2002). However, it must be

remembered that irrespective of their potential benefits, these agents are not a cure. MS Patients treated with these immunomodulatory agents may continue to deteriorate and suffer a wide variety of disease-induced symptoms, such as urinary and bowel incontinence, fatigue, cognitive deficits, spasticity and NPP, that ultimately affects overall quality of life (Thompson 2001; Beiske, Pedersen et al. 2004; D'Amico, La Mantia et al. 2004).

Magnetic Resonance Imaging

The first MRI studies in patients with MS were reported in 1981, and since then, MRI has become an valuable tool in the diagnosis and follow-up of the disease (Paty 1997; Horsfield and Jones 2002; Boissy and Fox 2007). At present, it plays a major role in the understanding of the natural history of the disease and better defines lesion pathology. Normal and normal appearing white matter (**NAWM**) has a dark, low intensity signal on T2-weighted images (Mattson 2002; Jenkins and Thompson 2009; Mallam and Scolding 2009). The signal is caused by the relaxation of water molecules, which is facilitated by their proximity to proteins in the myelin sheath. In myelin, the water is bound between the myelin bi-layers (Laule, Leung et al. 2006). In MS plaques, myelin breakdown occurs and water is freed from its compartment. "Free" water has a longer relaxation time, producing an increased signal on T2-weighted images (Horsfield and Jones 2002). In addition to demyelination, a range of other pathological processes (edema, gliosis, and inflammation) can also increase the signal on T2-

weighted images. Consequently, MRI sensitivity in detecting abnormalities is adequate but not specific for MS (Filippi, Rovaris et al. 1998; Filippi, Tortorella et al. 1999; Rovaris and Filippi 1999; Matthews and Arnold 2001).

Gadopentetic acid (in the form of gadopentetate dimeglumine or **Gd-DTPA**) is a paramagnetic contrast agent for MRI used for more sensitive detection of MS plaques. The earliest event in the generation of an MS plaque is characterized by inflammation, which is visualized on MRI as a high intensity lesion with Gd-DTPA enhanced T1 weighted image and on T2 weighted image (Ford, Ceckler et al. 1990; Miller, Barkhof et al. 1993). Under normal circumstances, the inflammatory phase lasts several weeks to months. The temporary reduction in Gd-DTPA enhancement correlates well with clinical improvement and the decrease in myelin breakdown (Paty 1997; Rausch, Hiestand et al. 2003; Serres, Anthony et al. 2009). MS lesions frequently show an ellipsoid or ovoid shape on MRI and are primarily localized to periventricular areas, corpus callosum, the temporal lobes, infra-tentorium, optic nerve, and sub cortical U-fibers (Filippi, Rovaris et al. 1998; Karlik, Munoz et al. 1999; Rausch, Hiestand et al. 2003). Several studies have shown that this form of MRI detects 5-10 times more disease activity than is clinically suspected. As a result, this form of MRI has been extensively used to monitor disease activity in clinical trials (Filippi, Rovaris et al. 1998; Filippi 1999; Rovaris and Filippi 1999; Matthews and Arnold 2001)

Classifications of MS

The majority (80%) of patients are initially diagnosed with symptoms characteristic of relapsing-remitting MS (**RRMS**). RRMS is characterized by acute attacks followed by full or partial recovery and lack of disease progression in periods between the attacks (Mattson 2002). Interestingly, about 80% of RRMS patients go on to develop secondary progressive MS (**SPMS**). Secondary Progressive MS is characterized by a progressive course of the disease with or without relapses (McDonald WI, Halliday AM).

Primary Progressive MS is characterized by disease progression from theinitial presentation. Progressive Relapsing MS is characterized by disease progression from the onset of the symptoms with subsequent overlapping relapses (McDonald, Compston et al. 2001; Jenkins and Thompson 2009; Mallam and Scolding 2009).

Clinical Signs and Symptoms

Since MS “plaques” can occur in many parts of the CNS, there is a wide variety of signs and symptoms that characterizes the disease. The most frequent signs and symptoms at the initial presentation are:

1) sensory loss (30-50%) usually involving the legs and includes numb and tingling sensations,

2) optic neuritis, which is the initial presentation in up to 16% of patients and manifests as a decrease in visual acuity, sometimes associated with poor colour discrimination and pain with eye movement (O'Connor 2002; O'Connor and Group 2002),

3) fatigue, is experienced by 20% of patients and is frequently described as extreme tiredness (O'Connor 2002; O'Connor and Group 2002),

4) weakness and stiffness, mainly in the legs, which accounts for 10% of cases presenting with an initial complaint (Lublin and Reingold 1996; McDonald, Compston et al. 2001; O'Connor and Group 2002).

During the course of the disease, the frequency and variation of these symptoms increases, ultimately being experienced by the majority of patients. For example, fatigue is experienced by up to 90% of patients, optic neuritis is experienced by up to 65%, and sensory loss or weakness mainly in the legs is experienced in about 90% of patients (Lublin and Reingold 1996; O'Connor and Group 2002; Melanson, Grossberndt et al. 2010). In addition, cognitive deficits manifest in a high percentage (70%) of patients with MS. Characteristics of cognitive deficits include poor concentration, difficulty expressing themselves and poor short-term memory (Paty 1997; O'Connor 2002; O'Connor and Group 2002).

The most frequent signs and symptoms at initial presentation as listed by Paty and Ebers from MS Clinics in Canada, are sensory abnormalities (including

neuropathic pain: 30%), fatigue (20%), optic neuritis (16%), nystagmus (20%), vertigo (4-14%), gait disturbances (18%), sensory loss (30-50%), weakness in the legs (10%), and spasticity (10%) (Paty 1997; Namaka, Turcotte et al. 2008). Signs and symptoms seen at any time during the disease course are fatigue (80-90%), sensory abnormalities (including neuropathic pain) 70%, cognitive changes (70%), optic neuritis (65%), vertigo (about 50%), ataxia (50%), and sensory loss (90%) as well as weakness in the legs and spasticity (90%), euphoria (10–60%), depression (25–54%), optic atrophy (77%), retinal nerve fiber loss (80%), dysarthria (50%), limb ataxia (50%), ataxia of the gait and trunk (50–80%), cramps (50%), amyotrophy (50%), bladder disturbance (80%), and sexual disturbance (50% in women, 75% in men) (Lublin and Reingold 1996; Paty 1997; McDonald, Compston et al. 2001; O'Connor 2002; O'Connor and Group 2002; Namaka, Turcotte et al. 2008; Melanson, Grossberndt et al. 2010).

Drug Treatment

During the past decade, major advances in therapies of patients with RRMS have been developed. In Canada there are four main types of disease-modifying drugs offered to treat patients with MS. These treatments include IFNβ (Betaseron®, Rebif®, and Avonex®) and glatiramer acetate (Copaxone®) (Namaka, Pollitt-Smith et al. 2006; Boissy and Fox 2007; Namaka, Turcotte et al. 2008; Frost, Pillai et al. 2010). These therapies have a beneficial effect on specific disease measures, such as the frequency and severity of attacks and/or MRI activity. However, efficacy varies from patient to patient. On average, the

drugs decrease the frequency of attacks by approximately 30% as well as decrease the burden of T2 lesions on an MRI (Namaka, Pollitt-Smith et al. 2006; Rudick, Stuart et al. 2006; Boissy and Fox 2007; Rudick and Cutter 2007; Namaka, Turcotte et al. 2008). Tysabri® (Natalizumab) represents another new targeted treatment approach for RRMS (Johnson 2007; Di Pauli, Berger et al. 2009). Natalizumab is a humanized IgG4 monoclonal antibody that is known to block α4 integrin activity. It is administered i.v. at monthly intervals. Natalizumab blocks the activity of α4β1, thereby preventing the adherence of activated leucocytes to cEC of the BBB, a critical step in the infiltration of leukocytes into the CNS (Gordon, Hamilton et al. 2002; Correale and Villa 2007; Kappos, Bates et al. 2007). In addition, by inhibiting interactions between α4-integrin and its ligands, natalizumab may reduce immune-cell activation and promote the apoptosis of lymphocytes (Yednock, Cannon et al. 1992; Cannella and Raine 1995). The net effect is an inhibition of local inflammatory mediator migration and activity at the level of the BBB. Consequently, fewer T-cells are able to cross the BBB in MS patients treated with Natalizumab and fewer MS lesions develop. MS patients treated with Natalizumab have a significant reduction in the frequency of attacks and show significant improvements in lesion activity, as confirmed by MRI (Johnson 2007). This can be seen by the results of two large, randomized, double-blind, placebo controlled, phase-3, 24 month trials. The AFFIRM trial assessed natalizumab (300 mg iv every 28 days) as monotherapy and the SENTINEL trial assessed natalizumab (300 mg iv every 28 days) as add on therapy to Avonex 30 µg/week im (Polman, O'Connor et al.

2006; Rudick, Stuart et al. 2006). In the AFFIRM study, sustained increased disability after 2 years was reduced by 42% and annualized relapse rate was reduced by 68% with natalizumab therapy (both P<0.001 versus placebo). The proportion of relapse free patients at 2 years was 67% in the treatment group as compared to 42% in the placebo group (P<0.001). Also, an 83% reduction in mean numbers of new and enlarging lesions on T2 imaging was seen in the treatment group as compared to placebo group (P<0.001). In a 2 year period, no new or enlarging T2 lesions developed in 57% of the treatment group as compared to 15% of the placebo group. Further, the numbers of gadolinium enhancing lesions were decreased by 92% with natalizumab therapy (P<0.001) (Polman, O'Connor et al. 2006). Results of the SENTINEL study were similar. Combination therapy was found to reduce annualized relapse rates by 54% (P<0.001), to reduce new or enlarging lesions on T (2)-weighted magnetic resonance imaging by 83% and to reduce the mean number of gadolinium-enhancing lesions by 89% (P<0.001) (Rudick, Stuart et al. 2006).

Clinical Pathological Presentation

MS is characterized by the presence of hallmark areas of demyelination and axonal loss in the CNS. These characteristic lesions tend to be most common in certain areas of white matter, such as the periventricular area in the brain and cervical spinal cord. MS lesions are divided into three major types, including acute, chronic active and chronic silent (Rodriguez 1992; Trapp, Bo et al. 1999; Lassmann, Bruck et al. 2001; O'Connor and Group 2002; Kornek and Lassmann

2003; Ludwin 2006). Acute lesions are of recent origin. They are uniform in appearance and show intense inflammatory activity with early onset of demyelination and axon damage (Raine and Wu 1993). Chronic active lesions are already established. Their centers show little or no evidence of ongoing inflammatory activity. However, their borders are histopathologically similar to those of acute lesions (Rodriguez 1992; Ludwin 2006). Chronic silent lesions are the oldest lesions and show no significant evidence of ongoing inflammatory activity (Wolswijk 1998; Trapp, Bo et al. 1999; Ludwin 2006). Recently, the importance of axon injury in MS lesion development has been described (Trapp, Bo et al. 1999; Bitsch, Schuchardt et al. 2000). Using immunohistochemical (**IHC**) staining and confocal microscopy, injured (transected) axons have been shown to be a "consistent" feature of MS lesions (Trapp, Peterson et al. 1998; Trapp, Bo et al. 1999). This is particularly true for acute lesions and the peripheral borders of chronic active lesions. These findings are consistent with other results that have identified the expression of an amyloid precursor protein, which is a marker of axonal damage (Chalmers, Wilcock et al. 2005; Buttner, Rohrmoser et al. 2006; Hortobagyi, Wise et al. 2007). A putative marker of axonal integrity [N-Acetyl Aspartate (**NAA**)] has been shown to be reduced in MS lesions as well as surrounding NAWM and cortical grey matter, where it may be a precursor to lesion formation (Kapeller, McLean et al. 2001; Chard, Griffin et al. 2002; Sarchielli, Presciutti et al. 2002; Tiberio, Chard et al. 2006)**.**

MS Summary

MS is a chronic disease of the CNS characterized by areas of demyelination and axonal injury associated with inflammatory activity. The cause of this disease remains uncertain. However, epidemiological studies indicate that race, geography and environmental factors are involved in the development of MS (Compston 1999; Kurtzke 2000; Warren, Warren et al. 2003; Basso, Campi et al. 2004; Kalman and Leist 2004; Czlonkowska, Ciesielska et al. 2005; Ebringer, Rashid et al. 2005; Koch, Goedde et al. 2005; Ransohoff 2005). MS is present in approximately twice as many women as men, (Jacobs, Wende et al. 1999; Coyle 2005). Genetic factors play a significant role in MS as indicated by an increased risk of developing MS in close relatives to the patients (Kurtzke 2000; Warren, Warren et al. 2003).

Though the causative agent responsible for the development of the disease remains unknown, it is widely accepted that an autoimmune mechanism underlies the pathology of MS. The latter is supported by the presence of immune cells and elevated levels of immune cell products in the “MS plaques”. The sequence of events that leads to development of MS includes:

a) Th-cells in the peripheral blood become activated on interaction with a CNS myelin protein, or an antigenic protein that shares similar amino acid sequence homology to known CNS myelin proteins. Such interactions may occur in response to either viral or bacterial infection or exposure to some other unknown environmental stimulus.

b) The activated Th-cells attach to the BBB via adhesive molecules such as adhesins, selectins, and integrins. The clustering of activated T-cells at the BBB facilitates the release of inflammatory cytokines, which subsequently activate MMPs and compromise the integrity of the BBB. Thus facilitating Th-cell migration across the BBB and entry into the CNS.

c) Once inside the CNS, Th-cells become reactivated in response to CNS myelin proteins. These T-cells continue to secrete key mediators of inflammation that subsequently activate the phagocytic cells that will directly attack and destroy CNS myelin.

A key defining feature of MS is that lesions are disseminated in both space and time [i.e. they occur in different areas of the brain and spinal cord and occur at least twice any time in the patient's life (at least one month apart)] in order for a confirmatory diagnosis of RRMS. The most frequent symptoms are sensory abnormalities and weakness of the lower extremities.

MRI has provided a great improvement in the diagnosis of MS. MRI can identify and locate CNS lesions in living patients gadolinium (**Gd**) enhanced MRI's detect five to ten times more disease activity than is clinically suspected. Consequently, MRI has been used in clinical trials to monitor disease activity (Miller, Barkhof et al. 1993; Miller, Albert et al. 1996; Filippi, Rovaris et al. 1998). Currently, there are four main immunomodulatory therapies for patients with MS as discussed above (Frost, Pillai et al. 2010). These treatments decrease the

frequency of relapses by about 30% and decrease disease activity as indicated by burden of T2 lesions (Galetta, Markowitz et al. 2002). Despite the potential benefits of using the T-cell adhesion blocker Tysabri for RRMS, reports of progressive multifocal leukoencephalopathy in 1:1000 patients warrants its classification as a second line agent for RRMS until further safety data becomes available (Yousry, Major et al. 2006). Currently, there are no effective therapies used for PPMS or SPMS.

Dorsal Root Ganglia

The dorsal root ganglia (**DRG**) are located immediately adjacent to the spinal cord residing in between the vertebrae along the entire length of the vertebral column. The DRG house many subtypes of sensory neurons, which are responsible for relaying specific types of peripheral sensory stimuli to the spinal cord for subsequent processing in the brain. Specifically, the synaptic connections from primary afferents that link the PNS to the CNS via the DRG represent an integral pathway essential for connecting the brain with various environmental external sensory stimuli. Interestingly, the subpopulations of sensory neurons involved in this intricate signaling pathway rely on various different neurotrophic and non-neurotrophic factors for survival and maintenance of function. Some of these factors include nerve growth factor (**NGF**), brain-derived neurotrophic factor (**BDNF**), neurotrophin-3 (**NT-3**), neurotrophin-4 (**NT-4**) and glial cell line-derived neurotrophic factor (**GDNF**) (Bennett, Michael et al. 1998; Josephson, Widenfalk et al. 2001).

The dorsal root is a fascicle or bundle of axons, which connect the DRG to the spinal cord (Devor 1999). The roots enter the spinal cord at the dorsal root entry zone. The cells that form dorsal roots are a unique type of neuron called pseudo-unipolar sensory neurons. Pseudo-unipolar neurons have only one axon extending from their cell body. However, this axon divides into two a short length from the cell body. One end of the axon reaches out to the periphery and the other end extends in towards the spinal cord (Devor 1999). The cell bodies of sensory pseudo-unipolar neurons lie within the DRG. The dorsal roots convey afferent sensory information from the sensory neurons housed within the DRG, while the ventral roots carry efferent motor fibers that innervate skeletal muscles and autonomic effectors (Devor, Wall et al. 1984; Scallow 1989; Tandrup 1995; Devor 1999; Hiura 2000). In a similar fashion, the ventral root exit zone is an exit to the fascicles that form the ventral root. The cell bodies from which ventral roots originate are located in the grey matter of the spinal cord (Hung and Zhao 2003).

Sensory Neurons

The various subpopulations of DRG sensory neurons have been classified according to different parameters, such as neuronal diameter, axonal diameter, presence or absence of myelin, conduction velocity, and neurotrophin specificity (Devor 1999).

There are three main types of cutaneous afferent fibers including Aβ, Aδ and C. The Aβ afferents are large myelinated axons with a rapid conduction velocity of 30 – 100 m/sec. The Class Aδ consists of intermediate sized, thinly myelinated sensory axons with conduction velocity of 4 – 30 m/sec. The unmyelinated small diameter sensory axons, with slow conduction velocity of less than 2.5 m/sec, are the C fibers (Gasser 1950; Coleridge, Coleridge et al. 1973; Treede, Meyer et al. 1995; Treede, Meyer et al. 1998; Djouhri and Lauson 2004). Aβ fibers normally are responsive to the transmission of touch vibration, pressure and other modes of non-noxious, low intensity mechanical stimuli. They are not normally involved in the transmission of nociceptive stimuli. Conversely, Aδ and C fibers are able to transmit nociceptive information (Florian Beissner, et al. 2010). There are two main classes of Aδ fibers. The first class, known as Type I, is activated by high intensity mechanical stimuli in the noxious range (e.g. pinch). These mechanoreceptors are weakly responsive to high intensity heat, cold and chemical stimuli. However, repeated thermal stimulation may eventually render these receptors refractory to continued heat stimulus. Type II Aδ fibers have a much slower conduction velocity. These fibers have a lower threshold to noxious heat stimuli and are more responsive to cooling than to mechanical stimuli (Treede, Meyer et al. 1995; Treede, Meyer et al. 1998; Djouhri and Lauson 2004). The C fibers are activated by chemo-specific nociceptors, thermoreceptors as well as low threshold mechanoreceptors. The three subtypes of cutaneous afferents can also be classified according to the area they to which they project in the dorsal horn of the spinal cord (i.e. the Aβ fibers are

relayed to all regions of the dorsal horn except for the marginal layer). The mechanical and thermal noxious stimuli transmitted by Aδ fibers project to I, II, III, IV and V layers of the dorsal horn. Finally, the noxious stimulus transmitted by C fibers predominantly project to lamina I and II (Willis 1985; Nakanishi, Tamaki et al. 1986; Stankus, Dlugopolski et al. 2000; Namaka, Gramlich et al. 2004).

Size Classification

DRG neurons are relatively large, round cells that range from 20 to 150 µm in diameter (Devor 1999). DRG neurons are difficult to classify. Consequently, they are identified by neurotrophin specificity and by the soma size of the sensory cell body (Warrington and Griffith 1904). Three main classes of neurons have evolved that include:

- Small<25 µm
- Medium 25-50 µm.
- Large >50 µm.

The diameter of the cell body determines axonal size (Devor 1999). Axonal size determines extent of myelination (Duchen and Scaravilli 1977; Berthold, Nilsson et al. 1983; Windebank, Wood et al. 1985). Consequently,, in DRG, large cells have a large axon, which is usually myelinated while small cells have an unmyelinated axon (Duncan 1934). These two classifications generally correspond to A and C fibers, respectively. The medium sized neurons are generally classified as Aδ fibers. The surface of many DRG neurons, particularly

the larger ones, is coated with a dense layer of microvilli (Pannese 1981; Pannese, Ledda et al. 1990; Devor 1999). These are embedded within the lamella of ensheathing satellite cells, greatly increasing the membrane surface area of the neuron (Pannese 1981; Pannese, Ledda et al. 1990; Devor 1999). During early developmental stages, DRG neurons are in direct contact with one another (Fulton 1995; Devor 1999). In the early developmental stages, Schwann cell-like glia migrate into the spaces between the neurons, subsequently wrapping them in a sheath of satellite cells (Pannese 1981; Shinder, Admir et al. 1998; Devor 1999). As a result, the cell bodies of most adult DRG neurons are separated from one another by up to two layers of satellite cell cytoplasm. Satellite cells are integral for the trophic support for the enveloped neurons (Devor 1999).

Neurotrophin Classification of Neuronal subtype

There are five major distinct phenotypic subtypes of neurons in the DRG that transmit receptor-mediated stimuli of different sensory modalities, such as touch, temperature and pain. They differ based on neurotrophin specificity for selective receptor subtypes (Josephson, Widenfalk et al. 2001). Neurotrophins are a closely related family of proteins that were first identified as survival factors for sensory neurons (Skaper 2008). NGF was first identified in the 1950's (Levi-Montalcini and Hamburger 1951; Levi-Montalcini, Meyer et al. 1954), and BDNF was identified in the early 1980's (Barde, Edgar et al. 1982). Subsequently, two

other members of the family were identified and characterized, NT-3 and NT-4/5 (Ernfors, Ibanez et al. 1990; Skaper 2008).

NGF is essential for the survival, differentiation and maintenance of many sensory neurons (Skaper 2008). It is mainly synthesized and secreted by non-neuronal cells that are in contact with NGF-sensitive neurons. NGF is taken up by the target neuron at the nerve terminal and retrogradely transported along the axon to the cell body. The effects of NGF are mediated by both a low-affinity p75 receptor (**p75NTR**) and a high-affinity TrkA receptor (Silos-Santiago, Greenlund et al. 1995; Silos-Santiago, Molliver et al. 1995; Zheng, Stewart et al. 1995; Pezet, Onteniente et al. 1999; Li, Beng et al. 2000). The role of p75NTR remains unknown since most of the biological effects of NGF are observed in the absence of this receptor. In contrast, the TrkA receptor mediates most of the biological functions of NGF. Sensory neurons expressing TrkA are predominantly small and medium in size. In an adult rat DRG, TrkA-expressing neurons are reported to represent 40-45% of the total neurons found in the ganglia. Interestingly, the percentage of TrkA-expressing neurons appears to be higher during early stages of development and stabilizes as the animal reaches maturity.

BDNF is integral to the support and survival of a second subtype of sensory neurons (Barde, Edgar et al. 1982; Lindsay, Thoenen et al. 1985). It is structurally similar to NGF and other members of the neurotrophin family (Barde, Edgar et al. 1982). BDNF preferentially binds to the TrkB receptor and

preferentially supports the survival and development of intermediate size (25-40 μm) sensory neurons, which are myelinated afferent fibers. Two additional members of the neurotrophin family are NT-3 and NT-4/5. Like BDNF, NT-4/5 signals through the high affinity TrkB receptor, but is functionally different from BDNF. For example, BDNF is needed for the survival of vestibular afferents, whereas the action of NT-4/5 is necessary for the survival of D-hair receptors (Ernfors, Lee et al. 1994; Jones, Farinas et al. 1994; Conover, Erickson et al. 1995). In order for NT-3 to exert its effect, it must bind both the high affinity TrkC receptor and the low affinity p75 receptor (Barker and Murphy 1992). NT-3 supports the survival, development and maintenance of medium and large myelinated afferent fibers via TrkC mediated receptor interactions (Ernfors, Lee et al. 1994; Chalazonitis 1996; Snider and McMahon 1998; Guan, Puthenveedu et al. 2003). In addition to its specific functions within DRG sensory neurons, the signaling of NT-3 through the TrkC receptor is also required for the survival of the muscle proprioceptors, such as the Ia muscle spindle afferents, Ib Golgi tendon organ afferents, cutaneous mechanoreceptor afferents and Merkel cells (Ernfors, Lee et al. 1994; Farinas, Jones et al. 1994; Jones, Farinas et al. 1994; Fundin, Silos-Santiago et al. 1997; Butowt and von Bartheld 2001; Sieber-Blum, Szeder et al. 2004). As noted with the other neurotrophins, NT-3 is also transported from peripheral targets through connecting proprioceptive afferent fibers in a retrograde fashion to the cell body of NT3-TrkC responsive sensory neurons (Howe and Mobley 2005). This specific subtype of sensory cell predominantly displays immunoreactivity for neuropeptide Y, RT-97, the enzyme

carbonic anhydrase (**CA**) and parvalbumin. RT-97 is a monoclonal antibody that recognizes the 200 kDa subunit of a neurofilament protein characteristically expressed by large diameter sensory neurons (Ishii, Miyashita et al. 2004). The survival effects of NT-3 on sensory neurons found in ganglia are not restricted to NT-3/TrkC expressing neurons but also extends to that of both TrkA/NGF and TrkB/BDNF responsive neurons (Kaplan and Miller 1997).

Sensory neurons, which contain the GDNF receptor and the Ret receptor are the last major subpopulation of DRG sensory neurons that are primarily responsive to GDNF. GDNF is a member of the transforming growth factor-β (**TGFβ**) superfamily. It was initially isolated on the basis of its potent trophic effect on midbrain dopaminergic neurons *in vitro* (Lin, Doherty et al. 1993). This specific subclass of sensory neurons was identified during studies on early DRG development. During early development, all small DRG neurons require NGF for survival and express the NGF receptors, TrkA and p75 (Mendell 1995; Silos-Santiago, Molliver et al. 1995; Skaper 2008). However, by postnatal day 15 in the rat, a subset of small DRG neurons loses the expression of p75 and TrkA and the ability to transport NGF retrogradely (Verge, Merlio et al. 1992; Wright and Snider 1995; Molliver, Wright et al. 1997). Instead, these neurons begin to express GDNF receptors and are identified as the GDNF phenotypic subtype. In order to exert its maximal biological effect, GDNF must bind to a both high affinity Ret receptor and a low affinity GDNF receptor (Mason 1996). GDNF production is increased after peripheral nerve injury in nerves both distal to the site of injury

and in the DRG. GDNF provides trophic support to neurons (Bar, Saldanha et al. 1998; Popper, Lopez et al. 1999; Acosta, Fabrega et al. 2001; Zaccaro, Ivanisevic et al. 2001).

Microglia/Macroglia

Microglia, the immune cells of the CNS, are mobilized in response to injury, infection or disease (Benveniste 1997; Li, Field et al. 1997). Microglia are phagocytic cells, which facilitate non-specific immune reactions by ingesting and digesting foreign substances and/or cell debris. Neutrophils, monocytes (in the blood) and macrophages (in the tissue) are phagocytic cells that are also collectively known as APCs. APCs, such as macrophages, which exist in the DRG, are known to express MHCII. The presentation of this receptor on their cell surface is essential for the recognition and attachment to foreign antigens and represents a component essential to initiation of the immune response (Graus, Campo et al. 1990). The monoclonal antibodies, EBMII (a monoclonal antibody to human macrophages) and Leu-M3 (a monocyte differentiation antigen associated with the monocytelmacrophage maturation pathway) were found by some researchers to be good IHC markers for monocytes/macrophage lineage cells in all human DRG's (Gidlund, Rossi et al. 1988; Lendon, Davies et al. 1991). According to the specific morphological characteristics of the cells, those identified as immuno-positive for these markers were found to be elongated in shape and localized to interstitial tissue between satellite cells (Esiri and McGee 1986; Hutchins, Dickson et al. 1992; Williams, Bar-Or et al. 1992;

Raymon, Thode et al. 1999; Dodge, Rahimtula et al. 2002; Zhou and Zhao 2002).

In addition, other morphological sub-phenotypes of macrophages have been reported that include: ramified, amoeboid, and leipolar phenotypes. Alternatively, macroglial cells, such as Schwann cells and satellite cells, are also contained within DRG (Devor 1999).

Neuropathic Pain

NPP is a chronic pain syndrome of unknown etiology that has been associated with drug, disease or injury-induced damage or destruction to the sensory afferent fibers of the PNS, and/or central fibers of the CNS involved in the synaptic transmission of pain (Choi, Yoon et al. 1994; Koltzenburg and Scadding 2001; Zimmermann 2001; Campana and Myers 2003; Ji and Strichartz 2004; Miao, Melanson et al. 2004; Namaka, Gramlich et al. 2004; Romanelli and Espsotp 2004; Coutaux, Adam et al. 2005; Vanderah 2007; Costigan, Scholz et al. 2009; Namaka, Leong et al. 2009; Olechowski, Truong et al. 2009). This damage or destruction of sensory neurons leads to the abnormal synaptic rewiring of Aβ, Aδ, and C fibers at the level of the spinal cord, creating a state of chronic pain. This type of synaptic rewiring is referred to as central sensitization (Namaka, Gramlich et al. 2004; Vanderah 2007). During central sensitization, drug, disease or injury-induced destruction of C fibers results in reduced synaptic connections to lamina II of the spinal cord. As a result, Aβ fibers, which are not

normally involved in nociception begin to form collateral sprouts, which innervate the vacant areas of lamina II previously occupied by C fibers. In addition, these collateral sprouts also undergo a phenotypic change with synthesis of the same nociceptive chemical messengers as those synthesized by Aδ and C fibers. They include substance P and calcitonin gene related peptide (**CGRP**). This dual change results in the conversion of normal touch or movement signals to that of intense chronic pain, creating a state of allodynia and hyperalgesia (Millan 1999; Vanderah 2007).

Regardless of cause, the characteristic clinical symptoms of NPP include the feeling of pins and needles, burning, shooting, throbbing and/or numbness (Koltzenburg and Scadding 2001; Zimmermann 2001; Romanelli and Espsotp 2004; Costigan, Scholz et al. 2009). The relative ease of confirmatory diagnosis based on these classical symptoms may often be followed by frustration in selecting an appropriate and effective treatment strategy that is flexible to the dynamic needs of the patient. This frustration has lead to the realization that the pharmacological management of NPP has proven to be a difficult task (Barkin and Fawcett 2000; Moulin 2006). Though the primary goal is to alleviate pain, clinicians recognize that the most appropriate treatment strategy may only be able to reduce pain to a more tolerable level. Despite the numerous treatment options available, residual pain still remains problematic (Barkin and Fawcett 2000; Moulin 2006). Since standard analgesics provide only temporary and/or partial relief, other alternative, off-label agents have been tested by clinical trial.

Alternative treatments have targeted the molecular mechanisms underlying the pain-processing loop, by either suppressing neuronal excitability at the level of the spinal cord or by potentiating the effects of the various anti-nociceptive substances released via the central descending systems (Barkin and Fawcett 2000; Moulin 2006).

The Role of TNFα in Neuropathic Pain

Inflammatory cytokines, such as TNFα, have been shown to play an integral role in facilitating the development of NPP (Junger and Sorkin 2000; Vogel, Lindenlaub et al. 2000; Inglis, Nissim et al. 2005; Spengler, Sud et al. 2007). Several studies involving the use of cytokine inhibitors, knock-out mice, or direct application of cytokines with subsequent investigation of electrical activity and behavioral changes, support the involvement of TNFα in the development of chronic *NPP* (Sommer, Schmidt et al. 1998; Vogel, Lindenlaub et al. 2000; Zhang, Li et al. 2002; Schafer, Svensson et al. 2003). TNFα acts via two receptors, the p55 TNF receptor type 1 (**TNFR1**) and the p75 TNFα receptor 2 (**TNFR2**) (Barbacid 1995; Baud and Karin 2001). Both receptors are present in rat DRG, though the cellular distribution remains controversial (Pollock, McFarlane et al. 2002; Schafers, Sokin et al. 2003). However, specific increases in neuronal TNFR1 expression were reported following the administration of intraperitoneal (**i.p.**) lipopolysaccharide (**LPS**), suggesting a direct effect for TNFα on nociceptive pathways via TNFR1 (Li, Ji et al. 2004).

