INSTITUTE OF NEUROLOGY
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Myelin Basic Protein Isoform Expression: A Comparison of Developing Rat Brain and Myelinating and Remyelinating Rat Brain Aggregate Cell Cultures

A dissertation submitted in partial satisfaction of the
requirements for the degree of Master of Philosophy in Medicine

by

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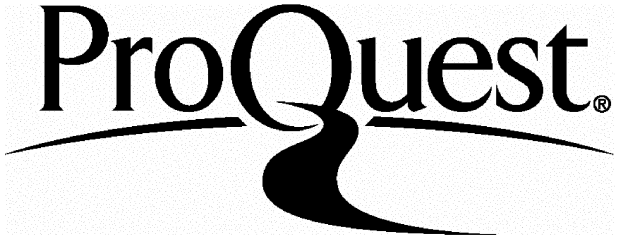

ABSTRACT OF THE DISSERTATION

Recent evidence suggests myelin basic protein (MBP) exon-2 containing isoforms play a significant role in the onset of myelination as they are more abundant during early development. The pattern of expression of MBP exon-2 containing forms was studied in rat brain aggregate cultures during myelination in order to draw comparisons with the developing brain and upon remyelination following demyelinative treatment. The pattern of MBP isoform expression in the aggregate cultures was found to be similar to that of the brain and was recapitulated following demyelination with anti-myelin antibodies. Macrophage enrichment, resulting in increased accumulation of total MBP in the cultures, did not alter the isoform distribution. Both control and enriched cultures expressed a 16 kDa protein (26 +/- 9.8% of total MBP for control samples) which reacted with MBP antisera at the onset of myelination (day in vitro 14) but was barely detectable by day in vitro 21. The expression of this protein, also present in postnatal day 11 rat brain but no longer by day 15, has been predicted by RT-PCR; however, detection of a corresponding protein with the same developmental pattern and appropriate molecular weight has not been previously reported. The results of this study reinforce the value of the aggregate culture system as a versatile yet accurate model of myelination and remyelination.

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ABBREVIATIONS

Ab: antibody

APS: ammonium persulfate

BAME: benzoyl-L-arginine methyl ester

BSA: bovine serum albumin

cDNA: complementary DNA

CNP or CNPase: 2',3'-cyclic nucleotide 3'-phosphodiesterase

CNS: central nervous system

cpm: counts per minute

DEPC: diethylpyrocarbonate

DIG: digoxigenin

DIV: day(s) in vitro

DNA: deoxy ribonucleic acid

DTT: dithiothreitol

EBSS: Earle's Balanced Salt Solution

EDTA: ethylenediaminetetraacetic acid

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GPS: guinea pig serum

HRP: horse radish peroxidase

IgG: immunoglobulin G

MAG: myelin-associated glycoprotein

MBP: myelin basic protein

MOG: myelin/oligodendrocyte glycoprotein

MOPS: 3-[N-Morpholino] propanesulfonic acid

mRNA: messenger ribonucleic acid

MS: multiple sclerosis

PBS: phosphate buffered saline

PLP: proteolipid protein

PMSF: phenylmethylsulfonyl fluoride

PNS: peripheral nervous system

PVDF: polyvinylidene fluoride

RIA: radio-immunoassay

SDS: sodium lauryl sulfate

SSC: solution of NaCl and sodium citrate

TAME: p-tosyl-L-arginine methyl ester

TBS: Tris buffered saline

TEMED: N, N, N', N'-tetramethylethylenediamine

TPCK: L-1-tosylamide-2 phenylethylchloromethyl ketone

TTBS: Tris buffered saline with Tween 20

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PUBLICATIONS AND PRESENTATIONS

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Chapter I. Introduction

The purpose of this project was twofold: (1) to compare myelination in the rat brain aggregate cell culture system to that of the developing rat brain in order to verify the system as an accurate model of myelination for future studies, and (2) to compare myelination with remyelination in control and macrophage enriched culture preparations to learn more about the remyelinative process and whether it recapitulates myelination. The hope is that this new knowledge may help further the understanding of and development of new treatments for demyelinatve disorders such as multiple sclerosis. A brief introduction to the topics necessary for understanding this project as well as the rationale for its focus follows.

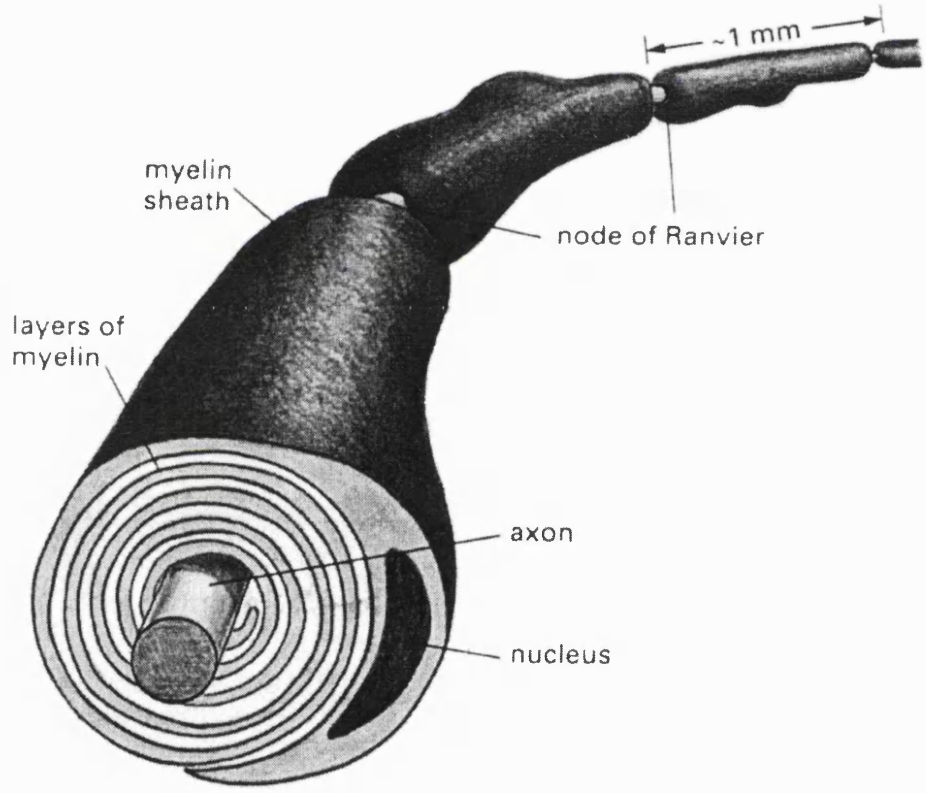
Myelin

Axons in both the central nervous system (CNS) and peripheral nervous system (PNS) are myelinated in order to insulate and speed conduction along the nerve fibers. Greater conduction speed can also be achieved by increasing the diameter of the axon, but not nearly as effectively. For example, myelination allows mammalian nerve fibers which are generally not larger than 20 μm in diameter to conduct more than twice as quickly as squid axons with a 600 μm diameter (Smith, 1996). The compact structure of myelinated nerves allows for the formation of complex neural networks. Myelin consists of extensions of specialized glial cell membranes which are wrapped in concentric layers around the axons and serve as an electrical insulator. Each sheath is approximately 1-2 mm long and is separated from the next sheath by a node of Ranvier (see Figure 1 below, Junquiera et al., 1998, pp. 170-171) . Only the axonal membrane in the region of the nodes contains the Na+ and K+ channels which respond to and propagate depolarisation. The action potential therefore 'jumps' from gap to gap, resulting in what is called saltatory conduction. In this way, the depolarisation moves at a constant rate down the axon, and its amplitude does not dissipate.

The myelin sheath is produced by oligodendrocytes in the CNS which can each send extensions to up to 50 different axons (Smith, 1996, pp 134). Schwann

cells perform the same function in the peripheral nervous system, but each is associated with only one axon (see Figure 1 below).

A


~1 mm
myelin sheath
node of Ranvier
layers of myelin
axon
nucleus

B

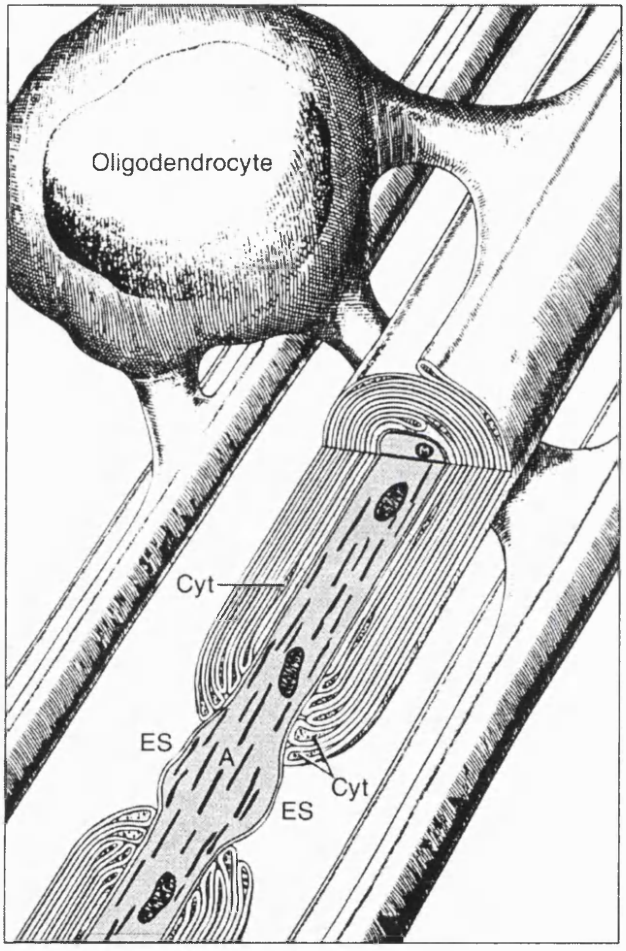
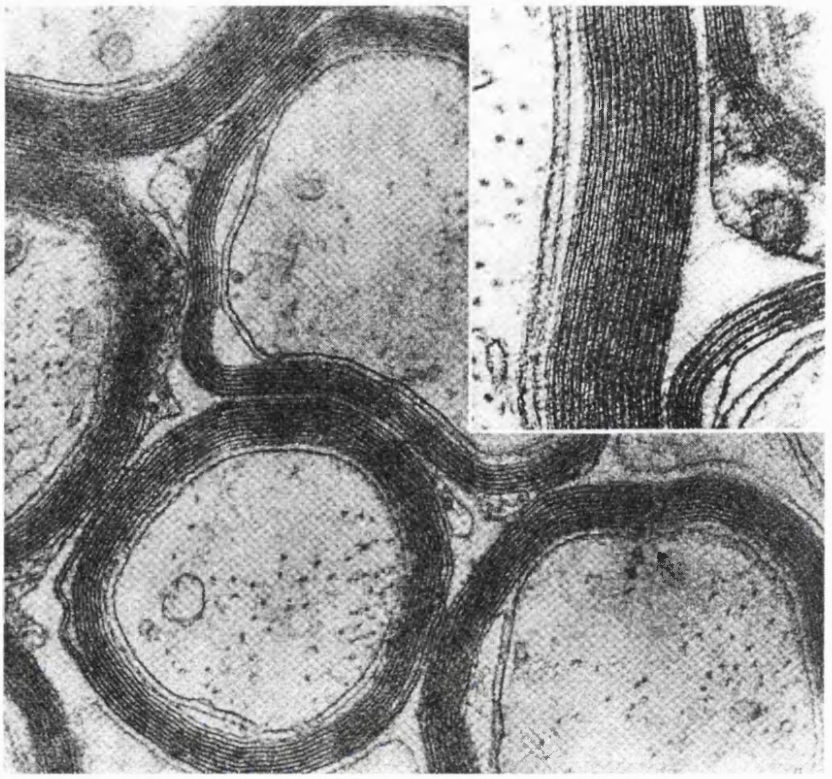


Figure 1. (A) A myelinated peripheral nerve showing nodes of Ranvier. Reproduced from Alberts et al., *Molecular Biology of the Cell*, 3rd Edition, p. 532. **(B)** Myelinated central nervous system axons showing that the same oligodendrocyte may send out processes to several different axons. Reproduced from Junqueira et al., *Basic Histology*, p. 164.

The structure of myelin results from interactions between specific proteins located on either side of the membrane. Myelin basic protein which is rich in positively charged arginine and lysine residues is present on the intracellular side and thought to aid in myelin compaction by holding the negatively charged intracellular faces of the membrane together (Smith, 1996, p. 137). This results in formation of the major dense lines which are concentric electron dense lines seen in electron micrographs of myelin (Figure 2a below). In the central nervous system, a second major myelin protein is proteolipid protein (PLP). PLP has three transmembrane helices with a large extracellular domain between helices two and three. This domain is thought to intertwine with a PLP molecule on an adjacent whorl of myelin (Smith, 1996, p. 138) to provide stability between adjacent layers of the sheath. This forms the intraperiod line which alternates with the major dense line. A protein called P_0 performs a similar function in PNS myelin. Many other proteins present in minor amounts have also been identified in myelin including myelin associated glycoprotein and myelin oligodendrocyte glycoprotein, but their functions are not yet well characterised.

A



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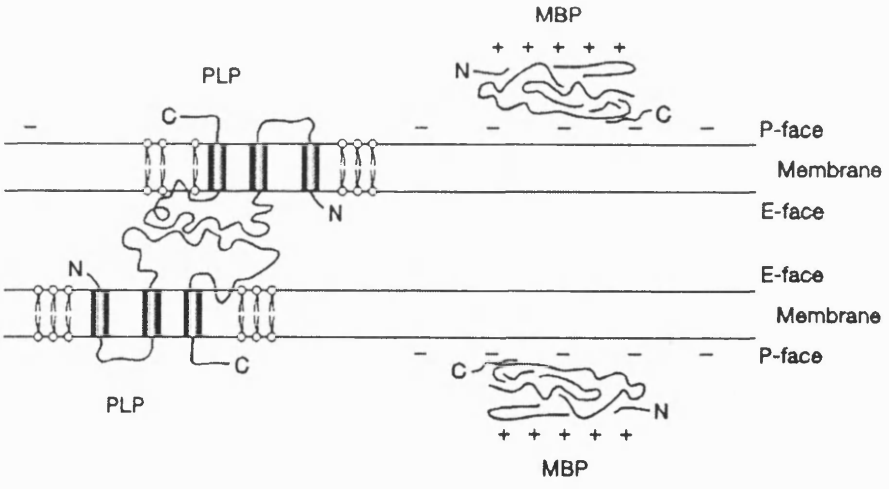

Figure 2. (A) Transmission electron micrograph rat optic nerve demonstrating the major dense and intraperiod lines of myelin (Reproduced from Smith, 1996, p. 137). (B) Arrangement of major myelin proteins in central nervous system myelin (Reproduced from Smith, 1996, p. 136).

The synthesis of several myelin proteins is developmentally regulated at the transcriptional level. Different isoforms are produced as the animal matures by alternative RNA splicing. MBP, which consists of at least seven exons and has at least four major isoforms in most mammals, is one of the most studied of these proteins. Expression of the forms containing exon-2 peak in the initial stages of myelination, possibly helping to direct the initial steps of myelin compaction, while those lacking exon-2 increase later and then decrease less rapidly (Jordan et al., 1990). Exon-2 containing transcripts are also considerably upregulated at the onset of remyelination making it possible for their presence to be used as a marker for this process (Nagasato et al., 1997). Myelin-associated glycoprotein (MAG), a minor myelin protein which shows significant sequence homology to neural cell adhesion molecule (N-CAM), and PLP, the major integral membrane protein in CNS myelin, also have early and adult forms. MAG transcripts lacking exon-12 predominate postnatally while transcripts containing exon-12 predominate in the adult. The early form of PLP, known as DM20, lacks a portion of exon-3 (Sutcliffe, 1987).

Multiple Sclerosis

Demyelinating diseases are characterised by a preferential loss of myelin with relative sparing of the nerve axons; however, secondary damage to the nerves may occur as the disease progresses (Cotran et al., 1999). One of the most common of these diseases is multiple sclerosis (MS) with an incidence of approximately 1/1000 in the United States and European populations (Cotran et al., 1999). It is a putative autoimmune disease of the central nervous system with unknown cause but both environmental and genetic factors have been implicated.

Remyelination has been observed in acute MS plaques; however, minimal functional repair is achieved as the disease progresses (Miller et al., 1996). The disease course varies widely between affected individuals, but repeated episodes of myelin destruction ultimately result in the chronically demyelinated lesions characteristic of the disease. In contrast, immune mediated myelin damage in the peripheral nervous system often results in complete repair. For example, in Guillain-Barre syndrome, which is an acute illness characterized by weakness of the distal limbs which rapidly advances to affect more proximal muscles and can result in death

by respiratory paralysis, results in full recovery in the vast majority of patients (Cotran et al., 1999, pp. 1275-6). In addition, extensive remyelination in the central nervous system with return to normal function is often seen in animal models where a monophasic demyelinating disease has been induced by ingestion of toxin, and/or autoimmune attack, or viral infection (Miller et al., 1996). By studying such animal models, one can gain a better understanding of how remyelination occurs and why it ultimately fails in chronic diseases such as MS with the hope of developing strategies to encourage myelin repair in the early stages of disease onset.

Macrophages

In the brain, microglia are small, elongated cells with irregular processes that represent the mononuclear phagocytic system. They are thought to be derived from cells in the bone marrow and are involved in both inflammation and repair. Similar to peripheral macrophages, these cells can become activated and release proteases and free radicals, serve as antigen presenting cells, secrete immunoregulatory cytokines, and clear cell debris from damaged tissue (Junquiera et al., 1998). They can also proliferate and migrate to the site of injury (Cuzner, 1997).

Macrophages have long been implicated in the MS disease process because once activated they can release reactive oxygen intermediates, proteases, and inflammatory mediators leading to CNS damage. For example, products such as hydrogen peroxide and nitric oxide have been shown to cause neuronal cell injury in cocultures (Thery et al., 1991), and nitric oxide may cause damage to oligodendrocytes directly (Merrill et al., 1993) leading to demyelination. Proteases such as elastase and plasminogen activator can catalyze the degradation of MBP (Cuzner, 1997), the major extrinsic protein of myelin. Inflammatory mediators released by activated microglia may attract circulating lymphocytes and monocytes which with proper antigen presentation could lead to T cell activation and significant myelin destruction (Cuzner, 1997).