Further, following peripheral nerve axotomy, TNFα is produced in response to Wallerian degeneration contributes to the damage of sensory neurons via the mitogen-activated protein kinases (**MAPKs**) and NF-κB pathways (**Figure 2**) (Baud and Karin 2001; Shamash, Reichert et al. 2002; George, Buehl et al. 2004; Ji and Strichartz 2004). Studies using both NF-κB cytoplasmic and nuclear staining indicate nuclear activation (Ma and Bisby 1998), and $p38^{MAPK}$ as a second messenger of IL-1β and TNFα receptor activation (Myers, Sekiguchi et al. 2003), suggesting alternative pathways by which TNFα may exert its destructive effects on sensory neurons. This is confirmed using activating transcription factor three (**ATF-3**) immunoreactivity as a marker of neuronal injury (Tsujino, Tsujino et al. 2000; Zelenka, Schafers et al. 2005) (**Figure 2**). In addition, the effects of TNFα have also been linked to the intracellular signaling pathways that play a role in the pathogenic activation of DRG cells following inflammation or injury (Takahashi, Kikuchi et al. 2006). TNFα has also been shown to directly induce neuronal production of neuropeptides and inflammatory agents such as substance P and CGRP in the DRG and spinal cord (Ohtori, Takahashi et al. 2004). The effects of CGRP and Substance P on NPP are well known (Hokfelt 1991; Ding, Hart et al. 1995).

Alternatively, TNFα has also been reported to sensitize nociceptive neurons indirectly via the induction of a pro-inflammatory cytokine cascade involving IL-1β, IL-6, and IL-8. This cytokine cascade results in the release of prostaglandins

and other inflammatory mediators from immune cells (Cunha, Lorenzetti et al. 1991; Ferreira, Lorenzetti et al. 1993; Woolf, Allchorne et al. 1997; Scholz and Woolf 2007; Woolf and Ma 2007). In addition, TNFα is also capable of directly inducing pain. For example, when TNFα was injected directly into the sciatic nerve it was found to cause pain (Wagner and Myers 1996; Junger and Sorkin 2000). Electrophysiological studies have confirmed the effects of TNFα in inducing pain. Subcutaneous (s.c.) injections of low dose TNFα have been shown to induce ectopic activity in nociceptive neurons within 2 minutes, while at higher doses, TNFα was found to produce significant mechanical and thermal hyperalgesia by 15 minutes (Sorkin, Xiao et al. 1997; Junger and Sorkin 2000; Zelenka, Schafers et al. 2005). Interestingly, studies examining the time course of TNFα activity within the DRG and spinal cord have identified a transient period of elevated TNFα expression, which is much shorter than that required for injury evoked mechanical allodynia and thermal hyperalgesia (Xu, Xin et al. 2006). Consequently, TNFα is thought to be responsible for the initiation rather than the maintenance of NPP (Ji and Strichartz 2004).

While the molecular mechanisms underlying MS-induced NPP are poorly understood, the role of inflammatory cytokines in injury-induced models of NPP is better known (Baud and Karin 2001; Ammit, Lazaar et al. 2002; Myers, Sekiguchi et al. 2003; van Vliet, Bukczynska et al. 2005). TNFα is recognized as a principle modulator of early degenerative changes involved in the initial induction of pain that occurs following peripheral nerve injury (Mayers and

Johnson 1998; Ruohonen, Jagodi et al. 2002; Shubayev and Myers 2002; Schmeichel, Schmelzer et al. 2003; Miao, Madec et al. 2008). The induction and maintenance of NPP appears to be regulated at the level of transcription with activation of a distinct set of genes, such as those that encode TNFα, BDNF, galanin, NPY, in injured DRG sensory neurons (Mayers and Johnson 1998; Ruohonen, Jagodi et al. 2002; Shubayev and Myers 2002; Schmeichel, Schmelzer et al. 2003; Miao, Madec et al. 2008). TNFα and IL-1β are thought to be integral components in the upstream initiation of persistent NPP, while IL-6 is thought to have an important role in the maintenance of NPP (Mayers and Johnson 1998; Ruohonen, Jagodi et al. 2002; Shubayev and Myers 2002; Schmeichel, Schmelzer et al. 2003; Miao, Melanson et al. 2004; Miao, Madec et al. 2008). Interestingly, TNFα is known to stimulate the secretion of IL-6, providing further evidence for its role as an upstream effector of pain induction (Baud and Karin 2001; Lee, Lee et al. 2004). Further, it is known that the binding of TNFα to its receptors causes activation of two major transcription factors, NF-κB and AP-1, and that in turn activates genes involved in chronic and acute inflammatory responses (Baud and Karin 2001; Lee, Lee et al. 2004). Studies using TNFα antagonists following peripheral nerve injury showed dramatic reduction of injury-induced mechanical allodynia, providing further evidence for the critical role of this cytokine in the induction of NPP (Svensson, Schafers et al. 2005; Kato, Liu et al. 2009).

The published temporal cytokine expression profiles associated with NPP differ according to the tissue type and location, and the injury or disease model used. For example, studies have shown that the induction of cytokines in the DRG appears to be more rapid than in the spinal cord in two different models of NPP (Lee, Lee et al. 2004; Melanson, Miao et al. 2009). This suggests that cytokines play different functional roles in the context of the intricate cell signaling pathways involved in the development of NPP. The differential expression of cytokines in the DRG may account for the tremendous variation in type, severity, intensity and location of clinical sensory symptoms described by patients that often makes the confirmatory diagnosis of NPP problematic. Interestingly, the cytokine profile associated with NPP is similar to that associated with the early inflammatory stage of MS (Cannella and Raine 1995; Millan 1999; Ransohoff 1999; Filippi and Rocca 2003; Poisbeau, Patte-Mensah et al. 2005; Fox, Kivisakk et al. 2006; Giovannoni 2006; Pleasure, Soulika et al. 2006; Bar-Or 2008). However, the up-regulation of inflammatory cytokines in DRG and/or spinal cord leading to NPP in nerve injury models of NPP, does not lead to demyelination and subsequent induction of MS. This may be attributed to the localized production of cytokines in response to a focal injury, rather than activation of the entire immune system that occurs during the early stages of MS (Benveniste 1997; Rausch, Hiestand et al. 2003; Hauser and Oksenberg 2006). Based on the important role of TNFα in MS and NPP, it has also been hypothesized that MS patients suffering a peripheral nerve injury may be at a significantly higher

risk for accelerated progressive of their MS (DeLeo, Rutkowski et al. 2000; Ji and Strichartz 2004).

The specific cell signaling pathways that link inflammatory cytokines to both MS and NPP is not the only factor relevant to this hypothetical link between both chronic diseases. For example, the highly vascularized and permeable DRG facilitates the bi-directional transport of blood-borne inflammatory cytokines with the DRG. The proteins can subsequently be transported to the spinal cord via the dorsal root point of entry in the dorsal horn of the cord (Altar and DiStefano 1998; Devor 1999; Shubayev and Myers 2002; Obata and Noguchi 2006; Ng, Chen et al. 2007; Wang, Meng et al. 2008). As a result, we have developed a model to describe how the DRG may function as a pivotal reservoir for MS-induced inflammatory cytokines (Melanson, Miao et al. 2009; Zhu, Frost et al. 2010). We hypothesize that besides their direct effect on sensory neurons of the DRG, these cytokines also undergo *axoplasmic anterograde transport* into the CNS via the dorsal root port of entry. Once in the spinal cord they subsequently facilitate induction of pain at the dorsal horn level of the spinal cord (Altar and DiStefano 1998; Obata and Noguchi 2006; Salio, Averill et al. 2007; Wang, Meng et al. 2008). Although there is no dispute that Th1 activation is a critical event in MS pathogenesis, the indirect effects of liberated inflammatory cytokines such as TNFα on the DRG and spinal cord have not been studied as a potential source for MS-induced NPP.

MS Induced Neuropathic Pain

Pain is a well-known characteristic of MS. Despite many years of research, the nature, scope and impact of MS associated pain remains unclear. Numerous studies have attempted to clarify the role of pain in the disease process of MS. However there is substantial variation in the literature on the extent and prevalence of MS associated pain (Ehde, Osborne et al. 2006). The most common site of pain (74% of adult MS patients) is the legs, with 59% of patients reporting pain in the lower back, 52% the neck and 49% the shoulders. Pain in the hands and feet are the next most common anatomical sites with 48% and 47% respectively (Ehde, Osborne et al. 2006; Kenner, Menon et al. 2007). *Studies have shown that NPP, such as trigeminal neuralgia, may be an early indicator of MS* (Sommer, Schmidt et al. 1998; Barker, Middleton et al. 2001; Hermann, Rogers et al. 2001; Zimmermann 2001; Marchettini, Formaglio et al. 2006; Kenner, Menon et al. 2007).

Approximately 75 % of all MS patients suffer NPP, placing it as the 2nd worst disease induced symptom (Svendsen, Jensen et al. 2003; Beiske, Pedersen et al. 2004; Ehde, Osborne et al. 2006; Kenner, Menon et al. 2007; Jones, Pohar et al. 2008; Osterberg and Boivie 2009). NPP syndrome has moved to the forefront of priority concerns, warranting its classification as a clinically recognized priority initiative for patients with MS (*http://www.mult-sclerosis.org/ms_world.html)*. In addition, the identification of NPP as a *potential diagnostic marker* of the early stages of MS is an intriguing concept, which could well lead to earlier diagnosis

of MS. Earlier diagnosis could well lead to earlier treatment with immunomodulatory agents, which would result in decreased disease progression and increased patient health and well-being.

There is significant evidence for neuroimmune modulation of pain (Keller, Beggs et al. 2007; Abbadie, Bhangoo et al. 2009; Olechowski, Truong et al. 2009; Leung and Cahill 2010). In addition, inflammatory cytokines have been implicated in the development of MS (Lindberg, De Groot et al. 2001; Jurewicz, Matysiak et al. 2003; Melanson, Miao et al. 2009). Thus, it is feasible that an experimental animal model for MS may represent an optimal model for understanding the pathophysiology of NPP (Racz, Nadal et al. 2008).

During normal sensory functioning, the DRG is involved in the regulation and maintenance of sensory homeostasis. However, throughout the relapsing remitting course of MS, rapid and sustainable bursts of inflammatory cytokines from within DRG may serve as the abnormal stimulus that eventually disrupts this sensory equilibrium (Groves, Barnett et al. 1993; Feiguin, Ferreira et al. 1994; Hermann, Rogers et al. 2001; Gabay and Tal 2004; George, Buehl et al. 2004; Fernyhough, Smith et al. 2005). This response may be further augmented by the highly permeable capillaries that surround the DRG, which permits the bi-directional transport of cytokines between circulating blood and DRG. The DRG is primed by the activation of the peripheral immune system. This leads to sensory neuron production of inflammatory cytokines and neurotrophins that are

transported along the dorsal root to the spinal cord (Obata and Noguchi 2006). Spinal cord neurons are activated by the anterogradely transported inflammatory mediators, leading to activation of pain signalling pathways (Millan 1999; Coutaux, Adam et al. 2005; Geranton, Fratto et al. 2008). This model represents a very plausible and novel pathway for the development of MS-induced NPP (Melanson, Miao et al. 2009). According to this MS model of NPP, the DRG could function as a pivotal reservoir and relay station for MS-induced inflammatory cytokines, accounting for direct effects on sensory neurons and the subsequent DRG induced effects on dorsal horn neurons (Portenoy, Yang et al. 1988; Jakeman, Guan et al. 2000; Obata and Noguchi 2006).

Base on this intriguing concept, we developed a model for MS-induced NPP, see **Figure 3** (Melanson, Miao et al. 2009). According to our model, antigen induced activation of Th1 cells in the peripheral blood results in the elevated production of inflammatory cytokines such as TNFα, IL-12 and IFNγ. As the activated Th-cells and/or cytokines migrate from the blood to the DRG, they interact directly with the sensory neurons. The cytokines produced within the DRG can then undergo axoplasmic transport directly into the CNS via the dorsal root port of entry to the spinal cord where they can participate in direct cellular interaction with dorsal horn neurons (Zhu, Frost et al. 2010). Based on this model, the antigen-induced surge in cytokine production and subsequent interaction with the sensory and dorsal horn neurons may catalyze the initial step

involved in the development of chronic NPP that accounts for peripheral and central sensitization known to occur in patients with MS.

We thus developed the hypothesis that “MS induced inflammatory cytokine mRNA expression levels are elevated within DRG leading to a subsequent increase in the respective inflammatory protein which can be transported into the CNS via axoplasmic anterograde transport through the dorsal root port of entry, for subsequent activation of dorsal horn neurons of the spinal cord”.

Preliminary IHC studies in the DRG of human MS patients showed apparent increases in TNFα protein expression compared to age matched non-MS control tissue, shown in

APPENDIX I. These initial studies in humans formed the impetus to launch a full investigative research study in an EAE animal model of MS.

There is no dispute that Th1 activation is a critical event in the pathogenesis of MS (Prineas 1975; Portenoy, Yang et al. 1988; Balashov, Smith et al. 1997; Martino and Hartung 1999; Sorensen, Tani et al. 1999; Comi, Leone et al. 2000;

Ziemssen, Neuhaus et al. 2001; Ziemssen, Kumpfel et al. 2002; Harp, Lovett-Racke et al. 2008; Hohlfeld, Meinl et al. 2008; Zeis, Graumann et al. 2008; Ingram, Hakobyan et al. 2009; Prendergast and Anderton 2009; Skihar, Silva et al. 2009; Segal 2010). The indirect effects of liberated inflammatory cytokines on DRG and spinal cord have not been studied as a potential source for MS-induced NPP. We undertook further studies into the cellular mechanisms involving MS induced cytokine expression and NPP using the well established EAE animal model of MS. This study was designed to investigate the temporal changes of TNFα expression in the DRG and/or spinal cord of EAE animals and to correlate these changes with neurological disability scoring used to detect the onset of early inflammatory stage of MS.

OVERALL AIM

To determine if the gene and/or protein expression levels of TNFα are up-regulated within the DRG and/or spinal cord of Lewis rats induced to a state of encephalomyelitis, and to correlate these changes with the temporal changes in neurological disability scoring.

HYPOTHESIS

The elevated production of inflammatory cytokines in the early inflammatory stages of MS are responsible for the initial activation of DRG and/or spinal cord

that facilitate the downstream cellular cascade of events involved in the underlying pathogenesis of MS.

EXPERIMENTAL DESIGN

To test our hypothesis we induced EAE in female adult Lewis rats. Female Lewis rats were specifically chosen for this study due to the known higher incidence of MS in females compared to males (Kurtzke 2005; Namaka, Turcotte et al. 2008).

EAE is the primary animal model utilized to study MS (Swanborg 1988; Link, Soderstrom et al. 1994; Petry, Boullerne et al. 2000). It shares several similarities with human MS including an MHC linked susceptibility, female predominance, the presence of myelin-reactive T-cells and antibodies in the CNS, and associated elevation of inflammatory cytokines such as TNFα and IFN-γ (Link and Xiao 2001). Based on our experimental design, we induced EAE via MBP (Gold, Hartung et al. 2000). This specific model was chosen as it allows us to explore the role of TNFα in the acute antigen induced inflammatory phase which occurs prior to demyelination. According to our in house experience with this model, by day 9, animals receiving the full inoculation, start to develop clinical symptoms of EAE, but symptoms are usually mild (tail weakness or paralysis), if present at all (Swanborg 1988) (**Figure 4**). However, by day 12 to

13 all animals experience a full range of clinical symptoms that include hind-limb paralysis (Hillel Panitch and Carlo Ciccone 1981; Stepaniak, Gould et al. 1995).

Animals were sacrificed at specific, predetermined time points (3, 6, 9, 12 and 15 days post disease induction). These time points represent the onset, progression and remission stages of acute inflammation. Specifically, 3 animals were sacrificed at each time point, to assess the levels of TNFα gene and protein expression in the DRG and spinal cord. The entire spinal cord, and the DRG along the entire length of the cord, was removed for immediate RNA isolation by qRT-PCR analysis. The changes in TNFα gene expression were correlated with changes disease progression, as depicted via the neurological disability scoring. For protein expression studies, animals were perfusion fixed and tissue was cryopreserved for subsequent immunohistochemical analysis.

RESEARCH METHODS

Development of Lewis rat model of EAE

We established an animal model of EAE for this study, using a previously described MBP Lewis rat model (Hillel Panitch and Carlo Ciccone 1981). This model uses Freund's incomplete adjuvant (Sigma-Aldrich) and pertussis toxin (**PT**) during immunization. (Ahn, Kang et al. 2001). These co-injections of PT and CFA are used to prime the immune system. Priming the immune system

results in increased incidence and severity of the disease induced by immunization with neuroantigens.

A total of 66 adult female Lewis rats (Charles River, Pointe-Claire, QC) were divided into 3 experimental groups (see **Table 1**): naïve control, active control and active EAE (**aEAE**). Naïve control animals (n=6) did not receive any injections. In order to prime the immune system, active control animals (n=30) received two i.p. injections of PT (0.3 µg in 200 µl of phosphate buffered saline (**PBS**) at the pre-determined time point's day 0 & 2. In addition, these animals also received 2 x 50 µl s.c. injections of Freund's incomplete adjuvant (**FA**) + 500 µg of *Mycobacterium Tuberculosis* (**MT**) H37Ra + saline into the area above the base of the tail at day 0. *aEAE animals* (n=30) received the same PT regimen administered to *active controls* plus full inoculation with FA + 500 µg MT + 100 µg Guinea pig MBP given as 2 x 50 µl s.c. injections into the area above base of the tail.

Neurological Disability Analysis

All aEAE animals were assessed for neurological disability every day post disease induction, and compared to pre-disease induction neurological parameters. To reduce inter-operative differences, the neurological disability scoring was conducted 3 times for each animal, on three separate occasions throughout each day. Disability scoring was conducted by the lab technician Mrs. Kim Madec. Disability was assessed according to the parameters detailed in Table 2.

All animal experiments were conducted in accordance with protocols approved by the University of Manitoba Protocol Management and Review Committee in full compliance with the Canadian Council on Animal Care.

General Histological Analysis

Female Sprague Dawley rats were deeply anaesthetized using an i.p. injection of Ketamine (Biospacific Emeryville, CA) 30mg/100g and Xylazine (Bayer Health Care, Toronto, Ontario) 3 mg/100g body weight diluted in saline. Full body perfusion was performed via intra-cardiac cannulation using a pre-fixative solution containing 1 U/ml heparin (LEO Pharma Inc., Thornhill, Ontario) and 1% sodium nitrate (ThermoFisher Scientific, Ottawa, Ontario) in 0.9% sodium chloride (Sigma Aldrich, Oakville, Ontario) at a volume equal to 1/3 of the animal's body weight. The animals were then perfused with a 4% paraformaldehyde (Sigma Aldrich) in 0.1% $NaPO_4$ (ThermoFisher Scientific) fixative buffer at a volume equal to twice the body weight of the animal. The whole vertebral column was removed, dissected free of surrounding soft tissue, and fixed in 4% paraformaldehyde for 24 hrs at 4°C. Tissue samples were then washed in PBS for 24 hours and placed into a 10% sucrose storage solution in 0.05 M phosphate buffer and stored at 4°C until processed (Begum, Zhu et al. 2010). The DRG and spinal cord were dissected free of the lamina, embedded in OCT, and frozen on dry ice. Samples were stored at -80°C. Lumbar spinal cord

and DRGs were cryostat sectioned at a 10 µm thickness. Sectioning was performed by Mrs. Kim Madec, laboratory technician.

Immunofluorescent Analysis

Polyclonal antibodies against TNFα (1:100; R&D Systems), were used according to previously established in house procedures (Namaka, Sawchuk et al. 2001). In order to determine the cellular source (s) of TNFα production, triple-labeled immunofluorescence with monoclonal antibodies against neurofilament-160 (NF-160) (1:40; Sigma) or the neuronal nuclear marker NeuN (1:1000; Chemicon) and satellite cell polyclonal antibodies against GFAP (1:100; R&D Systems) were used in conjunction with the cytokine marker for TNFα. All primary antibodies were added at the specified concentrations in conjunction with the appropriate blocking serum as determined by the host of the secondary antibody. The sections were immersed in a combination of primary antibody, 1% blocking serum and PBS-T (0.3 % Triton-X-100) and were incubated at 4°C for 48 hours in an incubation chamber. The tissue was then washed in PBS-Tween (**PBS-T**) for 3 x 30 minute prior to the addition of specific secondary antibodies (FITC 1:50; Jackson Laboratory; Alexa-568 1:500; Molecular Probes) with 1% blocking serum. Sections were incubated in secondary antibody for 1.5 hours at room temperature. The slides were then washed once for 20 minutes with PBS-T and 2 x 20 minutes with 50 mM Tris-HCL. Following the application of one drop of mounting medium, the slides were cover-slipped for subsequent imaging

using an Olympus IX70 microscope, coupled to a monochrome digital camera and computerized image capturing system (described below).

Image analysis

Cell counting was performed in a double-blinded procedure. Tissue was sectioned and slides labeled using a coding system by the first researcher (Mrs. Kim Madec). IHC staining was then performed on each set of slides, and images collected using Image Pro Plus software v 7.0.1 (MediaCybernetics, Bethesda, MD, USA) by the second researcher (Dr. Maria Melanson). Cell counts were then performed by the first researcher, on images coded by the second researcher. Results were decoded, and cell count analysis was then assigned to the correct tissue sample prior to statistical analysis.

Fully Quantitative Real Time PCR Analysis

Fully Quantitative RT-PCR was used to allow us analysis of gene expression from a very small amount of RNA. It also allows us to obtain highly specific, sensitive and reproducible data. The DRG and spinal cord from the remaining 33 animals were harvested (within 30-45 minutes from the time of death) for subsequent qRT-PCR analysis (see **Table 1**). Specifically, 3 animals from each of the *active control* and *aEAE* groups were sacrificed at each pre-determined time point (day 3, 6, 9, 12 & 15) with 3 additional animals being sacrificed from the *naïve control* group at the age equivalent to day 15.

*Ribonucleic acid (**RNA**) isolation and purification:* Total RNA was isolated according to the manufacturer's recommendation outlined for TRIzol LS (Invitrogen). All tissue to be analyzed was placed in TRIzol LS solution and homogenized by Polytron. RNA was then extracted by phenol/chloroform and precipitated by ethanol.

RT-PCR for reverse transcription

Total RNA was used for the synthesis of 1st strand complementary deoxyribonucleic acid (**cDNA**) by RT-for PCR kit (Clontech). 1 µg of total RNA was mixed with Oligo (dT) primers, dNTP and reverse transcriptase. The reaction mix was then incubated for 1 hour at 42°C. At the end of the procedure, the reaction solution was heated to 94°C for 5 minutes to deactivate the enzyme.

Quantitative RT-PCR

During the PCR amplification of *TNFα* gene, 50 ng of the 1st strand cDNA solution was used per reaction. The PCR reaction was performed using a Light-Cycler-DNA master SYBR green 1 kit (Roche). The specific primer pairs for *TNFα* (Genbank No. NM AF329982) were synthesized by Invitrogen. The quantitative PCR reaction was set according to the manufacturer's instructions. The PCR cycle was 94°C for 2 minutes followed by 25 cycles of denaturing at 94°C for 30 seconds, annealing at specified Tm temperatures for 30 seconds and

elongation at 72°C for 1 minute followed by a final elongation step at 72°C for 5 minutes using a Roche Light-Cycler.

Statistical analysis

Data analysis was performed using GraphPad Prism version 4.08 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

All the data were tested with one way analysis of variance (ANOVA) model to compare the means of all groups based on two independent variables: day and group. The process design was to detect the difference between the main effects by day and group values and also to look at the interaction effect of day X group values.

Post-hoc analysis was conducted using Tukey's test of a single step multiple comparison in conjunction with the ANOVA in order to determine which means were significantly different from one another. The criteria of $P<0.05$ was considered a significant difference.

Differences between means were assessed using unpaired Student's *t*-test, or a paired Student's *t*-test where applicable. The paired *t*-test was used to compare changes in data obtained from the same sample over a period of time, or after specific treatments. The unpaired *t*-test was used when data was obtained from two separate independent samples.

RESULTS

Global neurological disability assessments

All animals in the aEAE groups were assessed for neurological disability according to the global neurological disability assessment tool. Neurological disability clinical assessment scoring was conducted in accordance to previous outlined methods described in **Table 2.** EAE animals were assigned a summated clinical score based on the cumulative total score assigned to each of the six clinical assessment sub-domains outlined in **Table 2** that include tail, bladder, right forelimb, left forelimb, right rear limb and left rear limb. Prior to day 9 post-EAE induction, all animals displayed no clinical neurological deficits thereby scoring 0 at days 0, 3 and 6 respectively (**Figure 4**). However, by day 9 animals started to display clinical signs of neurological disability that progressively worsened upon daily assessment scoring. Reaching peak disability at day 12, with clinical scores subsequently subsiding by day 15 as the animals entered the remission phase of disease (**Figure 5** and **Figure 4**). Interestingly, the bell shaped distribution of the peak global neurological disability scores at the various time points outlined in

Figure 4, appear to share the same trend that was suggested by the preliminary IHC analysis depicted in **Figure 7**, **Figure 8**, and **Figure 9**, which suggests peak TNFα immunoreactivity at day 12 post EAE induction. Data was

analyzed using ANOVA followed by Tukey's Multiple Comparison Test. Tukey's tests all possible 2-way comparisons between the multiple groups in the analysis. This analysis showed that values obtained for day 9 (effectively normal levels) were not significantly different from day 10, but were highly significantly different from days 11 and 15 ($p<0.01$), and very highly significantly different from days 12, 13 and 14 ($p<0.001$). Day 11 neurological disability was numerically the highest, and Tukey's test showed that day 13 scoring differed very highly significantly from days 9, 10, 11 and 15 ($p<0.001$), but was not significantly different from days 12 or 14 (**Figure 5**).

Immunohistochemical analysis of TNFα in DRG

Comparative IHC analysis was conducted on 10 µm cryostat sections of spinal cord and DRG obtained from aEAE, naïve control and active control animals. Lumbar tissue was chosen as the initial symptoms appear in the tail and hind limbs, suggesting lumbar spinal cord and DRG involvement.

Western blot analysis of the TNFα antibody (**Figure 6**), confirmed the specificity of the TNFα antibody used in the IHC analysis. This antibody detected the 17 kDa isoform of TNFα, which acts via the TNFR1, in the tissue being analyzed (Cawthorn and Sethi 2008; Chadwick, Magnus et al. 2008).

Comparative IHC analysis of aEAE versus active control animals euthanized at 6, 9, 12 and 15 days post induction (**dpi**), revealed more pronounced TNFα immunoreactivity in the aEAE animal tissues at days 6 through day 15 relative to that displayed in the active control group at the same time points (**Figure 7**). Interestingly, we showed increased immunoreactivity for TNFα in the cytoplasm of the sensory neurons as well as in the satellite cells of the DRG. The overall increase in TNFα immunoreactivity in the satellite cells and neuronal cells appears to reach a peak around 12 dpi (**Figure 8**). Due to the apparent increase for TNFα immunoreactivity at 12 dpi, further comparative analysis amongst the experimental groups was conducted with naïve control DRG samples (**Figure 8**).

The results of this comparative analysis among animal groups confirmed pronounced TNFα immunoreactivity in the aEAE day 12 group relative to the other 2 experimental groups at the same time point. Specifically, the images depicted for the aEAE group in the lower panel of **Figure 8** at a total magnification of 200X and the images depicted in **Figure 9 (**400X total magnification) reveal enhanced TNFα immunoreactivity from both neuronal and non-neuronal cell types (as described above). However, the pronounced TNFα immunoreactivity depicted in the aEAE day 12 group relative to all other groups appears to have resulted from increased neuronal expression of TNFα. For example, based on a double blind analysis of the aEAE group relative to both control groups (naïve and active control), we show that the percentage of

neurons identified as TNFα positive (+) increased from 45.36% (day 6) to a relative peak of 81.28% at 12 dpi, which ultimately subsided back down to 65.97% by 15 dpi (**Figure 10**). However, the same increase in neuronal expression of TNFα was not seen in the active control groups as depicted in 10 **Figure 10**, **Figure 11**, and **Figure 12**. A sub-analysis of the TNFα+ neurons identified in the aEAE group reveal a predominant cell size distribution that is reflective of small diameter (<30 μm) sensory neurons which corresponds to C and Aδ fibers respectively (**Figure 13**, **Figure 14**, **Figure 15**, and **Figure 16**).

Cell Soma Length Analysis

Naïve Control Cells

Results were obtained from a total of 328 sections from naïve control rats. The average naive control cell soma length was 35.6 ± 14.6 μm. The average naïve control cell soma lengths of the small sized cells (<30 μm); medium sized cells (30-45 μm) and large cells (>45 μm) were 21.4 ± 4.1 μm, 38.4 ± 4.1 μm and 55.1 ± 7.2 μm, respectively.

All results were compared to naïve control results.

Day 6 Analysis

Results were obtained from a total of 134 sections from active control rats, and 97 sections from aEAE rats.

The average active control cell soma length was 21.6 ± 3.7 μm. The average aEAE cell soma length was 23.4 ± 4.1 μm.

The average active control cell soma lengths of the small sized cells (<30 μm); medium sized cells (30-45 μm) and large cells (>45 μm) were 21.6 ± 3.7 μm, 36.6 ± 3.0 μm and 54.2 ± 9.0 μm, respectively. The average aEAE cell soma lengths of the small sized cells (<30 μm), medium sized cells (30-45 μm) and large sized cells (>45 μm) were 23.4 ± 4.1 μm, 36.6 ± 3.1 μm and 55.9 ± 7.6 μm, respectively (**Figure 13**).

Day 9 Analysis

Results were obtained from a total of 177 sections from active control rats, and 229 sections from aEAE rats.

The average aEAE cell soma length was 22.6 ± 4.1 μm. The average active control cell soma length was 24.2 ± 3.4 μm.

The average active control cell soma lengths of the small sized cells (<30 μm); medium sized cells (30-45 μm) and large cells (>45 μm) were 24.2 ± 3.4 μm, 36.1 ± 4.1 μm and 56.0 ± 8.3 μm, respectively. The average aEAE cell soma lengths of the small sized cells (<30 μm), medium sized cells (30-45 μm) and large sized cells (>45 μm) were 22.6 ± 4.2 μm, 35.6 ± 3.9 μm and 51.7 ± 4.7 μm, respectively (**Figure 14**).

Day 12 analysis

Results were obtained from a total of 105 sections from active control rats, 203 sections from active rats.

The average aEAE cell soma length was 20.2 ± 5.0 μm. The average active control cell soma length was 24.9 ± 3.2 μm.

The average active control cell soma lengths of the small sized cells (<30 μm); medium sized cells (30-45 μm) and large cells (>45 μm) were 24.9 ± 3.2 μm, 33.5 ±4.1 μm and 50.7 ± 3.8 μm, respectively. The average aEAE cell soma lengths of the small sized cells (<30 μm), medium sized cells (30-45 μm) and large sized cells (>45 μm) were 20.2 ± 5.0 μm, 36.4 ± 4.2 μm and 49.0 ± 3.6 μm, respectively (**Figure 15**).

Day 15 Analysis

Results were obtained from a total of 155 sections from active control rats, and 144 sections from aEAE rats.

The average active control cell soma length was 24.5 ± 3.9 μm. The average aEAE cell soma length was 24.3 μm ± 2.7 μm.

The average active control cell soma lengths of the small sized cells (<30 μm); medium sized cells (30-45 μm) and large cells (>45 μm) were 24.5 ± 3.9

μm, 36.2 ± 4.3 μm and 57.1 ± 9.8 μm, respectively. The average aEAE cell soma lengths of the small sized cells (<30 μm), medium sized cells (30-45 μm) and large sized cells (>45 μm) were 24.3 ± 2.7 μm, 31.3 ± 7.7 μm and 52.0 ± 3.4 μm, respectively (**Figure 16**).

RT-PCR and Real Time PCR analysis (DRG, spinal cord, brain)

We used comparative semi-quantitative RT-PCR to provide additional support to the results depicted in the IHC analysis discussed above. Comparative RT-PCR analysis amongst the 3 experimental groups (naïve control, active control and aEAE) was initially conducted using sensory ganglia obtained from the entire vertebral column for all animals designated for RT-PCR at all pre-determined experimental time points. Specifically RT-PCR was conducted to evaluate the expression levels of TNFα mRNA in parallel with that of the normal housekeeping gene known to be found in all tissue GAPDH (Overbergh, Valckx et al. 1999). As expected the mRNA expression for GAPDH was consistent across all tissue samples analyzed at all experimental time points (**Figure 17**). However, comparative analysis of TNFα mRNA within the DRG sensory ganglia obtained from the 3 experimental groups, revealed peak expression in the aEAE group at day 12 compared to all other experimental groups (**Figure 18**). Following the completion of RT-PCR, full quantification was conducted using Real Time PCR. **Figure 19** represents the graphical depiction of the Real Time PCR results that conclusively demonstrates that TNFα

expression of DRG for animals of the aEAE group, euthanized at day 12, were significantly higher than other groups ($p<0.05$). However, no significant difference was noted between the naïve control and active control animal groups euthanized at any of the time points. The RT-PCR and qRT-PCR data for the spinal cord and brain tissue obtained from the respective animals in each group did not identify any significant changes between groups, at any of the described time points. These findings provided strong qualitative evidence to support the initial findings presented in the IHC assessment analysis of the sensory ganglia. These results taken together with the IHC analysis prove that the detectable peak expression of mRNA for TNFα in the aEAE animal at day 12 is in fact translated to TNFα protein that is detectable via IHC analysis.

Following the detailed analysis of the DRG, RT-PCR was also undertaken on the spinal cord and brain tissue. The spinal cord and brain tissue obtained from 3 separate animals in each respective experimental group at the various experimental time points revealed variable results without any significant trend or pattern identified with respect to TNFα expression (data not shown). Due to the variable results during the 1st analysis, a 2nd subsequent analysis on the tissue was conducted. Similar variations in TNFα expression was noted thereby preventing a definitive conclusion to be drawn from this latter analysis.

DISCUSSION

Although traditional animal models of MS (EAE, cuprizone, MHV induced) have been extensively studied to explore the underlying pathogenesis of MS, the exact cellular etiology still remains elusive (Yasuda, Tsumita et al. 1975; Johnson and Ludwin 1981; Frost, Nielsen et al. 2003; Mix, Meyer-Rienecker et al. 2008; Mannie, Swanborg et al. 2009). Recent research has expanded current strategies to explore the implications of the immune system in the development of MS (Hohlfeld, Meinl et al. 2008; Lees, Iwakura et al. 2008; Mix, Meyer-Rienecker et al. 2008; Proudfoot, de Souza et al. 2008; Prendergast and Anderton 2009; El-Behi, Rostami et al. 2010). Our initial experimental findings have identified significant changes in the levels of TNFα mRNA and protein in the DRG of rats with active MS at 12 days post induction of disease. The absence of a similar trend in regard to mRNA expression for TNFα in the spinal cord, suggests that the DRG are responsible for synthesizing TNFα protein for subsequent axonal transport to the spinal cord. Recent research involving the anterograde and retrograde autosomal transport mechanisms of proteins such as NGF between the DRG and spinal cord support this proposed concept (Richardson and Riopelle 1984; Yip and Johnson 1986; Averill, Robson et al. 2004; Zweifel, Kuruvilla et al. 2005). Furthermore, in addition to protein transport, other research has focused on mRNA transport by mRNA binding proteins via the microtubule system. For example, the established transport

mechanisms for BDNF or NT-3 mRNA, provides support for the anterograde transport of mRNA between DRG and spinal cord (Melrose, Kinloch et al. 2007; Qiao, Gulick et al. 2008; Wang, Meng et al. 2008). The literature supporting the bi-directional trafficking of protein and/or mRNA between the DRG and spinal cord correlates well with our current experimental findings and our published DRG model of MS (Melanson, Miao et al. 2009). Interestingly, our experimental findings that link the DRG to the development of MS are also supported by previous theories proposed by Nelson (1992 &1993) (Nelson 1992; Nelson 1993).

According to our published immune activation model for MS (Melanson, Miao et al. 2009), Th1 cells are activated by the presence of MBP in the circulating blood and subsequently release inflammatory cytokines, such as TNFα. As the disease progresses, the inflammatory cytokines then pass through the permeable capillaries surrounding the DRG (Sekerkova, Malatova et al. 1996). Once inside the DRG, the TNFα activates small to medium sized sensory neurons to produce further TNFα, which can pass back into the capillaries to the blood to activate more Th-cells. This results in a positive feedback loop between the DRG and the immune system. In addition, we hypothesize that activated Th1 cells migrate into the DRG, crossing the endothelium of the capillaries in the same manner as cells crossing the BBB (described above) (Tsiang, Lycke et al. 1989; Nelson 1993). The dual invasion of inflammatory cytokines and activated Th1 cells continues to facilitate the positive feedback loop between the DRG and

the activated immune system. Following this initial priming phase of the DRG sensory neurons, the TNFα protein would then undergo anterograde transport along the microtubule system of the dorsal roots to the dorsal root entry zone of the dorsal horn. The DRG derived TNFα thus activates nociceptive neurons synapsing in laminae II of the dorsal horn, as well as the innate microglial cells. Subsequent synthesis and secretion of TNFα by the microglia and/or dorsal horn neurons results in the synchronous hyper-excitability of the nociceptive laminae II neurons, which is an established hallmark characteristic of the inflammatory phase of MS (Cuellar, Montesano et al. 2004; Wei, Zang et al. 2007; Kawasaki, Zhang et al. 2008; Youn, Wang et al. 2008). In addition, once this connective pathway between the DRG and spinal cord dorsal horn neurons is established, the delivery of DRG derived TNFα protein facilitates the initial development of chronic pain. Thus, it is our belief that TNFα is involved in the initial induction and short term maintenance of chronic NPP. During the third, chronic phase of pain, we hypothesize that instead of transporting protein via the established pathogenic pathway between DRG and spinal cord, a switch from protein to mRNA transport via mRNA binding proteins to the spinal cord occurs. In this manner, the pathogenic pathway involving local translation of transported mRNA provides a more efficient mechanism for pathogenic maintenance of TNFα signaling through interaction with dorsal horn neurons. Further experiments correlating alterations in pain sensitivity with neurological disability score, and TNFα expression changes, are required to confirm our hypothesis. However, the results presented from the current study support the concept of the DRG being a

critical source of TNFα. Our findings identified significant elevations of TNFα mRNA in DRG obtained from aEAE day 12 that were not were not detected in spinal cord during the entire 15 day disease period. As a result, future experiments using a mouse model of relapsing remitting EAE, conducted over a longer period of 30 to 45 days, are planned to encompass the second phase of disease induction.

The advantage of converting future experiments to the mouse model is that the use of knockout mice could facilitate advanced research into specific cellular mechanisms described in our model. In addition, the use of *in situ* hybridization to detect mRNA anterograde transport along nerve axons to the spinal cord would also be crucial in providing additional support for this model at the extended time point of 45 days. Further, although this research focused primarily on the DRG, the ability to perform western blot and/or ELISA to provide some quantification as to the protein levels anticipated in the spinal cord would also represent an essential step to support our model. Thus, it would be important to assess protein levels of TNFα in the dorsal horn, and correlate them with the expression of mRNA at specific locations with the spinal cord. Since CD3 and CD2 are excellent markers for activated T-lymphocytes (Bimal, Singh et al. 2008; Ren, Wu et al. 2008), the ability to conduct qRT-PCR on DRG, spinal cord and brain tissue at the various time points would assist in confirming the role of Th-cells in the induction of MS.

The antigen-induced expression of TNFα within the DRG, and potential anterograde microtubule transport into the CNS, may represent a key element in the conversion of peripheral immune activation to central induction of dorsal horn neurons. This peripheral-central link is essential to the cellular cascade of inflammation preceding MS. The present study presents intriguing data to support a proposed interaction between the immune system and the DRG. This interaction is thought to be integral in pain transmission, and normally protects us from external noxious stimuli. However, in diseases such as MS where the immune system is already hyper-excited due to the introduction of a foreign antigen, the sustained production of inflammatory cytokines such as TNFα may be a critical player in the disease progression. Although the cellular mechanisms of MS are unknown, the present research implicates the DRG in the induction and possible maintenance of this chronic pain syndrome.

To the best of our knowledge, this is the first research conducted in an EAE rat model depicting an acute attack of MS that establishes a conclusive link between the immune activation associated with MS and cellular changes in DRG sensory neurons. In addition, we have provided conclusive evidence that the small to medium diameter sensory neurons in the DRG-spinal cord are involved in the TNFα signaling pathway. This finding indicates a novel target for early intervention strategies aimed at the attenuation of the early disease process. The present research may also facilitate the advancement of research into other autoimmune disorders that are associated with chronic NPP such as type-1

diabetes (Fried, Persson et al. 2008; Otto-Buczkowska, Kazibutowska et al. 2008; White and Wilson 2008).

The transient effects of TNFα are most likely the result of few different mechanisms which may include but are not limited to: 1) TNFα receptor being internalized by monocytes 2) extracellular TNFα can be degraded by a variety of proteolytic enzymes 3) TNFα's ability to undergo retrograde and anterograde transport migrating from the site of its production 4) the biological half life of TNFα is short and estimated to be about 18-20 minutes (Oliver, Bland et al. 1993). Potential treatments may need to focus on this narrow window of opportunity as a critical step toward interrupting downstream cellular events involved in the early inflammatory pathophysiolology of MS and NPP.

The sensation of pain protects the body from further external nociceptive trauma (Millan 1999). Our results link the induction of pain to the early inflammatory stages of MS. MS patients frequently report sensory abnormalities prior to their diagnosis [Melanson and Namaka, unpublished clinical observation]. Thus NPP may serve as a pre-diagnostic warning of prolonged immune system activation. As a result, this information may offer clinical relevance for patients undergoing baseline screening for MS. Often these patients present with sensory abnormalities in conjunction with the risk factors thought to be associated with the development of MS. Further studies are required to confirm our hypothesis.

CONCLUSION

Previous studies have indicated that TNFα plays an important role in the induction of MS (Issazadeh, Ljungdahl et al. 1995; Ahn, Kang et al. 2001; Glabinski, Bielecki et al. 2004). A preliminary study in our lab, showed that TNFα expression is upregulated in the DRG of human MS patients compared to age matched control DRG. We established an animal model of EAE in order to more closely confirm changes in TNFα expression in DRG and spinal cord. The role of inflammatory cytokines, such as TNFα, in the development of MS is well known. TNFα has been implicated in the development of demyelination in a variety of animal models (Merrill 1992; Segal and Cross 2000; Jurewicz, Matysiak et al. 2003; Ruffini, Chojnacki et al. 2006). The results of our research clearly indicate that peripheral TNFα plays a critical role in DRG activation following the antigenic activation of Th-cells. The transient up-regulation of TNFα expression within the DRG suggests that this inflammatory cytokine is a key upstream mediator in the development MS. The variable TNFα expression within the spinal cord and brain, described above, suggests that the short term nature of the study (<15 days) meant that we missed TNFα changes in these respective tissues. However, the research does suggest that during an MS attack when inflammatory cytokines are in abundance, early cellular changes within the DRG may be the first step in the chronic pain cascade. The research also suggests that there is a narrow therapeutic window of opportunity to treat or possibly

prevent the wind-up phenomena of the dorsal horn neurons so that synaptic changes don't become permanent. Hence, although MS clinicians need to treat the primary disease, they should also consider the earlier aggressive intervention of pharmacotherapy for NPP in an attempt to prevent the synchronous excitation of dorsal horn neurons. As a result, the DRG and/or spinal cord represent plausible targets for early treatment strategies aimed at attenuating the effects of inflammatory cytokines during the initial active stages of MS. This research represents a significant step towards improving quality of life for Manitobans living with MS by addressing their primary symptomatic concern of NPP.

Future Directions

It is evident from numerous studies, that the nature of the molecular processes underlying inflammation in MS are highly complex (Codarri, Fontana et al. 2010). Several studies, including this one, have linked TNFα to MS induction (Segal and Cross 2000; Glabinski, Bielecki et al. 2004; Codarri, Fontana et al. 2010). Another protein that has been shown to be significant in the progression of MS is the neurotrophin brain derived neurotrophic factor (**BDNF**) (Ziemssen, Kumpfel et al. 2002; De Santi, Annunziata et al. 2009). The immunomodulatory agent glatiramer acetate (**GA**), has demonstrated neuroprotective effects in MS patients (Namaka, Turcotte et al. 2008; Arnon and Aharoni 2009), via production of neurotrophins particularly BDNF (Aharoni, Eilam et al. 2005; Arnon and Aharoni 2009). The use of GA in the treatment of MS patients result in significantly increased serum BDNF levels compared to normal healthy control

serum (Antel and Miron 2008). GA has been shown to increase T-cell BDNF expression (Ziemssen, Kumpfel et al. 2002), and also to reduce inflammation and neuronal damage in an EAE mouse model of MS (Arnon and Aharoni 2009). These findings can be attributed to the potential neuroprotective increase in BDNF expression induced by this therapeutic agent (Azoulay, Vachapova et al. 2005). Interestingly, studies have also shown that TNF-α induces the expression of BDNF in CNS tissue (Aloe, Properzi et al. 1999; Saha, Liu et al. 2006). A recent study also showed that BDNF plays a role in neuroprotection in MS (Linker, Lee et al. 2010). In addition, TNFα induces expression of nerve growth factor (**NGF**) expression (Gadient, Cron et al. 1990; Kuno, Yoshida et al. 2006) and regulates NGF signaling (Takei and Laskey 2008; Takei and Laskey 2008). NGF is known to ameliorate the clinical symptoms of EAE (Villoslada, Hauser et al. 2000; Kuno, Yoshida et al. 2006), though the mechanisms underlying this suppression remain unclear. Other studies suggest NGF can act as an anti-inflammatory mediator and interfere with the functions of MHC class II positive cells that mediate autoimmune processes in the CNS (Villoslada, Hauser et al. 2000). Thus, NGF may exert its function not only through direct neuroprotection, but also via suppressive effects on glial cells. Interestingly, NGF also induces TNFα expression in neurons and immune cells (Xiong, Futamura et al. 2002; Takei and Laskey 2008). TNFα expressing neurons depend on NGF for survival; if they fail to obtain sufficient levels of NGF they undergo apoptosis (Barker, Middleton et al. 2001). The endogenous TNF-α does not kill neurons in the presence of NGF. However, when NGF is withdrawn, endogenous TNF-α

becomes cytotoxic, via TNFR1 (Barker, Middleton et al. 2001; Takei and Laskey 2008). BDNF acts as a chemo-attractant directional cue for NGF (Dasari, Spomar et al. 2007; Lykissas, Batistatou et al. 2007) which subsequently promotes preferential TNFα signaling via TNFR2. Thus, the signaling effects of TNFα can be protective rather than pathogenic.

Therefore, our future studies will investigate changes in BDNF and NGF expression in the DRG and spinal cord of EAE animals, at the gene and protein level. We will correlate expression patterns with neurological disability, as with TNFα.

Further, inflammatory cytokines have been shown to play an *integral role in facilitating the development of NPP* (Sommer, Schmidt et al. 1998; Hermann, Rogers et al. 2001; Zimmermann 2001). Studies using cytokine inhibitors, knock-out mice, or direct application of cytokines, with subsequent investigation of electrical activity in neurons and animal behavioral changes, confirm the involvement of TNFα in the development of chronic NPP (Sommer, Schmidt et al. 1998; Vogel, Lindenlaub et al. 2000; Zhang and Liu 2002). TNFα is also capable of directly inducing pain, as evident by its role in producing a state of painful neuropathy when injected directly into the sciatic nerve (Myers, Sekiguchi et al. 2003). In addition to cytokine involvement, BDNF also appears to be as a central modulator of pain (Merighi, Salio et al. 2008). It is an important modulator of sensory neurotransmission in nociceptive pathways both at spinal and supraspinal

levels, and a key player in the central sensitization that underlies many forms of hyperalgesia. Therefore, we will use behavioural studies to assess sensory abnormalities in the EAE rat model, and correlate these sensory changes with expression changes of TNFα, BDNF and NGF.

REFERENCES

Abbadie, C., Bhangoo, S., De Koninck, Y., Malcangio, M., Melik-Parsadaniantz, S. and White, F. A. (2009). "Chemokines and pain mechanisms." *Brain Res Rev* **60** (1): 125-34.

Acosta, C. G., Fabrega, A. R., Masco, D. H. and Lopez, H. S. (2001). "A sensory neuron subpopulation with unique sequential survival dependence on nerve growth factor and basic fibroblast growth factor during development." *J Neurosci* **21** (22): 8873-85.

Aharoni, R., Eilam, R., Domev, H., Labunskay, G., Sela, M. and Arnon, R. (2005). "The immunomodulator glatiramer acetate augments the expression of neurotrophic factors in brains of experimental autoimmune encephalomyelitis mice." *Proc Natl Acad Sci U S A* **102** (52): 19045-50.

Ahn, M., Kang, J., Lee, Y., Riu, K., Kim, Y., Jee, Y., Matsumoto, Y. and Shin, T. (2001). "Pertussis toxin-induced hyperacute autoimmune encephalomyelitis in Lewis rats is correlated with increased expression of inducible nitric oxide synthase and tumor necrosis factor alpha." *Neurosci Lett* **308** (1): 41-4.

Aloe, L., Properzi, F., Probert, L., Akassoglou, K., Kassiotis, G., Micera, A. and Fiore, M. (1999). "Learning abilities, NGF and BDNF brain levels in two lines of TNF-alpha transgenic mice, one characterized by neurological disorders, the other phenotypically normal." *Brain Res* **840** (1-2): 125-37.

Altar, C. A. and DiStefano, P. S. (1998). "Neurotrophin trafficking by anterograde transport." *Trends Neurosci* **21** (10): 433-7.

Ammit, A. J., Lazaar, A. L., Irani, C., O'Neill, G. M., Gordon, N. D., Amrani, Y., Penn, R. B. and Panettieri, R. A., Jr. (2002). "Tumor necrosis factor-alpha-induced secretion of RANTES and interleukin-6 from human airway smooth muscle cells: modulation by glucocorticoids and beta-agonists." *Am J Respir Cell Mol Biol* **26** (4): 465-74.

Antel, J. P. and Miron, V. E. (2008). "Central nervous system effects of current and emerging multiple sclerosis-directed immuno-therapies." *Clin Neurol Neurosurg* **110** (9): 951-7.

Argani, P., Zakowski, M. F., Klimstra, D. S., Rosai, J. and Ladanyi, M. (1998). "Detection of the SYT-SSX chimeric RNA of synovial sarcoma in paraffin-embedded tissue and its application in problematic cases." *Mod Pathol* **11** (1): 65-71.

Arnon, R. and Aharoni, R. (2009). "Neuroprotection and neurogeneration in MS and its animal model EAE effected by glatiramer acetate." *J Neural Transm* **116** (11): 1443-9.

Averill, S., Robson, L. G., Jeromin, A. and Priestley, J. V. (2004). "Neuronal calcium sensor-1 is expressed by dorsal root ganglion cells, is axonally transported to central and peripheral terminals, and is concentrated at nodes." *Neuroscience* **123** (2): 419-27.

Azoulay, D., Vachapova, V., Shihman, B., Miler, A. and Karni, A. (2005). "Lower brain-derived neurotrophic factor in serum of relapsing remitting MS: reversal by glatiramer acetate." *J Neuroimmunol* **167** (1-2): 215-8.

Balashov, K. E., Smith, D. R., Khoury, S. J., Hafler, D. A. and Weiner, H. L. (1997). "Increased interleukin 12 production in progressive multiple sclerosis: induction by activated CD4+ T cells via CD40 ligand." *Proc Natl Acad Sci U S A* **94** (2): 599-603.

Bar-Or, A. (2008). "The immunology of multiple sclerosis." *Semin Neurol* **28** (1): 29-45.

Bar, K. J., Saldanha, G. J., Kennedy, A. J., Facer, P., Birch, R., Carlstedt, T. and Anand, P. (1998). "GDNF and its receptor component Ret in injured human nerves and dorsal root ganglia." *Neuroreport* **9** (1): 43-7.

Barbacid, M. (1995). "Structural and functional properties of the TRK family of neurotrophin receptors." *Ann N Y Acad Sci* **766**: 442-58.

Barde, Y. A., Edgar, D. and Thoenen, H. (1982). "Purification of a new neurotrophic factor from mammalian brain." *EMBO J* **1** (5): 549-53.

Barker, P. A. and Murphy, R. A. (1992). "The nerve growth factor receptor: a multicomponent system that mediates the actions of the neurotrophin family of proteins." *Mol Cell Biochem* **110** (1): 1-15.

Barker, V., Middleton, G., Davey, F. and Davies, A. M. (2001). "TNFalpha contributes to the death of NGF-dependent neurons during development." *Nat Neurosci* **4** (12): 1194-8.

Barkin, R. L. and Fawcett, J. (2000). "The management challenges of chronic pain: the role of antidepressants." *Am J Ther* **7** (1): 31-47.

Barten, D. M. and Ruddle, N. H. (1994). "Vascular cell adhesion molecule-1 modulation by tumor necrosis factor in experimental allergic encephalomyelitis." *J Neuroimmunol* **51** (2): 123-33.

Basso, O., Campi, R., Frydenberg, M., Koch-Henriksen, N., Bronnum-Hansen, H. and Olsen, J. (2004). "Multiple sclerosis in women having children by multiple partners. A population-based study in Denmark." *Mult Scler* **10** (6): 621-5.

Baud, V. and Karin, M. (2001). "Signal transduction by tumor necrosis factor and its relatives." *Trends Cell Biol* **11** (9): 372-7.

Baumann, N. and Pham-Dinh, D. (2001). "Biology of oligodendrocyte and myelin in the mammalian central nervous system." *Physiol Rev* **81** (2): 871-927.

Begum, F., Zhu, W., Namaka, M. P. and Frost, E. E. (2010). "A novel decalcification method for adult rodent bone for histological analysis of peripheral-central nervous system connections." *Journal of Neuroscience Methods* **187** (1): 59-66.

Beiske, A. G., Pedersen, E. D., Czujko, B. and Myhr, K. M. (2004). "Pain and sensory complaints in multiple sclerosis." *Eur J Neurol* **11** (7): 479-82.

Beissner F, et al,. "Quick Discrimination of Aδ and C Fiber Mediated Pain Based on Three Verbal Descriptors." *PLoS ONE* 5(9): e12944.

Bennett, D. L., Michael, G. J., Ramachandran, N., Munson, J. B., Averill, S., Yan, Q., McMahon, S. B. and Priestley, J. V. (1998). "A distinct subgroup of

small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury." *J Neurosci* **18** (8): 3059-72.

Benveniste, E. N. (1997). "Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis." *J Mol Med* **75** (3): 165-73.

Berthold, C. H., Nilsson, I. and Rydmark, M. (1983). "Axon Diameter and Myelin Sheath Thickness in Nerve-Fibers of the Ventral Spinal Root of the 7th Lumbar Nerve of the Adult and Developing Cat." *Journal of Anatomy* **136** (May): 483-508.

Bimal, S., Singh, S. K., Sinha, S., Pandey, K., Sinha, P. K., Ranjan, A., Bhattacharya, S. K. and Das, P. (2008). "Leishmania donovani: role of CD2 on CD4+ T-cell function in Visceral Leishmaniasis." *Exp Parasitol* **118** (2): 238-46.

Bitsch, A., Schuchardt, J., Bunkowski, S., Kuhlmann, T. and Bruck, W. (2000). "Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation." *Brain* **123 (Pt 6)**: 1174-83.

Boissy, A. and Fox, R. J. (2007). "Current treatment options in multiple sclerosis." *Curr Treat Options Neurol* **9** (3): 176-86.

Brosnan, C. F., Selmaj, K. and Raine, C. S. (1988). "Hypothesis: a role for tumor necrosis factor in immune-mediated demyelination and its relevance to multiple sclerosis." *J Neuroimmunol* **18** (1): 87-94.

Bunge, R. P. (1968). "Glial cells and the central myelin sheath." *Physiol Rev* **48** (1): 197-251.

Buntinx, M., Gielen, E., Van Hummelen, P., Raus, J., Ameloot, M., Steels, P. and Stinissen, P. (2004). "Cytokine-induced cell death in human oligodendroglial cell lines. II: Alterations in gene expression induced by interferon-gamma and tumor necrosis factor-alpha." *J Neurosci Res* **76** (6): 846-61.

Butowt, R. and von Bartheld, C. S. (2001). "Sorting of internalized neurotrophins into an endocytic transcytosis pathway via the Golgi system: Ultrastructural analysis in retinal ganglion cells." *J Neurosci* **21** (22): 8915-30.

Buttner, A., Rohrmoser, K., Mall, G., Penning, R. and Weis, S. (2006). "Widespread axonal damage in the brain of drug abusers as evidenced by accumulation of beta-amyloid precursor protein (beta-APP): an immunohistochemical investigation." *Addiction* **101** (9): 1339-46.

Campagnoni, A. T. (1988). "Molecular Biology of Myelin Proteins from the Central Nervous System." *Journal of Neurochemistry* **51** (1): 1-14.

Campana, W. M. and Myers, R. R. (2003). "Exogenous erythropoietin protects against dorsal root ganglion apoptosis and pain following peripheral nerve injury." *Eur J Neurosci* **18** (6): 1497-506.

Cannella, B., Hoban, C. J., Gao, Y. L., Garcia-Arenas, R., Lawson, D., Marchionni, M., Gwynne, D. and Raine, C. S. (1998). "The neuregulin, glial growth factor 2, diminishes autoimmune demyelination and enhances remyelination in a chronic relapsing model for multiple sclerosis." *Proc Natl Acad Sci U S A* **95** (17): 10100-5.

Cannella, B. and Raine, C. S. (1989). "Cytokines up-regulate Ia expression in organotypic cultures of central nervous system tissue." *J Neuroimmunol* **24** (3): 239-48.

Cannella, B. and Raine, C. S. (1995). "The adhesion molecule and cytokine profile of multiple sclerosis lesions." *Ann Neurol* **37** (4): 424-35.

Cawthorn, W. P. and Sethi, J. K. (2008). "TNF-alpha and adipocyte biology." *FEBS Lett* **582** (1): 117-31.

Chadwick, W., Magnus, T., Martin, B., Keselman, A., Mattson, M. P. and Maudsley, S. (2008). "Targeting TNF-alpha receptors for neurotherapeutics." *Trends Neurosci* **31** (10): 504-11.

Chalazonitis, A. (1996). "Neurotrophin-3 as an essential signal for the developing nervous system." *Mol Neurobiol* **12** (1): 39-53.

Chalmers, K., Wilcock, G. and Love, S. (2005). "Contributors to white matter damage in the frontal lobe in Alzheimer's disease." *Neuropathol Appl Neurobiol* **31** (6): 623-31.

Chard, D. T., Griffin, C. M., McLean, M. A., Kapeller, P., Kapoor, R., Thompson, A. J. and Miller, D. H. (2002). "Brain metabolite changes in cortical grey and normal-appearing white matter in clinically early relapsing-remitting multiple sclerosis." *Brain* **125** (Pt 10): 2342-52.

Choi, Y., Yoon, Y. W., Na, H. S., Kim, S. H. and Chung, J. M. (1994). "Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain." *Pain* **59** (3): 369-76.

Codarri, L., Fontana, A. and Becher, B. (2010). "Cytokine networks in multiple sclerosis: lost in translation." *Curr Opin Neurol* **23** (3): 205-11.

Coleridge, H. M., Coleridge, J. C., Dangel, A., Kidd, C., Luck, J. C. and Sleight, P. (1973). "Impulses in slowly conducting cagal fibers from afferent endings in the veins, atria and arteries of dogs and cats." *Circ Res.* **33** (1): 87-97.

Comi, C., Leone, M., Bonissoni, S., DeFranco, S., Bottarel, F., Mezzatesta, C., Chiocchetti, A., Perla, F., Monaco, F. and Dianzani, U. (2000). "Defective T cell fas function in patients with multiple sclerosis." *Neurology* **55** (7): 921-7.

Compston, A. (1999). "THe genetic dpidemiology of multiple sclerosis." *Philos Trans R Soc Lond B Biol Sci* **354** (1390): 1623-34.

Confavreux, C., Aimard, G. and Devic, M. (1980). "Course and prognosis of multiple sclerosis assessed by the computerized data processing of 349 patients." *Brain* **103** (2): 281-300.

Conover, J. C., Erickson, J. T., Katz, D. M., Bianchi, L. M., Poueymirou, W. T., McClain, J., Pan, L., Helgren, M., Ip, N. Y. and Boland, P. (1995). "Neuronal deficits, not involving motor neurons in mice lacking BDNF and/or NT4." *Nature* **375** (6528): 235-8.

Correale, J. and Villa, A. (2007). "The blood-brain-barrier in multiple sclerosis: functional roles and therapeutic targeting." *Autoimmunity* **40** (2): 148-60.

Costa, O., Divoux, D., Ischenko, A., Tron, F. and Fontaine, M. (2003). "Optimization of an animal model of experimental autoimmune

encephalomyelitis achieved with a multiple MOG (35-55)peptide in C57BL6/J strain of mice." *J Autoimmun* **20** (1): 51-61.

Costigan, M., Scholz, J. and Woolf, C. J. (2009). "Neuropathic pain: a maladaptive response of the nervous system to damage." *Annu Rev Neurosci* **32**: 1-32.

Cousins, D. J., Lee, T. H. and Staynov, D. Z. (2002). "Cytokine Coexpression During Human Th1/Th2 Cell Differentiation: Direct Evidence for Coordinated Expression of Th2 Cytokines." *J Immunol* **169** (5): 2498-2506.

Coutaux, A., Adam, F., Willer, J.-C. and Le Bars, D. (2005). "Hyperalgesia and allodynia: peripheral mechanisms." *Joint Bone Spine* **72** (5): 359-371.

Coyle, P. K. (2005). "Gender issues." *Neurol Clin.* **23** (1): 39,60.

Cuellar, J. M., Montesano, P. X. and Carstens, E. (2004). "Role of TNF-alpha in sensitization of nociceptive dorsal horn neurons induced by application of nucleus pulposus to L5 dorsal root ganglion in rats." *Pain* **110** (3): 578-87.

Cunha, F. Q., Lorenzetti, B. B., Poole, S. and Ferreira, S. H. (1991). "Interleukin-8 as a mediator of sympathetic pain." *Br J Pharmacol.* **104** (3): 765-7.

Czlonkowska, A., Ciesielska, A., Gromadzka, G. and Kurkowska-Jastrzebska, I. (2005). "Estrogen and cytokines production - the possible cause of gender differences in neurological disease." *Curr Pharm Des.* **11** (8): 1017-30.

D'Amico, D., La Mantia, L., Rigamonti, A., Usai, S., Mascoli, N., Milanese, C. and Bussone, G. (2004). "Prevalence of primary headaches in people with multiple sclerosis." *Cephalalgia* **24** (11): 980-4.

Dasari, V. R., Spomar, D. G., Gondi, C. S., Sloffer, C. A., Saving, K. L., Gujrati, M., Rao, J. S. and Dinh, D. H. (2007). "Axonal remyelination by cord blood stem cells after spinal cord injury." *J Neurotrauma* **24** (2): 391-410.

De Santi, L., Annunziata, P., Sessa, E. and Bramanti, P. (2009). "Brain-derived neurotrophic factor and TrkB receptor in experimental autoimmune encephalomyelitis and multiple sclerosis." *J Neurol Sci* **287** (1-2): 17-26.

DeLeo, J. A., Rutkowski, M. D., Stalder, A. K. and Campbell, I. L. (2000). "Transgenic expression of TNF by astrocytes increases mechanical allodynia in a mouse neuropathy model." *neuroreport* **11** (3): 599-602.

Devor, M. (1999). "Unexplained peculiarities of the dorsal root ganglion." *Pain* **Suppl 6**: S27-35.

Devor, M., Wall, P. D. and McMahon, S. B. (1984). "Dichotomizing somatic nerve fibers exist in rats but they are rare." *Neurosci Lett.* **49** (1-2): 187-92.

Dhib-Jalbut, S., Jiang, H. and Williams, G. J. (1996). "The effect of interferon beta-1b on lymphocyte-endothelial cell adhesion." *J Neuroimmunol* **71** (1-2): 215-22.

Di Pauli, F., Berger, T. and Reindl, M. (2009). "Monoclonal Antibodies in the Treatment of Multiple Sclerosis." *Curr Med Chem*.

Diaz-Villoslada, P., Shih, A., Shao, L., Genain, C. P. and Hauser, S. L. (1999). "Autoreactivity to myelin antigens: myelin/oligodendrocyte glycoprotein is a prevalent autoantigen." *J Neuroimmunol* **99** (1): 36-43.

Ding, M., Hart, R. P. and Jonakait, G. M. (1995). "Tumor necrosis factor-alpha induces substance P in sympathetic ganglia through sequential induction of interleukin-1 and leukemia inhibitory factor." *J Neurobiol* **28** (4): 445-54.

Djouhri, L. and Lauson, S. N. (2004). "Abeta-fiber nociceptive primary afferent neurons: A review of incidence and properties in relation to other afferent A-fiber neurons in mammals." *Brain Res Brain Res Rev.* **46** (2): 131-45.