However, much evidence suggests that macrophages also play a crucial role in repair and remyelination. The clearance of cellular debris is necessary for efficient repair, and released growth factors may directly or indirectly affect oligodendrocytes and their progenitor cells. For example, uncleared myelin debris has been shown to

prevent axonal regrowth (Schnell and Schwab, 1990). In fact, one explanation of why CNS axons do not regenerate as well as those in the PNS is that macrophage recruitment to the site of injury is delayed or insufficient (Perry et al., 1987). Studies on transected rat optic nerve, which does not normally regenerate, have demonstrated that repair can occur if macrophages are transplanted locally to the damaged site (Lazarov-Spiegler et al., 1996). However, the technique is only successful if the macrophages are first appropriately activated. Incubation with sciatic nerve prior to transplant leads to recovery but exposure to optic nerve does not, suggesting that the CNS environment may have an inhibitory effect on the macrophages. Thus, while macrophages can play a crucial role in repair, they may only be able to do so if present in sufficient number and appropriately activated. Clearly, further study is needed to understand the complex role macrophages play in the CNS and how they can be manipulated to promote optimal recovery in trauma and disease states.

Myelin Basic Protein

Myelin basic protein (MBP) is a major structural protein in myelin and thought to be responsible for compaction and stabilisation of the major dense line; however, recent studies have shown it is likely to have a wider role in myelinogenesis. Classical MBP consists of seven exons; however, MBP is now known to be part of a larger gene and include at least four more exons 5' to the original seven. This has been termed Golli-mbp (Campagnoni et al., 1993). Alternative splicing of MBP exons 2, 5 and 6 results in at least five major isoforms in rodent (Newman et al., 1987). Expression of isoforms containing the exon-2 sequence (21.5 and 17.2 kDa in rodents) have been found to peak in the initial stages of myelination and again at the onset of remyelination in mouse and human studies (Jordan et al., 1990 and Capello et al., 1997). In addition, immunocytochemical studies have shown them to be distributed diffusely in the cytoplasm rather than oligodendrocyte processes and actively transported into the nucleus (Pedraza et al., 1997) suggesting they may play a regulatory role in early myelination. Isoforms lacking exon-5 (20.2 and 17.3 in mouse) may also be important in early myelination. Using RT-PCR, forms lacking this exon have been found to peak just before birth and are barely detectable by the time of peak myelination (postnatal day 18 in mice)

indicating they may be associated with the genesis or differentiation of immature oligodendrocytes (Nakajima et al., 1993).

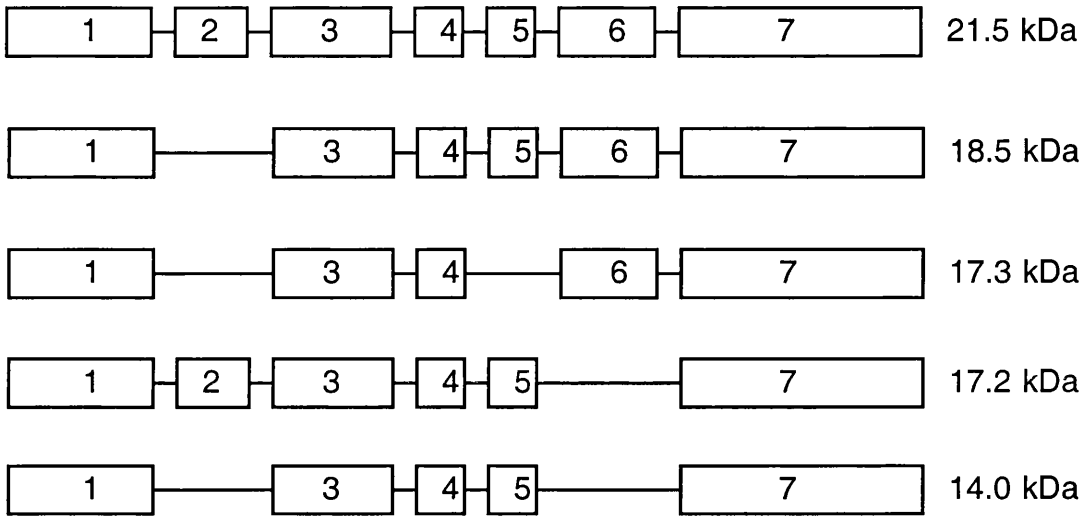

Figure 3. Exons present in major MBP isoforms, reproduced from Newman et al., 1987.

The major MBP isoforms present and their pattern of expression vary widely between species (see Table 1 below). This list is likely not complete because new isoforms are being characterised frequently.

Molecular Weight (kDa)	Humans	Rats	Mice
21.5	X	X	X
20.2	X		X
18.5	X*	X*	X*
17.3	X		X
17.2		X	X
14		X*	X*

Table 1. Predominate and well characterised MBP isoforms in mice, rats and humans. The '*' indicates the most prevalent isoforms in adult CNS myelin.

The Rat Brain Aggregate Cell Culture Model

Foetal rat telencephalon cells are maintained under constant rotation to form three-dimensional aggregates. An organised tissue including synapses and compact myelin around axons forms in a fashion similar to that seen in brain development *in vivo* (Honegger, 1985). Once developed, demyelination can be induced by adding cytokines such as interferon-γ and interleukin-1α or by adding antibodies such as anti-myelin oligodendrocyte glycoprotein plus complement. The aggregate culture model provides several distinct advantages for studying myelination and remyelination over other available systems. More control over the experimental environment is possible than with studies using intact animals because growth factors, cytokines, or other molecules of interest can be easily added and maintained at specific concentrations. The cultures can be enriched or potentially depleted of specific cell types in order to better characterise their role in myelination. Aggregates, while more difficult to set up and maintain, have advantages over traditional cell culture and cell lines also: they contain all the cell types normally present in the brain and in an architecture that mimics what is seen *in vivo*. The aggregate culture system is not be confused with neurospheres which are self

renewing epidermal growth factor-responsive multipotential neural progenitor cell populations generally derived from embryonic brain (Milward et al., 1997). Neurospheres are an excellent system for studying the differentiation of neural progenitor cells into neurons and glia and a possible source of cells for transplantation, but are not as useful for studying myelination and remyelination as the aggregate model.

It has previously been shown with the rat brain aggregate culture system that the addition of macrophages at the beginning of the culture period leads to an increased rate of accumulation of myelin basic protein, and electron microscopy indicates that the myelin is axonally associated (Loughlin et al., 1997). 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), used as an index of oligodendrocyte number, remained stable indicating that the increased accumulation of MBP was probably due to enhanced capacity for myelin production rather than oligodendrocyte proliferation. During remyelination following demyelination, macrophage enriched cultures had a greater amount of MBP, but the rate of accumulation did not differ significantly from control cultures.

Transcription levels of exon-2 containing mRNAs and the patterns of expression of major MBP isoforms have not previously been investigated in myelinating and remyelinating aggregate cell cultures. Knowledge of whether the patterns of gene expression in the aggregates mirror that seen *in vivo* will be necessary for determining the suitability of the 3-D culture system for future studies and may help to elucidate whether remyelination recapitulates embryonic myelination. Comparing MBP isoform expression in developing and macrophage enriched aggregate cultures should lead to a better understanding of their roles in the developing and damaged brain.

Chapter II. Materials and Methods

Standard laboratory chemicals and reagents unless stated otherwise were obtained from Sigma (Sigma-Aldrich, Poole, UK) or BDH (Merck Limited, Lutterworth, UK).

A. RNA Isolation

This procedure was modified from that published in Chomczynski and Sacchi, 1987. Tissue was collected, immediately frozen on dry ice, and kept at -70 °C until homogenised in the solution below. Aggregates were harvested, rinsed in PBS, and frozen before homogenisation. Approximately 750 µl of guanidine thiocyanate homogenisation buffer was used for every 50 mg of sample. This was done on ice in the fumehood using a 23 gauge needle and sterile syringe to disrupt the tissue. All water used to make solutions was treated with diethyl pyrocarbonate (DEPC) to inactivate any RNAase (made by adding 1 ml DEPC per liter of distilled water in the fumehood, stirring for one hour, and autoclaving).

Guanidine Thiocyanate Homogenisation Solution (25 ml):

12.5g guanidine thiocyanate (4.215 M)
12.5 ml DEPC treated water
625 µl of 1M, pH 7 stock sodium citrate (25 mM)
625 µl of 20% stock N-lauroyl sarcosine (0.5%)
175 µl of 14.26 M β-mercaptoethanol (0.1 M)
Filter sterilised before use.

All subsequent solution volumes in this procedure assume a 750 µl volume of homogenisation buffer was used. After homogenisation, 75 µl sodium acetate (2 M sodium acetate, 2 M acetic acid, pH 4), 750 µl of saturated phenol and 150 µl of chloroform/isoamyl alcohol (24:1 ratio) were added sequentially. The solution was vortex mixed gently before adding the phenol, and all steps were done in the fume hood. Following addition of all ingredients, each sample was vortex mixed for 10 seconds, placed on ice for 15 minutes, and then centrifuged at 8000g for 15 minutes

at 4 °C to separate the aqueous and organic layers. The aqueous phase was collected in a fresh tube, and the RNA was precipitated by adding 750 µl of isopropanol, vortex mixing for 10 seconds, and incubating overnight at -20 °C.

The samples were centrifuged for 30 min at 8000g and 4 °C to pellet the RNA. The supernatant was discarded, and the pellet was redissolved in 750 µl of guanidine hydrochloride (see below). It was then gently vortex mixed and placed on ice for 15 minutes to make sure the entire pellet had dissolved. After adding 375 µl of absolute ethanol, the samples were vortex mixed and stored overnight at -20 °C to re-precipitate the RNA.

Guanidine Hydrochloride (10 ml):
5.73 g guanidine hydrochloride (6 M)
500 µl of 2 M sodium acetate, pH 4 (0.1 M)
50 µl of 160 mg/ml DTT stock (5 mM)
5.1 ml of DEPC treated water
Filter sterilised before use.

The samples were spun as above for 20 minutes, and the supernatant was discarded. The pellet was washed successively with 70%, 80%, and absolute ethanol by spinning it briefly with each alcohol and discarding all supernatants. After the final spin, all traces of absolute ethanol were removed with a fine glass pipette. The pellet was then air dried briefly in a laminar flow hood, resuspended in 40 µl of water, gently vortex mixed, heated to 60 °C for 10 minutes, and frozen. Its concentration and purity were determined by thawing, adding 3 µl to 400 µl of water in a quartz cuvette and reading the optical density at 260 and 280 nm. The RNA was considered sufficiently pure if the 260:280 ratio was close to 2. The RNA concentration was calculated using the following formula:

$$\frac{(\text{O.D. at 260 nm}) * (400\mu l / 3\mu l * 40\mu g \text{ RNA} / (ml * \text{O.D.}))}{1000\mu l / ml} = \mu g/\mu l \text{ RNA}$$

The term 400µl / 3µl corrects for the dilution, and 40µg RNA / ml is the RNA concentration that would give an O.D. reading of 1.

B. Northern Blotting

I. Sample preparation

Based on the known RNA concentrations, the desired amount of RNA (usually 5-20 µg) was calculated and placed in a fresh Eppendorf tube. Enough cold DEPC water was added to make the final volume 100 µl. To that, 10 µl of autoclaved 3 M sodium acetate (not pH'd) and 200 µl absolute ethanol were added. After vortex mixing, the solution was left overnight at -20 °C to allow RNA to precipitate. The RNA pellet was collected by centrifuging at 8000g and 4 °C for 30 minutes. All traces of remaining ethanol were removed with a fine glass pipette, and the following were added sequentially to each sample:

3.5 µl formaldehyde
10 µl formamide
2 µl 10x MOPS
4.5 µl DEPC treated water

Samples were briefly centrifuged and placed in a water bath at 65 °C for 10 minutes to make sure the RNA was completely dissolved and denatured (vortex mixed after 5 minutes), and quenched on ice. Two µl of bromophenol blue dye (0.25% in water) were then added, and the samples were stored on ice until ready to load gel.

II. Gel Preparation

The gel tank was set up in the fumehood and leveled. A 1% agarose gel containing 1X MOPS (3-[N-Morpholino] propane sulfonic acid)-EDTA-sodium acetate buffer and 2.2 M formaldehyde was prepared by first adding the agarose to the total amount of water needed and microwaving until dissolved. MOPS-EDTA-sodium acetate and formaldehyde were then added from their stock solutions (10X and 12.3M respectively). A 300 ml gel, for example, required 3 g of agarose, 216 ml water, 30 ml 10X MOPS, and 54 ml formaldehyde. The solution was mixed by swirling and poured into a leveled gel tray. The comb was inserted, and all bubbles

were removed using a sterile needle.

III. Running Gel

The gel tank was filled with enough 1X MOPS-EDTA-sodium acetate buffer to cover just the edges of the gel. Samples were loaded, and the gel was run at 120 V until the samples entered the gel (approximately 5-10 minutes). Then additional buffer was added to cover the gel, and it was run for approximately 4 hours at 100-120 V. The running buffer was kept circulating during this time.

IV. Blotting

Excess gel was removed, leaving at least a centimeter on all sides surrounding the samples. It was then rinsed in DEPC water for 2 minutes, and equilibrated in two 15 minute washes of 20X SSC (175.3 g NaCl and 88.2 g of sodium citrate in 1 L water, pH 7.0). A nylon membrane (Nytran-Plus, Schleicher and Schuell, Dassel, Germany) was cut to be 0.5 cm smaller than the gel on all sides, prewet in DEPC treated water for 5 minutes, and rinsed in 10X SSC for 5 minutes. A sheet of 3 MM Whatman paper was cut oblong to serve as a wick along with 5 pieces cut to the same size as the membrane. Five pieces of Whatman paper were also cut to the same size as the membrane. The wick was wetted in 20X SSC and placed smoothly over a glass plate. The ends of the wick were in a tray containing 20X SSC. The gel was then inverted onto the wick, and all bubbles were removed using a glass pipette. Four pieces of parafilm were placed over the gel edges being careful not to cover any of the sample wells. The nylon membrane was placed over this, and again all bubbles present were removed by rolling with a glass pipette. The surface was flooded with 20X SSC, and the sheets of Whatman paper, blotting paper, a glass plate, and a 500 g weight were placed sequentially on top. The gel was allowed to blot overnight at 4 ^{o}C by capillary action.

The next day, the paper and parafilm were removed carefully so that the membrane remained attached to the gel. The gel and membrane were then flipped over onto a fresh piece of Whatman paper so that the positions of the wells could be marked with a pencil. The gel was then stained with ethidium bromide to verify that

the transfer was complete and discarded. The membrane was rinsed in 2X SSC, blotted on Whatman paper, then wrapped in cling film. The RNA was cross-linked to the membrane by exposing each side to UV light for 3 minutes. The gel was then stained with methylene blue to visualize the 28S and 18S ribosomal bands. To do this, the membrane was first fixed in a solution of 5% acetic acid for 2 minutes, then placed in a solution of 0.4% methylene blue and 0.5 M sodium acetate (pH 5.2) for 5 minutes. Then the filter was washed thoroughly in DEPC treated water to remove excess stain.

V. Hybridisation and Detection

All hybridisations were done using the protocol from Boehringer Mannheim (Mannheim, Germany) and a high SDS concentration hybridisation solution containing:

7% SDS

50% deionized formamide

2% blocking reagent (Boehringer Mannheim, Mannheim, Germany)

50 mM sodium phosphate, pH 7.0

0.1% n-lauroylsarcosine

The buffer was stored in frozen aliquots until use and warmed to the desired hybridisation temperature before adding to membranes.

The membrane was pre-wet in 2X SSC, and then prehybridised for one hour with 5 ml of fresh hybridisation solution in a rotating tube in a hybridisation oven. In all experiments, 55 °C was used for oligonucleotide probes and 50 °C for cDNA probes. The appropriate amount of digoxigenin (DIG) labelled probe (prepared and quantified according to Boehringer Mannheim's kit instructions) was added to 5 ml of hybridisation solution. In the case of oligonucleotide probes, Boehringer Mannheim recommends adding between 0.1 and 2.0 pmol of probe per ml of solution. Here 2 pmol/ml was used. Hybridisation was allowed to occur overnight. Probe solutions were collected after use, frozen at -20 °C and reused multiple times.

The membrane was briefly rinsed with 2X SSC wash solution (2X SSC with 0.1% SDS) and transferred to a plastic dish. The membrane was then washed twice

for 5 minutes at room temperature with 2X SSC wash solution, followed by two 15 minute washes at 68 °C in the hybridisation oven using pre-heated wash solution. The membrane was then rinsed at room temperature for 3 minutes in buffer 1 (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, DEPC treated). The membrane was blocked for 30 minutes in blocking buffer (10 ml block [Boehringer Mannheim] to 90 ml buffer 1) before adding the anti-DIG antibody conjugated to alkaline phosphatase (Fab fragment, Boehringer Mannheim [Mannheim, Germany]) diluted 1:10,000 in blocking buffer. The membrane was then rinsed in buffer 1 with 0.3% Tween 20 and washed two times for 15 minutes in this same solution at room temperature. Finally, the membrane was rinsed briefly in buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5), washed in buffer 3 for at least three minutes, and then exposed to chemiluminescent substrate (CPDStar, Boehringer Mannheim, Mannheim, Germany; 1:100 dilution in Buffer 3) for 5 minutes in a hybridisation bag in the dark. The membrane was then blotted on a piece of Whatman paper and sealed in a fresh hybridisation bag for exposure to X-ray film.

VI. Membrane Stripping

Membranes were stripped of probe according to Boehringer Mannheim (Mannheim, Germany) protocol. Briefly, the membrane was rinsed in water for 1 minute and then incubated for 10 minutes with agitation in 100 ml of 0.1% SDS heated to just below its boiling point. This was followed by a 5 minute wash in buffer 1 with 0.3% tween 20 and a rinse in 2X SSC.

C. Protein Quantification

The Lowry method (Lowry et al., 1951) was routinely used; however, the Bradford method (Bradford, 1976) which is somewhat faster was also used.