Dodge, M. E., Rahimtula, M. and Mearow, K. M. (2002). "Factors contributing to neurotrophin-independent survival of adult sensory neurons." *Brain Res* **953** (1-2): 144-56.

Duchen, L. W. and Scaravilli, F. (1977). "Structure and Composition of Peripheral-Nerves and Nerve Roots in Sprawling Mouse." *Journal of Anatomy* **123** (Jul): 763-775.

Duncan, D. (1934). "The Importance of Diameter as a Factor in Myelination." *Science* **79** (2051): 363.

Dyer, C. A. (2002). "The structure and function of myelin: from inert membrane to perfusion pump." *Neurochem Res* **27** (11): 1279-92.

Ebringer, A., Rashid, I., Wilson, C., Boden, R. and Thompson, E. (2005). "A possible link between multiple sclerosis and creutzfeldt-jakob diseas based on clinical genetic pathological and immunological evidence involving acinetobacter bacteria." *Med Hypotheses* **64** (3): 487-94.

Ehde, D. M., Osborne, T. L., Hanley, M. A., Jensen, M. P. and Kraft, G. H. (2006). "The scope and nature of pain in persons with multiple sclerosis." *Mult Scler* **12** (5): 629-38.

El-Behi, M., Rostami, A. and Ciric, B. (2010). "Current Views on the Roles of Th1 and Th17 Cells in Experimental Autoimmune Encephalomyelitis." *J Neuroimmune Pharmacol* **5** (2): 189-98.

Elenkov, I. J., Wilder, R. L., Bakalov, V. K., Link, A. A., Bimitrov, M. A., Fisher, S., Crane, M., Kanik, K. S. and Chrousos, G. P. (2001). "IL-12, TNF-alpha, and hormonal changes during late pregnancy and early postpartum: Implications for auto immune diseas activity during these times." *J Clin Endocrinol Metab* **86** (10): 4933-8.

Ernfors, P., Ibanez, C. F., Ebendal, T., Olson, L. and Persson, H. (1990). "Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain." *Proc Natl Acad Sci U S A* **87** (14): 5454-8.

Ernfors, P., Lee, K. F. and Jaenisch, R. (1994). "Mice lacking brain-derived neurotrophic factor develop with sensory deficits." *Nature* **368** (6467): 147-50.

Ernfors, P., Lee, K. F., Kucera, J. and Jaenisch, R. (1994). "Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents." *Cell* **77** (4): 503-12.

Esiri, M. M. and McGee, J. O. (1986). "Monoclonal antibody to macrophages (EMB/11) labels macrophages and microglial cells in human brain." *J Clin Pathol* **39** (6): 616-21.

Evangelou, N., Jackson, M., Beeson, D. and Palace, J. (1999). "Association of the APOE epsilon4 allele with diseas activity in multiple sclerosis." *J Neurol Neurosug Psychiatry* **67** (2): 203-5.

Farinas, I., Jones, K. R., Backus, C., Wang, X. Y. and Reichardt, L. F. (1994). "Severe sensory and sympathetic deficits in mice lacking neurotrophin-3." *Nature* **369** (6482): 658-61.

Feiguin, F., Ferreira, A., Kosik, K. S. and Caceres, A. (1994). "Kinesin-mediated organelle translocation revealed by specific cellular manipulations." *J Cell Biol* **127** (4): 1021-39.

Fernyhough, P., Smith, D. R., Schapansky, J., Van Der Ploeg, R., Gardiner, N. J., Tweed, C. W., Kontos, A., Freeman, L., Purves-Tyson, T. D. and Glazner, G. W. (2005). "Activation of nuclear factor-kappaB via endogenous tumor necrosis factor alpha regulates survival of axotomized adult sensory neurons." *J Neurosci* **25** (7): 1682-90.

Ferreira, S. H., Lorenzetti, B. B., Cunha, F. Q. and Poole, S. (1993). "Bradykinin release of TNF-alpha plays a key role in the development of inflammatory hyperalgesia." *Agents Actions* **38 Spec No**: C7-9.

Ffrench-Constant, C. (1994). "Pathogenesis of multiple sclerosis." *Lancet* **343** (8892): 271-5.

Filippi, M. (1999). "Megnetization transfer imaging to monitor the evolution of multiple sclerosis." *Ital J Neurol Sci* **20** (5 Suppl): S232-40.

Filippi, M. and Rocca, M. A. (2003). "MRI aspects of the "inflammatory phase" of multiple sclerosis." *Neurol Sci* **24 Suppl 5**: S275-8.

Filippi, M., Rovaris, M., Capra, R., Gasperini, C., Yousry, T. A., Sormani, M. P., Prandini, F., Horsfield, M. A., Martinelli, V., Bastianello, S., kuhne, I., Pozzilli, C. and Comi, G. (1998). "A multicentre longitudinal study comparing the sensitivity of monthly MRI after standard and triple dose gadolinium-DTPA for monitoring disease activity in multiple sclerosis implications for phase II clinical trials." *Brain* **121** ((pt 10) (pt 10)): 2011-20.

Filippi, M., Tortorella, C. and Bozzali, M. (1999). "Normal-appearing white matter changes in multiple sclerosis: The contribution of magnetic resonance techniques." *Mult Scler* **5** (4): 273-82.

Ford, C. C., Ceckler, T. L., Karp, J. and Herndon, R. M. (1990). "Magnetic resonance imaging of experimental demyelinating lesions." *Magn Reson Med* **14** (3): 461-81.

Fox, R. J., Kivisakk, P., Lee, J. C., Tucky, B., Lucchinetti, C., Rudick, R. A. and Ransohoff, R. M. (2006). "Chemokine receptors as biomarkers in multiple sclerosis." *Dis Markers* **22** (4): 227-33.

Frei, K., Fredrikson, S., Fontana, A. and Link, H. (1991). "Interleukin-6 is elevated in plasma in multiple sclerosis." *J Neuroimmunol* **31** (2): 147-53.

Fried, K., Persson, A. K., Xu, X. J. and Wiesenfeld-Hallin, Z. (2008). "[Genetics of neuropathic pain]." *Lakartidningen* **105** (39): 2678-82.

Frost, E. E., Nielsen, J. A., Le, T. Q. and Armstrong, R. C. (2003). "PDGF and FGF2 regulate oligodendrocyte progenitor responses to demyelination." *J Neurobiol* **54** (3): 457-72.

Frost, E. E., Pillai, P. P., Begum, F., Esfahani, F., Doupe, M. and Namaka, M. (2010). "The cellular pathology of multiple sclerosis and prospects for disease modifying therapies." *Current Opinion in Investigational Drugs*

Fuller, K. G., Olson, J. K., Howard, L. M., Croxford, J. L. and Miller, S. D. (2004). "Mouse models of multiple sclerosis: Experimental autoimmune encephalomyelitis and theiler's virus-induced demyelinating disease." *Methods Mol Med* **102**: 339-61.

Fulton, B. P. (1995). "Gap junctions in the developing nervous system." *Perspect Dev Neurobiol* **2** (4): 327-34.

Fundin, B. T., Silos-Santiago, I., Ernfors, P., Fagan, A. M., Aldskogius, H., DeChiara, T. M., Phillips, H. S., Barbacid, M., Yancopoulos, G. D. and Rice, F. L. (1997). "Differential dependency of cutaneous mechanoreceptors on neurotrophins, Trk receptors, and P75 LNGFR." *Dev Biol* **190** (1): 94-116.

Gabay, E. and Tal, M. (2004). "Pain behavior and nerve electrophysiology in the CCI model of neuropathic pain." *Pain* **110** (1-2): 354-60.

Gadient, R. A., Cron, K. C. and Otten, U. (1990). "Interleukin-1 beta and tumor necrosis factor-alpha synergistically stimulate nerve growth factor (NGF) release from cultured rat astrocytes." *Neurosci Lett* **117** (3): 335-40.

Galetta, S. L., Markowitz, C. and Lee, A. G. (2002). "Immunomodulatory agents for the treatment of relapsing multiple sclerosis: A systematic review." *Arch Intern Med* **162** (19): 2161-9.

Gasser, H. S. (1950). "Unmedullated fibers originating in dorsal root ganglia." *J Gen Physiol* **33** (6): 651-90.

George, A., Buehl, A. and Sommer, C. (2004). "Wallerian degeneration after crush injury of rat sciatic nerve increases endo- and epineurial tumor necrosis factor-alpha protein." *Neurosci Lett.* **372** (3): 215-9.

George, A., Buehl, A. and Sommer, C. (2004). "Wallerian degeneration after crush injury of rat sciatic nerve increases endo- and epineurial tumor necrosis factor-alpha protein." *Neurosci Lett* **372** (3): 215-9.

Geranton, S. M., Fratto, V., Tochiki, K. K. and Hunt, S. P. (2008). "Descending serotonergic controls regulate inflammation-induced mechanical sensitivity and methyl-CpG-binding protein 2 phosphorylation in the rat superficial dorsal horn." *Mol Pain* **4**: 35.

Gidlund, M., Rossi, P., Cotran, P., Ramstedt, U. and Wigzell, H. (1988). "In human monocytes a strong correlation exists between expression of the M3 antigen, Fc-mediated phagocytic activity and failure to participate in extracellular antibody-dependent cytotoxicity." *Eur J Immunol* **18** (3): 477-80.

Giovannoni, G. (2006). "Multiple sclerosis cerebrospinal fluid biomarkers." *Dis Markers* **22** (4): 187-96.

Glabinski, A. R., Bielecki, B., Kawczak, J. A., Tuohy, V. K., Selmaj, K. and Ransohoff, R. M. (2004). "Treatment with soluble tumor necrosis factor receptor (sTNFR):Fc/p80 fusion protein ameliorates relapsing-remitting

experimental autoimmune encephalomyelitis and decreases chemokine expression." *Autoimmunity* **37** (6-7): 465-71.

Glabinski, A. R., O'Bryant, S., Selmaj, K. and Ransohoff, R. M. (2000). "CXC chemokine receptors expression during chronic relapsing experimental autoimmune encephalomyelitis." *Ann N Y Acad Sci* **917**: 135-44.

Gold, R., Hartung, H. P. and Toyka, K. V. (2000). "Animal models for autoimmune demyelinating disorders of the nervous system." *Mol Med Today* **6** (2): 88-91.

Gordon, F. H., Hamilton, M. I., Donoghue, S., Greenlees, C., Palmer, T., Rowley-Jones, D., Dhillon, A. P., Amlot, P. L. and Pounder, R. E. (2002). "A pilot study of treatment of active ulcerative colitis with natalizumab, a humanized monoclonal antibody to alpha-4 integrin." *Aliment Pharmacol Ther* **16** (4): 699-705.

Graus, F., Campo, E., Cruz-Sanchez, F., Ribalta, T. and Palacin, A. (1990). "Expression of lymphocyte, macrophage and class I and II major histocompatibility complex antigens in normal human dorsal root ganglia." *J Neurol Sci* **98** (2-3): 203-11.

Griffiths, I., Klugmann, M., Anderson, T., Thomson, C., Vouyiouklis, D. and Nave, K. A. (1998). "Current concepts of PLP and its role in the nervous system." *Microsc Res Tech* **41** (5): 344-58.

Groves, A. K., Barnett, S. C., Franklin, R. J., Crang, A. J., Mayer, M., Blakemore, W. F. and Noble, M. (1993). "Repair of demyelinated lesions by

transplantation of purified O-2A progenitor cells." *Nature* **362** (6419): 453-5.

Guan, W., Puthenveedu, M. A. and Condic, M. L. (2003). "Sensory Neuron Subtypes Have Unique Substratum Preference and Receptor Expression before Target Innervation." *J. Neurosci.* **23** (5): 1781-1791.

Harp, C. T., Lovett-Racke, A. E., Racke, M. K., Frohman, E. M. and Monson, N. L. (2008). "Impact of myelin-specific antigen presenting B cells on T cell activation in multiple sclerosis." *Clin Immunol* **128** (3): 382-91.

Hauser, S. L. and Oksenberg, J. R. (2006). "The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration." *Neuron* **52** (1): 61-76.

Hermann, G. E., Rogers, R. C., Bresnahan, J. C. and Beattie, M. S. (2001). "Tumor necrosis factor-alpha induces cFOS and strongly potentiates glutamate-mediated cell death in the rat spinal cord." *Neurobiol Dis* **8** (4): 590-9.

Hesselgesser, J. and Horuk, R. (1999). "Chemokine and chemokine receptor expression in the central nervous system." *J Neurovirol* **5** (1): 13-26.

Hillel Panitch and Carlo Ciccone (1981). "Induction of recurrent experimental allergic encephalomyelitis with myelin basic protein." *Annals of Neurology* **9** (5): 433-438.

Hiura, A. (2000). "Neuroanatomical effects of capsaicin on the primary afferent neurons." *Arch Histol Cytol* **63** (3): 199-215.

Hohlfeld, R., Meinl, E. and Dornmair, K. (2008). "B- and T-cell responses in multiple sclerosis: Novel approaches offer new insights." *Journal of the Neurological Sciences* **274** (1-2): 5-8.

Hohlfeld, R. and Wekerle, H. (2001). "Immunological update on multiple sclerosis." *Curr Opin Neurol* **14** (3): 299-304.

Hokfelt, T. (1991). "Neuropeptids in perspective: The last ten years." *Neuron* **7** (6): 867-79.

Horsfield, M. A. and Jones, D. K. (2002). "Applications of diffusion-weighted and diffusion tensor MRI to white matter diseases - a review." *NMR Biomed* **15** (7-8): 570-7.

Hortobagyi, T., Wise, S., Hunt, N., Cary, N., Djurovic, V., Fegan-Earl, A., Shorrock, K., Rouse, D. and Al-Sarrag, S. (2007). "Traumatic axonal damage in the brain can be detected using beta-APP immunohistochemistry wihtin 35 min after head injury to human adults." *Neuropathol Appl Neurobiol* **33** (2): 226-37.

Howe, C. L. and Mobley, W. C. (2005). "Long-distance retrograde neurotrophic signaling." *Curr Opin Neurobiol* **15** (1): 40-8.

Huitinga, I., Ruuls, S. R., Jung, S., Van Rooijen, N., Hartung, H. P. and Dijkstra, C. D. (1995). "Macrophages in T cell line-mediated, demyelinating, and chronic relapsing experimental autoimmune encephalomyelitis in Lewis rats." *Clin Exp Immunol* **100** (2): 344-51.

Hung, L. K. and Zhao, X. (2003). "Relationship of cervical spinal rootlets nad the inferrior vertebral notch." *Clin Orthop Relat Res* ((409) (409)): 131-7.

Hutchins, K. D., Dickson, D. W., Rashbaum, W. K. and Lyman, W. D. (1992). "Localization of microglia in hte human fetal cervical spinal cord." *Brain Res Dev Brain Res* **66** (2): 270-3.

Huxley, A. F. and Stampfli, R. (1949). "Evidence for saltatory conduction in peripheral myelinated nerve fibres." *J Physiol* **108** (3): 315-39.

Imrich, H. and Harzer, K. (2001). "On the role of peripheral macrophages during active experimental allergic encephalomyelitis (EAE)." *J Neural Transm* **108** (4): 379-95.

Inglis, J. J., Nissim, A., Lees, D. M., Hunt, S. P., Chernajovsky, Y. and Kidd, B. L. (2005). "The differential contribution of tumour necrosis factor to thermal and mechanical hyperalgesia during chronic inflammation." *Arthritis Res Ther* **7** (4): R807-16.

Ingram, G., Hakobyan, S., Robertson, N. P. and Morgan, B. P. (2009). "Complement in multiple sclerosis: its role in disease and potential as a biomarker." *Clin Exp Immunol* **155** (2): 128-39.

Ishii, M., Miyashita, T., Tsuchiya, K., Ueda, K., Umemura, A. and Honda, T. (2004). "Histological distribution and ultrastructural features of immunoreactive terminals against RT97, a monoclonal antibody to a 200 kD neurofilament, in the spinal dorsal horn of a rat." *Fukushima J Med Sci* **50** (2): 65-74.

Issazadeh, S., Ljungdahl, A., Höjeberg, B., Mustafa, M. and Olsson, T. (1995). "Cytokine production in the central nervous system of Lewis rats with experimental autoimmune encephalomyelitis: dynamics of mRNA

expression for interleukin-10, interleukin-12, cytolysin, tumor necrosis factor [alpha] and tumor necrosis factor [beta]." *Journal of Neuroimmunology* **61** (2): 205-212.

Jacobs, L. D., Wende, K. E., Brownsheidle, C. M., Apatoff, B., Coyle, P. K., Goodman, A., Gottesman, M. H., Granger, C. V., Greenberg, S. J., Herbert, J., Krupp, L., Lava, N. S., Mihai, C., Miller, A. E., Perel, A., Smith, C. R. and Snyder, D. H. (1999). "A profile of multiple sclerosis: The new york state multiple sclerosis consortium." *Mult Scler* **5** (5): 369-76.

Jahn, O., Tenzer, S. and Werner, H. B. (2009). "Myelin proteomics: molecular anatomy of an insulating sheath." *Mol Neurobiol* **40** (1): 55-72.

Jakeman, L. B., Guan, Z., Wei, P., Ponnappan, R., Dzwonczyk, R., Popovich, P. G. and Stokes, B. T. (2000). "Traumatic spinal cord injury produced by controlled contusion in mouse." *J Neurotrauma* **17** (4): 299-319.

Jenkins, T. M. and Thompson, A. J. (2009). "Diagnosing and managing multiple sclerosis." *Practitioner* **253** (1721): 25-30, 2-3.

Ji, R. R. and Strichartz, G. (2004). "Cell signaling and the genesis of neuropathic pain." *Sci STKE* **2004** (252): reE14.

Johnson, E. S. and Ludwin, S. K. (1981). "The demonstration of recurrent demyelination and remyelination of axons in the central nervous system." *Acta Neuropathol (Berl)* **53** (2): 93-8.

Johnson, K. P. (2007). "Natalizumab (tysabri) treatment for relapsing multiple sclerosis." *Neurologist* **13** (4): 182-7.

Jones, C. A., Pohar, S., Warren, S., Turpin, K. and Warren, K. (2008). "The burden of multiple sclerosis: A community health survey." *Health and Quality of Life Outcomes* **6** (1): 1.

Jones, K. R., Farinas, I., Backus, C. and Reichardt, L. F. (1994). "Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development." *Cell* **76** (6): 989-99.

Josephson, A., Widenfalk, J., Trifunovski, A., Widmer, H. R., Olson, L. and Spenger, C. (2001). "GDNF and NGF family members and receptprs in human fetal and adult spinal cord and dorsal root ganglia." *J Comp Neurol* **440** (2): 204-17.

Junger, H. and Sorkin, L. S. (2000). "Nociceptive and inflammatory effects of subcutaneous TNFalpha." *Pain* **10** (4): 201-15.

Jurewicz, A., Matysiak, M., Tybor, K. and Selmaj, K. (2003). "TNF-induced death of adult human oligodendrocytes is mediated by c-jun NH2-terminal kinase-3." *Brain* **126** (Pt 6): 1358-70.

Kalman, B. and Leist, T. P. (2004). "Familial multiple sclerosis and other inherited disorders of hte white matter." *Neurologist* **10** (4): 201-15.

Kapeller, P., McLean, M. A., Griffin, C. M., Chard, D., Parker, G. J., Barker, G. J., Thompson, A. J. and Miller, D. H. (2001). "Preliminary evidence for neuronal damage in cortical grey matter and normal appearing white matter in short duration relapsing-remitting multiple sclerosis: A quantitative MR spectroscopic imaging study." *J Neural* **248** (2): 131-8.

Kaplan, D. R. and Miller, F. D. (1997). "Signal transduction by the neurotrophin receptors." *Curr Opin Cell Biol* **9** (2): 213-21.

Kaplan, M. R., Meyer-Franke, A., Lambert, S., Bennett, V., Duncan, I. D., Levinson, S. R. and Barres, B. A. (1997). "Induction of sodium channel clustering by oligodendrocytes." *Nature* **386** (6626): 724-8.

Kappos, L., Bates, D., Hartung, H. P., Havrdova, E., Miller, D., Polman, C. H., Ravnborg, M., Hauser, S. L., Rudick, R. A., Weiner, H. L., O'Connor, P. W., King, J., Radue, E. W., Yousry, T., Major, E. O. and Clifford, D. B. (2007). "Natalizumab treatment for multiple sclerosis: recommendations for patient selection and monitoring." *Lancet Neurol* **6** (5): 431-41.

Karim, S. A., Barrie, J. A., McCulloch, M. C., Montague, P., Edgar, J. M., Kirkham, D., Anderson, T. J., Nave, K. A., Griffiths, I. R. and McLaughlin, M. (2007). "PLP overexpression perturbs myelin protein composition and myelination in a mouse model of Pelizaeus-Merzbacher disease." *Glia* **55** (4): 341-51.

Karlik, S. J., Munoz, D., St Louis, J. and Strejan, G. (1999). "Correlation between MRI and clinico-pathological manifestations in Lewis rats protected from experimental allergic encephalomyelitis by acylated synthetic peptide of myelin basic protein." *Magn Reson Imaging* **17** (5): 731-7.

Kato, K., Liu, H., Kikuchi, S. I., Myers, R. R. and Shubayev, V. I. (2009). "Immediate anti-tumor necrosis factor-alpha (etanercept) therapy enhances axonal regeneration after sciatic nerve crush." *J Neurosci Res* **88** (2): 360-368.

Kawamura, K., Yamamura, T., Yokoyama, K., Chui, D. H., Fukui, Y., Sasazuki, T., Inoko, H., David, C. S. and Tabira, T. (2000). "HIa-DR2-restricted responses to proteolipid protein 95-116 peptide cause autoimmune encephalitis in transgenic mice." *J Clin Invest* **105** (7): 977-84.

Kawasaki, Y., Zhang, L., Cheng, J. K. and Ji, R. R. (2008). "Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord." *J Neurosci* **28** (20): 5189-94.

Keller, A. F., Beggs, S., Salter, M. W. and De Koninck, Y. (2007). "Transformation of the output of spinal lamina I neurons after nerve injury and microglia stimulation underlying neuropathic pain." *Mol Pain* **3**: 27.

Kenner, M., Menon, U. and Elliott, D. G. (2007). "Multiple sclerosis as a painful disease." *Int Rev Neurobiol* **79**: 303-21.

Kerlero de Rosbo, N., Milo, R., Lees, M. B., Burger, D., Bernard, C. C. and Ben-Nun, A. (1993). "Reactivity to myelin antigens in multiple sclerosis. Peripheral blood lymphocytes respond predominantly to myelin oligodendrocyte glycoprotein." *J Clin Invest* **92** (6): 2602-8.

Klein, G. M., Rose, M. S. and Seland, T. P. (1994). "A prevalence study of multiple sclerosis in the Crowsnest Pass region of southern Alberta." *Can J Neurol Sci* **21** (3): 262-5.

Koch, S., Goedde, R., Nigmatova, V., Epplen, J. T., Muller, N., De Seze, J., Vermersch, P., Mamot, T., Schmidt, R. E. and Witte, T. (2005).

"Assotiation of multiple sclerosis with ILT6 deficiency." *Genes Immun* **6** (5): 445-7.

Koltzenburg, M. and Scadding, J. (2001). "Neuropathic pain." *Curr Opin Neurol* **14** (5): 641-7.

Kornek, B. and Lassmann, H. (2003). "Neuropathology of multiple sclerosis-new concepts." *Brain Res Bull* **61** (3): 321-6.

Kumar, V. and Sercarz, E. (1998). "Induction or protection from experimental autoimmune encephalomyelitis depends on the cytokine secretion profile of TCR peptide-specific regulatory CD4 T cells." *J Immunol* **161** (12): 6585-91.

Kuno, R., Yoshida, Y., Nitta, A., Nabeshima, T., Wang, J., Sonobe, Y., Kawanokuchi, J., Takeuchi, H., Mizuno, T. and Suzumura, A. (2006). "The role of TNF-alpha and its receptors in the production of NGF and GDNF by astrocytes." *Brain Res* **1116** (1): 12-8.

Kurtzke, J. F. (2000). "Epidemiology of multiple sclerosis. does this really point toward an etiology? lectio doctoralis." *Neurol Sci* **21** (6): 383-403.

Kurtzke, J. F. (2005). "Epidemiology and etiology of multiple sclerosis." *Phys Med Rehabil Clin N Am* **16** (2): 327-49.

Kurtzke, J. F., Page, W. F., Murphy, F. M. and Norman, J. E., Jr. (1992). "Epidemiology of multiple sclerosis in US veterans. 4. age at onset." *Neuroepidemiology* **11** (4-6): 226-35.

Lassmann, H., Bruck, W. and Lucchinetti, C. (2001). "Heterogeneity of multiple sclerosis pathogenesis: Implications for diagnosis and therapy." *Trends Mol Med* **7** (3): 115-21.

Lassmann, H. and Ransohoff, R. M. (2004). "The CD4-Th1 model for multiple sclerosis: a critical [correction of crucial] re-appraisal." *Trends Immunol* **25** (3): 132-7.

Laule, C., Leung, E., Lis, D. K., Traboulsee, A. L., Paty, D. W., MacKay, A. L. and Moore, G. R. (2006). "Myelin water imaging in multiple sclerosis: quantitative correlations with histopathology." *Mult Scler* **12** (6): 747-53.

Lee, H. L., Lee, K. M., Son, S. J., Hwang, S. H. and Cho, H. J. (2004). "Temporal expression of cytokines and their receptors mRNAs in a neuropathic pain model." *Neuroreport* **15** (18): 2807-11.

Lees, J. R., Iwakura, Y. and Russell, J. H. (2008). "Host T cells are the main producers of IL-17 within the central nervous system during initiation of experimental autoimmune encephalomyelitis induced by adoptive transfer of Th1 cell lines." *J Immunol* **180** (12): 8066-72.

Lendon, C. L., Davies, M. J., Born, G. V. and Richardson, P. D. (1991). "Atherosclerotic plaque caps are locally weakened when macrophages density is increased." *Atherosclerosis* **87** (1): 87-90.

Leung, L. and Cahill, C. M. (2010). "TNF-alpha and neuropathic pain--a review." *J Neuroinflammation* **7**: 27.

Levi-Montalcini, R. and Hamburger, V. (1951). "Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo." *J Exp Zool* **116** (2): 321-61.

Levi-Montalcini, R., Meyer, H. and Hamburger, V. (1954). "In vitro experiments on the effects of mouse sarcomas 180 and 37 on the spinal and sympathetic ganglia of the chick embryo." *Cancer Res* **14** (1): 49-57.

Li, L., Beng, Y. S. and Zhou, X. F. (2000). "Downregulation of TrkA expression in primary sensory neurons afetr unilateral lumbar spinal nerve transection and som rescuing effects of nerve growth factor infusion." *Neurosci Res* **38** (2): 183-91.

Li, Y., Field, P. M. and Raisman, G. (1997). "Repair of adult rat corticospinal tract by transplants of olfactory ensheathing cells." *Science* **277** (5334): 2000-2.

Li, Y., Ji, A., Weihe, E. and Schafer, M. K. (2004). "Cell-specific expression and lipopolysaccharide-induced regulation of tumor necrosis factor alpha (TNFalpha) and TNF receptors in rat dorsal rood ganglion." *J, Neurosci* **24** (43): 9623-31.

Libbey, J. E., McCoy, L. L. and Fujinami, R. S. (2007). "Molecular mimicry in multiple sclerosis." *Int Rev Neurobiol* **79**: 127-47.

Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S. and Collins, F. (1993). "GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons." *Science* **260** (5111): 1130-2.

Lindberg, R. L., De Groot, C. J., Montagne, L., Freitag, P., van der Valk, P., Kappos, L. and Leppert, D. (2001). "The expression profile of matrix

metalloproteinases (MMPs) and their inhibitors (TIMPs) in lesions and normal appearing white matter of multiple sclerosis." *Brain* **124** (Pt 9): 1743-53.

Lindert, R. B., Haase, C. G., Brehm, U., Linington, C., Wekerle, H. and Hohlfeldd, R. (1999). "Multiple sclerosis: B- and T-cell responses to the extracellular domain of the myelin oligodendrocyte glycoprotein." *Brain* **122** ((pt 11) (pt 11)): 2089-100.

Lindsay, R. M., Thoenen, H. and Barde, Y. A. (1985). "Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor." *Dev Biol* **112** (2): 319-28.

Link, H. and Xiao, B. G. (2001). "Rat models as tool to develop new immunotherapies." *Immunol Rev* **184**: 117-28.

Link, J., Soderstrom, M., Olsson, T., Hojeberg, B., Ljungdahl, A. and Link, H. (1994). "Increased transforming growth factor-beta, interleukin-4, and interferon-gamma in multiple sclerosis." *Ann Neurol* **36** (3): 379-86.

Linker, R. A., Lee, D. H., Demir, S., Wiese, S., Kruse, N., Siglienti, I., Gerhardt, E., Neumann, H., Sendtner, M., Luhder, F. and Gold, R. (2010). "Functional role of brain-derived neurotrophic factor in neuroprotective autoimmunity: therapeutic implications in a model of multiple sclerosis." *Brain* **133** (Pt 8): 2248-63.

Lublin, F. D. (1984). "Role of myelin antigens in murine relapsing experimental allergic encephalomyelitis." *J Clin Lab Immunol* **13** (4): 179-82.

Lublin, F. D. and Reingold, S. C. (1996). "Defining the clinical course of multiple sclerosis: Results of an international survey. national multiple sclerosis society (USA) advisory committee on clinical trials of new agents in multiple sclerosis." *Neurology* **46** (4): 907-11.

Lucchinetti, C., Bruck, W. and Noseworthy, J. (2001). "Multiple sclerosis: Recent developments in neuropathology, pathogenesis, magnetic resonance imaging studies and treatment." *Curr Opin Neurol* **14** (3): 259-69.

Lucchinetti, C., Bruck, W., Rodriguez, M. and Lassmann, H. (1996). "Distinct patterns of multiple sclerosis pathology indicates heterogeneity on pathogenesis." *Brain Pathol* **6** (3): 259-74.

Ludwin, S. K. (2006). "The pathogenesis of multiple sclerosis: relating human pathology to experimental studies." *J Neuropathol Exp Neurol* **65** (4): 305-18.

Lykissas, M. G., Batistatou, A. K., Charalabopoulos, K. A. and Beris, A. E. (2007). "The role of neurotrophins in axonal growth, guidance, and regeneration." *Curr Neurovasc Res* **4** (2): 143-51.

Lyman, W. D., Roth, G. A., Brosnan, C. F., Bornstein, M. B. and Raine, C. S. (1988). "Quantitation of antigen-specific T-cell-induced demyelination in vitro." *J Neuroimmunol* **17** (2): 175-80.

Ma, W. and Bisby, M. A. (1998). "Increased activation of nuclear factor kappa B in rat lumbar dorsal rood ganglion neurons following partial sciatic nerve injuries." *Brain Res* **797** (2): 243-54.

Maimone, D., Reder, A. T. and Gregory, S. (1993). "T cell lymphokine-induced secretion of cytokines by monocytes from patients with multiple sclerosis." *Cell Immunol* **146** (1): 96-106.

Mallam, E. and Scolding, N. (2009). "The Diagnosis of MS." *Int MS J* **16** (1): 19-25.

Mannie, M., Swanborg, R. H. and Stepaniak, J. A. (2009). "Experimental Autoimmune Encephalomyelitis in the rat." *Current Protocols in Immunology* **Apr.** (15.2): 15.2.

Marchettini, P., Formaglio, F. and Lacerenza, M. (2006). "Pain as heralding symptom in multiple sclerosis." *Neurol Sci* **27** (Supplement 4): s294-s296.

Martino, G. and Hartung, H. P. (1999). "Immunopathogenesis of multiple sclerosis: the role of T cells." *Curr Opin Neurol* **12** (3): 309-21.

Mason, I. I. (1996). "The GDNF receptor: Recent progress and unanswered questions." *Mol Cell Neurosci* **8** (2/3): 112-9.

Matthews, P. M. and Arnold, D. L. (2001). "Magnetic resonance imaging of multiple sclerosis: New insights linking pathology to clinical evolution." *Curr Opin Neurol* **14** (3): 279-87.

Mattson, D. H. (2002). "Update on the diagnosis of multiple sclerosis." *Expert Rev Neurother* **2** (3): 319-28.

Mayers, I. and Johnson, D. (1998). "The nonspecific inflammatory response to injury." *Can J Anaesth* **45** (9): 871-9.

McDonald, W. I., Compston, A., Edan, G., Goodkin, D., Hartung, H. P., Lublin, F. D., McFarland, H. F., Paty, D. W., Polman, C. H., Reingold, S. C.,

Sandberg-Wollheim, M., Sibly, W., Thompson, A. J., van den Noort, S., Weinshenker, B. Y. and Wolinsky, J. S. (2001). "Recommended diagnostic criteria for multiple sclerosis: Guidelines from the international panel on hte diagnosis of multiple sclerosis." *Ann Neurol* **50** (1): 121-7.

McDonald, W. I., Compston, A., Edan, G., Goodkin, D., Hartung, H. P., Lublin, F. D., McFarland, H. F., Paty, D. W., Polman, C. H., Reingold, S. C., Sandberg-Wollheim, M., Sibly, W., Thompson, A. J., van den Noort, S., Weinshenker, B. Y. and Wolinsky, J. S. (2001). "Recommended diagnostic criteria for multiple sclerosis: Guidelines from the international panel on the diagnosis of multiple sclerosis." *Ann Neurol* **50** (1): 121-7.

Melanson, M., Grossberndt, A., Klowak, M., Leong, C., Frost, E. E., Prout, M., LeDorze, J., Gramlich, C., Doupe, M., Wong, L. and Namaka, M. (2010). "Fatigue and cognition in patients with relapsing multiple sclerosis treated with interferon beta

" *International Journal of Neuroscience* **in press**.

Melanson, M., Miao, P., Eisenstat, D., Gong, Y., Gu, X., Au, K., Zhu, W., Begum, F., Frost, E. and Namaka, M. (2009). "Experimental autoimmune encephalomyelitis-induced upregulation of tumor necrosis factor-alpha in the dorsal root ganglia." *Mult Scler* **15** (10): 1135-1145.

Melrose, H. L., Kinloch, R. A., Cox, P. J., Field, M. J., Collins, D. and Williams, D. (2007). "[3H] pregabalin binding is increased in ipsilateral dorsal horn following chronic constriction injury." *Neurosci Lett* **417** (2): 187-92.

Mendel, I., Kerlero de Rosbo, N. and Ben-Nun, A. (1995). "A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor V beta expression of encephalitogenic T cells." *Eur J Immunol* **25** (7): 1951-9.

Mendell, L. M. (1995). "Neurotrophic factors and the specification of neural function." *The Neuroscientist* **1** (1): 26-34.

Menon, K. K., Piddlesden, S. J. and Bernard, C. C. (1997). "Demyelinating antibodies to myelin oligodendrocyte glycoprotein and galactocerebroside induce degradation of myelin basic protein in isolated human myelin." *J Neurochem* **69** (1): 214-22.

Merighi, A., Salio, C., Ghirri, A., Lossi, L., Ferrini, F., Betelli, C. and Bardoni, R. (2008). "BDNF as a pain modulator." *Progress in Neurobiology* **85** (3): 297-317.

Merrill, J. E. (1992). "Proinflammatory and antiinflammatory cytokines in multiple sclerosis and central nervous system acquired immunodeficiency syndrome." *J Immunother* **12** (3): 167-70.

Miao, P., Madec, K., Gong, Y., Shen, H., Eisenstat, D., Melanson, M., Gu, X., Leong, C., Klowak, M. and Namaka, M. (2008). "Axotomy-induced up-regulation of tumor necrosis factor-alpha in the dorsal root ganglia." *Neurol Res* **30** (6): 623-31.

Miao, P., Melanson, M. and Namaka, M. (2004). "Injury-induced expression of infammatory cytokines within rat dorsal root ganglia."

Millan, M. J. (1999). "The induction of pain: An integrative review." *Prog Neurobiol* **57** (1): 1-164.

Miller, D. H., Albert, P. S., Barkhof, F., Francis, G., Frank, J. A., Hodgkinson, S., Lublin, F. D., Paty, D. W., Reingold, S. C. and Simon, J. (1996). "Guidelines for the use of magnetic resonance techniques in monitoring the treatment of multiple sclerosis. US national MS society task force." *Ann Neurol* **39** (1): 6-16.

Miller, D. H., Barkhof, F. and Nauta, J. J. (1993). "Gadolinium enhancement increases the sensitivity of MRI in detecting disease activity in multiple sclerosis." *Brain* **116** ((pt 5) (pt 5)): 1077-94.

Mix, E., Meyer-Rienecker, H. and Zettl, U. (2008). "Animal models of multiple sclerosis for the development and validation of novel therapies – potential and limitations." *Journal of Neurology* **255** (0): 7-14.

Mobius, W., Patzig, J., Nave, K. A. and Werner, H. B. (2008). "Phylogeny of proteolipid proteins: divergence, constraints, and the evolution of novel functions in myelination and neuroprotection." *Neuron Glia Biol* **4** (2): 111-27.

Molliver, D. C., Wright, D. E., Leitner, M. L., Parsadanian, A. S., Doster, K., Wen, D., Yan, Q. and Snider, W. D. (1997). "IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life." *Neuron* **19** (4): 849-61.

Mondino, A., Khoruts, A. and Jenkins, M. K. (1996). "The anatomy of T-cell activation and tolerance." *Proceedings of the National Academy of Sciences of the United States of America* **93** (6): 2245-2252.

Mosmann, T. R. and Sad, S. (1996). "THe expanding universe of T-cell subsets: Th1, Th2 and more." *Immunol Today* **17** (3): 138-46.

Moulin, D. (2006). "Does acute pain associated with perpes zoster respond to treatment with gabapentin?" *Nat Clin Pract Neurol* **2** (6): 298-9.

Myers, R. R., Sekiguchi, Y., Kikuchi, S., Scontt, B., Medicherla, S., A., P. and Campana, W. M. (2003). "Inhibition of p38 MAP kinase activity enhances axonal regeneration." *Exp Neurol* **184** (2): 606-14.

Myers, R. R., Sekiguchi, Y., Kikuchi, S., Scott, B., Medicherla, S., Protter, A. and Campana, W. M. (2003). "Inhibition of p38 MAP kinase activity enhances axonal regeneration." *Exp Neurol* **184** (2): 606-14.

Nakanishi, T., Tamaki, M., Mizusawa, H., Akatsuka, T. and Kinoshita, T. (1986). "An experimental study for analyzing nerve conduction velocity." *Elecroencephalogr Clin Neurophysiol* **63** (5): 484-7.

Nakazawa, T., Nakazawa, C., Matsubara, A., Noda, K., Hisatomi, T., She, H., Michaud, N., Hafezi-Moghadam, A., Miller, J. W. and Benowitz, L. I. (2006). "Tumor necrosis factor-alpha mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma." *J Neurosci* **26** (49): 12633-41.

Namaka, M., Crook, A., Doupe, A., Kler, K., Vasconcelos, M., Klowak, M., Gong, Y., Wojewnik-Smith, A. and Melanson, M. (2008). "Examining the

evidence: complementary adjunctive therapies for multiple sclerosis." *Neurol Res* **30** (7): 710-9.

Namaka, M., Gramlich, C. R., Ruhlen, D., Melanson, M., Sutton, I. and Major, J. (2004). "A treatment algorithm for neuropathic pain." *Clin Ther* **26** (7): 951-79.

Namaka, M., Kapoor, S., Simms, L., Leong, C., Grossberndt, A., Prout, M., Frost, E., Esfahani, F., Gomori, A. and Mulvey, M. R. (2010). "Molecular Mimicry and Multiple Sclerosis." *J. Neurological Research* **submitted**.

Namaka, M., Leong, C., Grossberndt, A., Klowak, M., Turcotte, D., Esfahani, F., Gomori, A. and Intrater, H. (2009). "A treatment algorithm for neuropathic pain: an update." *Consult Pharm* **24** (12): 885-902.

Namaka, M., Pollitt-Smith, M., Gupta, A., Klowak, M., Vasconcelos, M., Turcotte, D., Gong, Y. and Melanson, M. (2006). "The clinical importance of neutralizing antibodies in relapsing-remitting multiple sclerosis." *Curr Med Res Opin* **22** (2): 223-39.

Namaka, M., Turcotte, D., Leong, C., Grossberndt, A. and Klassen, D. (2008). "Multiple sclerosis: etiology and treatment strategies." *Consult Pharm* **23** (11): 886-96.

Namaka, M. P., Sawchuk, M., MacDonald, S. C., Jordan, L. M. and Hochman, S. (2001). "Neurogenesis in postnatal mouse dorsal root ganglia." *Exp Neurol* **172** (1): 60-9.

Naugler, W. E. and Karin, M. (2008). "The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer." *Trends Mol Med* **14** (3): 109-19.

Navikas, V. and Link, H. (1996). "Review: Cytokines and the pathogenesis of multiple sclerosis." *J Neurosci* **45** (4): 322-33.

Nelson, D. A. (1992). "Sensory symptoms of multiple sclerosis may be clues to causation: review and a hypothesis." *Del Med J* **64** (3): 205-13.

Nelson, D. A. (1993). "Dorsal root ganglia may be reservoirs of viral infection in multiple sclerosis." *Med Hypotheses* **40** (5): 278-83.

Ng, B. K., Chen, L., Mandemakers, W., Cosgaya, J. M. and Chan, J. R. (2007). "Anterograde Transport and Secretion of Brain-Derived Neurotrophic Factor along Sensory Axons Promote Schwann Cell Myelination." *J. Neurosci.* **27** (28): 7597-7603.

Nie, D. Y., Ma, Q. H., Law, J. W., Chia, C. P., Dhingra, N. K., Shimoda, Y., Yang, W. L., Gong, N., Chen, Q. W., Xu, G., Hu, Q. D., Chow, P. K., Ng, Y. K., Ling, E. A., Watanabe, K., Xu, T. L., Habib, A. A., Schachner, M. and Xiao, Z. C. (2006). "Oligodendrocytes regulate formation of nodes of Ranvier via the recognition molecule OMgp." *Neuron Glia Biol* **2**: 151-164.

O'Connor, K. C., Bar-Or, A. and Hafler, D. A. (2001). "The neuroimmunology of multiple sclerosis: Possible roles of T and B lymphocytes in immunopathogenesis." *J Clin Immunol* **21** (2): 81-92.

O'Connor, P. (2002). "Multiple Sclerosis: the facts you need." *Toronto: Key Porter* **N/A** (N/A): N/A.

O'Connor, P. and Group, C. M. S. W. (2002). "Key issues in the diagnosis and treatment of multiple sclerosis. an overview." *Neurology* **59** ((6 Suppl 3)): S1-33.

O'Connor, R. A., Taams, L. S. and Anderton, S. M. (2010). "Translational mini-review series on Th17 cells: CD4 T helper cells: functional plasticity and differential sensitivity to regulatory T cell-mediated regulation." *Clin Exp Immunol* **159** (2): 137-47.

Obata, K. and Noguchi, K. (2006). "BDNF in sensory neurons and chronic pain." *Neurosci Res* **55** (1): 1-10.

Ohtori, S., Takahashi, K., Moriya, H. and Myers, R. R. (2004). "TNF-alpha and TNF-alpha receptor type 1 upregulation in glia and neurons after peripheral nerve injury: Studies in murine DRG and spinal cord." *Spine* **29** (10): 1082-8.

Oka, N., Akiguchi, I., Mizutani, K., Satoi, H. and Kimura, J. (1998). "Tumor necrosis factor-alpha in peripheral nerve lesions." *Acta Neropathol (Berl)* **95** (1): 67-62.

Olechowski, C. J., Truong, J. J. and Kerr, B. J. (2009). "Neuropathic pain behaviours in a chronic-relapsing model of experimental autoimmune encephalomyelitis (EAE)." *Pain* **141** (1-2): 156-64.

Oliver, J. C., Bland, L. A., Oettinger, C. W., Arduino, M. J., McAllister, S. K., Aguero, S. M. and Favero, M. S. (1993). "Cytokine kinetics in an in vitro whole blood model following an endotoxin challenge." *Lymphokine Cytokine Res* **12** (2): 115-20.

Olsson, T., Zhi, W. W., Hojeberg, B., Kostulas, V., Jiang, Y. P., Anderson, G., Ekre, H. P. and Link, H. (1990). "Autoreactive T lymphocytes in multiple sclerosis determined by antigen-induced secretion of interferon-gamma." *J Clin Invest* **86** (3): 981-5.

Osterberg, A. and Boivie, J. (2009). "Central pain in multiple sclerosis - Sensory abnormalities." *Eur J Pain*.

Otto-Buczkowska, E., Kazibutowska, Z., Soltyk, J. and Machnica, L. (2008). "[Neuropathy and type 1 diabetes mellitus]." *Endokrynol Diabetol Chor Przemiany Materii Wieku Rozw* **14** (2): 109-16.

Overbergh, L., Valckx, D., Waer, M. and Mathieu, C. (1999). "Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR." *Cytokine* **11** (4): 305-12.

Ozenci, V., Kouwenhoven, M. and Link, H. (2002). "Cytokines in multiple sclerosis: Methodological aspects and pathogenic implications." *Mult Scler* **8** (5): 396-404.

Palma, A. E., Owh, P., Fredric, C., Readhead, C. and Moscarello, M. A. (1997). "Characterization of myelin basic protein charge microheterogeneity in developing mouse brain and in the transgenic shiverer mutant." *J Neurochem* **69** (4): 1753-62.

Panitch, H. S., Hirsch, R. L., Schindler, J. and Johnson, K. P. (1987). "Treatment of multiple sclerosis with gamma interferon: Exacerbations associated with activation of the immune system." *Neurology* **37** (7): 1097-102.

Pannese, E. (1981). "The satellite cells of the sensory ganglia." *Adv Anat Embryol Cell Biol* **65**: 1-111.

Pannese, E., Ledda, M., Conte, V. and Procacci, P. (1990). "The perikaryal projections of rabbit spinal ganglion neurons. A comparison of thin section reconstructions and scanning microscopy views." *Anat Embryol* **181** (5): 427-32.

Paty, D. W. (1997). "MRI as a method to reveal in-vivo pathology in MS." *J Neural Transm Suppl.* **49**: 211-7.

Pauly, S., Broll, K., Wittmann, M., Giegerich, G. and Schwarz, H. (2002). "CD137 is expressed by follicular dendritic cells and costimulates B lymphocyte activation in germinal centers." *J Leukoc Biol* **72** (1): 35-42.

Petry, K. G., Boullerne, A. I., Pousset, F., Caille, J. M. and Dousset, V. (2000). "Experimental allergic encephalomyelitis animal models for analyzing features of multiple sclerosis." *Pathol Biol (Paris)* **48** (1): 47-53.

Pezet, S., Onteniente, B., Grannec, G. and Calvino, B. (1999). "Chronic pain is associated with increased TrkA immunoreactivity in spinoreticular neurons." *J Neurosci* **19** (13): 5482-92.

Pleasure, D., Soulika, A., Singh, S. K., Gallo, V. and Bannerman, P. (2006). "Inflammation in white matter: clinical and pathophysiological aspects." *Ment Retard Dev Disabil Res Rev* **12** (2): 141-6.

Poisbeau, P., Patte-Mensah, C., Keller, A. F., Barrot, M., Breton, J. D., Luis-Delgado, O. E., Freund-Mercier, M. J., Mensah-Nyagan, A. G. and

Schlichter, R. (2005). "Inflammatory pain upregulates spinal inhibition via endogenous neurosteroid production." *J Neurosci* **25** (50): 11768-76.

Pollmann, W. and Feneberg, W. (2008). "Current management of pain associated with multiple sclerosis." *CNS Drugs* **22** (4): 291-324.

Pollock, J., McFarlane, S. M., Connell, M. C., Zehavi, U., Vandenabeele, P., MacEwan, D. J. and Scott, R. H. (2002). "TNF-alpha receptors simultaneously activate Ca2+ mobilisation and stress kinases in cultured sensory neurons." *Neuropharmacology* **42** (1): 93-106.

Polman, C. H., O'Connor, P., Havrdova, E., Hutchinson, M., Kappos, L., Miller, D. H., Phillips, J. T., Lublin, F. D., Giovannoni, G., Wajgt, A., Toal, M., Lynn, F., Panzara, M. A. and Sandrock, A. W. (2006). "AFFIRM Investigators. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis." *N Engl J med* **354** (9): 899-910.

Popper, P., Lopez, I., Beizai, P., Li, G., Kim, J., Micevych, P. E. and Honrubia, V. (1999). "Expression of BDNF and TrkB mRNAs in the crista neurosensory epithelium and vestibular ganglia following ototoxic damage." *Brain Res* **846** (1): 40-51.

Portenoy, R. K., Yang, K. and Thorton, D. (1988). "Chronic intractable pain: an atypical presentation of multiple sclerosis." *J Neurol* **235** (4): 226-8.

Poser, C. M. (1997). "Misdiagnosis of multiple sclerosis and beta-interferon." *Lancet* **349** (9069): 1916-1916.

Poser, C. M., Paty, D. W., Scheinberg, L., McDonald, W. I., Davis, F. A., Ebers, G. C., Johnson, K. P., Sibly, W., Silberberg, D. H. and Tourtellotte, W. W.

(1983). "New diagnostic criteria for multiple sclerosis: Guidelines for research protocols." *Ann Neurol* **13** (3): 227-31.

Pouly, S., Becher, B., Blain, M. and Antel, J. P. (2000). "Interferon-gamma modulates human oligodendrocyte susceptibility to Fas-mediated apoptosis." *J Neuropathol Exp Neurol* **59** (4): 280-6.

Prendergast, C. T. and Anderton, S. M. (2009). "Immune Cell Entry to Central Nervous System - Current Understanding and Prospective Therapeutic Targets." *Endocr Metab Immune Disord Drug Targets*.

Prineas, J. (1975). "Pathology of the early lesion in multiple sclerosis." *Hum Pathol* **6** (5): 531-54.

Proudfoot, A. E., de Souza, A. L. and Muzio, V. (2008). "The use of chemokine antagonists in EAE models." *J Neuroimmunol* **198** (1-2): 27-30.

Qiao, L. Y., Gulick, M. A., Bowers, J., Kuemmerle, J. F. and Grider, J. R. (2008). "Differential changes in brain-derived neurotrophic factor and extracellular signal-regulated kinase in rat primary afferent pathways with colitis." *Neurogastroenterol Motil* **20** (8): 928-38.

Racz, I., Nadal, X., Alferink, J., Banos, J. E., Rehnelt, J., Martin, M., Pintado, B., Gutierrez-Adan, A., Sanguino, E., Manzanares, J., Zimmer, A. and Maldonado, R. (2008). "Crucial role of CB (2) cannabinoid receptor in the regulation of central immune responses during neuropathic pain." *J Neurosci* **28** (46): 12125-35.

Raine, C. S., Moore, G. R., Hintzen, R. and Traugott, U. (1988). "Induction of oligodendrocyte proliferation and remyelination after chronic demyelination. Relevance to multiple sclerosis." *Lab Invest* **59** (4): 467-76.

Raine, C. S., Scheinberg, L. and Waltz, J. M. (1981). "Multiple sclerosis. Oligodendrocyte survival and proliferation in an active established lesion." *Lab Invest* **45** (6): 534-46.

Raine, C. S. and Wu, E. (1993). "Multiple sclerosis: remyelination in acute lesions." *J Neuropathol Exp Neurol* **52** (3): 199-204.

Ransohoff, R. M. (1999). "Mechanisms of inflammation in MS tissue: adhesion molecules and chemokines." *J Neuroimmunol* **98** (1): 57-68.

Ransohoff, R. M. (2005). "Immunologic correlates of MS pathologic subtypes." *Mult Scler* **11** (1): 101-2.

Rasminsky, M. and Sears, T. A. (1972). "Internodal conduction in undissected demyelinated nerve fibres." *J Physiol* **227** (2): 323-50.

Rausch, M., Hiestand, P., Baumann, D., Cannet, C. and Rudin, M. (2003). "MRI-based monitoring of inflammation and tissue damage in acute and chronic relapsing EAE." *Magn Reson Med* **50** (2): 309-14.

Raymon, H. K., Thode, S., Zhou, J., Friedman, G. C., Pardinas, J. R., Barrere, C., Johnson, R. M. and Sah, D. W. (1999). "Immortalized human dorsal root ganglion cells differentiate into neurons with nociceptive properties." *J Neurosci* **19** (13): 5420-8.

Ren, X. T., Wu, W. P., Xu, Q. G. and Huang, D. H. (2008). "[Change of CCR7 and CD45RA after blocking of Kv1.3 potassium channel of CD4+ T

lymphocytes in multiple sclerosis]." *Zhonghua Yi Xue Za Zhi* **88** (27): 1896-9.

Richardson, P. M. and Riopelle, R. J. (1984). "Uptake of nerve growth factor along peripheral and spinal axons of primary sensory neurons." *J Neurosci* **4** (7): 1683-9.

Rodriguez, M. (1992). "Central nervous system demyelination and remyelination in multiple sclerosis and viral models of disease." *J Neuroimmunol* **40** (2-3): 255-63.

Rodriguez, M. and Miller, D. J. (1994). "Immune promotion of central nervous system remyelination." *Prog Brain Res* **103**: 343-55.

Romagnani, S. (1999). "Th1/Th2 cells." *Inflammatory Bowel Diseases* **5** (4): 285-294.

Romanelli, P. and Espsotp, V. (2004). "The functional anatomy of neuropathic pain." *Neurosurgery* **15** (3): 257-68.

Rotteveel, F. T. and Lucas, C. J. (1990). "T lymphocytes in the cerebrospinal fluid of patients with multiple sclerosis." *Immunol Res* **9** (4): 287-97.

Rovaris, M. and Filippi, M. (1999). "Magnetic resonance techniques to monitor disease evolution and treatment trial outcomes in multiple sclerosis." *Curr Opin Neurol* **12** (3): 337-44.

Rudick, R. A. and Cutter, G. (2007). "Interferon-beta for multiple sclerosis: Long-term benefits?" *Ann Neurol* **61** (4): 283-5.

Rudick, R. A., Stuart, W. H., Calabresi, P. A., Confavreux, C., Galetta, S. L., Radue, E. W., Lublin, F. D., Weinstock-Guttman, B., Wynn, D. R., Lynn,

F., Panzara, M. A., Sandrock, A. W. and Investigators, S. (2006). "Natalizumab plus interferon beta-1a for relapsing multiple sclerosis." *N Engl J med* **354** (9): 911-23.

Ruffini, F., Chojnacki, A., Weiss, S. and Antel, J. P. (2006). "Immunobiology of oligodendrocytes in multiple sclerosis." *Adv Neurol* **98**: 47-63.

Ruohonen, S., Jagodi, M., Khademi, M., Taskinen, H. S., Ojala, P., Olsson, T. and M., R. (2002). "Contralateral non-operated nerve to transected rat sciatic nerve shows increased expression of IL-1beta, TGF-beta1,TNF-alpha nad IL-10." *J Neuroimmunol* **132** ((1-2)): 11-7.

Saha, R., Liu, X. and Pahan, K. (2006). "Up-regulation of BDNF in Astrocytes by TNF-α: A Case for the Neuroprotective Role of Cytokine." *Journal of Neuroimmune Pharmacology* **1** (3): 212-222.

Salio, C., Averill, S., Priestley, J. V. and Merighi, A. (2007). "Costorage of BDNF and neuropeptides within individual dense-core vesicles in central and peripheral neurons." *Developmental Neurobiology* **67** (3): 326-338.

Salzer, J. L. (2003). "Polarized Domains of Myelinated Axons." *Neuron* **40** (2): 297-318.

Sarchielli, P., Presciutti, O., Tarducci, R., Gobbi, G., Alberti, A., Pelliccioli, G. P., Chiarini, P. and Gallai, V. (2002). "Localized (1)H magnetic resonance spectroscopy in mainly cortical gray matter of patients with multiple sclerosis." *J Neurol* **249** (7): 902-10.

Scallow, G. (1989). "Efferent and afferent fibres in human sacral ventral nerve roots: Basic research and clinical implications." *Electroencephalogr Clin Neurophysiol* **29** (1): 33-53.

Schafer, M. K., Svensson, C. I., Sommer, C. and Sorkin, L. S. (2003). "Tumor necrosis factor-alpha induces mechanical allodynia after spinal nerve ligatation by activation of p38MAPK in primary sensory neurons." *J Neurosci* **23** (7): 2517-21.

Schafers, M., Sokin, L. S., Geis, C. and Shubayev, V. I. (2003). "Spinal nerve ligation induces transient upregulation of tumor necrosis factor receptors 1 and 2 in injured and adjacent uninjured dorsal rood ganglia in the rat." *Neurosci Lett.* **347** (3): 179-82.

Schmeichel, A. M., Schmelzer, J. D. and Low, P. A. (2003). "Oxidativ injury and apoptosis of dorsal rood ganglion neurons in chronic experimental diabetic neuropathy." *Diabetes* **52** (1): 165-71.

Scholz, J. and Woolf, C. J. (2007). "The neuropathic pain triad: neurons, immune cells and glia." *Nat Neurosci* **10** (11): 1361-8.

Scolding, N. J., Frith, S., Linington, C., Morgan, B. P., Campbell, A. K. and Compston, D. A. (1989). "Myelin-oligodendrocyte glycoprotein (MOG) is a surface marker of oligodendrocyte maturation." *J Neuroimmunol* **22** (3): 169-76.

Segal, B. M. (2010). "Th17 cells in autoimmune demyelinating disease." *Semin Immunopathol*.

Segal, B. M. and Cross, A. H. (2000). "Fas (t) track to apoptosis in MS: TNF receptors may suppress or potentiate CNS demyelination." *Neurology* **55** (7): 906-7.

Sekerkova, G., Malatova, Z. and Zigova, T. (1996). "Neovascularization of the dorsal root ganglia transplanted into the olfactory bulb of neonatal rats." *Arch Ital Biol* **134** (4): 291-304.

Selmaj, K. and Raine, C. S. (1988). "Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro." *Ann Neurol* **23** (4): 229-46.

Serres, S., Anthony, D. C., Jiang, Y., Campbell, S. J., Broom, K. A., Khrapitchev, A. and Sibson, N. R. (2009). "Comparison of MRI signatures in pattern I and II multiple sclerosis models." *NMR Biomed*.

Shamash, S., Reichert, F. and Rotshenker, S. (2002). "The cytokine network of wallerian degeneration: Tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta." *J Neurosci* **22** (8): 3052-60.

Shinder, V., Admir, R. and Devor, M. (1998). "Cross-excitation in dorsal root ganglia does not depend on close cell-to-cell apposition." *Neuroreport* **9** (18): 3997-4000.

Shubayev, V. I. and Myers, R. R. (2002). "Anterograde TNF[alpha] transport from rat dorsal root ganglion to spinal cord and injured sciatic nerve." *Neuroscience Letters* **320** (1-2): 99-101.

Sieber-Blum, M., Szeder, V. and Grim, M. (2004). "The role of NT-3 signaling in merkel cell development." *Prog Brain Res* **146**: 63-72.

Silos-Santiago, I., Greenlund, L. J., Johnson, E., M. Jr. and Snider, W. D. (1995). "Molecular genetics of neuronal survival." *Curr Opin Neurobiol* **5** (1): 42-9.

Silos-Santiago, I., Molliver, D. C., Ozaki, S., Smeyne, R. J., Fagan, A. M., Barbacid, M. and Snider, W. D. (1995). "Non- TrkA-expressing small DRG neurons are lost in TrkA deficient mice." *J Neurosci* **15** (9): 5929-42.

Skaper, S. D. (2008). "The biology of neurotrophins, signalling pathways, and functional peptide mimetics of neurotrophins and their receptors." *CNS Neurol Disord Drug Targets* **7** (1): 46-62.

Skihar, V., Silva, C., Chojnacki, A., Doring, A., Stallcup, W. B., Weiss, S. and Yong, V. W. (2009). "Promoting oligodendrogenesis and myelin repair using the multiple sclerosis medication glatiramer acetate." *Proc Natl Acad Sci U S A* **106** (42): 17992-7.

Slavin, A. J., Johns, T. G., Orian, J. M. and Bernard, C. C. (1997). "Regulation of myelin oligodendrocyte glycoprotein in different species throughout development." *Dev Neurosci* **19** (1): 69-78.

Snider, W. D. and McMahon, S. B. (1998). "Tackling pain at the source: new ideas about nociceptors." *Neuron* **20** (4): 629-32.

Sokolov, B. P. (2007). "Oligodendroglial abnormalities in schizophrenia, mood disorders and substance abuse. Comorbidity, shared traits, or molecular phenocopies?" *Int J Neuropsychopharmacol* **10** (4): 547-55.

Solly, S. K., Thomas, J. L., Monge, M., Demerens, C., Lubetzki, C., Gardinier, M. V., Matthieu, J. M. and Zalc, B. (1996). "Myelin/oligodendrocyte

glycoprotein (MOG) expression is associated with myelin deposition." *Glia* **18** (1): 39-48.

Sommer, C., Schmidt, C. and George, A. (1998). "Hyperalgesia in experimental neuropathy is dependent on the TNF receptor 1." *Exp Neurol* **151** (1): 138-42.

Sorensen, T. L., Tani, M., Jensen, J., Pierce, V., Lucchinetti, C., Folcik, V. A., Qin, S., Rottman, J., Sellebjerg, F., Strieter, R. M., Frederiksen, J. L. and Ransohoff, R. M. (1999). "Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients." *J Clin Invest* **103** (6): 807-15.

Sorkin, L. S., Xiao, W. H., Wagner, R. and Myers, R. R. (1997). "Tumor necrosis factor-alpha induces ectopic activity in nociceptive primary afferent fibres." *Neuroscience* **81** (1): 255-62.

Spengler, R. N., Sud, R., Knight, P. R. and Ignatowski, T. A. (2007). "Antinociception mediated by alpha (2)-adrenergic activation involves increasing tumor necrosis factor alpha (TNFalpha) expression and restoring TNFalpha and alpha (2)-adrenergic inhibition of norepinephrine release." *Neuropharmacology* **52** (2): 576-89.

Stankus, S. J., Dlugopolski, M. and Packer, D. (2000). "Management of herpes zoster (shingles) and postherpetic neuralgia." *Am Fam Physician.* **61** (8): 2437-44,2447-8.

Stepaniak, J. A., Gould, K. E., Sun, D. and Swanborg, R. H. (1995). "A comparative study of experimental autoimmune encephalomyelitis in Lewis and DA rats." *J Immunol* **155** (5): 2762-9.

Sun, J. B., Olsson, T., Wang, W. Z., Xiao, B. G., Kostulas, V., Fredrikson, S., Ekre, H. P. and Link, H. (1991). "Autoreactive T and B cells responding to myelin proteolipid protein in multiple sclerosis and controls." *Eur J Immunol* **21** (6): 1461-8.

Svendsen, K. B., Jensen, T. S., Overvad, K., Hansen, H. J., Koch-Henriksen, N. and Bach, F. W. (2003). "Pain in Patients With Multiple Sclerosis: A Population-Based Study." *Arch Neurol* **60** (8): 1089-1094.

Svensson, C. I., Schafers, M., Jones, T. L., Powell, H. and Sorkin, L. S. (2005). "Spinal blockade of TNF blocks spinal nerve ligation-induced increases in spinal P-p38." *Neurosci Lett* **379** (3): 209-13.

Swanborg, R. H. (1988). "Experimental allergic encephalomyelitis." *Methods Enzymol* **163**: 413-21.

Takahashi, N., Kikuchi, S., Shubayev, V. I., Campana, W. M. and Myers, R. R. (2006). "TNF-alpha and phosphorylation of ERK in DRG and spinal cord: Insights into mechanisms of sciatica." *Spine* **31** (5): 523-9.

Takei, Y. and Laskey, R. (2008). "Interpreting crosstalk between TNF-alpha and NGF: potential implications for disease." *Trends Mol Med* **14** (9): 381-8.

Takei, Y. and Laskey, R. (2008). "Tumor Necrosis Factor alpha Regulates Responses to Nerve Growth Factor, Promoting Neural Cell Survival but

Suppressing Differentiation of Neuroblastoma Cells." *Mol. Biol. Cell* **19** (3): 855-864.

Tandrup, T. (1995). "Are the neurons in the dorsal root ganglion pseudounipolar? A comparison of the number of neurons and the number of myelinated and unmyelinated fibres in the dorsal root." *J Comp Neurol* **357** (3): 341-7.

Thompson, A. J. (2001). "Symptomatic management and rehabilitation in multiple sclerosis." *J Neurol Neurosug Psychiatry* **71 Suppl 2**: ii22-7.