I. Lowry Protein Assay

Duplicate, 10 ml plastic tubes were labelled for standards and samples. Standards were prepared using a 0.2 mg/ml bovine serum albumin (BSA) stock solution as follows:

Total BSA (μg)	0	5	10	15	20	30
μl of dH_2O	150	125	100	75	50	0
μl of BSA stock	0	25	50	75	100	150

Previously homogenised samples, either in the homogenisation buffer described in section E. I. below for Western blotting or in the typical buffer used for aggregate biochemical studies (2 mM potassium phosphate with 1 mM EDTA), were diluted in 0.05 M NaOH. In general, 40 μl of homogenised aggregate or brain sample was diluted into 1000 μl of NaOH. Then 100μl of water was added to each sample tube plus 50 μl of the sample/NaOH solution. Finally, 50 μl of 0.4 M NaOH was added to all tubes, followed by a brief vortex mixing.

The steps following this were timed, and tubes were vortex mixed following each ingredient added. At T=0, 1 ml of solution X (see below) was added to all tubes. At T=15 minutes, 100 μl of Folin and Ciocalteau's Reagent diluted 1:1 in dH_2O was added. This reagent was added in the fumehood, and samples were wrapped in foil or placed in the dark after adding. At T=45 minutes, absorbance of samples was read at 750 nm.

Solution X

This was made by mixing two solutions just before use in the ratio of 99:1:

99 vol: 2% Na_2CO_3

0.02% Na K tartrate in 0.1 M NaOH

1 vol: 1% $CuSO_4 \cdot 5H_2O$

Standard curve and determination of protein concentrations were done as decribed below in the Bradford Assay.

II. Bradford Protein Assay

Duplicate standards were made in 10 ml plastic tubes (same as for Lowry assay) using a 1.0 mg/ml BSA stock solution. Standards containing 0, 5, 10, 20, and 30 μg per sample were made by adding 0, 5, 10, 20, and 30 μl of BSA stocks to the tubes, respectively. Then 5-20 μl of sample were added to duplicate tubes (diluting when necessary) followed by 1 ml Bradford reagent and vortex mixing. After 5 min at room temperature, sample absorbance was read at 595 nm.

Absorbance (y) vs. μg in standards (x), was plotted to determine the best linear fit (y=mx+b). The sample concentration was then computed using the following formula:

$$\frac{[(y-b)/m]*(\text{dilution of sample, if any})}{(\text{the volume of sample used in } \mu l)} = \text{protein concentration in } \mu g/\mu l \text{ or mg/ml}$$

D. Radioimmunoassay (RIA) for total MBP

I. ^{125}I Labelling of MBP

100 ml of a 1.0 M, pH 7.5 phophate buffer was prepared by mixing 18.6 ml of 1.0 M NaH_2PO_4 with 81.4 ml of 1.0 M Na_2HPO_4. This buffer was then used to make a solution containing 0.01 M HCl and 0.5 mg/ml calf thymus histones to equilibrate a PD10 Sephadex column (used to separate the labelled MBP from unreacted ^{125}I). In order to get the histones into solution, they were first dissolved in concentrated HCl (e.g. 50 mg histones into 87 μl HCl). Since both myelin basic protein and histones are highly basic proteins, the histones were used to block the column and prevent non-specific binding of MBP to the Sephadex beads which would decrease reaction yield.

A tube pre-coated with Iodo-gen (Pierce) was used for the reaction. 10 μl of 1 M phosphate buffer and 10 μl of a 1 mg/ml MBP stock solution were added carefully to the bottom of the tube. In an area approved for radioactive work behind appropriate shielding, 5 μl of ^{125}I supplied as sodium iodide in a sodium hydroxide solution (Amersham International plc, Little Chalfont, UK; typically 16.9 mCi/μg iodine) was added to the tube (~500 μCi). The reaction was incubated in the hood at

room temperature for 30 minutes, and then stopped by adding 0.5 ml phosphate buffer containing blue dextran (for colour) and 1mg/ml tyrosine (the protein is generally iodinated at tyrosine residues; therefore, tyrosine is a strong competitor). The tube contents were poured onto the Sephadex column, and the blue coloured solution was collected in 1 ml fractions as it exited the column. After the blue solution had entered the column, more phosphate buffer containing 0.01 M HCl and histones were be added to the top of the column. Counts per minute (CPM) of the collected fractions were determined by measuring 10 µl samples in a gamma-counter.

II. RIA

Duplicate RIA tubes (suitable for measurement in a gamma counter) were labelled for all standards and samples.

RIA Buffer:

0.05 M sodium phosphate buffer, pH 7 with
1.2% NaCl
0.5% CTMB
0.5 mg/ml histones (first dissolved in concentrated HCl)

Standards were prepared using a 1 mg/ml MBP stock solution which was diluted in RIA buffer to 1 ng/µl (2 µl stock into 98 µl buffer) and 0.05 ng/µl (50 µl of 1 ng/µl into 950 µl buffer). Standards were then prepared as follows:

Final MBP ng/ml Concentration	µl of 1 ng/µl stock	µl of 0.05 ng/µl stock	Buffer
100	30	---	270
50	15	---	285
20	---	120	180
10	---	60	240
5	---	30	270
2	---	12	288
1	---	6	294

Some trial and error of sample dilution was required to obtain a value within the linear portion of the standard curve, but 1/250 or 1/500 were generally appropriate with aggregate samples. For a 1/250 dilution, 2 µl of homogenised sample was added to 500 µl RIA buffer in a fresh Eppendorf tube. Samples were sonicated and vortex mixed immediately before diluting RIA buffer. The diluted samples were then vortex mixed, and 50 µl was added to each RIA tube. Tubes were kept on ice throughout these procedures. Tubes for non-specific binding (NSB) which measures binding in the absence of antibody and B_0 which measures maximal MBP binding in the absence of competing unlabeled MBP (i.e. sample) were also prepared by adding 100 and 50 µl of buffer to the NSB and B_0 tubes, respectively.

Anti-MBP antibody (a primary antibody raised in rabbit) was diluted 1:3000 in RIA buffer, and 50 µl was added to all tubes except NSB. Tubes were covered and incubated at 37 °C for 4 hours. An ^{125}I solution with 20,000 cpm per 50 µl was prepared (see section above), and 50 µl was added to each tube as well as to two 'total' tubes which were used to verify the total number of counts added to the samples. The tubes were incubated overnight at 4 °C. Then 100 µl of a suspension of cellulose beads coated with secondary antibody (anti-rabbit IgG, Sac-Cel, IDS Ltd., Boldon, UK) was added to all tubes except totals. Tubes with Sac-Cel were vortex mixed and allowed to incubate for 30 minutes before adding 1 ml of distilled water to all tubes (except totals) to stop the reaction. Samples were then centrifuged at 1000g for 5 minutes, and the supernatant was discarded. The cpm of each pellet was measured and compared to the standard curve to determine sample MBP concentration. The higher the amount of MBP present in the sample, the lower the cpm observed will be because there is more unlabelled MBP present to compete with the radiolabelled MBP.

E. Western Blotting

Western blotting was done using a modified version of procedure described by Laemmli, 1970.

I. Preparing samples

Samples for Western Blotting were either prepared fresh by homogenising in the solution below (adapted from Filliatreau et al., 1988), or taken from previously homogenised samples prepared for RIA.

Homogenisation solution (100 ml final volume):

Final Conc.	Material	amount	Notes
0.1 M	Pipes	3.02 g	
5 mM	MgCl2	1 ml of 0.5 M stock	
0.1 %	Triton X-100	100 µl	can vary
20%	glycerol	20 ml	
5 mM	EGTA	3 ml of 0.167 M	must add base to dissolve stock
	dH_2O	75.9 ml	

Addition of NaOH was required for solution to dissolve (final pH 6.9). This solution was stored at 4 °C with remaining ingredients added just before use: 100 µl of 0.1 M PMSF stock in isopropanol (added in fumehood) and 500 µl of protease inhibitor cocktail (see below) per 10 ml of homogenisation solution required.

20x Protease Inhibitor cocktail (10 ml):

Final Conc.	Substance	Stock Conc.	Amount
20 µg/ml	leupeptin	1 mg/ml	200 µl
20 µg/ml	pepstatin A	0.25 mg/ml	800 µl
200 µg/ml	benzoyl-L-arginine methyl ester (BAME)	100 mg/ml	20 µl
200 µg/ml	p-tosyl-L-arginine methyl ester (TAME)	10 mg/ml	200 µl
200 µg/ml	trypsin inhibitor	10 mg/ml	200 µl
200 µg/ml	TPCK	3.33 mg/ml	600 µl
200 µg/ml	aprotinin	10 mg/ml	200 µl
	autoclaved dH_2O	7.78 ml	

The protease cocktail solution was stored in frozen aliquots to prevent inactivation

by repeated freeze/thaw cycles. To homogenise by sonication, approximately 5X the sample volume was required.

III. SDS-PAGE with mini gels

A. Pre-cast gels

Pre-cast 15% acrylamide, Tris-HCl gels were obtained from Sigma (St. Louis, MO, USA) or Bio-Rad (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) to fit the Bio-Rad Protean II mini gel box. Gels were prepared according to package instructions by rinsing with water, removing tape over the bottom strip of the resolving gel, and rinsing the wells with deionised water.

B. Hand cast gels

Glass plates were cleaned with 100% EtOH and assembled onto holders with the short plate on the outside and 0.75 mm spacers between plates. After holders were snapped onto gel pouring stand, the system was tested with water for leaks. A mark on the outer glass plate 1 cm below the end of the teeth on the comb was made to mark the level for the top of the resolving gel.

The desired percentage resolving gel was made up in a disposable tube, swirling after adding each ingredient. The APS (free radical source) and TEMED (radical initiator) were added last and immediately before pouring. Recipes sufficient for 2 mini-gels are as follows:

15% Gel:

5.06 ml of 30% acrylamide (37.5:1 ratio acrylamide to bis-acrylamide; National Diagnostics, Hull, UK)

3.75 ml of 1 M Tris pH 8.8 (must be made from Tris base and pH'd)

1.09 ml of dH_2O

100 µl of 10% w/v SDS

100 µl of APS (10% w/v aliquots prepared fresh or from frozen aliquot)

10 µl of TEMED

12.5% Gel Recipe:

4.22 ml of 30% acrylamide (37.5:1 ratio acrylamide to bis-acrylamide)

3.75 ml of 1 M Tris pH 8.8 (must be made from Tris base and pH'd)

1.93 ml of dH_2O

100 µl of 10% w/v SDS

100 µl of APS

10 µl of TEMED

A glass pasteur pipette was used to pour the gel, stopping at the mark made for the resolving gel. A layer of butanol was immediately placed on top to prevent oxygen from inhibiting polymerization, and the gel was allowed to set for 30 minutes.

5% stacking gel:

650 µl of 30% acrylamide (37.5:1 ratio acrylamide to bis-acrylamide)

625 µl of 1 M Tris pH 6.8 (must be made up from Tris base)

3.28 ml of dH_2O

50 µl of 10% w/v SDS

37.5 µl of APS

5 µl of TEMED

Combs were cleaned with 100% ethanol and dried just before use. Once the resolving gel had polymerised, butanol was poured off, and the resolving gel was rinsed with tap water and dried using a paper towel as a wick. A pasteur pipette was used to layer on the stacking gel (recipe above). The comb was then gently slid in place, being careful not to trap any air bubbles. This gel was allowed to set for 15 minutes.

C. Sample Preparation

An aliquot of sample in homogenisation solution was prepared so that the desired amount of protein (usually 10-20 µg) would be contained in a volume in the range of 5-10 µl which was the same for all samples. The aliquot was then diluted in an equal volume of 2x sample buffer to obtain a final loading volume of 10-20 µl.

2X Sample Buffer:

Final Conc.	Material	for 20ml
2%	β-mercaptoethanol	400 µl
0.25 M	1M Tris, pH 6.8	5 ml
0.01%	bromophenol blue	a few crystals
10 mM	1M DTT	200 µl
2%	SDS	0.4 g
	water	12.4 ml
10%	glycerol	2 ml

Samples were boiled for 5 minutes (a hole in the top of each sample tube was made with a fine needle to relieve pressure), vortex mixed, and centrifuged for 1 minute at high speed. Left over samples were frozen and saved for reuse.

Gels were loaded into the gel box, and the central compartment was filled with 1X running buffer (see below). Once it was certain that the central compartment was not leaking, buffer was added to the outer compartment. Wells were rinsed with 1X running buffer using a Hamilton syringe to remove any residual water or unpolymerized material before loading the samples.

10X Running Buffer (1 liter, pH not adjusted):

0.25 M Tris base	30.3 g
1.92 M Glycine	144 g

1X Running Buffer (1 liter):

10x stock	100 ml
0.1% SDS	10 ml of 10% stock

Gels were loaded using a Hamilton syringe which was rinsed between samples with running buffer. A set of standards was loaded on either side of the samples in case the lanes ran unevenly. Following each use, the syringe was washed with water and and methanol. Gel was run at 200 V until the dye front reached the bottom of the gel. When prestained standards (Color Markers Low Range, Sigma,

St. Louis, MO, USA) were used, the gel was run until the bottom standard (6.5 kDa) had just left the bottom of the gel. This achieved better separation of the MBP isoforms which range in size from 14 to 21.5 kDa.

IV. Protein Transfer

A. Buffer

The buffer was made up in advance and placed in the freezer or refridgerator to cool. The higher the percentage gel, the more difficult protein transfer is; therefore, the lowest percentage gel which achieved the sufficient separation of the MBP isoforms was used. Basic proteins such as myelin basic protein are more easily transferred in a high pH buffer such as the sodium carbonate one listed below.

Transfer Buffer, pH ~8.3 (for normal use):

Final Conc.	Material	for 1L	for 2 L
25 mM	Tris	3.03g	6.06g
192 mM	Glycine	14.4g	28.8g
10%	Methanol	100 ml	200 ml
0.01%	10% SDS	1 ml	2 ml
	dH_2O	900 ml	1.8 L

Sodium Carbonate Transfer Buffer (Novex, San Diego, CA, USA)
pH ~9.9 (for basic protein transfer)

Final Conc.	Material	for 1L
3 mM	Na_2CO_3	0.318g
10 mM	$NaHCO_3$	0.840 g
	dH_2O	1L

B. Transfer set up

Gel was removed from the apparatus, and the plates were carefully pried apart with a spatula leaving the gel on one plate. The stacking gel was cut away and the top left hand corner was removed to mark gel orientation. In the case of precast

gels, the thick polymer strip at the bottom of the gel was also removed. Gel was placed in a tray of transfer buffer and allowed to equilibrate for 20 minutes.

A piece of PVDF (polyvinylidene fluoride) membrane (Immobilon-P, Millipore, Bedford, MA, USA) was cut to 8x10 cm, and two sheets of Whatman 3MM paper were cut to the exact size of the fiber pads supplied with the Bio-Rad Mini Trans-Blot apparatus (Bio-Rad Laboratories, Hemel Hempstead, UK). The membrane was activated by placing it in methanol for 30 seconds and then rinsing in distilled water for 5 minutes. It was then placed in transfer buffer for 20 min to equilibrate. Fiber pads and the sheets of 3MM paper were soaked in transfer buffer also.

Protein transfer was done according to manufacturer's instructions by placing a fiber pad on the black half of an open cassette, then a sheet of 3MM paper, and then the gel. Any trapped bubbles were removed with a gloved finger. The PVDF sheet was then laid on top of the gel and cut to the exact size of the membrane using a fresh scalpel blade once bubbles had been smoothed out. A further sheet of 3MM paper and fiber pad were placed on top. The cassette was assembled and placed in the gel box with the black side at the back (the proteins travel towards the positive electrode and get trapped on the sheet of PVDF). An ice pack and stirrer bar were added to prevent overheating during the 70 minute transfer at 60 V.

C. Verifying Transfer

Blots were dried immediately on 3MM Whatmann paper after removal from the transfer apparatus. Blots were either left overnight between sheets of Whatmann paper wrapped in saran wrap at 4 ^{o}C or immediately used for immunoblotting. Gels were either discarded or stained with Coomassie blue to verify transfer.

Coomassie Stain:

2.5 g	Coomassie brilliant blue
450 ml	methanol
450 ml	dH2O
100 ml	glacial acetic acid

Destain Solution:

100 ml	methanol
800 ml	water
100 ml	glacial acetic acid

The membrane was also occaisionally stained (reversibly as it is easily removed water or TTBS) with Ponceau S to visualize bands and verify transfer.

Ponceau S 10x Stock:

2g	Ponceau S
30g	trichloroacetic acid
30g	sulfonic acid
	water to 100 ml

V. Immunoblotting

For immunoblotting, standard Tris buffered saline (TBS) and Tris buffered saline with Tween 20 (TTBS) solutions were used.

10X TBS (1 L, pH 7.4):

Tris/HCl	12.1g
NaCl	87.7g

TTBS was made by adding 500 µl of tween 20 to 1 liter of 1X TBS. Blocking solution was made with low grade bovine serum albumin (BSA) at 3% w/v, dried milk at 5% w/v, or casein at 3% w/v.

Block:

100 ml	TTBS
3g	BSA

Blot was washed in TTBS for 10 min, then incubated in blocking solution with shaking for at least 30 minutes at room temperature. The blot was then briefly washed in TTBS before adding the primary antibody diluted in block. Antibody

was left on for 1-2 hours. Primary antibody preparations containing a small amount of sodium azide were stored at 4 °C and reused. The blot was washed three times in TTBS for 10 minutes each. Then the appropriate dilution (in block) of horse radish peroxidase linked (HRPL) secondary antibody (anti-rabbit IgG, Amersham Life Science Ltd, UK) was added and left to incubate for at least one hour. Secondary antibody was discarded after use since azide addition would inhibit the enzyme.

The blot was washed again using three 10 min changes of TTBS. During final wash, 8 ml of chemiluminescent substrate solution was placed in a square petri dish (either ECL reagent [Amersham Life Science Ltd, UK] or solution from the HRPL Luminescent Visualization Kit [National Diagnostics, Hull, UK]). Excess TTBS was drained from the blot by touching edges to a piece of 3 MM paper before incubating it for 1 min in the chemiluminescent substrate solution. The blot was briefly dried between two pieces of 3 MM paper, sealed in a hybridization bag or cling film, and taped into a x-ray cassette with the protein side face up. Exposure time usually ranged from 1-2 minutes.