Tiberio, M., Chard, D., Altmann, D. R., Davies, G., Griffin, C. M., McLean, M. A., Rashid, W., Sastre-Garriga, J., Thompson, A. J. and Miller, D. H. (2006). "Metabolite changes in early relapsing-remitting multiple sclerosis. A two year follow-up study." *J Neurol* **253** (2): 224-20.

Toulmond, S., Parnet, P. and Linthorst, A. C. (1996). "When cytokines get on your nerves: cytokine networks and CNS pathologies." *Trends Neurosci* **19** (10): 409-10.

Trapp, B. D., Bo, L., Mork, S. and Chang, A. (1999). "Pathogenesis of tissue injury in MS lesions." *J Neuroimmunol* **98** (1): 49-56.

Trapp, B. D., Peterson, J., Ransohoff, R. M., Rudick, R. A., Mork, S. and Bo, L. (1998). "Axonal transection in the lesions of multiple sclerosis." *N Engl J med* **338** (5): 278-85.

Traugott, U. and Lebon, P. (1988). "Multiple sclerosis: Involvement of interferons in lesion pathogenesis." *Ann Neurol* **24** (2): 243-51.

Treede, R. D., Meyer, R. A. and Campbell, J. N. (1998). "Myelinated mechanically insensitive afferents from monkey hairy skin: Heat-response properties." *J Neurophysiol* **80** (3): 1082-93.

Treede, R. D., Meyer, R. A., Raja, S. N. and Campbell, J. N. (1995). "Evidence for two different heat transduction mechanisms in nociceptive primary afferents innervating monkey skin." *J Physiol* **483** ((pt 3) (pt 3)): 747-58.

Trinchieri, G. (1995). "Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity." *Annu Rev Immunol* **13**: 251-76.

Tsiang, H., Lycke, E., Ceccaldi, P. E., Ermine, A. and Hirardot, X. (1989). "The anterograde transport of rabies virus in rat sensory dorsal root ganglia neurons." *J Gen Virol* **70 (Pt 8)**: 2075-85.

Tsuji, S., Hisaoka, M., Morimitsu, Y., Hashimoto, H., Shimajiri, S., Komiya, S., Ushijima, M. and Nakamura, T. (1998). "Detection of SYT-SSX fusion transcripts in synovial sarcoma by reverse transcription-polymerase chain reaction using archival paraffin-embedded tissues." *Am J Pathol* **153** (6): 1807-12.

Tsujino, Tsujino, H., Kondo, E., Fukuoka, T., Dai, T., Tokunaga, A., Miki, K., Yonenobu, K., Ochi, T. and Noguchi, K. (2000). "Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury." *Mol Cell Neurosci* **15** (2): 170-82.

van Vliet, C., Bukczynska, P. E., Puryer, M. A., Sadek, C. M., Shields, B. J., Tremblay, M. L. and Tiganis, T. (2005). "Selective regulation of tumor

necrosis factor-induced erk signaling by src family kinases and the T cell protein tyrosine phosphatase." *Nat Immunol* **6** (3): 253-60.

Vanderah, T. W. (2007). "Pathophysiology of pain." *Med Clin North Am.* **91** (1): 1-12.

Vassalli, P. (1992). "The pathophysiology of tumor necrosis factors." *Annu Rev Immunol* **10**: 411-52.

Verge, V. M., Merlio, P., Grondin, J., Ernfors, P., Persson, H., Riolell, R. J., Hokfelt, T. and Richardson, P. M. (1992). "Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF resector mRNA in primary sensory neurons: Responses to injury and infusion of NGF." *J Neurosci* **12** (10): 4011-22.

Villoslada, P., Hauser, S. L., Bartke, I., Unger, J., Heald, N., Rosenberg, D., Cheung, S. W., Mobley, W. C., Fisher, S. and Genain, C. P. (2000). "Human Nerve Growth Factor Protects Common Marmosets against Autoimmune Encephalomyelitis by Switching the Balance of T Helper Cell Type 1 and 2 Cytokines within the Central Nervous System." *J. Exp. Med.* **191** (10): 1799-1806.

Vogel, C., Lindenlaub, T., Tiegs, G., Toyka, K. V. and Sommer, C. (2000). Pain related behaviour in TNF-receptor deficient mice. 9th world congress on Pain, Progress in Pain Research and Management, Seattle, IASP Press.

Vogel, C., Lindenlaub, T., Tiegs, G., Toyka, K. V. and Sommer, C. (2000). "Pain related behaviour in TNF-receptor deficient mice. 9th world congress on

pain, progress in pain research and management." *Seattle: IASP Press* **n/a** (n/a): n/a.

Wagner, R. and Myers, R. R. (1996). "Endoneurial injection of TNF-alpha [roduces neuropathic pain behaviors." *Neuroreport* **7** (18): 2897-901.

Walker, E. J. and Rosenberg, G. A. (2009). "Divergent role for MMP-2 in myelin breakdown and oligodendrocyte death following transient global ischemia." *Journal of Neuroscience Research* **9999** (9999): NA.

Wang, T. H., Meng, Q. S., Qi, J. G., Zhang, W. M., Chen, J. and Wu, L. F. (2008). "NT-3 expression in spared DRG and the associated spinal laminae as well as its anterograde transport in sensory neurons following removal of adjacent DRG in cats." *Neurochem Res* **33** (1): 1-7.

Warren, S., Cockerill, R. and Warren, K. G. (1991). "Risk factors by onset age in multiple sclerosis." *Neuroepidemiology* **10** (1): 9-17.

Warren, S., Warren, K. G., Svenson, L. W., Schopflocher, D. P. and Jones, A. (2003). "Geographic and temporal distribution of mortality rates for multiple sclerosis in Canada, 1965-1994." *Neuroepidemiology* **22** (1): 75-81.

Warrington, W. G. and Griffith, F. (1904). "On the cells of the spinal ganglia and on the relationship of their histological structure to the axonal distribution." *Brain* **27**: 297-325.

Wei, X. H., Zang, Y., Wu, C. Y., Xu, J. T., Xin, W. J. and Liu, X. G. (2007). "Peri-sciatic administration of recombinant rat TNF-alpha induces mechanical

allodynia via upregulation of TNF-alpha in dorsal root ganglia and in spinal dorsal horn: the role of NF-kappa B pathway." *Exp Neurol* **205** (2): 471-84.

Weinshenker, B. G. (1996). "Epidemiology of multiple sclerosis." *Neurol Clin.* **14** (2): 291-308.

Weinshenker, B. G. (2006). "Review: magnetic resonance imaging alone is of limited usefulness in diagnosing multiple sclerosis." *Evid Based Med* **11** (5): 155.

White, F. A. and Wilson, N. M. (2008). "Chemokines as pain mediators and modulators." *Curr Opin Anaesthesiol* **21** (5): 580-5.

Williams, K., Bar-Or, A., Ulvestad, E., Olivier, A., Antel, J. P. and Yong, V. W. (1992). "Biology of adult human microglia in culture: Comparisons with peripheral blood monocytes and astrocytes." *J Neuropathol Exp Neurol* **51** (5): 538-49.

Willis, W. D. J. (1985). "The pain system. the neural basis of nociceptive transmissionin the mammalian nervous system." *Pain Headache* **8**: 1-346.

Windebank, A. J., Wood, P., Bunge, R. P. and Dyck, P. J. (1985). "Myelination determines the caliber of dorsal root ganglion neurons in culture." *J Neurosci* **5** (6): 1563-9.

Windhagen, A., Newcombe, J., Dangond, F., Strand, C., Woodroofe, M. N., Cuzner, M. L. and Hafler, D. A. (1995). "Expression of costimulatory molecules B7-1 (CD80) B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions." *J Exp Med* **182** (6): 1985-96.

Wolswijk, G. (1998). "Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells." *J Neurosci* **18** (2): 601-9.

Woodroofe, M. N. and Cuzner, M. L. (1993). "Cytokine mRNA expression in inflammatory multiple sclerosis lesions: Detection by non-radioactive in situ hybridization." *Cytokine* **5** (6): 583-8.

Woolf, C. J., Allchorne, A., Safieh-Garabedian, B. and Poole, S. (1997). "Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha." *Br J Pharmacol* **121** (3): 417-24.

Woolf, C. J. and Ma, Q. (2007). "Nociceptors--Noxious Stimulus Detectors." *Neuron* **55** (3): 353-364.

Wright, D. E. and Snider, W. D. (1995). "Neurotrophin receptor mRNA expression defines distinct populations of neurons in rat dorsal root ganglia." *J Comp Neurol* **351** (3): 329-38.

Xiong, H., Futamura, T., Jourdi, H., Zhou, H., Takei, N., Diverse-Pierluissi, M., Plevy, S. and Nawa, H. (2002). "Neurotrophins induce BDNF expression through the glutamate receptor pathway in neocortical neurons." *Neuropharmacology* **42** (7): 903-912.

Xu, J. T., Xin, W. J., Zang, Y., Wu, C. Y. and Liu, X. G. (2006). "The role of tumor necrosis factor-alpha in the neuropathic pain induced by lumbar 5 ventral root transection in rat." *Pain* **123** (3): 306-21.

Yasuda, T., Tsumita, T., Nagai, Y., Mitsuzawa, E. and Ohtani, S. (1975). "Experimental allergic encephalomyelitis (EAE) in mice. I. induction of the EAE with mouse spinal cord homogenate and myelin basic protein." *Jpn J Exp Med* **45** (5): 423-7.

Yednock, T. A., Cannon, C., Fritz, L. C., Sanchez-Madrid, F., Steinman, L. and Karin, N. (1992). "Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin." *Nature* **356** (6364): 63-6.

Yip, H. K. and Johnson, E. M., Jr. (1986). "Comparative dynamics of retrograde transport of nerve growth factor and horseradish peroxidase in rat lumbar dorsal root ganglia." *J Neurocytol* **15** (6): 789-98.

Youn, D. H., Wang, H. and Jeong, S. J. (2008). "Exogenous tumor necrosis factor-alpha rapidly alters synaptic and sensory transmission in the adult rat spinal cord dorsal horn." *J Neurosci Res* **86** (13): 2867-75.

Yousry, T. A., Major, E. O., Ryschkewitsch, C., Fahle, G., Fischer, S., Hou, J., Curfman, B., Miszkiel, K., Mueller-Lenke, N., Sanchez, E., Barkhof, F., Radue, E. W., Jager, H. R. and Clifford, D. B. (2006). "Evaluation of patients treated with natalizumab for progressive multifocal leukoencephalopathy." *N Engl J med* **354** (9): 924-33.

Zaccaro, M. C., Ivanisevic, L., Perez, P., Meakin, S. O. and Saragovi, H. U. (2001). "p75 co-receptors regulate ligand-dependent and ligang-independent trk receptor activation, in part by altering trk docking subdomains." *J Biol Chem* **276** (33): 31023-9.

Zamvil, S., Nelson, P., Trotter, J., Mitchell, D., Knobler, R., Fritz, R. and Steinman, L. (1985). "T-cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination." *Nature* **317** (6035): 355-8.

Zeis, T., Graumann, U., Reynolds, R. and Schaeren-Wiemers, N. (2008). "Normal-appearing white matter in multiple sclerosis is in a subtle balance between inflammation and neuroprotection." *Brain* **131** (1): 288-303.

Zelenka, M., Schafers, M. and Sommer, C. (2005). "Intraneural injection of interleukin-1beta and tumor necrosis factor-alpha into rat sciatic nerve at physiological doses induces signs of neuropathic pain." *Pain* **116** (3): 257-63.

Zhang, J. M., Li, H., Liu, B. and Brull, S. J. (2002). "Acute topical applicaiton of tumor necrosis factor alpha evokes protein kinase A-dependent responses in rat sensory neurons." *J Neurophysiol* **88** (3): 1387-92.

Zhang, W. and Liu, H. T. (2002). "MAPK signal pathways in the regulation of cell proliferation in mammalian cells." *Cell Res* **12** (1): 9-18.

Zheng, J. L., Stewart, R. R. and Gao, W. Q. (1995). "Neurotrophin-4/5, brain-derived neurotrophic factor, and neurotrophin-3 promote survival of cultured vestibular ganglion neurons and protect them against neurotoxicity of ototoxins." *J Neurobiol* **28** (3): 330-40.

Zhou, Y. and Zhao, Z. Q. (2002). "Effects of neomycin on high-threshold ca (2+) currents and tetrodotoxin-resistant na (+) currents in rat dorsal root ganglion neuron." *Eur Pharmacol* **450** (1): 29-35.

Zhu, W., Frost, E. E., Pillai, P. P., Begum, F., Au, K. and Namaka, M. (2010). "Antigen induced expression of brain derived neurotrophic factor in an autoimmune experimental encephalomyelitis (EAE) rat model of multiple sclerosis (MS)." *J Neurosci* **submitted**.

Ziemssen, T., Kumpfel, T., Klinkert, W. E., Neuhaus, O. and Hohlfeld, R. (2002). "Glatiramer acetate-specific T-helper 1- and 2-type cell lines produce BDNF: implications for multiple sclerosis therapy. Brain-derived neurotrophic factor." *Brain* **125** (Pt 11): 2381-91.

Ziemssen, T., Neuhaus, O. and Hohlfeld, R. (2001). "Risk-benefit assessment of glatiramer acetate in multiple sclerosis." *Drug Saf* **24** (13): 979-90.

Zimmermann, M. (2001). "Pathobiology of neuropathic pain." *Eur J Pharmacol* **429** (1-3): 23-37.

Zoja, C., Wang, J. W., Bettoni, S., Sironi, M., Renzi, D., Chiaffarino, F., Abboud, H. E., Van Damme, J., Mantovani, A. and Remuzzi, G. (1991). "Interleukin-1 beta and tumor necrosis factor-alpha induce gene expression and production of leukocyte chemotactic factors, colony-stimulating factors, and interleukin-6 in human mesangial cells." *Am J Pathol* **138** (4): 991-1003.

Zweifel, L. S., Kuruvilla, R. and Ginty, D. D. (2005). "Functions and mechanisms of retrograde neurotrophin signalling." *Nat Rev Neurosci* **6** (8): 615-25.

FIGURES

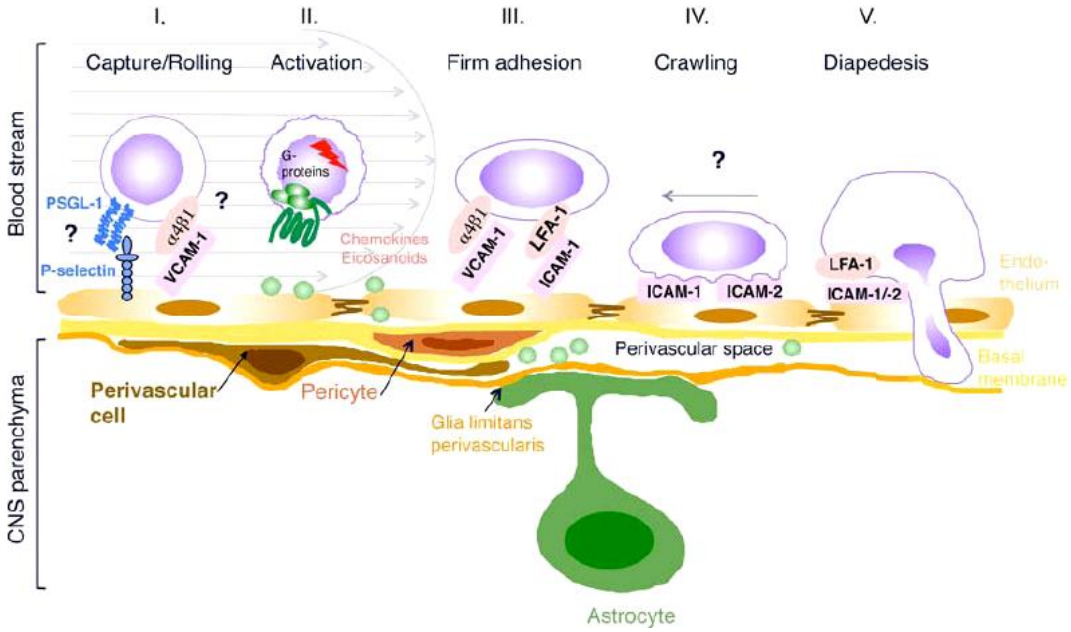


Figure 1: Molecular mechanisms involved in the multi-step recruitment of T-cells across the BBB

This schematic depicts an overview of the adhesion and signaling involved in T-cell migration across the BBB. The initial T-cell adhesion to the BBB is mediated by α4β1-integrin and VCAM-1. Subsequent G-protein dependent activation of α4β1-integrin and a second integrin, LFA-1, both mediate the attachment of T-cells to VCAM-1 and ICAM-1, respectively. T-cells crawl against the direction of flow to sites permissive for diapedesis. Finally, T-cells pass through the BBB in an LFA-1/ICAM-1 and ICAM-2 dependent manner, leaving tight junctions of the endothelium morphologically intact. After penetrating the BBB, T-cells migrate across the basement membrane and encounter antigen-presenting cells in the CSF drained perivascular space.

From Conference**:** Pharmacology and Toxicology of the Blood-Brain Barrier: State of the Art, Needs for Future Research and Expected Benefits for the EU. Brussels, Belgium, February 11 - 12, 2010. Engelhardt B (2010). Regulation of Immune Cell Entry into the Central Nervous System. *Conference Abstract: Pharmacology and Toxicology of the Blood-Brain Barrier: State of the Art, Needs for Future Research and Expected Benefits for the EU*. doi: 10.3389/conf.fphar.2010.02.00008

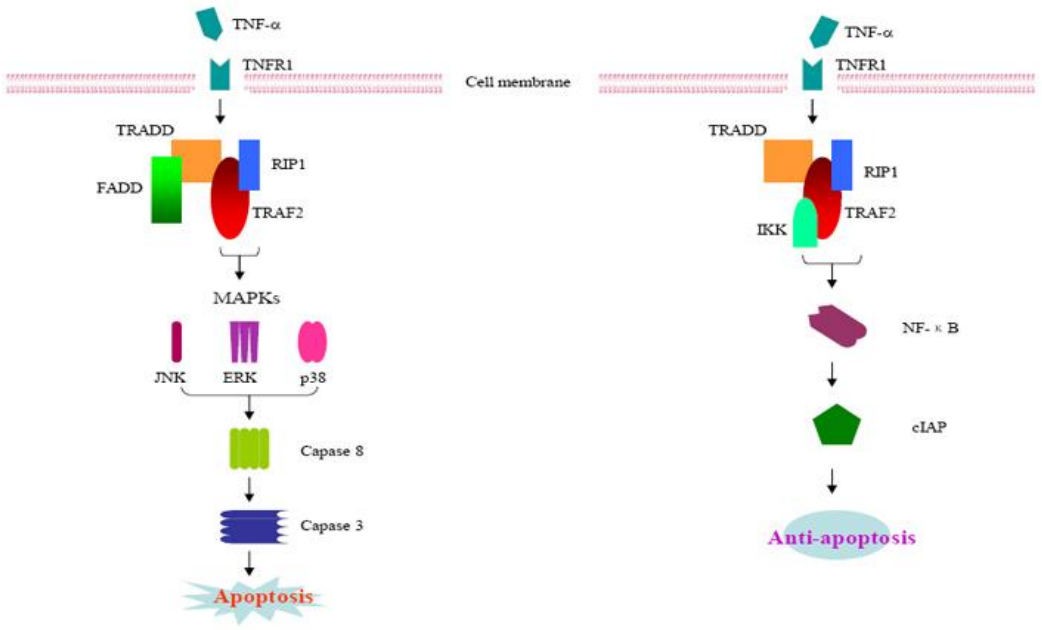


Figure 2: Sensory neuron damage via TNFα signaling pathway (s)

The TNFα ligand binds to the extracellular domain of the receptor, via domains referred to as Cysteine-Rich Domains (CRDs). This induces conformational changes in the receptor and activates the intracellular signaling pathways. Binding of TNFα with TNFR1 leads to the release of the inhibitory protein silencer of death domains (SODD) from TNFR1 intracellular death domain (DD). Release of SODD allows binding of TRADD (TNFR1-associated death domain protein) to the DD, which can further activate either the apoptotic pathway, via the Fas-associated death domain (FADD) protein, or the pro-inflammatory pathway, via TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein (RIP), resulting in the activation of nuclear factor-κB (NF-κB).

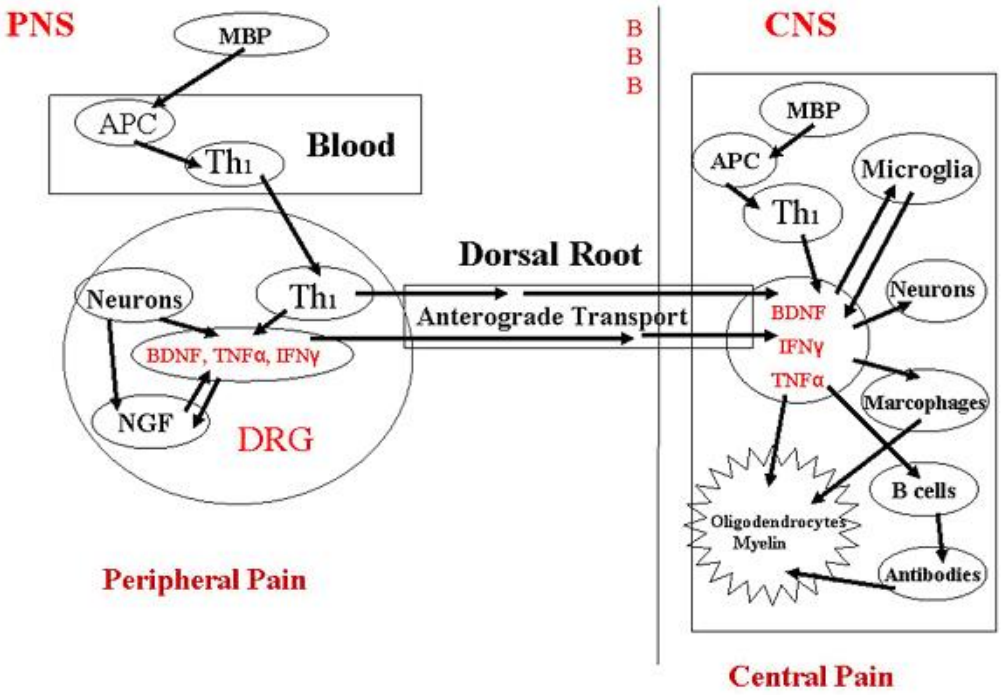
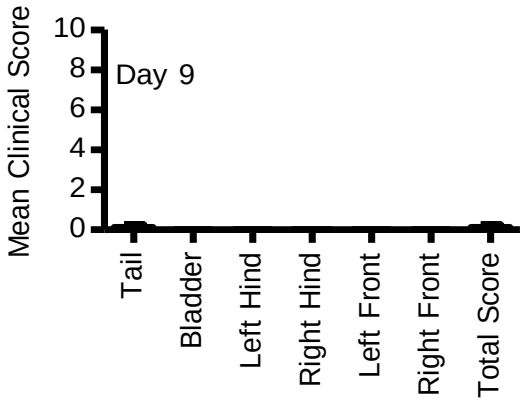


Figure 3: DRG/Spinal Cord Model of MS - Induced NPP


Mean Clinical Score
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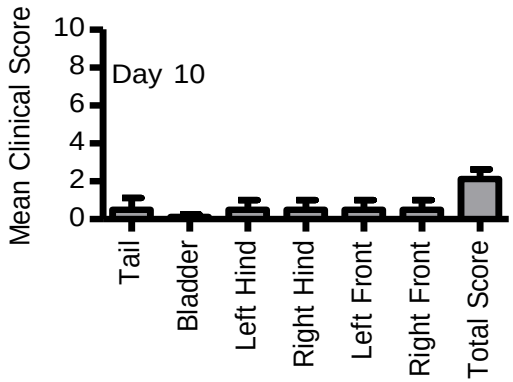
4
2
0
Day 9
Tail
Bladder
Left Hind
Right Hind
Left Front
Right Front
Total Score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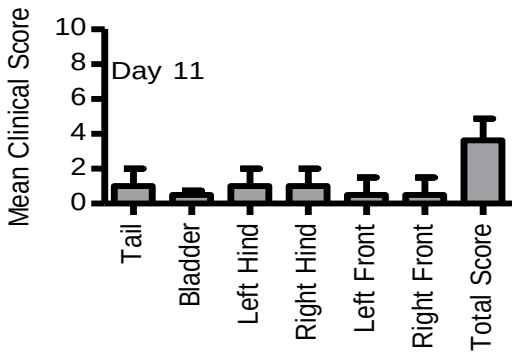
Total Score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Day 11
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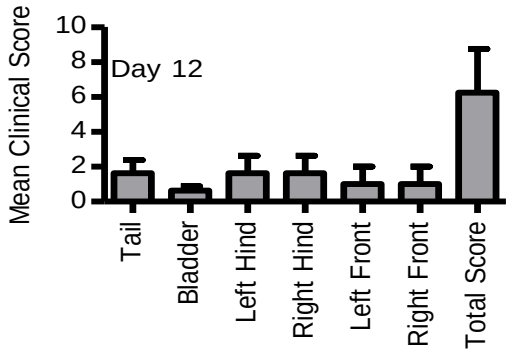
Total Score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Day 12
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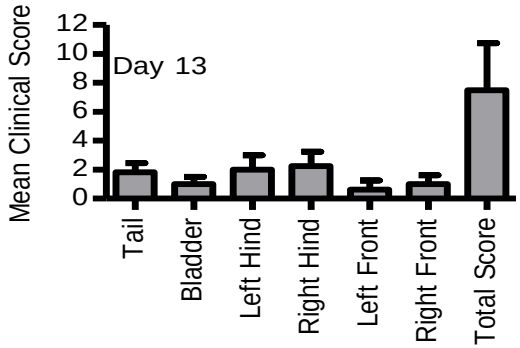
Right Front
Total Score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Day 13
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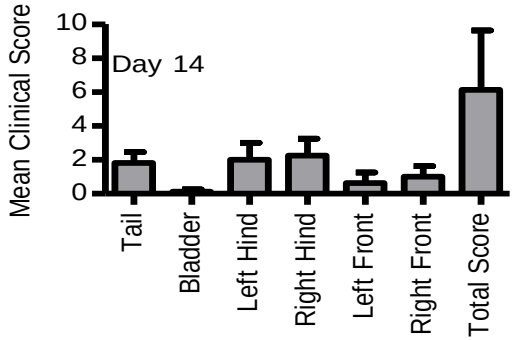
Total Score


Mean Clinical Score
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Day 14
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Bladder
Left Hind
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Left Front
Right Front
Total Score

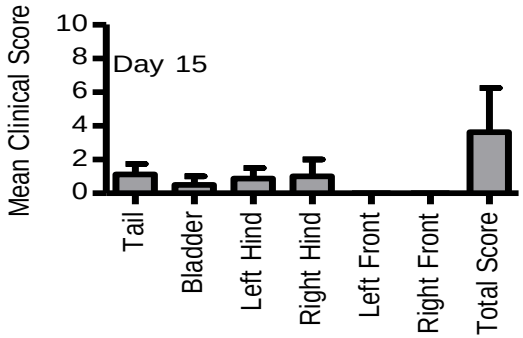
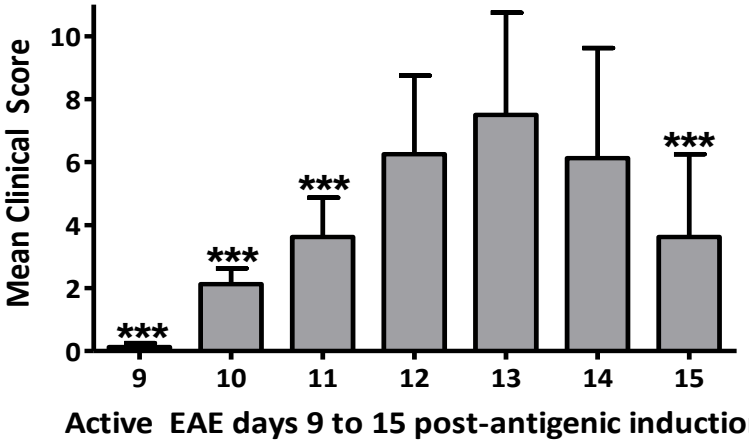


Figure 4: Detailed Neurological Disability Scores.

Detailed neurological disability scores for aEAE animals (N_T=18) at days 9 (n=6), 12 (n=6) & 15 (n=6), spanning the 6 specific domains of clinical disability assessment that include: *tail, bladder, left hind, right hind, left front and right front*. Total score represents the mean of the individual summated scores (n=6) of each specified time point for each of the 6 individual domains of clinical disability assessment. The mean clinical disability scores obtained at each predetermined time point range from 0 (no disability) to 15 (maximal disability).



**Note: The clinical scoring for all other treatment groups at all other times points = 0*

Figure 5: Global Neurological Disability Score for EAE animals (n=18) induced to a state of Experimental Autoimmune Encephalomyelitis.

Global Neurological Disability Score for EAE animals (n=18) induced to a state of encephalomyelitis. Disability scores range from a mean clinical disability score of 0 (no disability) to 15 (maximal disability). For example E9 = AEAE group at day 9 post-antigenic induction. The bell shaped distribution outlining peak neurological disability in response to EAE induction occurred at day 13 post-EAE induction.

Tukey's Multiple Comparison Test showed that Day 13 neurological disability score was very highly significantly different from the scores obtained for days 9, 10, 11 and 15. ***=$p<0.001$

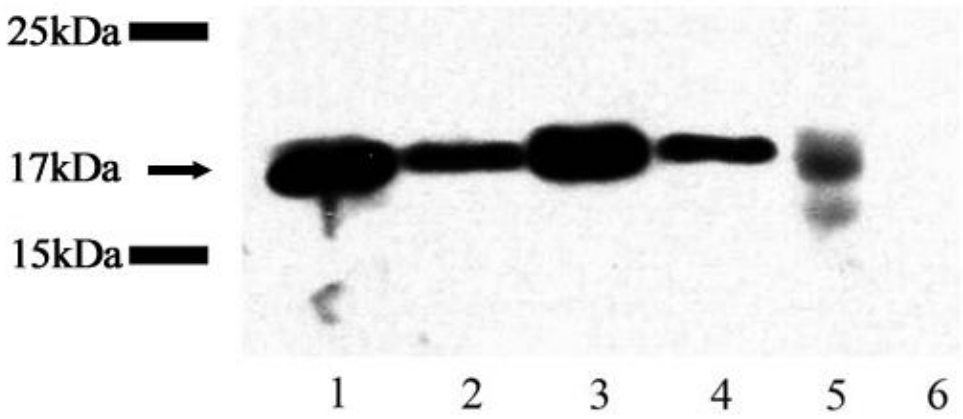


Figure 6: Western blot analysis showing specificity of TNFα antibody used for subsequent IHC analysis

Western blot analysis for TNFα antibody used in IHC analysis. ***Lane 1****:* Neat recombinant pure rat TNFα obtained from R&D Systems, Inc. 510-RT, ***Lane 2****:* 1:5 dilution of recombinant pure rat TNFα obtained from R&D Systems, Inc. 510-RT, ***Lane 3****:* Neat recombinant pure rat TNFα obtained from R&D Systems, Inc. 510-RT plus control cells (OL) that do not express TNFα, ***Lane 4****:* 1:5 dilution of recombinant pure rat TNFα obtained from R&D Systems, Inc. 510-RT plus control cells (OL) that do not express TNFα, ***Lane 5****:* Protein extracted from normal Lewis rat spleen known to contain TNFα protein, *Lane 6:* Control cells (OL that do not express TNFα).

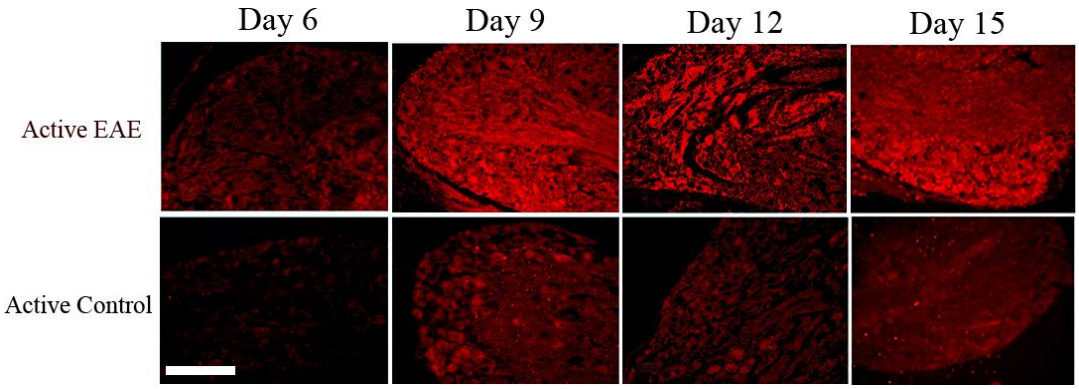


Figure 7: TNFα expression in the rat dorsal root ganglion (DRG) at different days after disease induction

Comparative IHC analysis of 10 μm sections of DRG obtained from active control, and aEAE assessed at three time points post-inoculation.

Day 0 = induction of EAE using MBP 100μg/animal and Mycobacterium Tuberculosis 500μg/animal in Freund's adjuvant.

Tissue sections were stained with TNFα (1:100; R & D Systems Minneapolis, MN). Specificity of TNFα antibody was confirmed with Western Blot Analysis (see Figure 6). Marked increases in TNFα immunoreactivity were noted in the aEAE animal group at day 9 relative to the active control group. Increased TNFα immunoreactivity was maintained at subsequent time points (days 12 and 15 post disease induction compared to active control DRG. Images were taken at a total magnification of 100x and were exposed for 700msec.

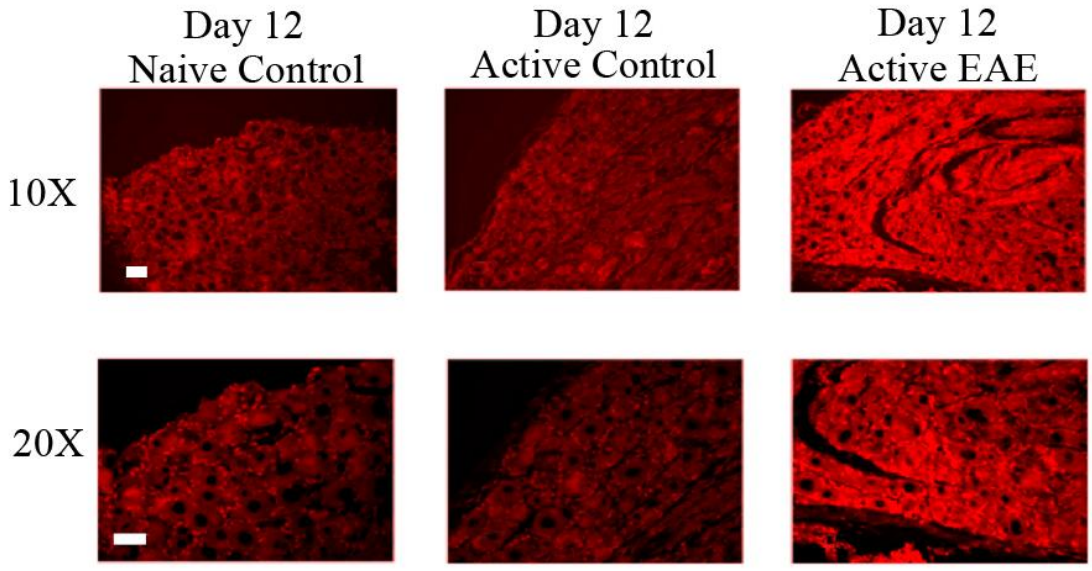


Figure 8: TNFα expression in EAE DRG

Comparative IHC analysis of 10 µm sections of DRG obtained from active control, and aEAE assessed at day 12 post-inoculation relative to naive control animals. Tissue sections were stained with TNFα (1:100; R & D Systems Minneapolis, MN). Specificity of TNFα antibody confirmed with Western Blot Analysis. Pronounced elevations in TNFα labeling were noted in the aEAE animal group at day 12 relative to all other treatment groups. Images were taken at a total magnification of 100x (top panel) and 200x (bottom panel) respectively and were exposed for 700msec @ 10x and 300 msec @ 20x respectively.

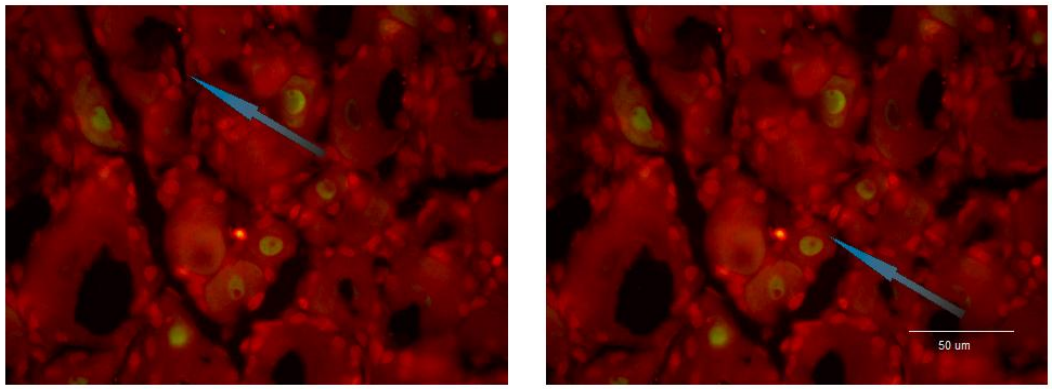


Figure 9: TNFα up-regulation occurs in neural soma of EAE induced rats at E12.

IHC analysis of 10 µm sections of DRG obtained from aEAE animals at day 12 post disease induction. Pronounced TNFα immunoreactivity was noted in the neural soma. Arrow in the left panel depicts TNFα negative neuron while arrow in the right panel depicts that of a TNFα positive neuron. All cells were also double labeled with a neuronal nuclear marker NeuN (green; 1:1000 Chemicon, Temecula, CA). Tissue sections were stained with TNFα (1:100; R & D Systems, Minneapolis, MN). Images were taken at a total magnification of 400x exposed for 500 msec.

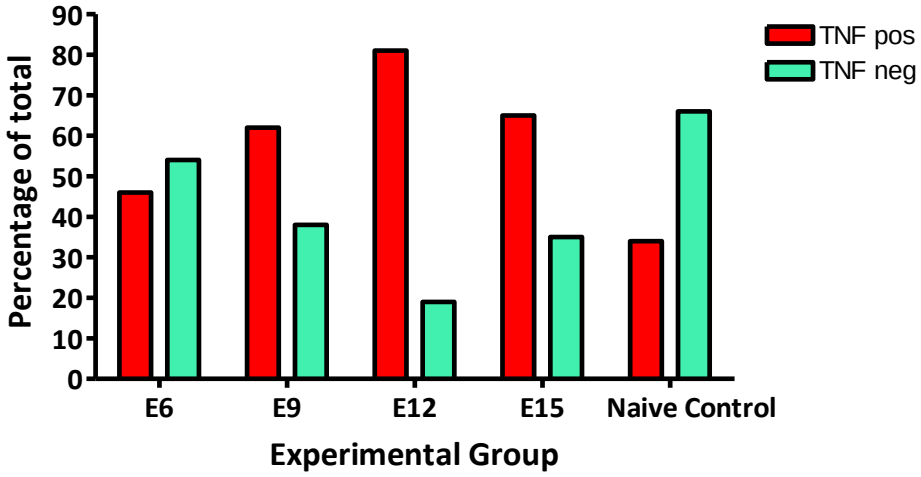


Figure 10: TNFα positive neurons as a percentage of the total number of neurons in aEAE animals.

Results were obtained from a total of 97 E-6 active rats, 229 E-9 active rats, 203 E-12 active rats, and 144 E-15 active rats.

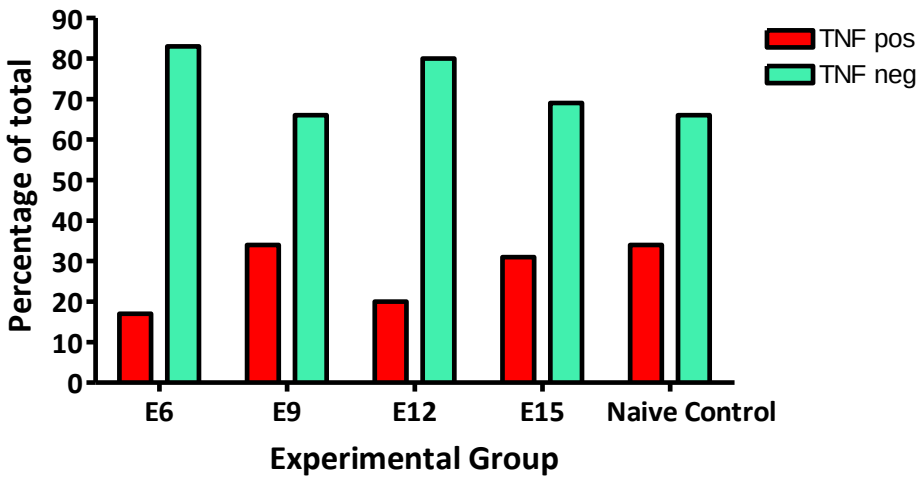


Figure 11: TNFα positive neurons as a percentage of the total number of neurons in active control animals

Results were obtained from a total of 134 E-6 active control rats, 177 E-9 active control rats, 105 E-12 active control rats and 155 E-15 active control rats.

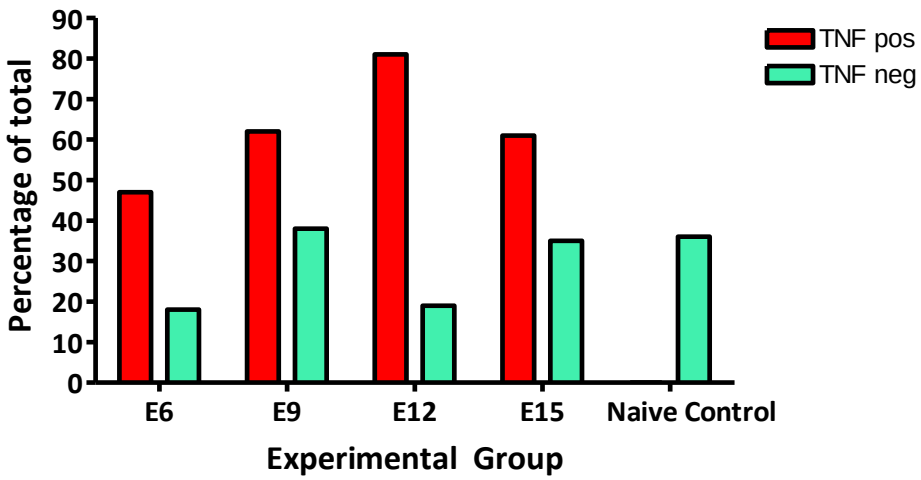


Figure 12: Percentage of total neurons that are TNFα positive comparing aEAE animals with active control animals.

Results were obtained from a total of 24 E-6 active control TNF positive rats, 44 E-6 active TNF positive rats, 55 E-9 active control TNF positive rats, 143 E-9 active TNF positive rats, 22 E-12 active control TNF positive rats, 165 E-12 active TNF positive rats, 50 E-15 active control TNF positive rats and 95 active TNF positive rats.

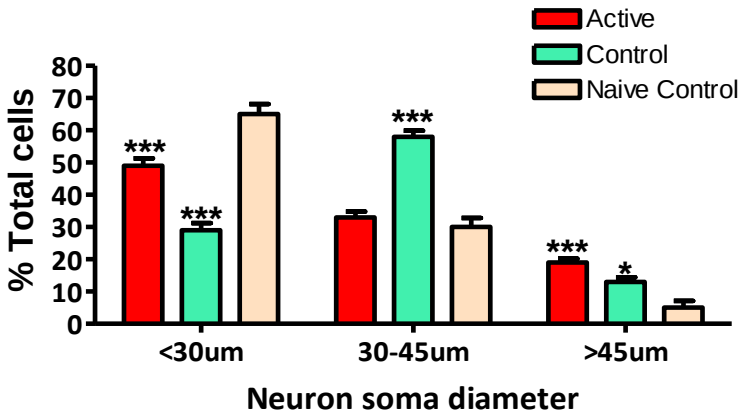


Figure 13: E6 neuron size

TNFα positive neurons, active control compared to aEAE and naïve control animals at 6 days post induction.

*=p<0.05 ***=p<0.001 vs naïve control % for the same cell size. Analyzed by Students *t*-test (unpaired).

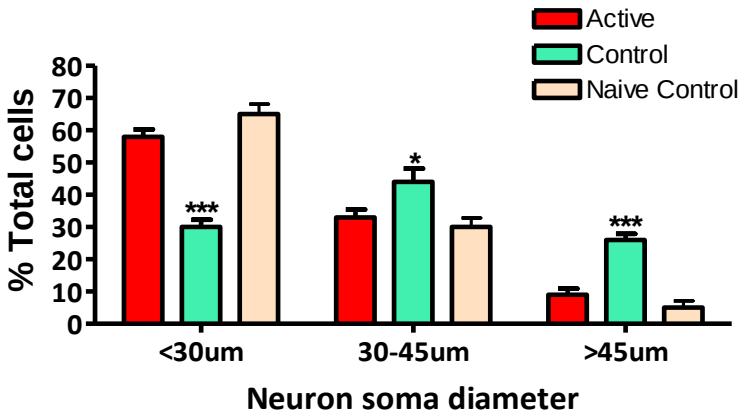


Figure 14: E9 neuron size

TNFα positive neurons, active control compared to aEAE and naïve control animals at 9 days post disease induction.

*=p<0.05 ***=p<0.001 vs naïve control % for the same cell size. Analyzed by Students *t*-test (unpaired).

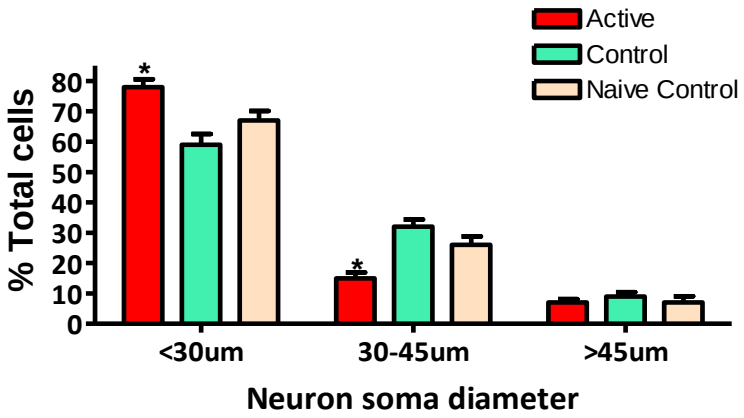


Figure 15: E12 neuron size

TNFα positive neurons, active control compared to aEAE and naïve control animals at 12 days post disease induction.

*=p<0.05 vs naïve control % for the same cell size. Analyzed by Students *t*-test (unpaired).

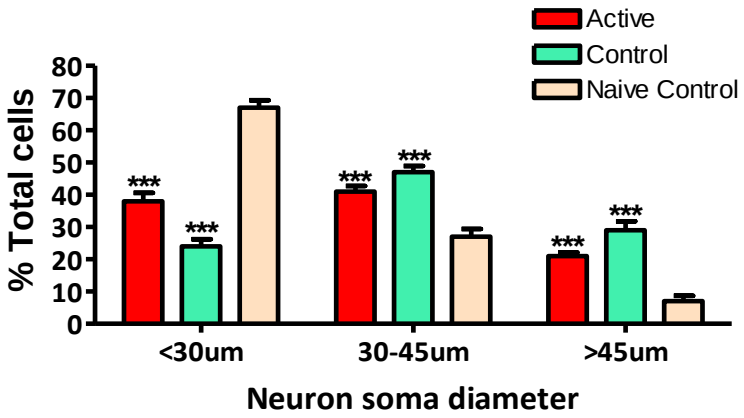


Figure 16: E15 neuron size

TNFα positive neurons, active control compared to aEAE and naïve control animals at 15 days post disease induction.

***=p<0.001 vs naïve control % for the same cell size. Analyzed by Students *t*-test (unpaired).

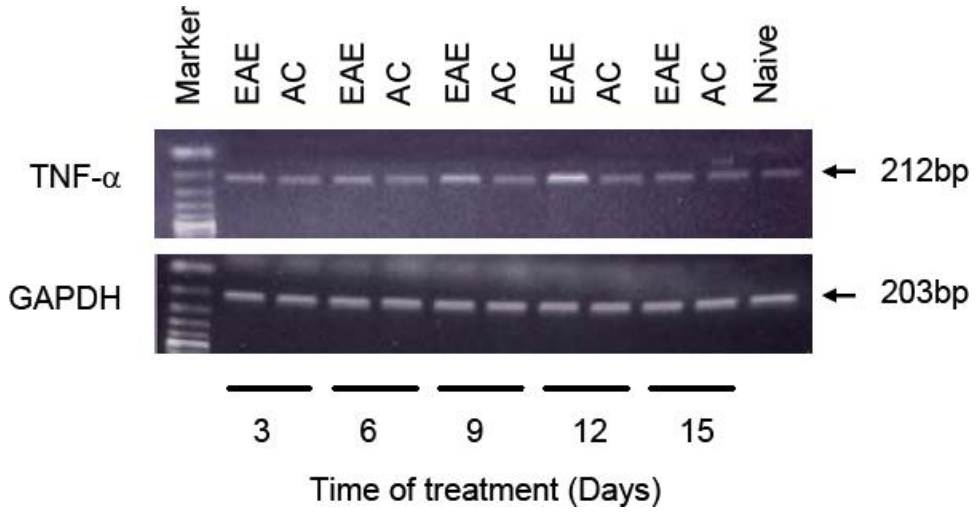


Figure 17: RT-PCR data depicting TNFα expression within the DRG sensory ganglia obtained from the aEAE, active control and naïve control groups.

Comparative analysis reveals peak expression in the aEAE group at day 12 compared to all other experimental groups at predefined time point assessments.

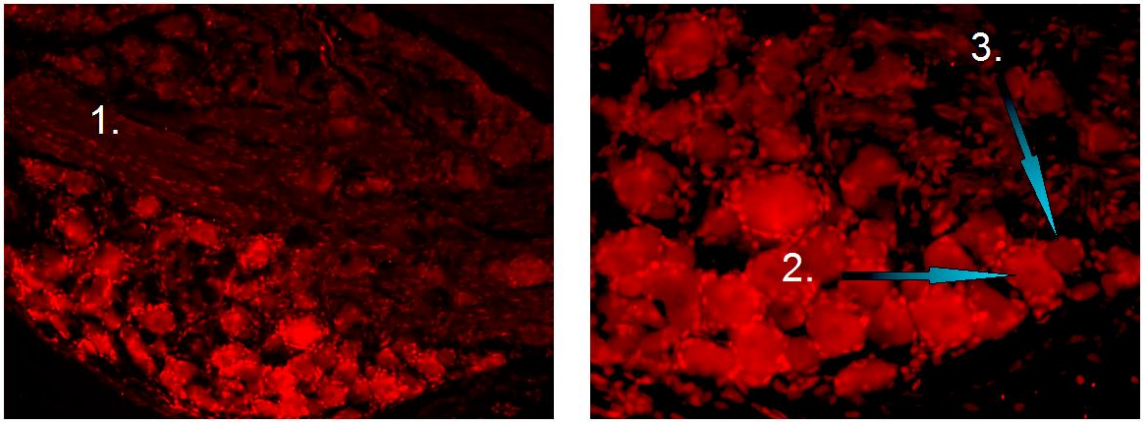


Figure 18: Identification of neuron positive TNFα expression in EAE induced rats

1. Axons of primary sensory neurons provide a visual reference for TNFα negative labeling
2. TNFα positive neuron
3. TNFα negative neuron

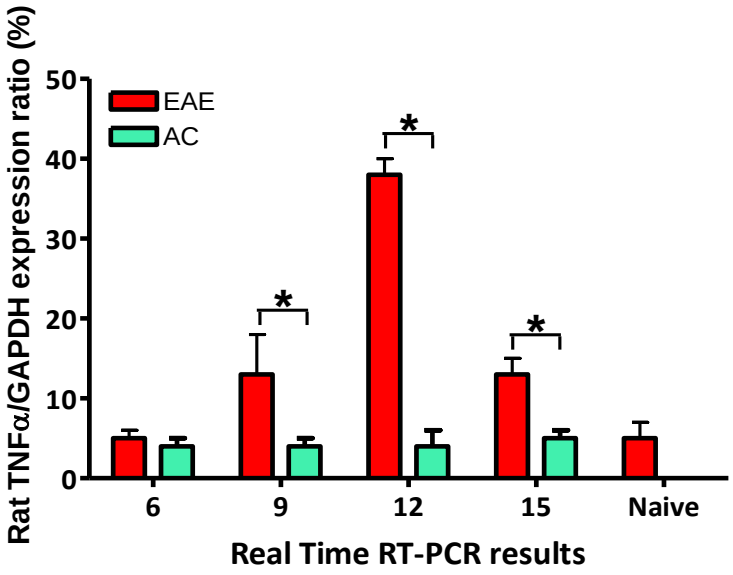


Figure 19: Real-time PCR results of TNFα expression within DRG sensory ganglia.

The TNFα expression of DRG for animals of the aEAE group, euthanized at day 12 is significantly higher than other groups (naïve control and active control (AC)) (*=p<0.05). There is no significant difference between the naïve control and active control animal groups euthanized at all of the time points (day 3, 6, 9, 12 and 15).

TABLES

Table 1 Animal usage: N=66

Group	Days post induction	Histology	qRT-PCR
Naïve Control	15	3	3
Active Control	3	3	3
	6	3	3
	9	3	3
	12	3	3
	15	3	3
AEAE	3	3	3
	6	3	3
	9	3	3
	12	3	3
	15	3	3

Table 2 Neurological Disability Clinical Scoring System for EAE animals induced to a state of MS.

The total score is the **sum** of the following individual scores obtained for each of the 6 specified clinical domains:

Tail:	**Bladder:**
0 – normal	0 – normal
1 – partially paralyzed, weakness	1 - incontinence
2 – completely paralyzed, limp	

Right hind limb:	**Left hind limb**:
0 – normal	0 – normal
1 – weakness	1 - weakness
2 – dragging with partial paralysis	2 - dragging with partial paralysis
3 – complete paralysis	3 – complete paralysis

Right forelimb:	**Left forelimb:**
0 – normal	0 - normal
1 – weakness	1 - weakness
2 – dragging, not able to support weight	2 – dragging, not able to support weight

3 – complete paralysis	3 – complete paralysis

APPENDIX I

Results of preliminary studies to investigate changes in TNFα protein and gene expression in the DRG of human MS patients.

To test the hypothesis that TNFα protein expression is upregulated in the DRG of human MS patients, we collected post mortem DRG tissue samples from a 55 year old male with active MS. In addition, DRG tissue samples were obtained from a similar age-sex matched control (60 year old male with a cord infarct). Ethics and PACT approvals are attached in

APPENDIX I.

Immunohistochemical Analysis of Human Tissue

De-paraffinizing and autofluorescence reduction protocol

Slides were deparaffinized prior to staining. Slides are dipped in histoclear for 2-5 minutes prior to rehydration in decreasing concentrations of ethanol (100%, 95%, 70% and 30%) to water.

Slides are then incubated in PBS, and PBS plus 0.3% Triton-X100. To reduce autofluorescence, the slides are incubated in PBST (phosphate buffered saline and 1% Triton X-100), for 2-4 days. This reduces the autofluorescence of cells surrounding neurons. However, the intense autofluorescence of neurons requires a stronger autofluorescent removal technique.

Staining protocol for human tissue

Following rehydration, slides are incubated in 10% normal sheep serum to block non-specific binding sites, followed by polyclonal anti-TNFα primary antibody (1:100; Serotec cat.# MCA747) then placed in 2% sheep serum, overnight at 4°C. Slides are washed 3 times in PBST (PBS plus 0.3% Triton X-100), prior to incubation in the fluorescently conjugated secondary antibody (Goat Anti-mouse CY-3; Jackson ImmunoResearch) at a dilution of 1:100 and 2% sheep serum in PBST). Sections are incubated at room temperature for 90 minutes, then washed 3 times in PBST prior to mounting on anti-fade medium

(VectaShield). Imaging was performed using an epifluorescent microscope followed by confocal imaging to ascertain correct immunoreactivity localization. Imaging was performed on an Olympus ZX51 inverted epifluorescent microscope attached to a dedicated imaging station equipped with monochromatic CCD camera and Image Pro 7 software. Confocal imaging was performed using an Olympus ZX81 scanning laser confocal microscope. Images were captured using Fluoview FV500 software. With the following settings:

Laser	Laser Label	Common Fluorochromes
Blue Argon	Argon 458,488,515	FITC, Alexa 488
Green Hene	Hene 543	Rhodamine Red
UV laser	Argon 351	DAPI

Real Time RT-PCR Analysis of Gene Expression

Human RNA Extraction from Archival Paraffin-Embedded Tissues

To isolate RNA from archived human tissue previously embedded in paraffin, 8-10 sections (4-5 µm), were collected in sterile 50 ml centrifuge tubes. The samples are deparaffinized in two xylene washes for 15 min each time, followed by 3 washes in 100% ethanol. After drying the tissue, pellets were re-suspended in 100 µl of lysis buffer (20 mM Tris-HCl, pH8.0, 20 mM EDTA, 2% SDS). The sample is homogenized with a hand homogenizer or two freeze-melt

cycles (liquid nitrogen/dry ice/ -70^{o}C -37^{o}C). The lysed sample was then transferred to a 1.5 ml sterile eppendorf tube and 10 µl of Proteinase K solution (50 mg/ml) added prior to incubation at 55°C overnight. 1 ml of TRIzol reagent (Invitrogen / Gibco BRL, Cat#: 15596-018) was added to each sample to extract RNA, per the manufacturer's manual: https://www.invitrogen.com/content/sfs/manuals/15596026.pdf (Argani, Zakowski et al. 1998; Tsuji, Hisaoka et al. 1998).

Isolated RNA was dissolved in DEPC-treated water, and the RNA concentration checked via the ratio of A260/A280. The RNA for each sample was then converted to cDNA via reverse transcription (**RT**) using an RT-for-PCR kit (BD Biosciences; Cat#: 639506) per the manufacturer's manual: http://www.clontech.com/clontech/techinfo/manuals/PDF/PT1107-1.pdf)

RT-PCR Reaction

After RT, 2.5-5 µl of the cDNA for each sample was then run through PCR analysis using a kit (Qiagen; Cat#: 201205), and the following cycles: 95°C for 5 min, followed by 28-36 cycles of 95°C for 30 seconds, Tm for 30 seconds followed by 72°C for 45 seconds. The samples were finally annealed at 72°C for 5 minutes, prior to resolution on a 1% agarose gel. Tm varies depending on the primer sets used, for TNFα was 61°C for a product length of 220bp, and GAPDH (standard housekeeping gene used to normalize the reactions) was 60°C for a product length of 276bp. Bands are visualized by soaking the agarose gel in

Ethidium Bromide (5 µg/ml) and imaged using a UV sensitive thermal imaging camera.

Results

Preliminary qualitative immunohistochemical data obtained from human post-mortem DRG tissue sections of patients with MS appeared to show positive immunoreactivity for TNFα within sensory neurons that are absent in age and sex matched controls without MS (**Figure 20**).

An attempt to quantify the TNFα expression observed in the human tissue RT-PCR was undertaken. Initially, GAPDH expression was conducted in control patients using spleen as a positive control as outlined in **Figure 21**. However, a consistent GAPDH signal could not be achieved amongst the various tissue samples analyzed, due to the fixation procedure used post-mortem. Therefore, we conducted RT-PCR for TNFα expression on the same tissue samples with the same inconsistent results, as depicted in **Figure 22**. Therefore, due to the inability to overcome the technical difficulties associated with the human post-mortem fixation procedure, no further RT-PCR or subsequent Real Time -PCR could be conducted on the human DRG neurons.

However, the initial albeit crude IHC data provided from our human DRG studies served as the basis for which our subsequent investigative research using an EAE animal model of MS.

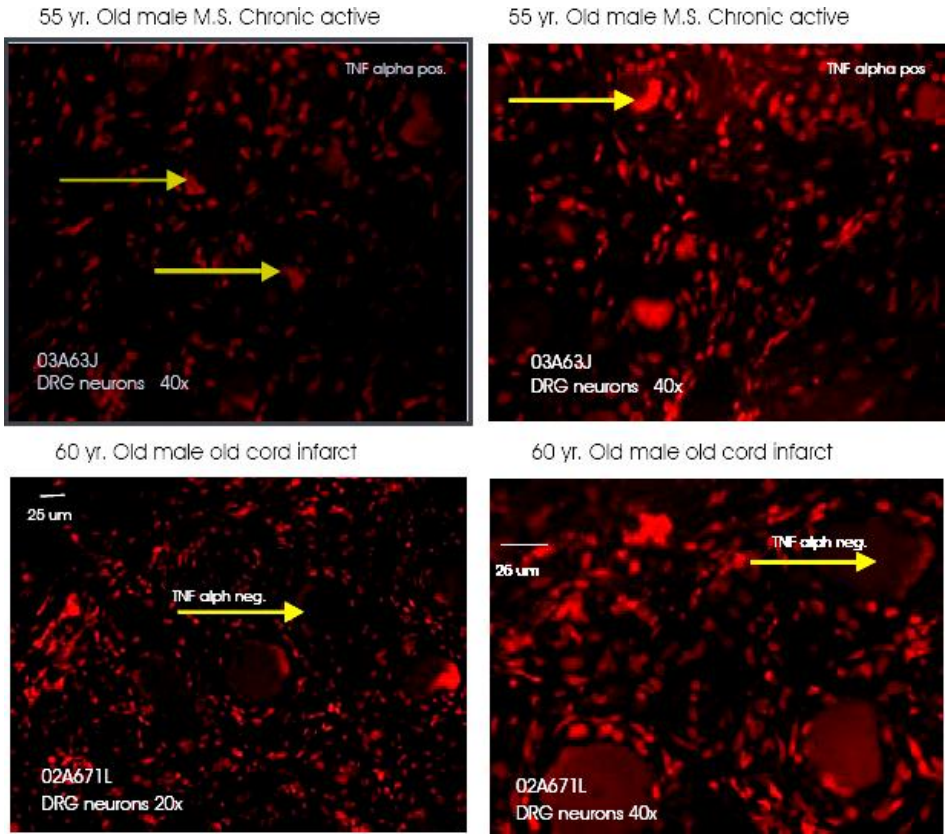


Figure 20: Age-Sex matched composite of human DRG sensory ganglia sectioned at 8 µm thickness at the specific identified magnifications.

DRG stained with TNFα primary monoclonal antibody (Serotec: 1:100) and secondary (goat-anti-mouse CY3: 1:100 Jackson). Arrows in top panel images depict *TNFα positive neurons* obtained from a 55 year old male with active MS. Arrows in bottom panel images depict *TNFα negative neurons* obtained from a similar age-sex matched control (60 year old male with a cord infarct).