F. Rat Brain Aggregate Cell Culture

I. Culture Set Up and Maintenance

Rat brain aggregate cell cultures were set up according to the techniques established by Honegger (Honegger, 1985) and also Jane Loughlin, who established the technique in the MS Lab. Animals were ordered on a Friday, three weeks prior to experimental day in vitro zero (DIV 0). The mating day was the following Tuesday, and the pups were dissected on embryonic day 16 (E16) which fell on the second Thursday thereafter. Each female typically had 10-14 pups. Females were sacrificed according to approved animal protocol and swabbed with ethanol. A lower abdominal incision was made to remove the string of pups. Pups were collected in a sterilised beaker containing 500 ml of D1 medium (made especially for our lab by Gibco, catalogue number 041-90999M, Life Technologies Ltd, Paisley, UK) with 1:100 dilution of penicillin-streptomycin (Gibco 15140-114 at 10,000 IU/ml,Life Technologies Ltd, Paisley, UK). The beaker was kept covered at all times with the lid of a sterile petri dish. The remaining procedure was done in a laminar flow

hood.

Autoclaved scissors, scalpel blades, fine forceps, and 200 and 115 μm sieves were prepared in advance. Dissecting platforms were also prepared by inverting metal dishes on baked glass trays filled with ice. On top of the platform in a shallow dish of D1 medium, each foetus was gently disengaged from its sac and collected in a clean dish of D1 medium. The brain was removed from surrounding tissue and collected in a dish with fresh medium. The hindbrain was cut away, and all frontal lobe pieces (telencephalon) were collected in a 50 ml centrifuge tube filled with D1 medium and kept on ice. Once all forebrains were collected and had settled to the bottom of the tube, the supernatant was poured off. The brains were washed twice in D1 medium and resuspended in 10 ml D1 medium after the second wash. The 200 μm sieve was placed into a fresh 50 ml centrifuge tube and wet with 10 ml of D1 medium. The forebrains were then poured in and forced through the sieve using a sterile plastic pipette. This was repeated with the 115 μm sieve. The cell suspension was then centrifuged at 300g at 4 ^{o}C for 15 minutes. The supernatant was aspirated and the pellet resuspended in 5 ml of D1 medium. The volume was made up to 50 ml, and the cells were counted using trypan blue and a haemocytometer as described below in section II. Cells were recentrifuged and resuspended in an appropriate volume of serum free complete DMEM (modified Dulbecco's Modified Eagle's Medium, Gibco catalogue number 41965, Life Technologies Ltd, Paisley, UK) so that $4x10^7$ cells per flask could be seeded. Each 25 ml De Long flask had a final volume of 4 ml of media.

Cells were kept at 37 ^{o}C in a humidified atmosphere under constant rotation with an atmosphere of 10% carbon dioxide and 90% air. The rotation speed was steadily increased as the cultures developed as follows:

DIV	revolutions per minute
0	68
0 (late afternoon)	72
1	74
2	76
5	78
6	80

On day 2, the cultures were transferred to 50 ml De Long flasks, and an additional 4 ml of prewarmed serum free complete DMEM was added to make the final volume up to 8 ml per flask. Cultures were split into two 50 ml De Long flasks at DIV 21, and each flask contained 8 ml of media for the remainder of the culture period.

The cultures were fed on days 5, 8, 11, 14, and every other day from then onwards. Medium was warmed to 37°C before adding. In order to remove old medium without losing the aggregates, flasks were removed from the incubator four at a time and placed in a tray at a slant to allow the aggregates to settle to one side. The cap was placed in a sterile dish containing 70% ethanol while medium was changed and air dried before replacing it. Medium was changed by removing and replacing 5 of the total 8 ml with fresh.

II. Macrophage Enrichment

Macrophages were obtained by peritoneal lavage of 10-12 week old male rats. Ice buckets, large forceps, and sharp scissors were required as well as the following for each animal:

50 ml syringe

20 ml syringe

19 gauge needle

50 ml conical centrifuge tube

Animals were sacrificed according to approved animal protocol, swabbed with 70% ethanol, and a midline abdominal incision was made to expose the abdomen. 50 ml

of chilled 1X EBSS (Earle's Balanced Salt Solution, without calcium, without magnesium and without phenol red; Gibco, Life Technologies Ltd, Paisley, UK) was injected into the peritoneal cavity. The abdominal wall was then lifted to make a small incision. A 20 ml syringe was inserted to collect the EBSS in a sterile centrifuge tube. It was possible to collect at least 40 ml per animal.

The collected EBSS was then centrifuged for 10 minutes at 300g at 4 °C to pellet the macrophages. The supernatant was discarded, and the pellet resuspended in 5 ml fresh EBSS. The final volume was then made up to 50 ml. The cells were again centrifuged and resuspended in 5 ml EBSS, and each preparation was assessed for bacterial contamination under the microscope. All clear preparations were pooled and resuspended in 30 ml to do a cell count using trypan blue and haemocytometer (Weber, B.S. 748, depth 0.1 mm, 1/400 mm^2). To do this, 10 µl of cell suspension was mixed with 90 µl of 0.1% Trypan blue in sterile PBS and bled onto a haemocytometer. Cells which did not take up the dye (living) were counted. The total number of living cells using our haemocytometer was determined as follows:

count of cells in 12 grids * 10^4 * dilution factor (10) * resuspension volume (30 ml)

10% macrophages ($4x10^6$ cells) were added to each culture flask which contained $4x10^7$ telencephalon derived cells. Macrophages were resuspended in serum free complete media before adding.

III. Demyelination

Demyelination was induced by adding cytokines or anti-myelin antibodies plus complement at varied time points during culture development. However, treatments were generally added between days 25 and 34 when cultures were fully myelinated. A 200 U/ml concentration of interleukin-1α (IL-1α, Genzyme, Cambridge, MA, USA) with 0.1% BSA (which acts as a stabiliser) in the culture flasks induced demyelination following two treatments which were 48 hours apart. Control cultures were treated with 0.1% BSA only. Cultures were also demyelinated

by adding 31.3 μg/ml α-MOG (anti-myelin oligodendrocyte glycoprotein) antibody derived from clone 8-18C5 (Linington et al., 1984) and 25 μl/ml guinea pig serum (GPS; Serotec, Oxford, UK) as a source of complement for 48 hr. Control cultures received a subclass-specific IgG_1 antibody (Sigma, St. Louis, MO, USA) plus GPS. All solutions were filter sterilised before addition to the cultures.

IV. Harvesting

Aggregates were evenly resuspended before removing samples. Samples were collected on ice in sterile, 10 ml centrifuge tubes. Once collected aggregates had settled, the supernatant was aspirated, and the pellet was rinsed twice in either chilled PBS or DEPC treated PBS (for samples to be used in RNA analysis). Then, in a small volume of PBS, aggregates were transferred to cryotubes or Eppendorf tubes. Supernatant was removed and samples were either frozen in dry ice or snap frozen in liquid nitrogen if they were to be used for RNA analysis. All samples were left on dry ice for at least half an hour before transferring to the -80 °C freezer for storage. Samples for biochemical analysis were homogenised in 500 μl of 2 mM potassium phosphate with 1 mM EDTA (see recipe below) by sonication and stored at -80 °C until use. The homogenisation buffer was prepared from a 1:1 mixture of the following two solutions:

2 mM $K_2HPO_4 \cdot 3H_2O$, 1 mM Na_2EDTA

2 mM KH_2PO_4, 1 mM Na_2EDTA

G. Myelin Isolation

Myelin was isolated from rat brain by mechanical homogenisation followed by separation in sucrose gradients. For example, approximately 14 g of tissue was homogenised in 0.32 M sucrose in TBS. The solution was brought up to 120 ml with the same solution. 20 ml of 0.8 M sucrose in TBS was then placed in the bottom of each of 8 centrifuge tubes, and 15 ml of homogenate was carefully layered on top of each one. These were centrifuged for 40 minutes at 20,000 rpm at 6 °C.

The middle (white) layer was then collected in a fresh tube and recentrifuged for 10 minutes at 20,000 rpm to pellet material. The supernatant was discarded and the pellet washed with 1X TBS, recentrifuged, and resuspended in a solution appropriate for the intended application. All steps were done on ice, and the yield was approximately 28 mg of myelin per gram of wet brain weight.

Chapter III. Establishing Methods

A. Northern Blot Analysis of Myelin Basic Protein Transcripts

Techniques for northern blot analysis were already established in the lab, (for detailed methods see Chapter II, section A). Probe choice, however, was difficult because the rat MBP exon-2 sequence was not available. Mouse and human exon-2 MBP had been sequenced; therefore, a region of exon-2 identical in mouse and human was chosen in the hope that it would be conserved in rat also (see Figure 4 below). A sequence from exon-1, which is present in all known MBP isoforms and has been sequenced in the rat (Schaich et al., 1986), was chosen to measure total MBP mRNA.

	1	2	3	4	5	6	7	8	9
HUMAN	Val	Pro	Trp	Leu	Lys	Pro	Gly	Arg	Ser
	GTA	CCC	TGG	CTA	AAG	CCG	GGC	CGG	AGC
	- - -	- - -	- - -	- - -	- - -	- A-	A - -	- - -	- - -
MOUSE	Val	Pro	Trp	Leu	Lys	Glu	Ser	Arg	Ser

	10	11	12	13	14	15	16	17	18
HUMAN	Pro	Leu	Pro	Ser	His	Ala	Arg	Ser	Gln
	CCT	CTG	CCC	TCT	CAT	GCC	CGC	AGC	CAG
	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- GT
MOUSE	Pro	Leu	Pro	Ser	His	Ala	Arg	Ser	Arg

	19	20	21	22	23	24	25	26
HUMAN	Pro	Gly	Leu	Cys	Asn	Met	Tyr	Lys
	CCT	GGG	CTG	TGC	AAC	ATG	TAC	AAG
	- - -	- - -	- - -	- - -	C- -	- - -	- - -	- - -
MOUSE	Pro	Gly	Leu	Cys	His	Met	Tyr	Lys

Figure 4. A comparison of mouse and human MBP exon-2 sequences (Reproduced from Kamholz et al., 1986). The identical region which was chosen for the probe is underlined.

Initially, four 30 base-pair oligonucleotide probes were ordered (Phil Marsh, Randall Institute, King's College, London) for MBP exon-1 and exon-2. One of each was anti-sense which should bind to the mRNA of interest and one sense which

would serve as a negative control. The sequences of the probes were as follows ('A' denotes the anti-sense probe):

MBP1:
5' GCC CGG CAT GGC TTC CTC CCA AGG CAC AGA 3'

MBP1A:
5' TCT GTG CCT TGG GAG GAA GCC ATG CCG GGC 3'

MBP2:
5' CGG AGC CCT CTG CCC TCT CAT GCC CGC AGC 3'

MBP2A:
5' GCT GCG GGC ATG AGA GGG CAG AGG GCT CCG 3'

All probes were DIG labelled using a 3' Tailing Kit from Boehringer Mannheim (Mannheim, Germany) according to their protocol. Labeling was quantified using DIG quantification teststrips and a labelled control probe, both from Boehringer Mannheim. All probes were initially diluted to 2.0 pmol of labelled probe per ml hybridisation solution and tested on adult and foetal rat brain RNA samples. An initial hybridisation temperature of 55 ^{o}C was chosen using estimation methods given by Boehringer Mannheim. The most stringent washing step involved two rinses with 0.1X SSC and 0.1% SDS at 68 ^{o}C for 15 minutes, which was standard procedure with other probes used in the lab. Unfortunately, under these conditions, no bands were visible with either the sense or anti-sense probes even after exposures as long as 90 minutes. It was decided that the washing procedure may have been too harsh, especially for the MBP2A probe which could potentially contain some mismatches to the rat mRNA. Instead of using 0.1X SSC for the washes at 68 ^{o}C, 2X SSC with 0.1% SDS was used for all washes. With this method, a band of the appropriate size was seen in the adult rat lane with the MBP1A

probe only. A band was not expected in the foetal rat brain sample because significant myelination does not occur until after birth. Northern blots were also run with extra rat brain aggregate cultures to verify that the conditions would work with that system (see Figure 5 below). Conditions were considered acceptably optimised for the MBP1A probe, but bands were still not visible with the MBP2A probe. A higher probe concentration of 10 pmol/ml hybridisation solution, lower hybridisation temperatures (50 and 40 ^{o}C) which should allow probe to bind even when mismatches are present, larger quanties of RNA loaded onto the gel (20 μg per lane instead of 10), longer exposures to x-ray film ranging from hours to days, and combinations of all of these were tried. Unfortunately, in all cases, either no signal with the MBP2A probe was observed or large amounts of background were present. The probe was also tested on mouse tissue to make sure there was not a problem in how it was synthesised or labeled. Bands of appropriate size were seen in mouse samples using probe at a concentration of 2 pmol/ml and the same conditions that were successful with the MBP1A probe on rat tissue.

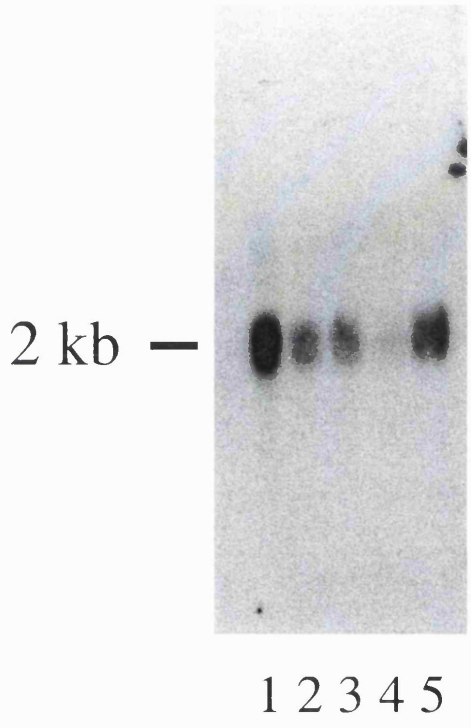

Figure 5. Northern blot using MBP1A oligonucleotide probe. Each lane contained 20 μg of RNA from adult rat (lane 1) and rat brain aggregate cell cultures (lanes 2-5). No bands were observed with MBP2A probe even with very long exposures.

It was decided that it would be more fruitful to test a different probe rather than continue to try to find suitable conditions for the MBP2A probe. Dr. Hans de Vries at the Laboratory of Physiological Chemistry, University of Groningen, the Netherlands published a paper demonstrating the successful use of a 78 nucleotide anti-MBP exon-2 probe to localize exon-2 containing mRNAs in cultured rat oligodendrocytes by *in situ* hybridisation (de Vries et al., 1997). Because the rat sequence was not available, they used the full 78 nucleotide sequence for mouse MBP exon-2. Dr. de Vries was kind enough to send an aliquot of the plasmid containing this anti-sense sequence for amplification and use on Northern blots. In order to do this, Epicurian Coli XL-1 blue supercompetent cells (Stratagene, Stratagene Europe, Amsterdam) were transformed and glycerol stocks were made by Lara Diemel according to standard protocol. Stocks were grown up overnight in LB medium at 37 °C while rotating at 150-200 rpm, and plasmid DNA was isolated using a Qiagen mini-prep (Qiagen, Crawley, UK) kit according to their protocol. The plasmids were then digested with HindIII and EcoRI restriction enzymes at 37 °C for 4 hours to free the exon-2 insert. Products were stored at 4 °C until run on a low molecular weight 1x TAE (40 mM Tris-acetate, 1mM EDTA), 1% agarose gel to separate the exon-2 segment from the plasmid. The appropriate band (78 nucleotides) was cut out, placed in a sterile Eppendorf tube, and heated to 70 °C to melt the agarose. The tube was then transferred to 45 °C and incubated with AgarACE (Promega, Southampton, UK) enzyme at one unit per 200 mg agarose for 1.5 hours to digest the agarose. Sodium acetate (3 M, pH 5.2) in an amount 0.1 times the reaction volume was added to stop the reaction. Then 2 times the reaction volume of 100% ethanol was added to precipitate the DNA at -20 °C for at least 2 hours. Samples were then spun down at high speed for 15 minutes and resuspended in water. The amount and quality of the DNA fragment obtained were assessed by running on a 1X TBE (90 mM Tris-borate, 2 mM EDTA), 1% agarose gel. Yield was low. The procedure was repeated except this time isolating larger amounts of plasmid using a Qiagen maxi prep kit (Qiagen, Crawley, UK). Enough exon-2 insert (see Figure 6B below) was obtained to label with digoxigenin (Random

Primed DNA Labeling Kit, Boehringer Mannheim, Mannheim, Germany) according to their protocol. The probe was tested on Northern blots containing developing rat brain of various ages as well as mouse brain. A probe concentration of 20 ng per ml hybridisation solution, 50 °C hybridisation temperature, and washes with 0.1X SSC wtih 0.1% SDS at 68 °C were used initially since that was standard protocol with other cDNA probes in the lab. Unfortunately, this resulted in high background, and no specific bands were seen, even in the mouse lanes which should have matched the sequence exactly. It was later determined that de Vries et al. used the probe by simply labelling the linearized plasmid rather than isolating the insert. In any case, after doing the amplification and purification steps multiple times, it was found to be more time and cost effective to have the probe for the 78 nucleotide exon synthesised rather than amplifying it in a plasmid.