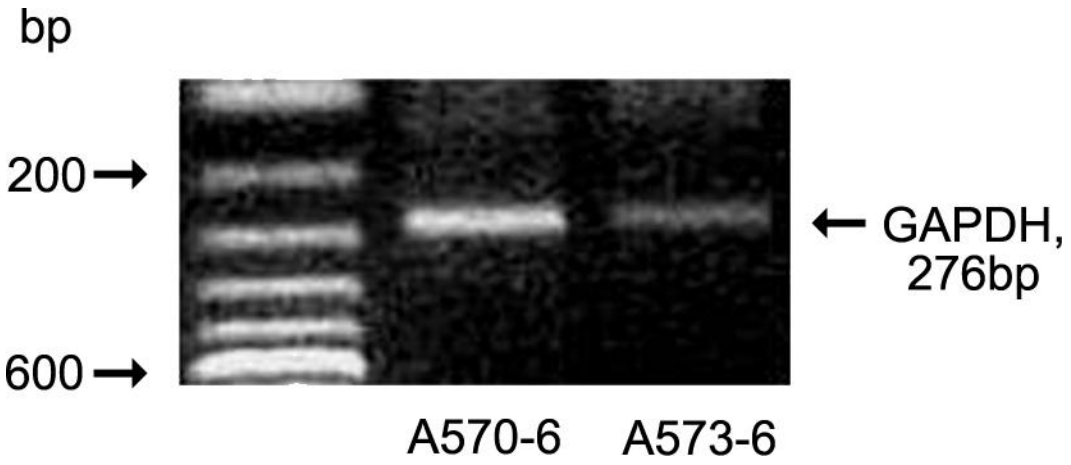


Figure 21: GAPDH PCR on spleen as positive control
Human GAPDH conducted on spleen as a positive control.
A570-6: Spleen, 50 years old, male, cardiac ischemia
A573-6: Spleen, 30 years old, male, sudden death

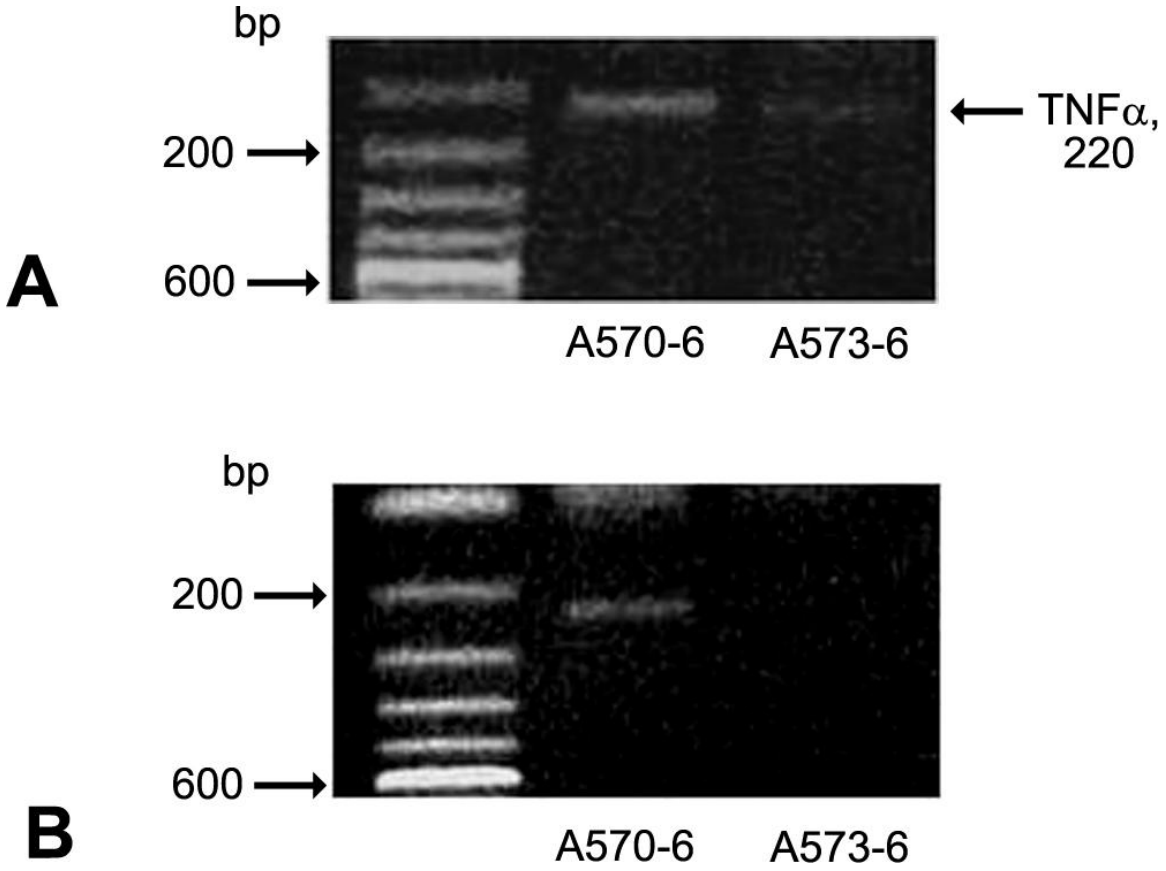


Figure 22: Two Consecutive trials on human TNFα conducted on spleen as a positive control

Two Consecutive Trials on Human TNFα conducted on spleen as a positive control.

A570-6: Spleen, 50 years old, male, cardiac ischemia

A573-6: Spleen, 30 years old, male, sudden death

Panel A – Temperature 61°C

Panel B – Temperature 61°C for 38 cycles

Attached:

- Ethics approval for human studies
- Pact Approval for human autopsy tissue collection

APPENDIX II

Statistical Analysis.

The differences between groups were compared using ANOVA univariant multiple factor analysis.

Differences were consider to be statistically significant at P value of <0.05.

Analysis was done using SPSS-16 software.

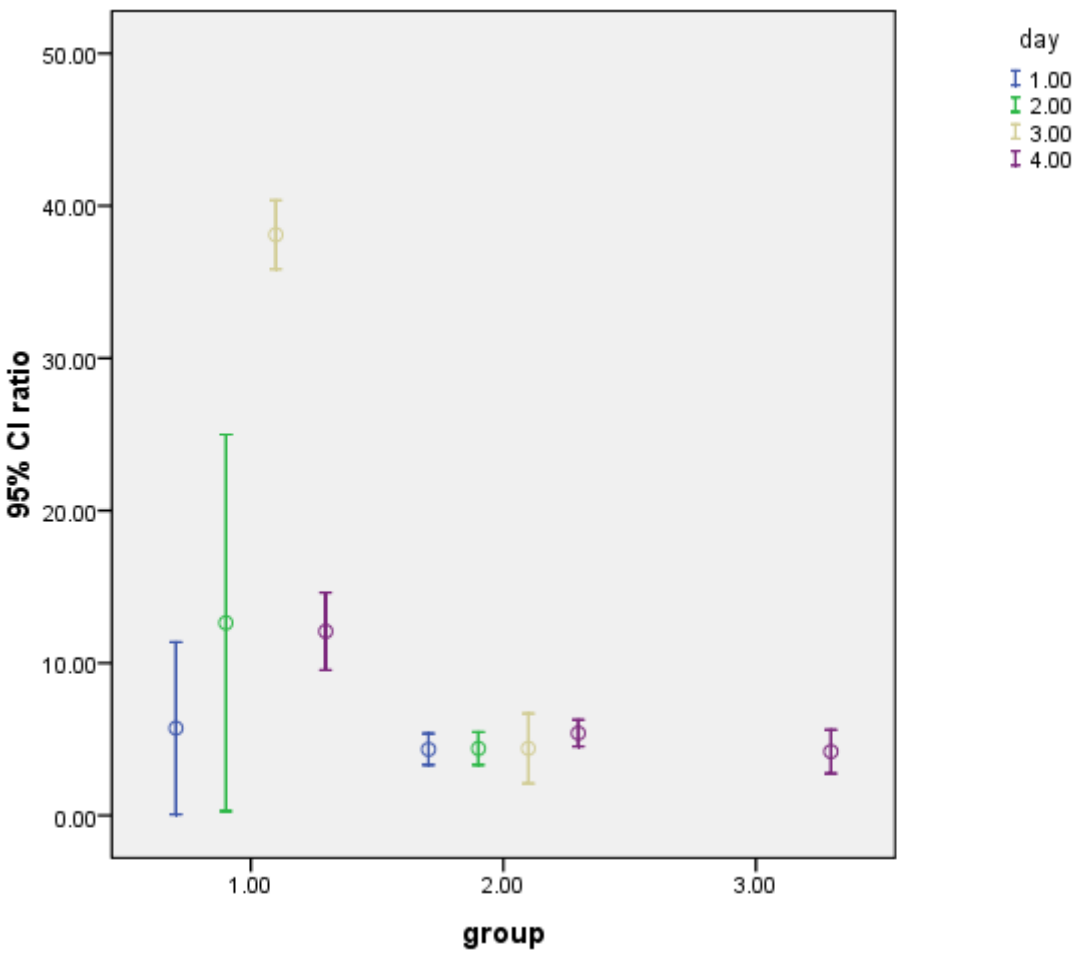
The data was clustered in two factors: first- day (indicating the day when animals were sacrifice d)

Day #1 all animals sacrificed on day 6, Day #2 all animals sacrificed on day #9, Day #3 all animals sacrificed on the day #12, Day #4 all animals sacrificed on the day #15.

Second clusters were the animals groups (active, active control and naïve control).

Graph #1

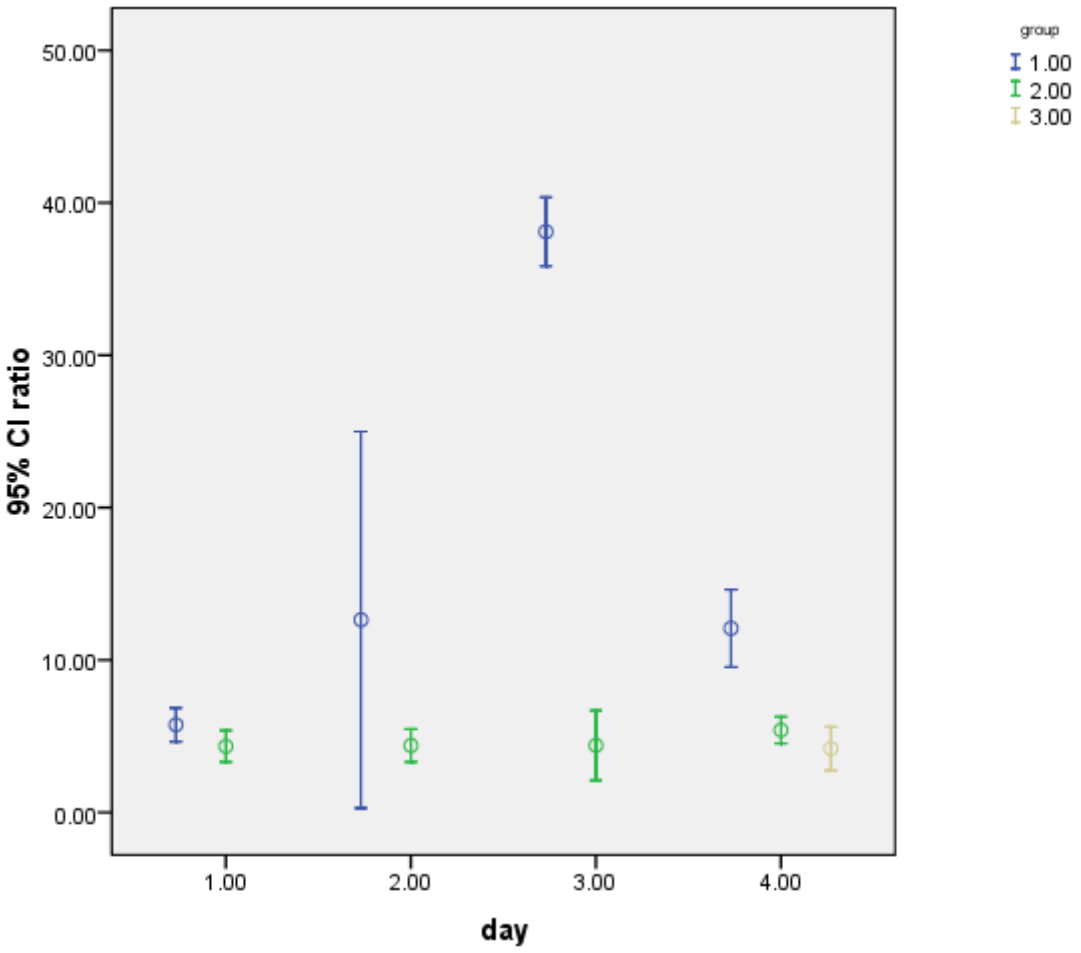
Error bar (CI 95) ratio by group by day



.

In the graph shown above there is a visible difference between group 3 (aEAE) compared other groups (1=naïve control, 2=active control).

Graph #2



mRNA production within the DRG appears to be elevated in group 3.

Univariate Analysis of Variance

Between-Subjects Factors		
		N
day	1	6
	2	6
	3	6
	4	9
group	1	12
	2	12
	3	3

Tests of Between-Subjects Effects					
Dependent Variable:ratio					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2905.740[a]	8	363.218	114.848	.000
Intercept	2161.954	1	2161.954	683.604	.000
day	910.450	3	303.483	95.961	.000
group	980.155	2	490.078	154.961	.000
day * group	936.952	3	312.317	98.754	.000
Error	56.926	18	3.163		
Total	5740.216	27			
Corrected Total	2962.667	26			
a. R Squared = .981 (Adjusted R Squared = .972)					

The univariate ANOVA analysis of variance reveled a statistically significant difference between the days factor (P value of 0.001)

There is also a statistically significant difference between groups factor (p value of 0.001). I have also found in this analysis statistically significant day by group interaction (P value of 0.001)

Since ANOVA analysis has shown statistical difference between day-day, group-group and day –group comparisons. I have proceeded to Post Hoc analysis.

To further analyze multiple comparisons. I have chosen to proceed with TUKEY analysis to be able to compare differences between groups.

Post Hoc Tests

Multiple Comparisons

ratio
Tukey HSD

(I) day	(J) day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-3.4717*	1.02674	.016	-6.3735	-.5698
	3	-16.2050*	1.02674	.000	-19.1069	-13.3031
	4	-2.1800	.93728	.129	-4.8290	.4690
2	1	3.4717*	1.02674	.016	.5698	6.3735
	3	-12.7333*	1.02674	.000	-15.6352	-9.8315

	4	1.2917	.93728	.528	-1.3574	3.9407
3	1	16.2050*	1.02674	.000	13.3031	19.1069
	2	12.7333*	1.02674	.000	9.8315	15.6352
	4	14.0250*	.93728	.000	11.3760	16.6740
4	1	2.1800	.93728	.129	-.4690	4.8290
	2	-1.2917	.93728	.528	-3.9407	1.3574
	3	-14.0250*	.93728	.000	-16.6740	-11.3760
Based on observed means. The error term is Mean Square (Error) = 3.163.						
*. The mean difference is significant at the 0.05 level.						

In the TUKEY multiple comparisons analysis all of the day factors were compared.

Day 1 was compared with days 2-3-4: there was statistical significance found between day 1 and day 2, as well as day 1 and 3. There was no statistical significance between day 1and 4.

Day 2 was compared with Day 1-3- 4. There was statistical significance between day 2 and 1 as well as 3 but no significance was found between days 2 and 4.

Day 3 was compared with Day 1-2 -4. There was statistically significant difference between day 3 and all other days.

Day 4 was compared with Day 1-2-3. There was a significant difference found between day 4 and 3 but no difference with days 1-2.

Homogeneous Subsets

ratio				
Tukey HSD				
day	N	Subset		
		1	2	3
1	6	5.0433		
4	9	7.2233	7.2233	
2	6		8.5150	
3	6			21.2483
Sig.		.156	.566	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 3.163.

Analysis by group

Multiple Comparisons						
ratio Tukey HSD						
(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	12.5083*	.72601	.000	10.6554	14.3612
	3	12.9508*	1.14793	.000	10.0211	15.8805
2	1	-12.5083*	.72601	.000	-14.3612	-10.6554
	3	.4425	1.14793	.922	-2.4872	3.3722
3	1	-12.9508*	1.14793	.000	-15.8805	-10.0211
	2	-.4425	1.14793	.922	-3.3722	2.4872
Based on observed means. The error term is Mean Square (Error) = 3.163.						
*. The mean difference is significant at the 0.05 level.						

In group analysis by Tukey, multiple comparisons have showed statistical significance between groups 1 and 2-3.

In comparison between group 2 and 1-3 there is significant statistical difference between groups 1 and 2 but not group 2 and 3.

Group 3 is statistically different then group 1 but there is no difference with group 2.

Homogeneous Subsets

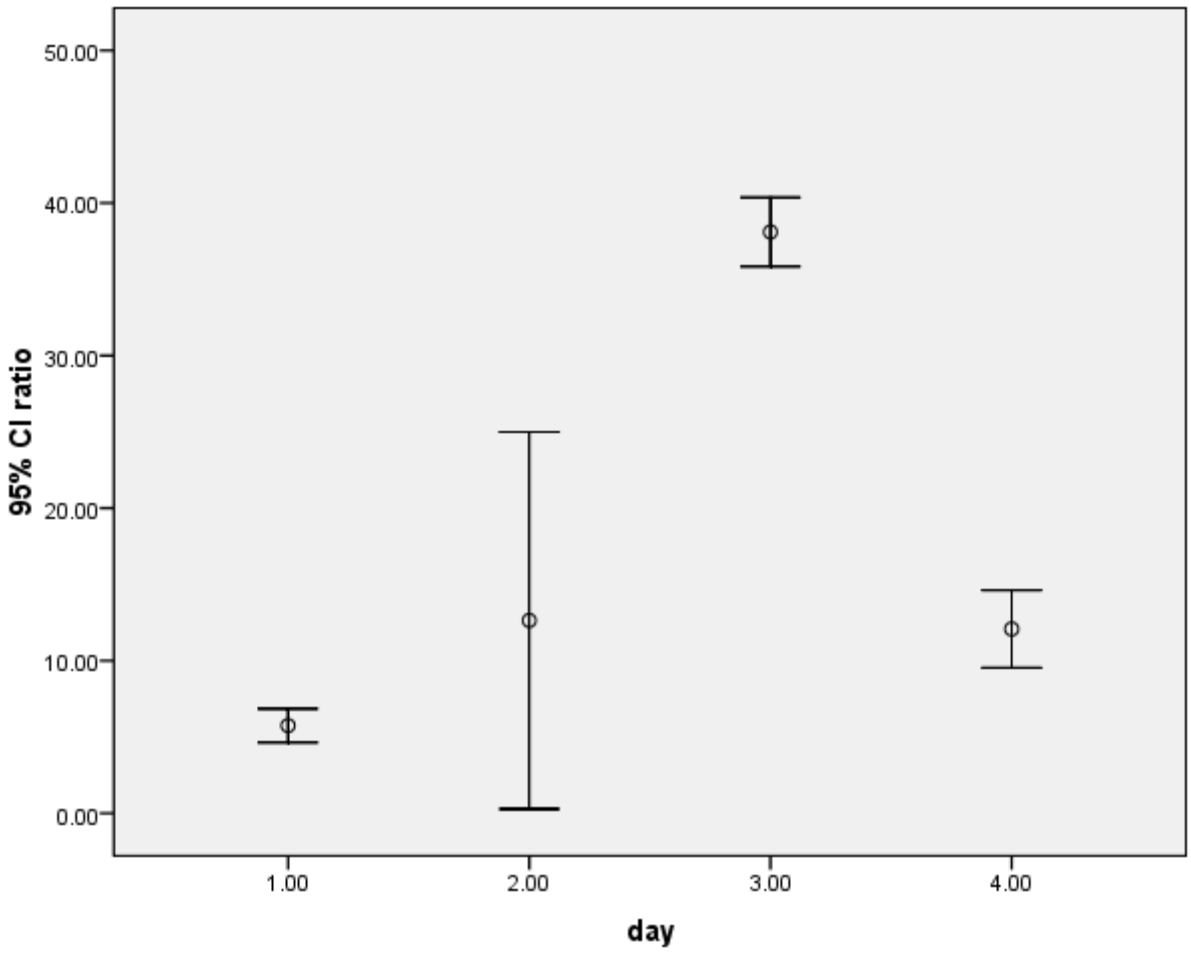
ratio			
Tukey HSD			
group	N	Subset	
		1	2
3	3	4.1900	
2	12	4.6325	
1	12		17.1408
Sig.		.903	1.000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 3.163.

```
UNIANOVA ratio BY day group
  /METHOD=SSTYPE (3)
  /INTERCEPT=INCLUDE
  /POSTHOC=day group (TUKEY)
  /CRITERIA=ALPHA (0.05)
  /DESIGN=day*group.
```

Univariate Analysis of Variance.

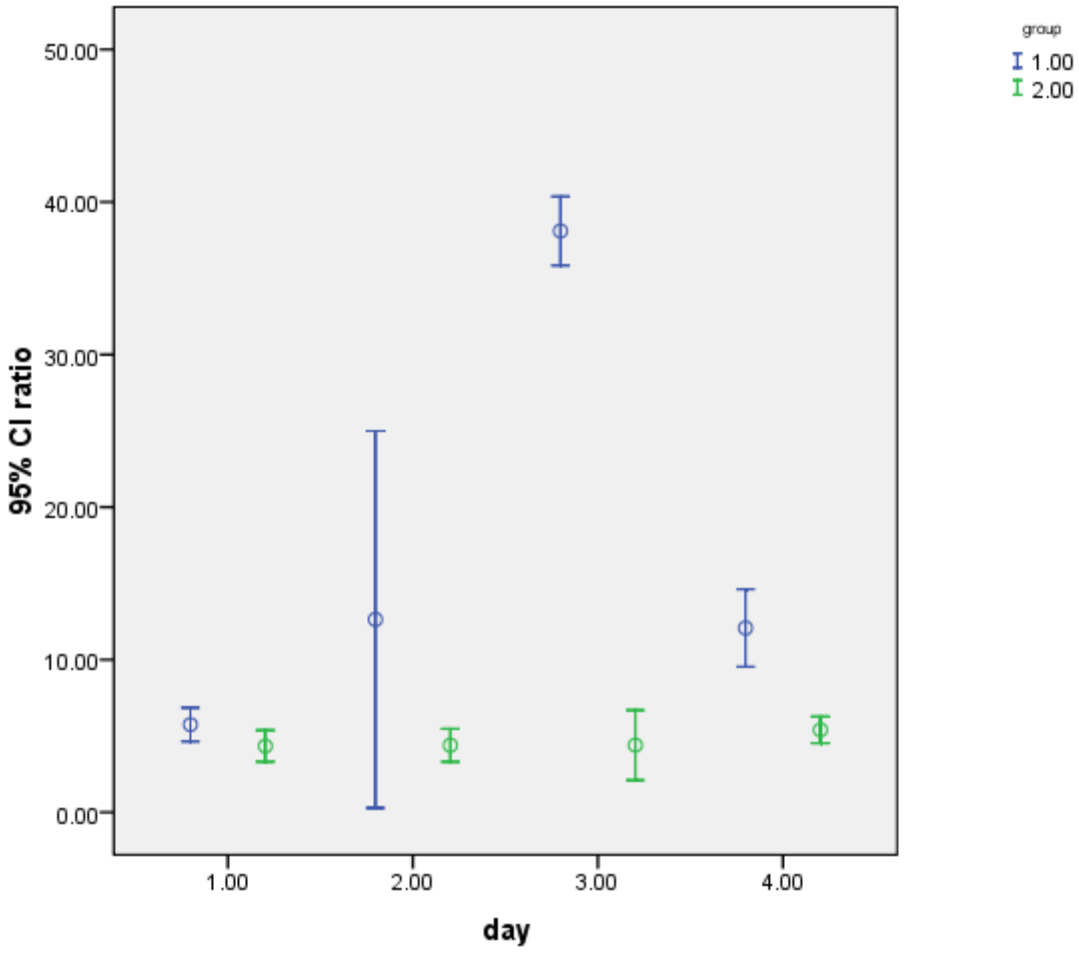
Graph



Ratio by day by group.

/Error Bar (CI 95) = ratio BY day BY group.

Graph



There is a difference between group 1 and 2, indicating that the TNFα mRNA is elevated in the DRG of active EAE animals, it is also time dependent.

```
UNIANOVA ratio BY day group
  /METHOD=SSTYPE (3)
  /INTERCEPT=INCLUDE
  /POSTHOC=day group (TUKEY)
  /CRITERIA=ALPHA (0.05)
  /DESIGN=day*group.
```

Univariate Analysis of Variance

Between-Subjects Factors			
		Value Label	N
day	1		6
	2		6
	3		6
	4		6
group <= 2 (FILTER)	1	Selected	24

Tests of Between-Subjects Effects					
Dependent Variable:ratio					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	910.450[a]	3	303.483	3.142	.048
Intercept	2844.468	1	2844.468	29.446	.000
day	910.450	3	303.483	3.142	.048
filter_$	.000	0	.	.	.
day * filter_$	.000	0	.	.	.
Error	1931.960	20	96.598		
Total	5686.879	24			
Corrected Total	2842.410	23			
a. R Squared = .320 (Adjusted R Squared = .218)					

Post Hoc Tests

Analysis by day

Multiple Comparisons						
ratio Tukey HSD						
(I) day	(J) day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-3.4717	5.67445	.927	-19.3541	12.4107
	3	-16.2050*	5.67445	.044	-32.0874	-.3226
	4	-3.6967	5.67445	.914	-19.5791	12.1857
2	1	3.4717	5.67445	.927	-12.4107	19.3541
	3	-12.7333	5.67445	.146	-28.6157	3.1491
	4	-.2250	5.67445	1.000	-16.1074	15.6574
3	1	16.2050*	5.67445	.044	.3226	32.0874
	2	12.7333	5.67445	.146	-3.1491	28.6157
	4	12.5083	5.67445	.156	-3.3741	28.3907
4	1	3.6967	5.67445	.914	-12.1857	19.5791
	2	.2250	5.67445	1.000	-15.6574	16.1074
	3	-12.5083	5.67445	.156	-28.3907	3.3741
Based on observed means. The error term is Mean Square (Error) = 96.598.						
*. The mean difference is significant at the 0.05 level.						

Homogeneous Subsets

ratio			
Tukey HSD			
day	N	Subset	
		1	2
1	6	5.0433	
2	6	8.5150	8.5150
4	6	8.7400	8.7400
3	6		21.2483
Sig.		.914	.146

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 96.598.

```
ONEWAY ratio BY group
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA (0.05).
```

Oneway

ANOVA					
ratio					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	938.750	1	938.750	10.849	.003
Within Groups	1903.660	22	86.530		
Total	2842.410	23			

```
T-TEST GROUPS=group (1 2)
  /MISSING=ANALYSIS
  /VARIABLES=ratio
  /CRITERIA=CI (.9500).
```

T-Test

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
ratio	1	12	17.1408	13.13781	3.79256
	2	12	4.6325	.67662	.19532

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
ratio	Equal variances assumed	25.557	.000	3.294	22	.003	12.50833	3.79759	4.63262	20.38405
	Equal variances not assumed			3.294	11.058	.007	12.50833	3.79759	4.15528	20.86139

```
USE ALL.
COMPUTE filter_$= ( day=3).
VARIABLE LABEL filter_$ ' day=3 (FILTER)'.
VALUE LABELS filter_$ 0 'Not Selected' 1 'Selected'.
FORMAT filter_$ (f1.0).
FILTER BY filter_$.
```

```
EXECUTE.
T-TEST GROUPS=group (1 2)
  /MISSING=ANALYSIS
  /VARIABLES=ratio
  /CRITERIA=CI (.9500).
```

T-Test

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
ratio	1	3	38.1000	.91000	.52539
	2	3	4.3967	.92338	.53311

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
ratio	Equal variances assumed	.016	.904	45.028	4	.000	33.70333	.74849	31.62518	35.78149
	Equal variances not assumed			45.028	3.999	.000	33.70333	.74849	31.62500	35.78166

```
USE ALL.
COMPUTE filter_$= ( day=4).
VARIABLE LABEL filter_$ ' day=4 (FILTER)'.
VALUE LABELS filter_$ 0 'Not Selected' 1 'Selected'.
FORMAT filter_$ (f1.0).
FILTER BY filter_$.
```

```
EXECUTE.
T-TEST GROUPS= group (1 2)
  /MISSING=ANALYSIS
  /VARIABLES=ratio
  /CRITERIA=CI (.9500).
```

T-Test

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
ratio	1	3	12.0800	1.02093	.58943
	2	3	5.4000	.35171	.20306

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
ratio	Equal variances assumed	3.795	.123	10.715	4	.000	6.68000	.62343	4.94908	8.41092
	Equal variances not assumed			10.715	2.468	.004	6.68000	.62343	4.43072	8.92928

```
USE ALL.
COMPUTE filter_$= ( day=2).
VARIABLE LABEL filter_$ ' day=2 (FILTER)'.
VALUE LABELS filter_$ 0 'Not Selected' 1 'Selected'.
FORMAT filter_$ (f1.0).
FILTER BY filter_$.
EXECUTE.
```

```
UNIANOVA ratio BY day filter_$
  /METHOD=SSTYPE (3)
  /INTERCEPT=INCLUDE
  /POSTHOC=day filter_$ (TUKEY)
  /CRITERIA=ALPHA (0.05)
  /DESIGN=day filter_$ day*filter_$.
```

Between-Subjects Factors			
		Value Label	N
day	2		6
day=2 (FILTER)	1	Selected	6

Tests of Between-Subjects Effects					
Dependent Variable:ratio					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.000[a]	0	.	.	.
Intercept	435.031	1	435.031	14.334	.013
day	.000	0	.	.	.
filter_$	.000	0	.	.	.
day * filter_$	.000	0	.	.	.
Error	151.752	5	30.350		
Total	586.783	6			
Corrected Total	151.752	5			
a. R Squared = .000 (Adjusted R Squared = .000)					

```
T-TEST GROUPS=group (1 2)
  /MISSING=ANALYSIS
  /VARIABLES=ratio
  /CRITERIA=CI (.9500).
```

T-Test

Group Statistics

	group	N	Mean	Std. Deviation	Std. Error Mean
ratio	1	3	12.6367	4.97215	2.87067
	2	3	4.3933	.43501	.25115

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
ratio	Equal variances assumed	11.290	.028	2.861	4	.046	8.24333	2.88164	.24263	16.24404
	Equal variances not assumed			2.861	2.031	.102	8.24333	2.88164	-3.97792	20.46458

```
USE ALL.
COMPUTE filter_$= ( day=1).
VARIABLE LABEL filter_$ ' day=1 (FILTER)'.
VALUE LABELS filter_$ 0 'Not Selected' 1 'Selected'.
FORMAT filter_$ (f1.0).
FILTER BY filter_$.
```

```
EXECUTE.
T-TEST GROUPS=group (1 2)
  /MISSING=ANALYSIS
  /VARIABLES=ratio
  /CRITERIA=CI (.9500).
```

T-Test

Group Statistics					
	group	N	Mean	Std. Deviation	Std. Error Mean
ratio	1	3	5.7467	.44658	.25783
	2	3	4.3400	.41388	.23896

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
ratio	Equal variances assumed	.000	.990	4.001	4	.016	1.40667	.35154	.43065	2.38269
	Equal variances not assumed			4.001	3.977	.016	1.40667	.35154	.42842	2.38491

UNIVERSITY OF MANITOBA | Faculty of Medicine

Department of Pathology
770 Bannatyne Avenue
D212 Dental Building
Winnipeg, Manitoba
Canada R3E 0W3
Telephone (204) 789-3538
Fax (204) 789-3931

Project #: 02-008

February 21, 2003

Project Title: Disease induced activation of Multiple Sclerosis via the dorsal root ganglia.

Project Principal Investigator: Dr. Mike Namaka

Dear Dr. Namaka:

The PACT Committee of the University of Manitoba has considered the request for access to tissue in relation to the above project.

The request is

- ☑ Approved
- ☐ Approved subject to acquiring REB approval
- ☐ Approved pending further information (see note below)
- ☐ Not approved

Please understand that as outlined on our website:

1. This approval relates to the overall project and intent of the study and that decisions concerning availability of sections or blocks from individual cases will reside with the designated study pathologist who will consider the clinical impact in specific cases.
2. That any costs involved in retrieval and sectioning will need to be determined by direct consultation with the relevant pathology department.
3. That the pathology department may request a copy of REB approval.

Sincerely,

[redacted]
Chair, PACT Committee

Notes: Further information that is required:

- ☐ identification of a pathologist involved and responsible for the tissue aspects of the project
- ☐ documentation of REB approval
- ☐ other

http://www.umanitoba.ca/faculties/medicine/units/pathology/PACT.htm

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UNIVERSITY OF MANITOBA

BANNATYNE CAMPUS
Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

APPROVAL FORM

Principal Investigator: Dr. Mike Namaka

Protocol Reference Number: H2005:022
Date of Approval: January 18, 2005
Date of Expiry: January 18, 2006

Protocol Title: **"Antigenic Induction of Multiple Sclerosis via the Dorsal Root Ganglia"**

The following is/are approved for use:

- **Proposal (submitted January 18, 2005)**

The above underwent expedited review and was **approved as submitted** on January 18, 2005 by Dr. K. Brown, MD, MBA, Chair, Health Research Ethics Board, Bannatyne Campus, University of Manitoba on behalf of the committee per your letter dated January 18, 2005. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations.*

This approval is valid for one year only. A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought form the relevant institution, if required.

Sincerely yours,

[redacted]
Chair, Health Research Ethics Board
Faculty of Medicine

Please quote the above protocol reference number on all correspondence.
Inquiries should be directed to REB Secretary
Telephone: (204) 789-3883 / Fax: (204) 789-3414