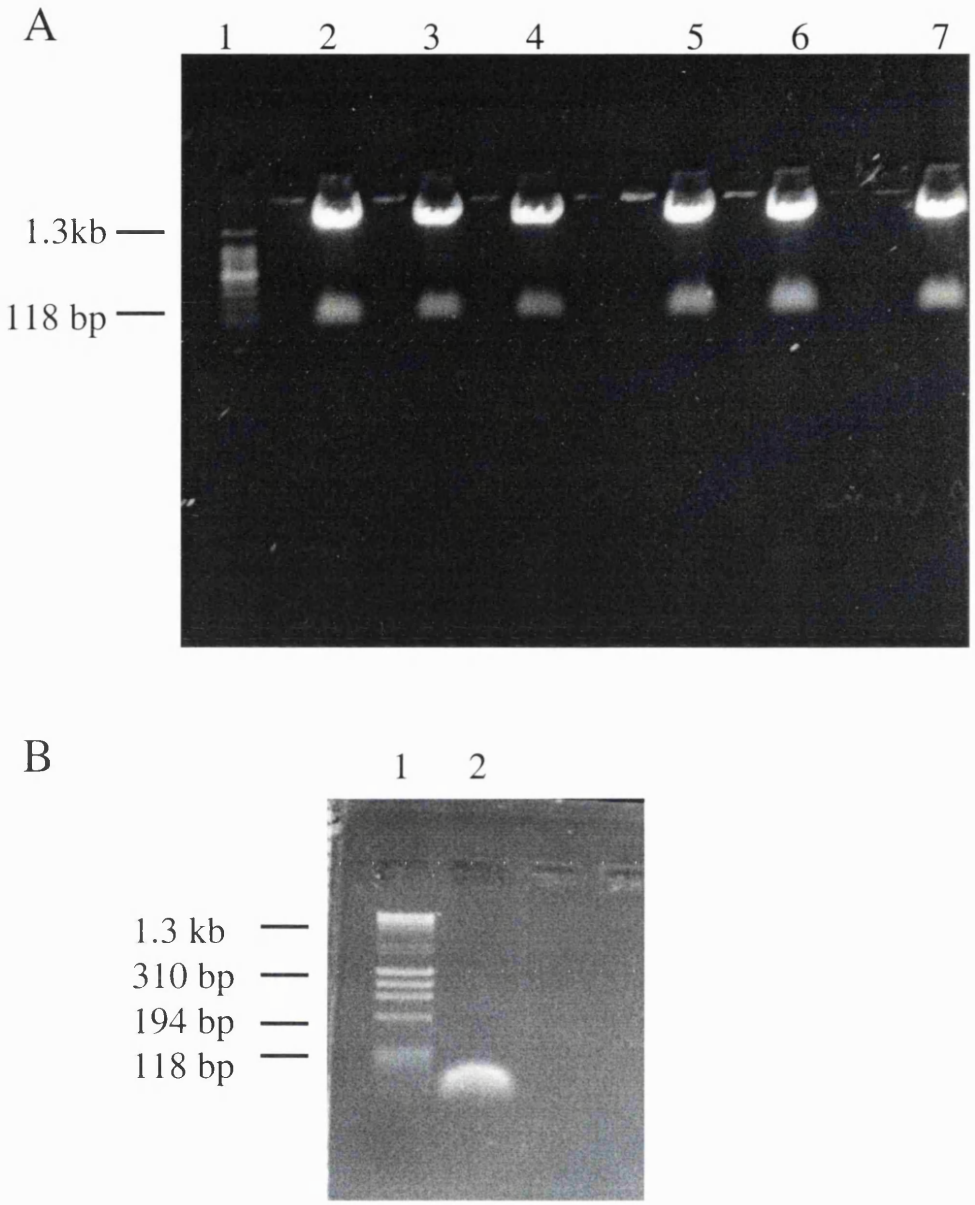

Figure 6. **(A)** A 1x TAE, 1% agarose gel demonstrating separation of the exon-2 MBP insert (lower band, 78 bp) from the plasmid (upper band) in lanes 2-7. Lane 1 contains a DNA ladder. **(B)** The exon-2 insert isolated from the previous gel is in lane 2 and a standard is shown in lane 1.

An abstract (Woodruff and Franklin, 1997) described the use of an anti-MBP exon-2 probe for *in situ* hybridisation on rat spinal cord tissue. Upon consultation we found out that this was the same 48 nucleotide probe previously used in mouse *in situ* hybridisation studies (Jordan et al., 1989). Rather than having an oligonucleotide synthesized for the entire exon-2 sequence, we decided to attempt using this one first. The sequence of the anti-sense probe is as follows:

5' TT GTA CAT GTG GCA CAG CCC AGG ACG GCT GCG GGC ATG AGA GGG CAG A 3'

This probe was tested initially on developing rat and mouse brain samples using the conditions optimized for the MBP1A oligonucleotide probe discussed above. A specific signal was seen using these conditions (as shown in Figure 7 below); therefore, they were not further optimised.

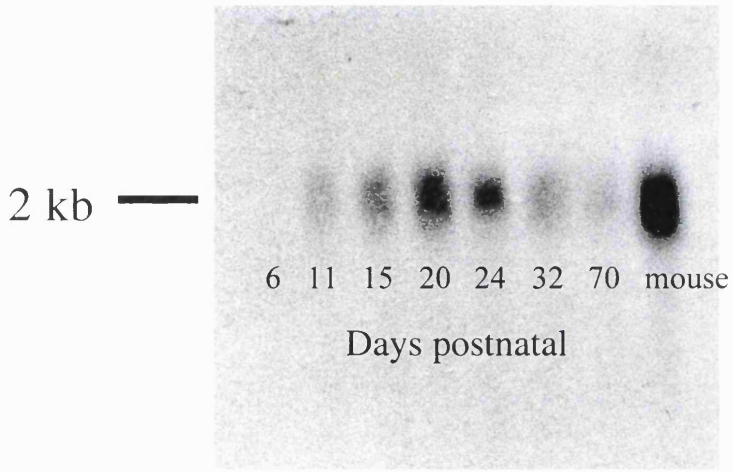

Figure 7. Northern blot results with the 48-mer anti-exon-2 MBP probe. From left to right, lanes contain 20 μg of RNA from rat brain samples from postnatal days 6, 11, 15, 20, 24, 32 and 70 and 24 day mouse brain. The mouse band is much darker because the probe sequence matches the mouse mRNA exactly.

An additional complication was that the mRNAs for the different MBP isoforms all localise to the same band on Northern blots. There is a 47 nucleotide 5' untranslated region and a 1486 nucleotide 3' untranslated region in addition to the poly(A) tail on all MBP mRNAs. Since the open reading frames for the major rat MBPs are only 128, 154, 169, and 195 respectively for the 14, 17, 18.5, and 21.5 kDa isoforms (Sutcliffe, 1987), all the MBP mRNAs are approximately 2 kilobases long. Because of this, the fraction of the total MBP mRNA which contained exon-2 could not be determined, only a comparison of the relative patterns of expression of total and exon-2 containing MBP mRNA could be made. This was done on the same blot once it had been stripped and reprobed. However, to be certain no residual probe remained, hybridisation with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (also DIG labelled) was done in between the MBP exon-1 and exon-2 hybridisations. The GAPDH mRNA band does not overlap with the MBP band, and levels could also be used to correct for any unequal loading of the gels.

B. Western Blot Analysis of MBP Isoforms

Standard SDS-PAGE (sodium lauryl sulfate--polyacrylamide gel electrophoresis, Laemmli, 1970) and immunoblotting techniques were learned from Paul Fernyhough at the Department of Pharmacology, St. Bartholomew's and Royal London Hospital School of Medicine and Dentistry, Queen Mary and Westfield College, University of London. A polyclonal anti-MBP antibody raised in rabbit (same as used in the MBP radioimmunoassay) and a donkey anti-rabbit IgG (Amersham Life Science Ltd., UK) were used for immunoblotting. All SDS-PAGE was performed using a Bio-Rad Mini-Gel system with 0.75 mm thick gels, and gels were transferred using a Bio-Rad Mini Trans-Blot system. Isolated rat myelin and purified MBP were used on some blots in addition to homogenised rat brain and rat brain aggregates to verify that the bands identified were MBP. For details of the final procedure used in all studies see chapter II, section E.

The MBP isoforms are relatively low molecular weight proteins (14 to 21.5 kDa) and are close in size; therefore, it was difficult to find conditions which would separate them into bands distinct enough to be analysed by densitometry. A homogenisation buffer recommended by Paul Fernyhough (adapted from Filliatreau

et al., 1988) with 0.5% Triton X-100 was initially tried, but bands in the region of interest were smeared, especially at the sides (see Figure 8, below). On the recommendation of Lara Diemel, the detergent concentration was dropped to 0.1%, and the bands became sharper. Lower concentrations of antibodies were also investigated after seeing the initial results. These initial blots were done using a semi-dry electro-blotter at Queen Mary and Westfield College which worked much more efficiently and effectively than the Bio-Rad Mini-Trans blot assembly available in our lab. Considerable additional optimisation was needed to get good protein transfer with our system as discussed below.

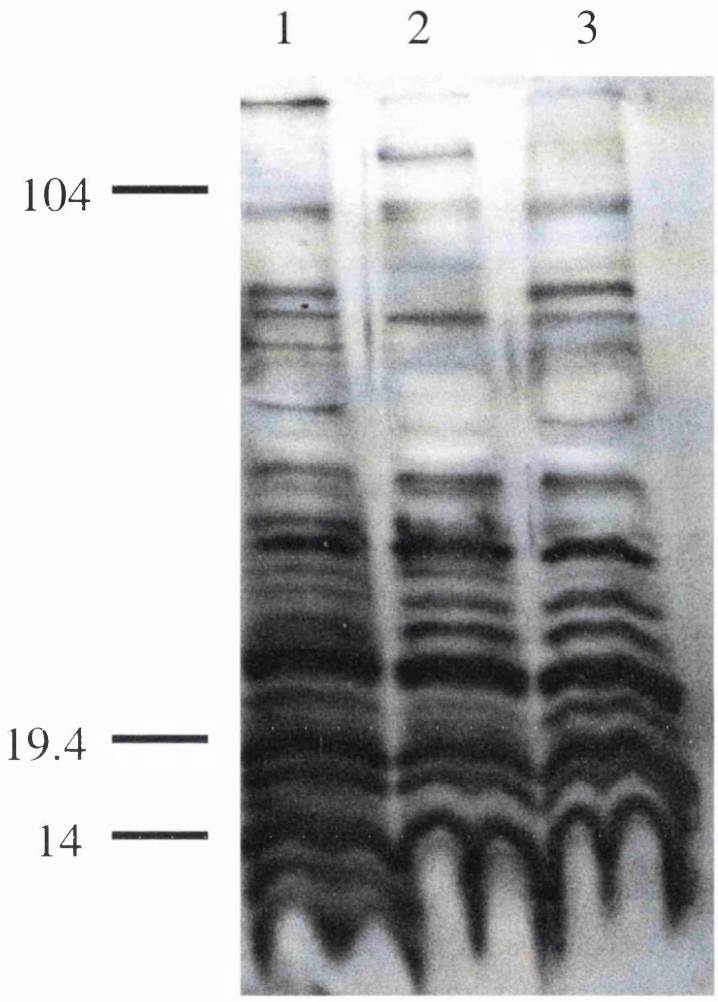

Figure 8. First attempt at Western blot showing smearing of the bands of interest. Lanes from left to right are adult rat brain(1), mouse brain (2) and 24 day old rat brain (3). Molecular weight markers in kilodaltons are indicated at left . Antibodies were diluted 1:500.

Hand cast gels containing 12.5, 13, 14, and 15% acrylamide were investigated. It is more difficult to transfer proteins from higher acrylamide percentage gels to membranes for immunoblotting, so we wanted to choose the lowest percentage gel that would separate the bands of interest. Unfortunately, none of the gels were able to achieve sufficient separation between the 17 and 18.5 kDa MBPs when the dye front was run to the bottom of the gel as is standard procedure. Pre-stained molecular weight standards were then obtained (low range colour markers, Sigma, St. Louis, MO, USA) so that the dye front could be run off the gel while making sure that proteins in the size range of MBP were not lost (14-21.5 kDa). Good separation was still not achieved with lower percentage gels, and the transfer with higher percentage gels was so poor that the bands were difficult to discern. A standard transfer buffer was being used (25 mM Tris, 192 mM glycine, 20% methanol, and 0.01% SDS) at 85 V for 1 hour as recommended by Bio-Rad. Transfer conditions voltage and transfer time were varied initially (e.g. 20 V overnight or 100 V for 1 hour), but transfer remained poor. Membranes were also placed in series next to the gel to verify that proteins were not passing through the membrane. Finally, the buffer was varied until an acceptable result was achieved according to the following the advice of Novex (San Diego, CA, USA) for optimising Western procedures:

1) 0.01-0.02% SDS can be added to increase protein movement out of the gel; however, it may decrease binding to the membrane. SDS imparts a uniform negative charge and keeps the protein in a soluble, unfolded state. If no SDS is added to the buffer, it will gradually dissociate from the proteins during transfer.

2) 10-20% methanol can be included in the transfer buffer to increase binding to the membrane, but it can decrease the amount of protein removed from the gel because it acts by stripping the proteins of SDS. Methanol should always be included in the transfer buffer if nitrocellulose is used as the membrane.

3)Basic proteins which have a positive charge can be more easily transferred if a buffer with a higher pH is used.

The buffer that was found to work best was a pH 9.9 sodium carbonate buffer (3 mM sodium carbonate, 10 mM sodium bicarbonate) recommended by Novex for transferring highly basic proteins such as histones. Fortunately, a polyvinylidene (PVDF) membrane (Immobilon P, Millipore) instead of nitrocellulose was already being used, so methanol was not needed for protein binding. Transfer conditions of 1 hour at 60 V were found to work best with this buffer. In order to achieve separation of the 17 and 18.5 kDa bands, the gel was run approximately 10 minutes at 200 V after the dye front left the gel at which time the bottom coloured standard (aprotinin at 6.5 kDa or 10.5 kDa including weight of bound dye) was about to run off the bottom of the gel.

Antibody dilutions in blocking solution were varied from 1:1000 to 1:10,000, and the following conditions were found to be optimal:

	1° Antibody	2° Antibody
Rat Brain Aggregates	1:2000	1:2000
Rat Brain	1:5000	1:5000

Concentrations as low as 1:1000 for primary and secondary antibodies were needed for samples early in development when MBP concentrations were very low.

Finally, while 15% acrylamide gels could achieve good separation of bands, it was found that the hand cast gels frequently yielded lanes of variable size which were often not straight and difficult to analyse by densitometry. Therefore, precast gels (15% acrylamide, Tris-HCl, Sigma, St. Louis, MO, USA) were used which yielded more consistent results. A typical Western blot result is shown in Figure 9 below.

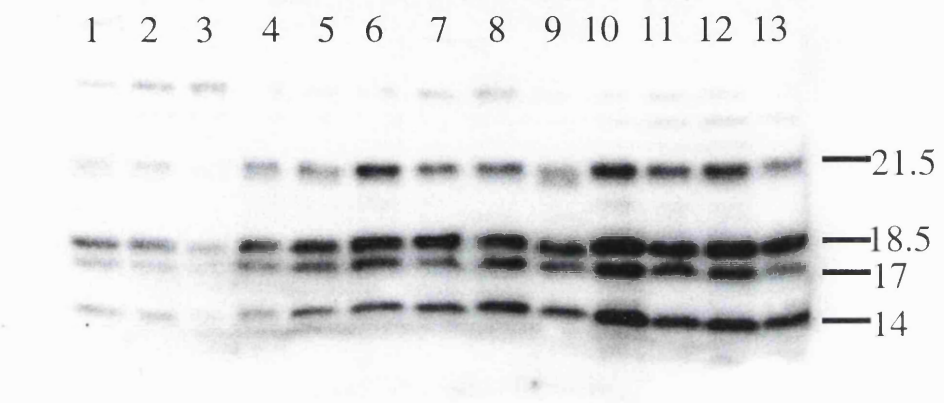

Figure 9. A typical Western Blot of samples isolated from developing rat brains containing 20 μg of total protein run on a 15% acylamide gel. Lanes from left to right contain rat brain from postnatal days 15 (lanes 1-3), 20 (lanes 4-6), 24 (lanes 7-9), 32 (lanes 10-12), and 70 (lane 13). The molecular weights (in kilodaltons) of the MBP isoforms are indicated at the margin.

C. Comparison to Previously Published Results

Results from developing rat brain Northern and Western blot analysis were compared to data previously obtained in mouse brain to verify the methods. Carson et al. (1983) published a paper on the developmental regulation of expression of MBP in mouse brain by quantifying the levels of MBP mRNAs and comparing them with the accumulation of MBP isoforms. mRNA levels were assessed by an *in vitro* translation system instead of by Northern blot, but an approximate comparison can be made with the present studies. They found a peak in MBP specific mRNA expression around postnatal day 20 in mouse brain which is identical to the peak observed in developing rat brain in the studies reported here (see Figure 10 below).

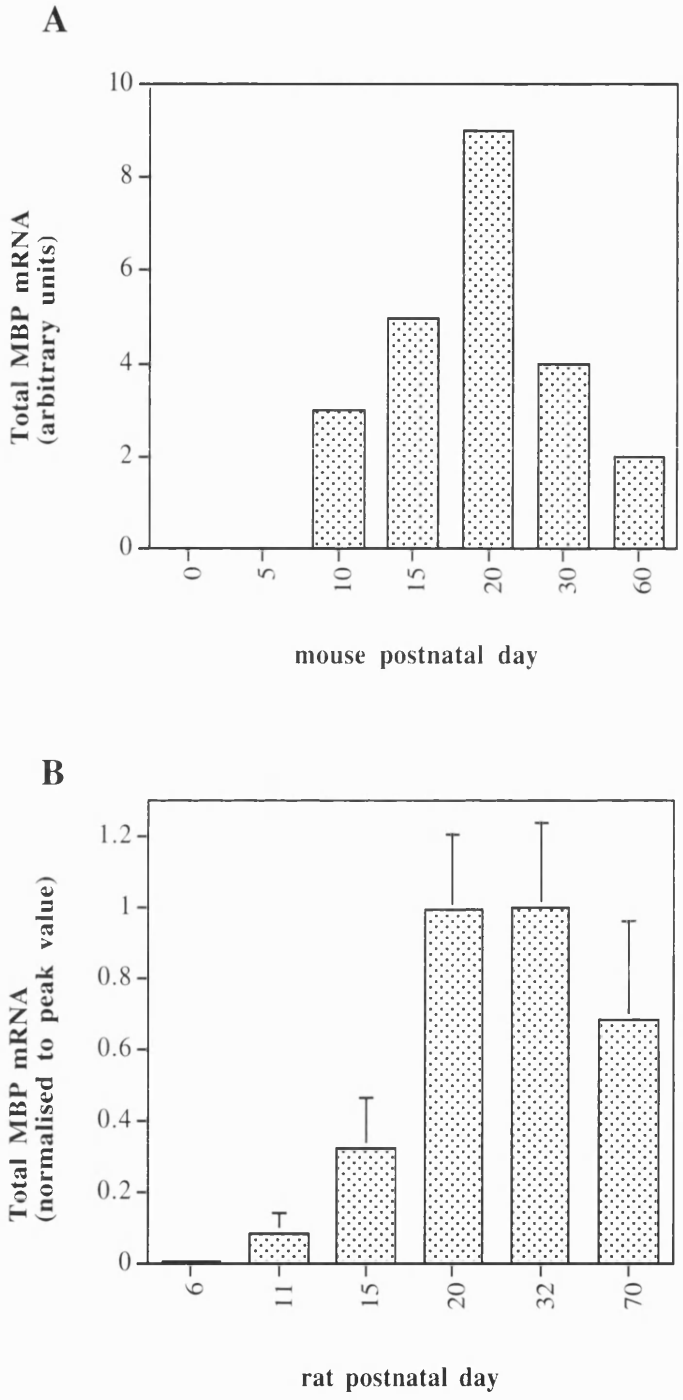

Figure 10. Comparison of mouse brain MBP mRNA data obtained by Carson et al., 1983 (A) with initial rat brain data obtained in my studies (B). Both species show a peak in MBP mRNA synthesis around postnatal day 20. No error bars were shown in the paper by Carson et al.

Data from Western blot analysis of Carson et al. (1983) were also compared to data reported here to verify the techniques. In both cases, total MBP levels were determined by radioimmunoassay and normalised to total protein concentration in whole brain homogenate. Also, in both studies total protein concentration was determined by the Lowry method of protein quantification (Lowry et al., 1951), and MBP isoforms were separated by SDS-PAGE and visualized by Western blotting. Relative levels of specific MBP isoforms were determined by densitometry, and then multiplied by the total MBP/mg protein to yield absolute values. Unfortunately, Carson et al. did not report these values directly. Instead, they reported the rates of accumulation of the different MBP isoforms over time. This data was used to approximate the total amounts of the different MBP isoforms accumulated by the time of harvest from the graphs published (days 5, 10, 15, 20, 25, 30 and 60 postnatal), but this required some estimation and undoubtedly introduced some error into the comparison. Nevertheless, the pattern of expression of the MBP isoforms was very similar between the rat (my studies) and the mouse (Carson et. al) as shown in Figure 11 below confirming that the methods were reliable. The absolute levels of the different isoforms varied somewhat between the studies, however. The amount of total MBP protein accumulated as measured by RIA was consistently less in the Carson et al. studies but on the same level of magnitude as is shown in Figure 11 C.

This could be due to a difference in the affinities of the antibodies used in the radioimmunoassay for MBP, storage conditions of the tissue after harvest, or the homogenisation buffer (they used only water rather than a buffer containing a protease inhibitor cocktail to protect the MBP from degradation as was used in my studies). In addition, there is a discrepancy between the total amount of MBP accumulated according to RIA data, and the amounts of the major isoforms accumulated in the data by Carson et al. (compare figure 11A with 11C). This is partly because they also calculated the rates of accumulation of other large molecular weight proteins which cross reacted with the MBP antibody (34, 30 and 26 kDa proteins). However, these proteins only make up a minor fraction of the total MBP; therefore, the discrepancy is thought to be due mainly to errors in estimation in converting their data (reported as rates of accumulation rather than

absolute accumulation of MBP) to a format similar to mine. RIA data were reported directly in the publication.

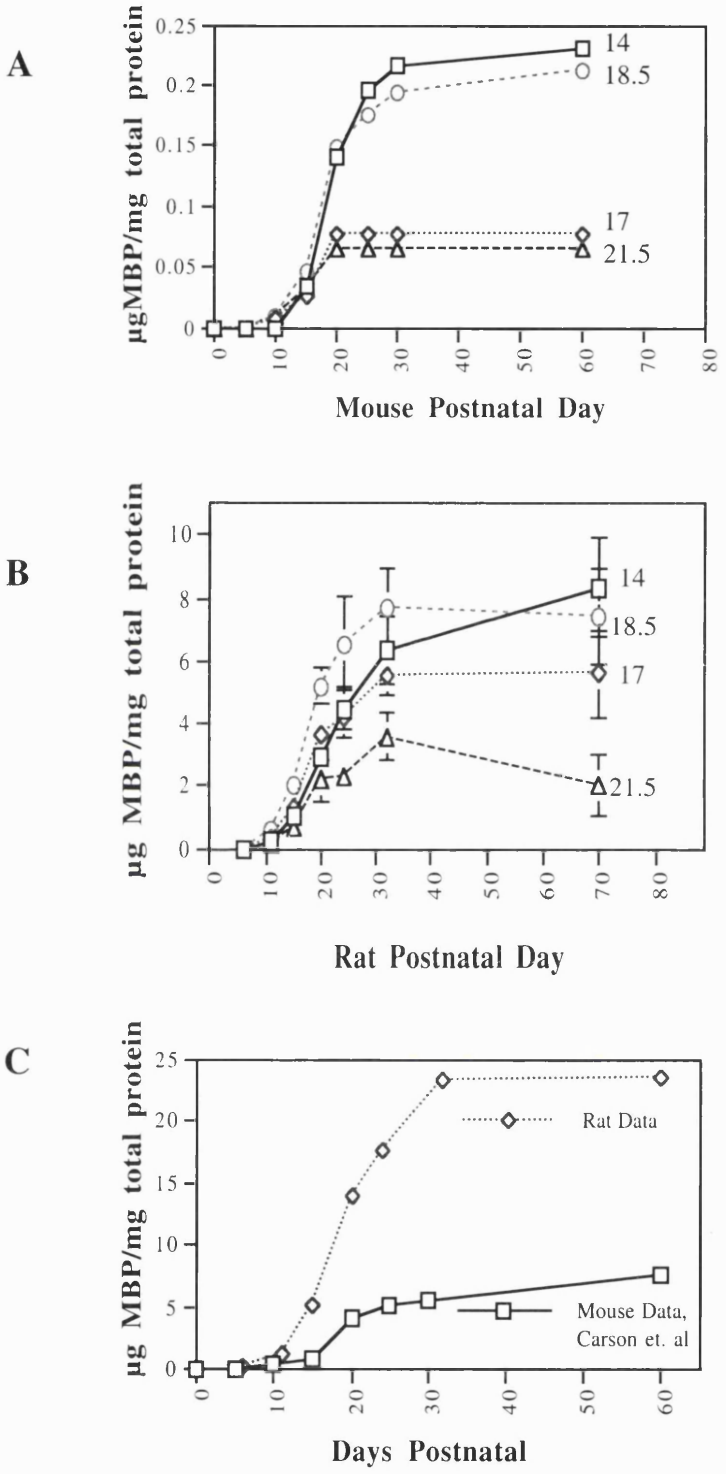

Figure 11. Comparison of the accumulation of different MBP isoforms by Western blot analysis between (A) developing mouse brain (Carson et al., 1983) and (B) developing rat brain (my results). Graph (C) shows a comparison between total MBP accumulation in these two systems. Error bars in graph B correspond to +/- one standard error of mean. No error bars were published by Carson et al. Molecular weights of the MBP isoforms in kilodaltons are shown on the right hand side of graphs A and B.

D. Discussion

The first step of this project was to establish and verify suitable methods to reproducibly determine levels of MBP mRNA and protein expression in rat brain tissue. This could then be used as a basis of comparison for the MBP expression in myelinating and remyelinating rat brain aggregate cell culture systems. Oligonucleotide probes were chosen and conditions were optimised to detect total and exon-2 containing MBP mRNA. Because all MBP mRNAs are approximately the same length, total and exon-2 specific probes hybridised to the same size band on the Nothern blot. Therefore, only relative patterns of expression rather than absolute amounts of different isoforms could be compared in this study. A more rigorous comparison could have been made by using an *in vitro* translation system as described by Carson et al., 1983 or by *in situ* hybridisation as used by Jordan et al., 1989; but it was not thought to be crucial to the objectives of this study. Standard Western Blotting techniques were optimised for measuring the relative amounts of different MBP protein isoforms. Absolute amounts of the MBP isoforms could then be determined from the total MBP protein concentration present (measured by RIA) and normalised to the amount of protein in the sample. In order to increase certainty that the correct bands were detected, isolated myelin was run adjacent to lanes containing whole rat brain.

Finally, initial data obtained on the patterns of MBP mRNA and protein isoform expression in the developing rat brain was compared to a published rodent study in order to verify the methods. A thorough study of the patterns of MBP expression in developing rat brain had not previously been published; therefore, a mouse paper was chosen instead. Mice and rats develop on a similar time scale and have been shown to express the same major MBP isoforms (Sutcliffe, 1987). In both my initial data and Carson's mouse data, the peak in MBP mRNA synthesis occurred at postnatal day 20, and an earlier rise in exon-2 containing MBP mRNAs was seen. MBP protein synthesis also followed a similar pattern with the 14 kDa isoform becoming the predominant isoform in adulthood. These comparisons indicate that the methods described here are likely yielding accurate results and should be

able to provide a reliable comparison between MBP expression in developing rat brain and myelinating and remyelinating rat brain aggregate cell cultures.

Chapter IV. Myelin Basic Protein Isoforms in Myelinating and Remyelinating Rat Brain Aggregate Cultures

Adapted from material previously published in:

G. M. Kruger, L. T. Diemel, C. A. Copelman, and M. L. Cuzner. 1999. "Myelin Basic Protein Isoforms in Myelinating and Remyelinating Rat Brain Aggregate Cultures," *Journal of Neuroscience Research* 56:241-7.

A. Abstract

Recent evidence suggests myelin basic protein (MBP) exon-2 containing isoforms play a significant role in the onset of myelination as they are more abundant during early development. The pattern of expression of MBP exon-2 containing forms was studied in rat brain aggregate cultures during myelination in order to draw comparisons with the developing brain and upon remyelination following demyelinative treatment. The pattern of MBP isoform expression in the aggregate cultures was found to be similar to that of the brain and was recapitulated following demyelination with anti-myelin antibodies. Macrophage enrichment, resulting in increased accumulation of total MBP in the cultures, did not alter the isoform distribution. Both control and enriched cultures expressed a 16 kDa protein (26 +/- 9.8% of total MBP for control samples) which reacted with MBP antisera at the onset of myelination (day in vitro 14) but was barely detectable by day in vitro 21. The expression of this protein, also present in postnatal day 11 rat brain but no longer by day 15, has been predicted by RT-PCR; however, detection of a corresponding protein with the same developmental pattern and appropriate molecular weight has not been previously reported. The results of this study reinforce the value of the aggregate culture system as a versatile yet accurate model of myelination and remyelination.

B. Introduction

Myelin basic protein (MBP) is a major structural protein in myelin thought to be primarily responsible for compaction and stabilisation of the major dense line. Recent studies, however, have shown it may also have a role in myelinogenesis (Pedraza et al., 1997). MBP consists of seven exons with alternative splicing of exons 2, 5 and 6 resulting in at least five major isoforms in rodent (Newman et al., 1987). Expression of isoforms containing the exon-2 sequence (21.5 and 17.2 kDa in rodents) have been found to peak during the initial stages of myelination and again at the onset of remyelination in mouse and human studies (Jordan et al., 1990; Capello et al., 1997). Where different MBP cDNAs are expressed in host cells such as Hela cells or shiverer oligodendrocytes, exon-2 containing isoforms were found diffusely in the cytoplasm or nucleus while the non-exon-2 containing forms, present later in development, were found associated with intracellular membranes (Pedraza, 1997). As the exon-2 containing forms appear to be actively transported to the nucleus, this observation combined with the timing of expression strongly suggests that they may play a role in the onset or regulation of myelination.

Dissociated rat telencephalon cells under appropriate conditions form highly organised three-dimensional aggregates developing and differentiating on a time scale and in a fashion similar to that in developing brain (Honegger, 1985). Demyelination can be induced in the aggregates by cytokines or antibodies directed against myelin-oligodendrocyte glycoprotein (MOG), and following removal of demyelinating agents MBP synthesis resumes (Loughlin et al., 1997). Furthermore, the coordinate expression of mRNA for myelin proteins in myelinating and remyelinating aggregates is consistent with deposition of structurally intact myelin.

The levels of exon-2 containing mRNAs and the patterns of expression of major MBP isoforms have not been investigated in aggregate cell cultures, a study of which may help to elucidate whether remyelination recapitulates myelinogenesis. The aims of the study were to compare myelinogenesis in aggregate cultures with that of the developing brain to validate this *in vitro* model of myelination and remyelination and to compare primary myelination with the remyelinative process.

C. Materials and Methods

I. Myelinating Aggregate Culture System

Serum-free, rotation-mediated aggregating cell cultures were prepared from foetal (16 days gestation) Sprague-Dawley rat telencephalon as previously described (Honegger, 1985).

II. Treatment of Aggregate Cultures with α-MOG

Cultures were demyelinated by adding 31.3 μg/ml α-MOG derived from clone 8-18C5 (Linington et al., 1984) and 25 μl/ml guinea pig serum (GPS) (Serotec, Oxford, UK) as a source of complement on day in vitro (DIV) 34 for 48 hours. Control cultures received a subclass-specific IgG_1 (Sigma, St. Louis, MO, USA) plus GPS. All solutions were filter sterilised before addition to the cultures.

III. Biochemical Analysis

Aggregates were sampled at pre-determined time points and homogenised for biochemical analysis as described previously (Kerlero de Rosbo et al., 1990). Rat brain samples were sonicated in homogenisation solution (0.1 M PIPES, 5 mM $MgCl_2$, 0.1% Triton X-100, 20% glycerol, 5 mM EGTA and 1 mM phenylmethylsulfonyl fluoride, adapted from Filliatreau et al., 1988) containing a protease inhibitor cocktail (20 μg/ml leupeptin and pepstatin A and 200 μg/ml benzoyl-L-arginine methyl ester, p-tosyl-L-arginine methyl ester, trypsin inhibitor, n-tosyl-L-phenylalanine chloromethyl ketone, and aprotinin). Protein concentrations of homogenates were measured by the Folin phenol method (Lowry et al., 1951) using BSA (0.2 mg/ml) as the standard. MBP content was measured by radioimmunoassay (RIA) (Barry et al., 1990) and expressed per mg of total protein in homogenates.

IV. Analysis of MBP Gene Expression by Northern Blotting

MBP exon-1 (MBP-1) and exon-2 (MBP-2) expression were assessed by Northern blot analysis of RNA isolated from tissue and aggregate cultures using cDNA or oligonucleotide probes. A 30-mer probe corresponding to nucleotides 97-

107 (Roach et al., 1983) for MBP-1 and a 48-mer probe corresponding to nucleotides 238-285 (Jordan et al., 1989) for MBP-2 were used. Either methylene blue staining of the 18S ribosomal band of transferred RNA or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Cambridge Bioscience, Cambridge UK) were used as positive controls to verify equal loading of gels. Oligonucleotide and cDNA probes were labelled with digoxigenin (DIG) according to the Boehringer Mannheim (Mannheim, Germany) protocol.

Total RNA was isolated from samples by homogenising in guanidine thiocyanate followed by phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Denatured RNA was electrophoresed through an agarose gel containing formaldehyde, transferred to a Nytran-Plus (Schleicher and Schuell, Dassel, Germany) membrane by overnight blotting, and cross-linked to the membrane by UV irradiation. Following hybridisation at 50°C with DIG-labelled probes and stringency washes, mRNA levels were detected with anti-DIG antibody conjugated to alkaline phosphatase and CDP-Star chemiluminescent substrate (Boehringer Mannheim, Mannheim, Germany). Prior to reprobing, the membrane was stripped and exposed to X-ray film to ensure that any luminescent signal was removed.

V. Analysis of MBP Isoform Distribution by Western Blotting

Samples were diluted in 2X reducing buffer (2% β-mercaptoethanol, 0.25 M Tris pH 6.8, 0.01% bromophenol blue, 10 mM DTT, 2% SDS, and 10% glycerol) and boiled for 5 min before loading on 15% acrylamide mini gels (Bio-Rad Laboratories, UK). For immunodetection, samples were transferred to poly(vinylidene fluoride) membranes (Millipore, Bedford, MA) using a mini trans-blot electrophoretic transfer cell (Bio-Rad Laboratories, UK) at 60 V for 70 min. A sodium carbonate transfer buffer (pH 9.9, Novex, San Diego, CA) was used to facilitate the transfer of basic proteins. Membranes were blocked for 30 minutes in Tris buffered saline with 0.5% Tween 20 (TTBS) and 3% bovine serum albumin as a blocking agent. Primary (MBP specific rabbit serum) and secondary (horseradish peroxidase linked [HRPL] anti-rabbit IgG, Amersham Life Science Ltd, UK) antibodies were diluted in blocking solution and left to incubate for at least one hour

with TTBS washes before and after addition of the secondary antibody. Blots were developed using the HRPL Luminescent Visualization Kit (National Diagnostics, UK).

Fractions of MBP contained in each of the major isoforms (21.5, 18.5, 17 and 14 kDa in all samples plus 16 kDa in DIV 14 samples) were determined by densitometry. The fractions were then multiplied by the value for total MBP determined by RIA for each sample to give the amount of each isoform per mg of total protein.

D. Results

I. Developing Rat Brain

Rats were sacrificed at 6, 11, 15, 20, 24, 32 and 70 days postnatal, and brain homogenates were analysed for MBP expression by Northern and Western blotting. Northern blot analysis, as shown in Figure 12, was performed using probes for exon-1 MBP (a measure of the total MBP and present in all known MBP transcripts), exon-2 MBP and GAPDH, which was used to correct for unequal loading of gels. Both MBP probes hybridised to a wide band of approximately 2.2 kb while GAPDH hybridised to a band at 1.4 kb (data not shown). Absolute values for exon-1 and 2 MBP mRNA expression could not be directly compared, but relative patterns of expression were demonstrated by normalising each set of values to its peak level. This occurred at day 20 for both total and exon-2 containing MBP transcripts. At day 15, exon-2 mRNA synthesis was 87.7 +/- 21.8 % of its peak while exon-1 mRNA synthesis was only 32.5 +/- 13.9% indicating an earlier rise in exon-2 mRNA.

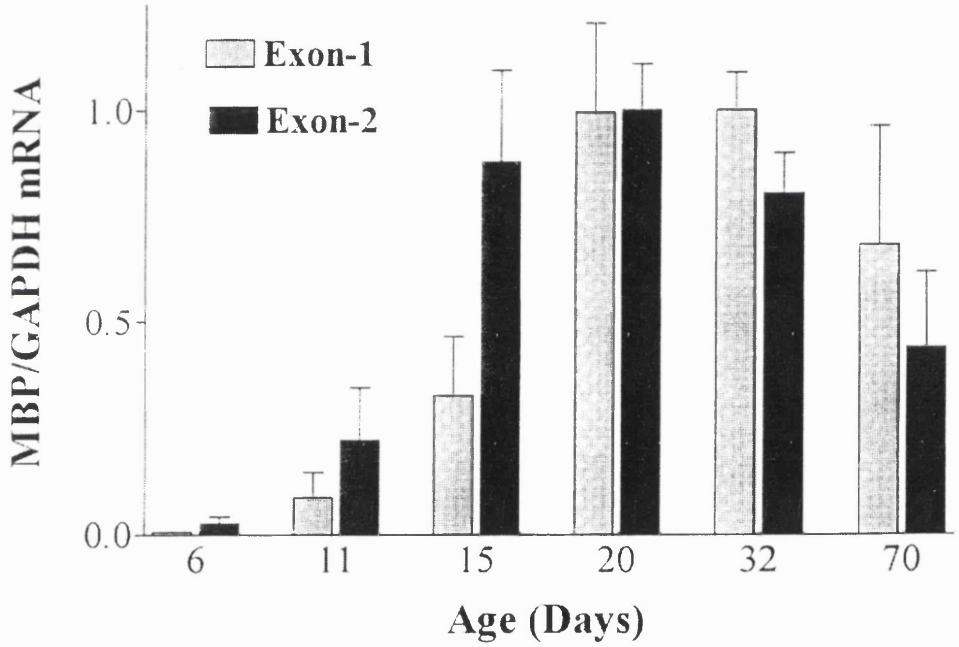

Figure 12. Relative levels of exon-1 and exon-2 containing MBP mRNAs as determined by Northern blot analysis. Values are expressed relative to GAPDH mRNA level and normalised to peak value (day 20 for both probes). Results are means +/- SEM of three to six brain samples.

GAPDH levels did not vary significantly over time (data shown in Figure 19).

Typical western blot lanes are shown in Figure 13A for rats aged 11 and 70 days. Isoforms present at all ages included the 21.5, 18.5, 17 and 14 kDa. A 16 kDa isoform was also readily visible on days 6 and 11 but was no longer detectable by day 20. Western blots were analyzed by densitometry and values were converted to μg MBP/mg protein by correction with RIA and Lowry data as shown in Figure 13B. Total MBP, as determined by RIA, increased as the animal reached adulthood from 0.17 μg/mg on day 6 to 23.5 μg/mg protein on day 70. Western data indicated that the fraction of MBP containing the 14 kDa isoform increased relative to the other major isoforms becoming predominant in adulthood.

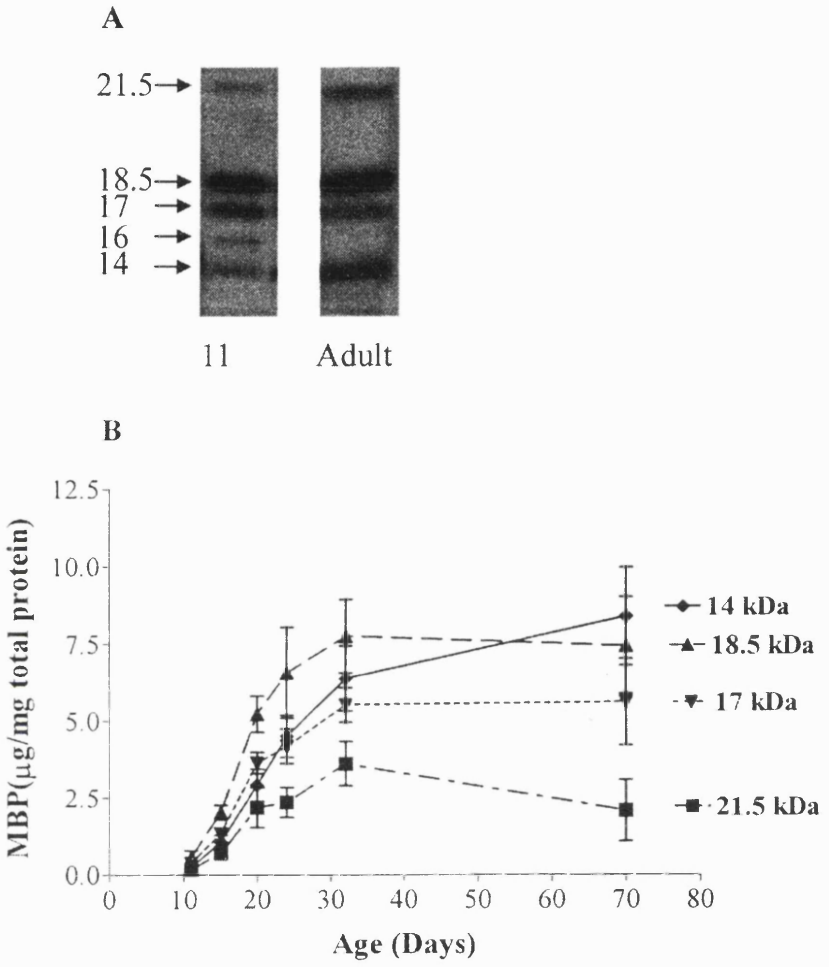

Figure 13. **(A)** Typical Western blot lanes from rats aged 11 and 70 days. **(B)** Accumulation of major MBP isoforms (21.5, 18.5, 17, and 14 kDa) in developing rat brain as determined by Western blot. Values expressed as means +/- SEM for four samples were calculated using the equation

$$\frac{\text{Isoform density}}{\text{Sum of major isoforms}} \quad \mathbf{X} \quad \frac{\text{MBP (µg)}}{\text{Protein (mg/ml)}}$$

The 21.5 and 17.2 kDa isoforms contain the exon-2 peptide. Total MBP (RIA) values were 0.17, 1.33, 5.09, 13.9, 17.6, 23.2, and 23.5 µg/ mg total protein for rats aged 6, 11, 15, 20, 24, 32, and 70 days respectively.

II. Aggregate Cultures

Rat aggregate brain cell cultures analysed for exon-1 and exon-2 MBP mRNA expression by Northern blot demonstrated a peak rate of MBP mRNA synthesis at day in vitro 21 for both probes as shown in Figure 14. Exon-2 containing MBP mRNA synthesis approached its peak level earlier than total MBP (exon-1). Values were 64.8 +/- 6.5 % and 49.7+/- 5.0 % of their peak for exon-2 and exon-1 probes, respectively, at DIV 14. The pattern of expression in macrophage enriched samples was similar to controls, however the timeframe for both transcripts was shifted forward by approximately five days (data not shown).

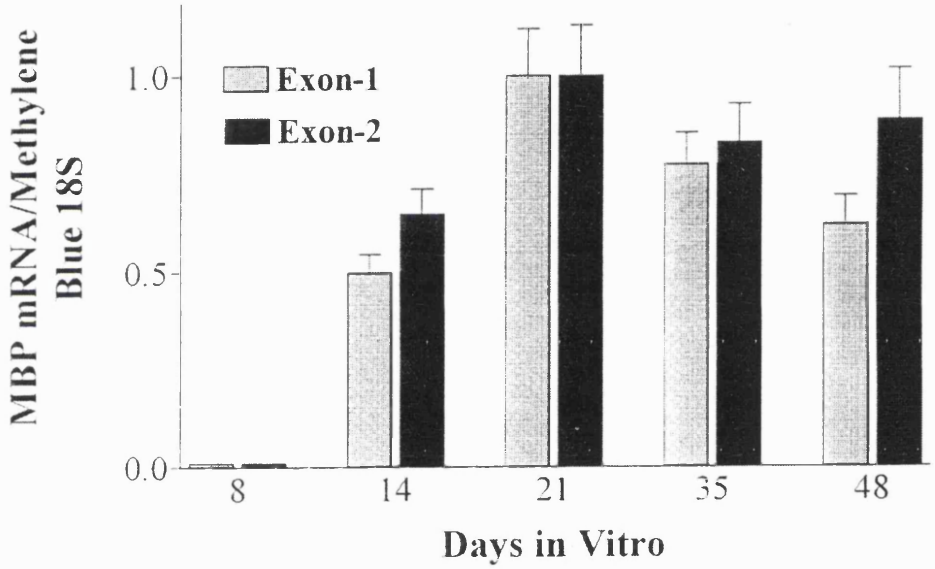

Figure 14. Northern blot analysis of myelinating rat brain aggregate cultures. MBP mRNA expression is shown relative to 18S methylene blue staining and normalised to peak value at DIV 21 for exon-1 and exon-2. Values are means +/- SEM for five to nine flasks.

Typical Western blot lanes from aggregate samples at DIV 14 and 48 are shown in Figure 15A. Major MBP bands present on all days tested were 21.5, 18.5, 17, and 14 kDa. A 16 kDa band was readily visible in control samples on DIV 14 at levels of 12.8 +/- 1.7 % of total MBP (or 0.12 +/- .01 μg/mg protein) which became undetectable by DIV 21. This observation was reproduced in a second aggregate culture preparation where the levels of the 16 kDa protein were 25.6 +/- 9.9 % of total MBP (or 0.12 +/- 0.03 μg/mg protein) for control samples and 19.7 +/- 2.8 % of total MBP (or 0.08 +/- 0.01 μg/mg protein) for macrophage enriched samples at DIV 14. The change in MBP isoform expression over time was determined in two different aggregate preparations harvested either for early or late time points as shown in Figure 15B. In the first preparation MBP (μg/mg total protein) increased from 1.06 +/- 0.20 at DIV 14 to 7.34 +/- 0.95 at DIV 21 and in the second preparation from 5.66 +/- 0.71 at DIV 34 to 12.8 +/- 1.1 at DIV 48. As shown in Figure 13B, the 14 kDa form became the predominant isoform over time in the cultures while the levels of other major isoforms reached a plateau between DIV 40 and 48. A similar pattern of expression was observed in macrophage enriched samples with the 14 kDa isoform increasing from 22.8 +/- 3.0 % of total MBP at DIV 14 to 36.2 +/- 0.8 % of total MBP at DIV 48 (data not shown).

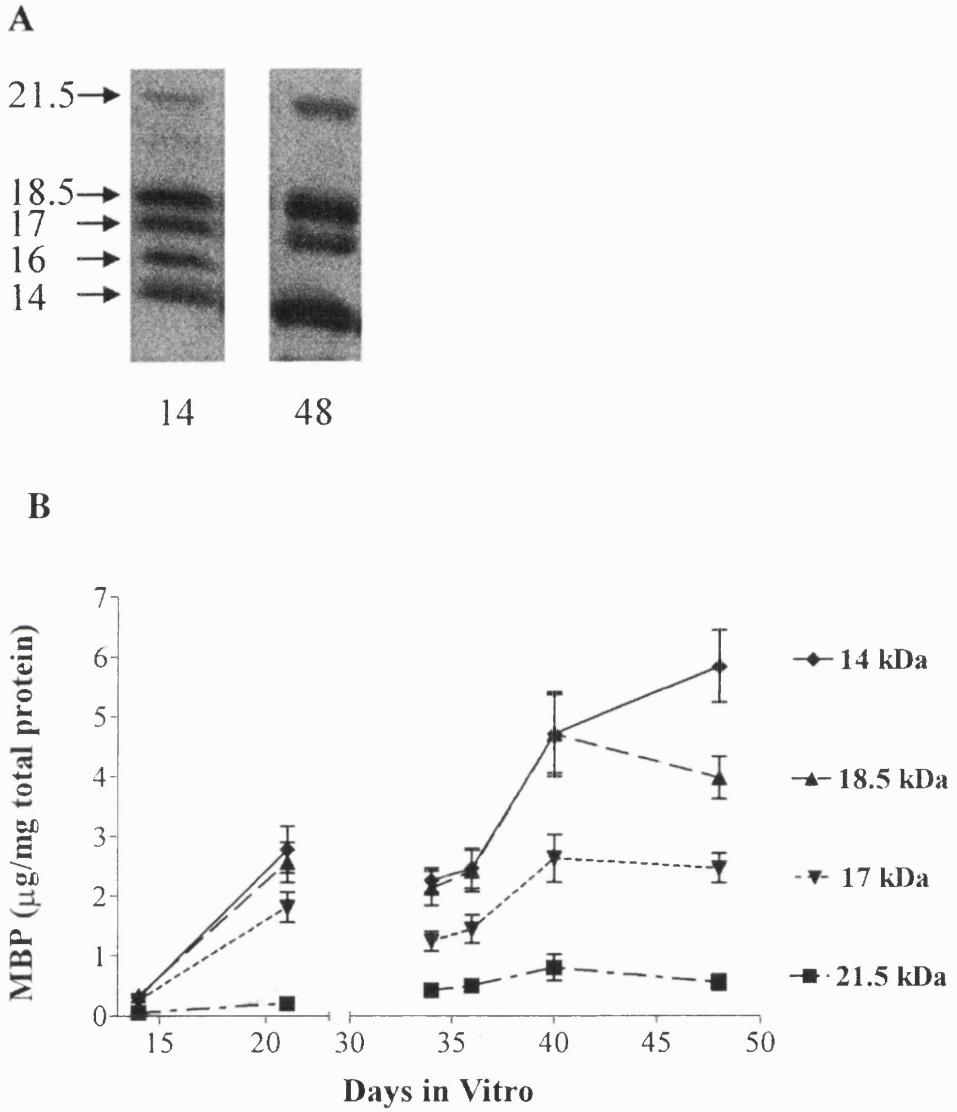

Figure 15. Western blot analysis of myelinating rat brain aggregate cultures. **(A)** Typical Western blot lanes from early (DIV 14) and late (DIV 48) time points. **(B)** Accumulation of major MBP isoforms as calculated in Figure 13. Data for early (DIV 14 and 21) and late (DIV 34, 36, 40, and 48) time points were taken from two separate culture preparations. The 16 kDa isoform was present in significant amounts only at DIV 14 and therefore not included. Results are expressed as means +/- SEM of 10 to 13 flasks for early time points and 5 to 8 flasks for later time points. Total MBP was 1.06, 7.34, 5.66, 6.79, 12.8, and 12.8 µg/mg protein on DIV 14, 21, 34, 36, 40 and 48 respectively.

Cultures treated with either α-MOG or control IgG_1 antibody plus complement were compared by Western blot analysis as shown in Figure 16. Following a 48 hour demyelinative treatment, total MBP values were significantly reduced ($P<0.001$) in demyelinated cultures at DIV 36 with values of 2.9 +/- 0.5 compared to 5.6 +/- 0.4 μg/mg total protein for control treated cultures. The 14 kDa form was predominant in both control and remyelinating cultures; however, the other major isoforms continued to increase up to DIV 48 in remyelinating cultures whereas they leveled off between DIV 40 and 48 in control cultures. Total MBP in control IgG_1 plus complement treated samples did not differ significantly from control untreated samples at any time point.

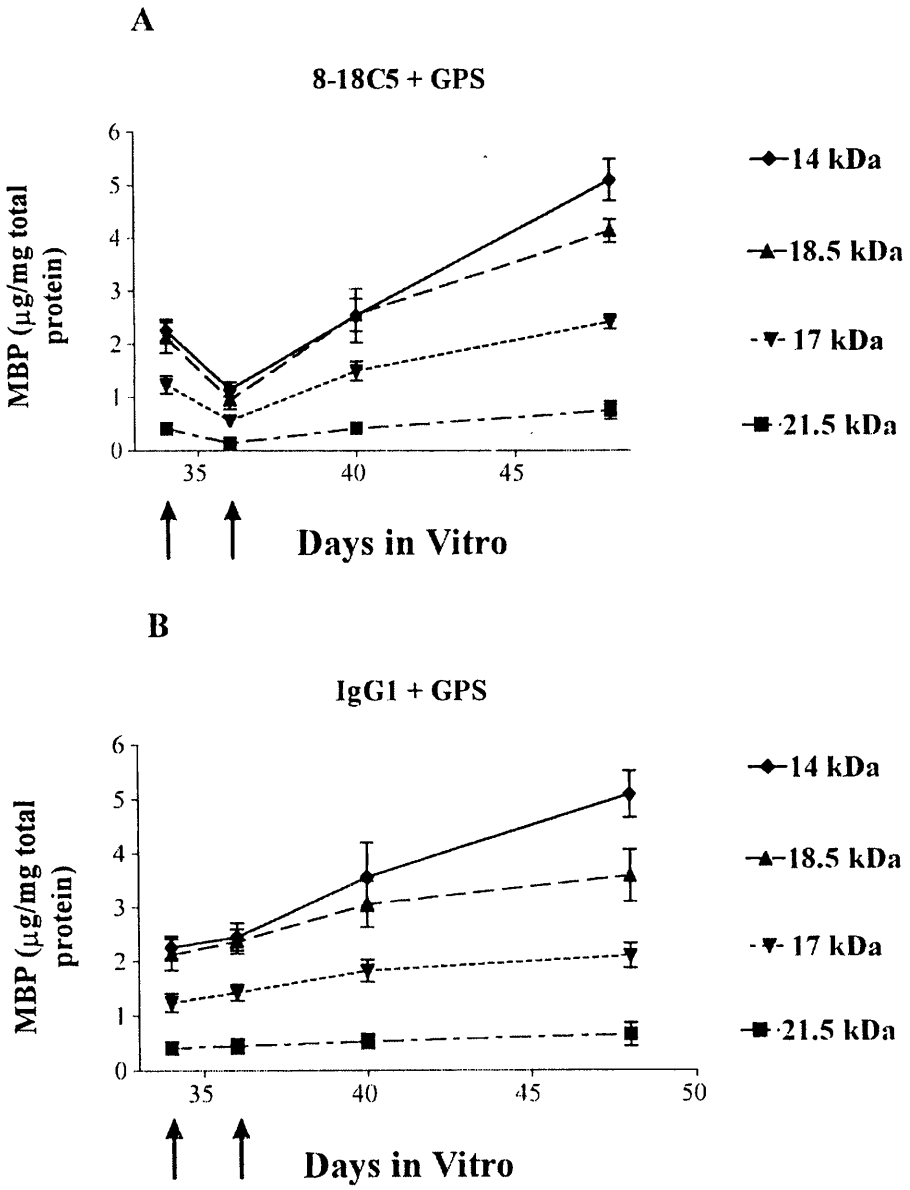

Figure 16. Distribution of major MBP isoforms before and after demyelinative or control treatments. The arrows indicate the treatment period. Values were calculated as in Figure 13 and expressed as means +/- SEM of 7 to 10 flasks. **(A)** Demyelinative treatment (α-MOG antibody 8-18C5 plus complement in the form of guinea pig serum (GPS)) and **(B)** IgG1 control antibody plus GPS were added on DIV 34 for 48 hours. Total MBP levels decreased significantly as determined by Student's two sample t-test ($P<0.001$) in α-MOG treated samples which contained 2.9 +/- 0.5 µg MBP/ mg total protein compared to 5.6 +/- 0.4.

E. Discussion

Expression of MBP exon-1 and exon-2 containing isoforms in myelinating/remyelinating control and macrophage-enriched rat brain aggregate cell cultures was compared to that of developing rat brain by Northern and Western blot analysis. The pattern of MBP mRNA and protein isoform expression in aggregate samples followed a similar pattern to that seen in developing rat brain, for which the results were in agreement with those reported in the literature in which peak MBP mRNA synthesis occurs at postnatal day 20 (Campagnoni and Macklin, 1988). Exon-2 containing MBPs in the myelinating aggregates approached their peak rate of synthesis before that for total MBP (exon-1) as in the developing brain where peak level of expression of exon-2 containing MBP mRNAs occurs earlier than total MBP mRNA (day 15 compared to day 20) indicating upregulated transcription earlier in development. Previous *in situ* hybridization studies in mouse brain (Jordan et al., 1989) have also shown a relative abundance of exon-2 mRNAs early in myelination.

Western blot results also confirmed that the time frame of myelinating aggregate development and the pattern of MBP gene expression is comparable to that of the developing rat brain and mouse brain (Carson et al., 1983). With time in culture, the proportion of 17 and 21 kDa isoforms falls relative to 14 and 18 kDa and the ratio of 14 to 18 kDa increases. In rat brain, levels of the 21.5, 18.5, and 17 kDa isoforms began to level off at day 32 while in aggregate cultures they began to level off by DIV 40 which is equivalent to postnatal day 35 in rat brain. As there is evidence of differences in the ratios of MBP isoforms from one region of the brain to another, the absence of a distinct peak in exon-2 containing proteins at the onset of myelination may have been masked in the analysis of whole brain homogenate. Only a portion of the oligodendrocyte precursors throughout the brain would be synthesising MBP exon-2 proteins at any given time unlike the distinct rise in exon-2 containing transcripts described by Jordan et al. (1990) who looked at discrete mouse brain regions. It has also been previously observed (Loughlin et al., 1997) that there is a rebound effect following demyelination with anti-myelin antibody, such that by 4 days after its removal the MBP content of the aggregates has virtually caught up with the controls. As in the myelinating cultures over the earlier time points, the rate of

synthesis of exon-1 isoforms overtakes that of exon-2 products with an increase in the 14:18 kDa ratio. Oligodendrocytes in the cultures appear to be spared as CNP-ase levels are unchanged; phagocytosis of myelin breakdown products by macrophages in the aggregates may stimulate myelin synthesis.

Of particular interest in this study was the detection of a protein of approximately 16 kDa present at early time points only (DIV 14 to 21 in aggregate samples and days 6 to 11 in rat brain). The 16 kDa MBP observed ephemerally both in the developing brain and in myelinating but not remyelinating cultures may correspond to a novel MBP transcript identified by reverse transcribed polymerase chain reaction (RT-PCR) (Nakajima et al., 1993) which showed similar patterns of expression and had a predicted molecular weight of 15.6 kDa. This transcript was found to contain exons 1, 2, 3, 4 and 7 and to be expressed at high levels from embryonic day 15 to postnatal day 8 with almost no expression by postnatal day 18. A recent study (Maata et al., 1997) has reported low levels of a 16 kDa protein in isolated rat MBP by immunoblotting and mass spectrometry which might be the protein product of this transcript. The failure to detect the 16 kDa isoform in aggregates recovering from demyelination may reflect the sparing of mature oligodendrocytes which retain the potential to increase the rate of myelin synthesis; alternatively the sampling times of remyelinating cultures may have been inappropriate for detection of the 16 kDa isoforms. The current study is the first report of a protein with the appropriate molecular weight and same developmental pattern of expression; however, further tests such as staining Western blots with exon-2 and exon-5 specific antibodies are needed in order to verify that this protein is indeed the one predicted by RT-PCR. In addition, there have been reports of another approximately 16 kDa protein which is also present exclusively in early development (Mathisen et al., 1993). It was found to be present from embryonic day 18 to postnatal day 6 and cross reacted with antibodies specific for both MBP exon-1 and an exon in the Golli region of MBP (Campagnoni et al., 1993). Thus the present study reinforces the value of the aggregate culture system for investigating in a controlled manner the process of myelination and for identifying features that may distinguish regeneration of myelin, with implications for therapy to demyelinating disease.

Chapter V. MBP Expression During Myelination and Remyelination in Rat Brain Aggregate Cell Cultures: Additional Data and the Role of Macrophage Enrichment

I. Introduction

The results presented in Chapter IV demonstrate that the pattern of MBP isoform expression in the rat brain aggregate cell culture system closely mimics that seen *in vivo*. Therefore, the patterns of MBP expression during myelination and remyelination in this system were further investigated in order to learn more about the remyelinative process. This chapter strengthens those results by demonstrating a similar pattern of events in multiple culture preparations.

In addition, data is presented on the effect of macrophage enrichment on the pattern of MBP expression in aggregate cultures which was not shown in Chapter IV. Microglia, the resident macrophages of the brain, are thought to play a role in both tissue destruction and repair. Previous studies in our lab have shown that enriching the cultures with peritoneal macrophages (10% of the total cell number) at the time of seeding the flasks results in increased accumulation of myelin in developing cultures as well as a stronger recovery following demyelinative treatment (Loughlin et al., 1997). Therefore, we wanted to further explore whether the macrophages alter the pattern of gene expression or upregulate any MBP isoforms specifically.

II. Materials and Methods

Aggregate culture preparations, protein quantification, RIA measurement of total MBP, Western Blotting, and Northern blotting were all performed as described in Chapter IV.

III. Results

A. Northern Blot Analysis

The pattern of MBP mRNA expression in developing control and macrophage enriched aggregate cultures is shown in Figure 17 below. These results

are from a different aggregate culture preparation than the data shown in Figure 14 but confirm the pattern of MBP mRNA expression: peak MBP mRNA synthesis was seen at DIV 21 for both total and exon-2 containing MBP. The data also indicate that while 10% macrophage enrichment increased the level of both total and exon-2 specific MBP mRNA expression over that of controls, it did not appear to alter the pattern. However, it is not possible to make absolute conclusions from this data about expression patterns because only a few time points were available with none before DIV 21. Thefore, a separate aggregate culture preparation with time points as early as DIV 5 was analysed as shown in Figure 18.

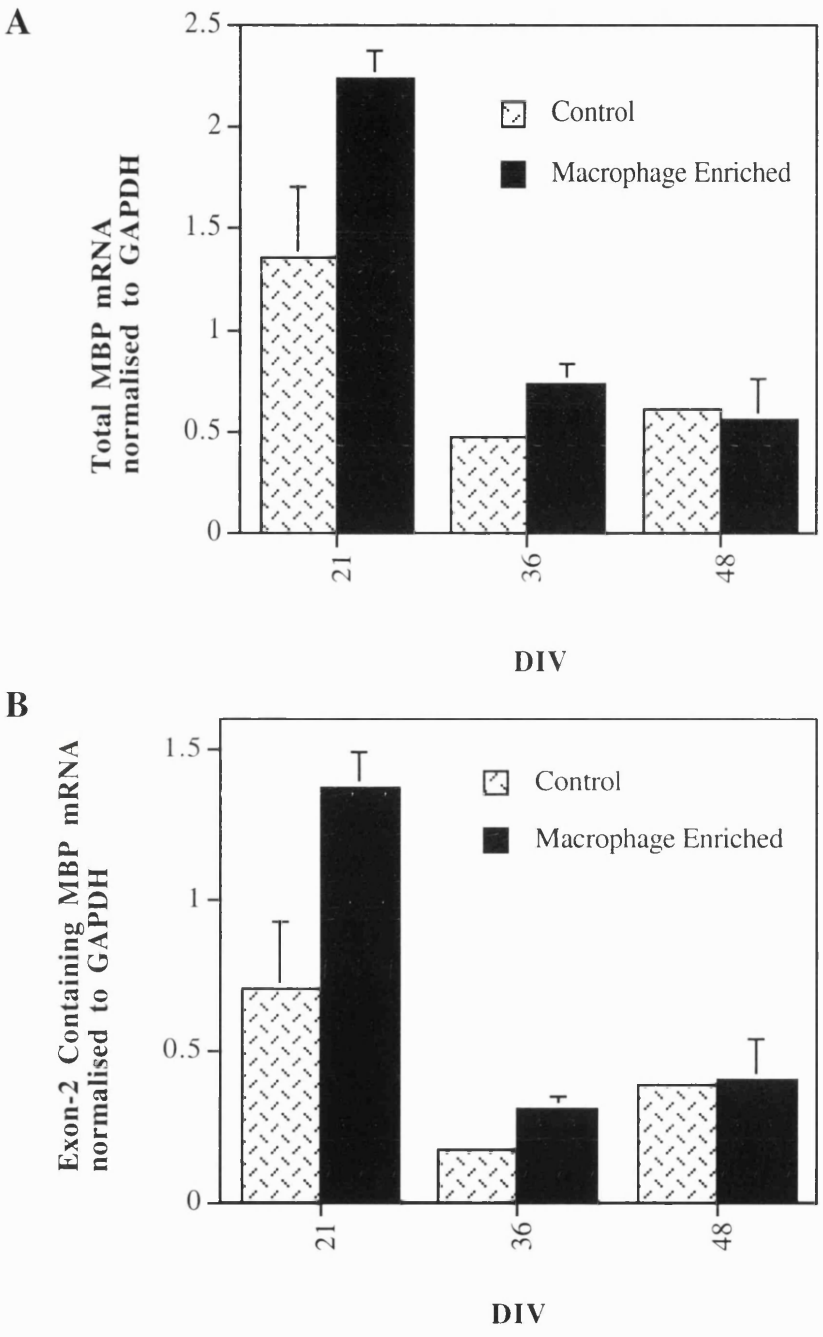

Figure 17. Results from aggregate preparation 28 Northern blot analysis. **(A)** Comparison of the pattern of total MBP mRNA expression in control and macrophage enriched cultures. **(B)** Comparison of exon-2 containing MBP mRNA expression in control and enriched cultures. Error bars are one standard error of the mean except for DIV 36 and 48 control cultures where only N=2 samples were available (no error shown).

Data from MBP mRNA expression at early time points in control and macrophage enriched cultures (preparation 30) was compared. Results demonstrate that although a similar pattern of expression was observed in control and macrophage enriched samples, expression appears to be shifted forward by about five days in macrophage enriched samples. At DIV 8, MBP mRNA levels were only detectable in macrophage enriched samples (see Figure 18 below) while control culture MBP mRNA became detectable by Nothern blot at DIV 14. It is possible that this is due to a limit in sensitivity of the system rather than an earlier onset of expression because macrophage enriched samples tended to have a higher level of MBP expression at all time points.

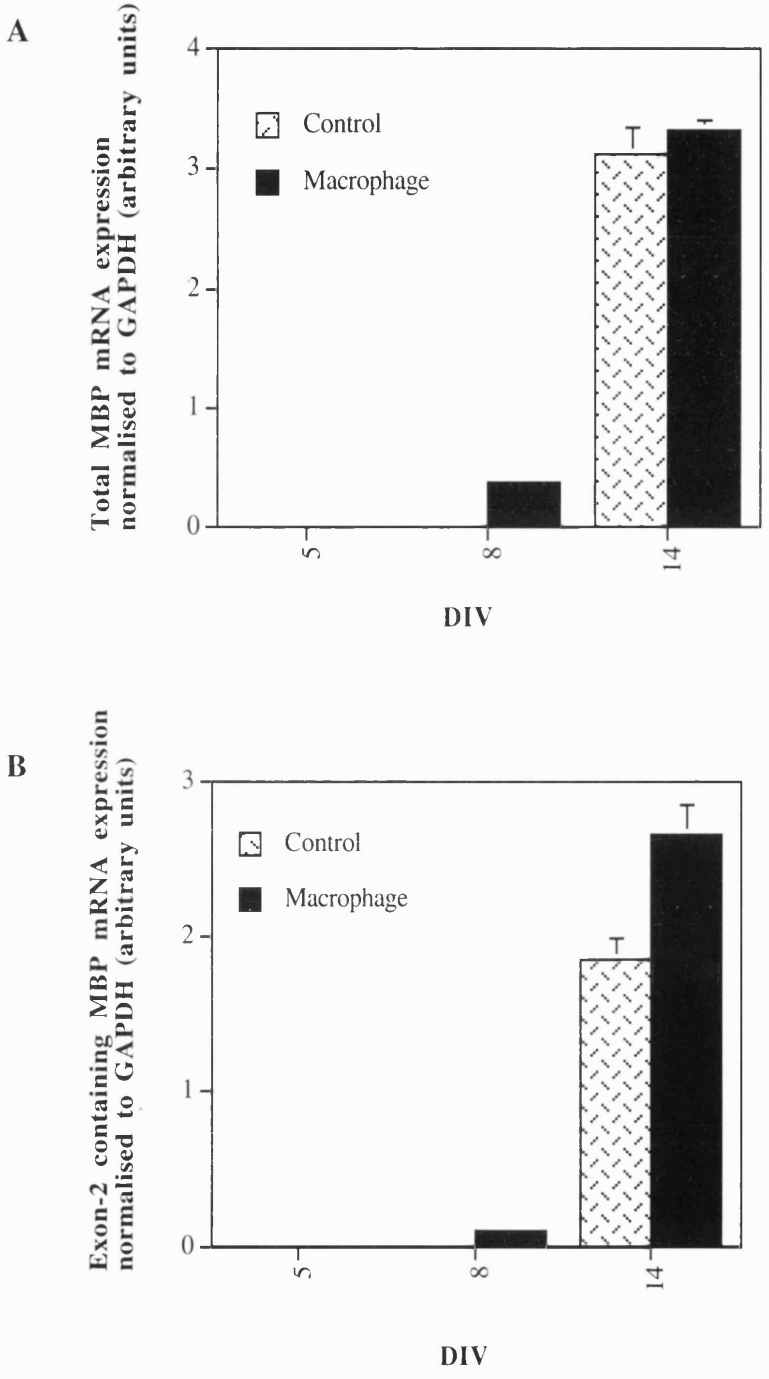

Figure 18. **(A)** Total and **(B)** exon-2 specific MBP mRNA expression in myelinating rat brain aggregate cultures at early time points. mRNA levels were corrected for any unequal loading by dividing by GAPDH mRNA level and expressed in arbitrary units. GAPDH levels were relatively constant between DIV 8 and 14 (see Figure 19B below). N was between 3 and 5 for all samples, and error is shown as one standard error of the mean.

Finally, an interesting pattern in GAPDH expression was seen in the developing aggregate cultures. GAPDH was expressed only at low levels between DIV 8 and 14 and then increased sharply by DIV 21. Levels then remained fairly constant between DIV 21 and 48 (see Figure 19 B below). In developing rat brain, GAPDH levels remained constant over the entire period of development studied from postnatal day 6 to adult (see Figure 19A below). Postnatal day 6 corresponds approximately to DIV 11; therefore, we had expected to see relatively constant levels of GAPDH expression in the aggregates over the time period studied.

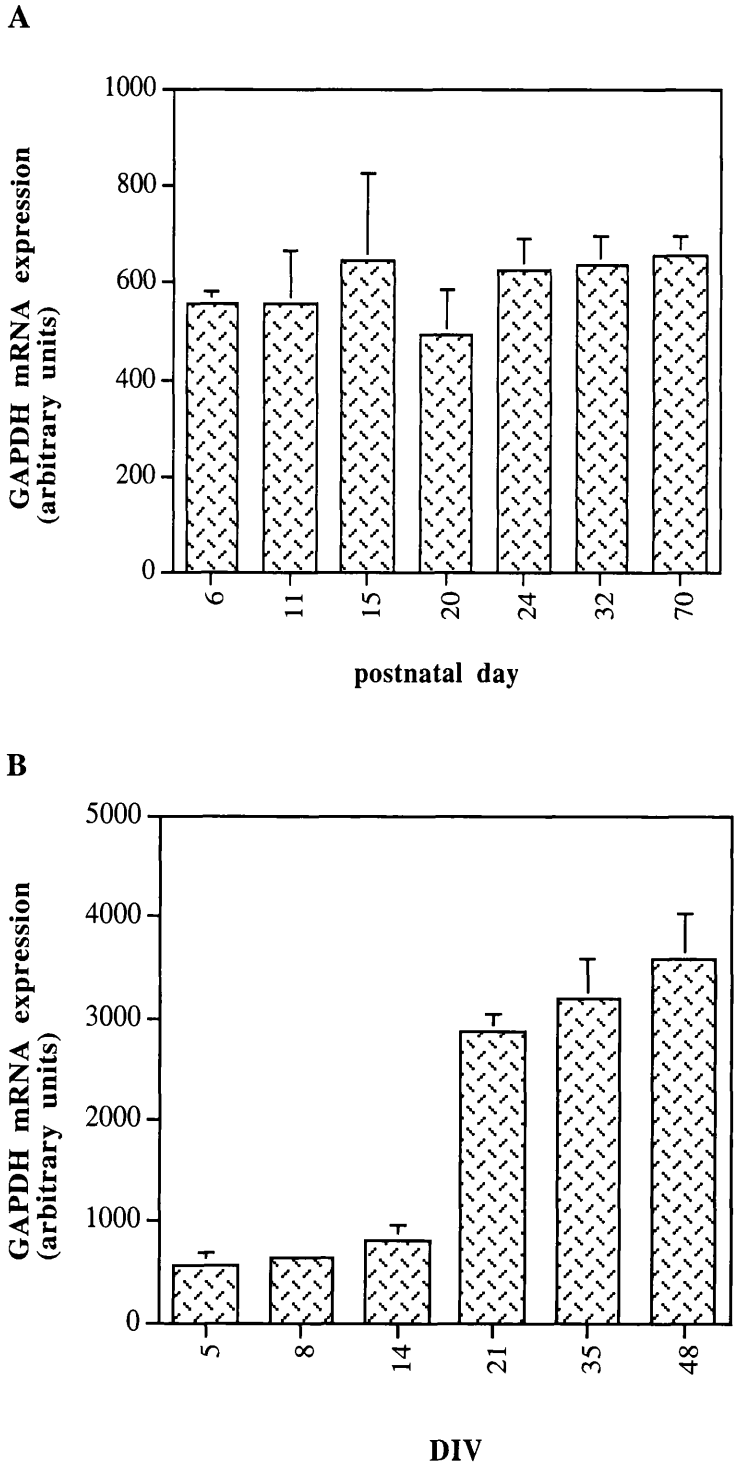

Figure 19. GAPDH mRNA levels in **(A)** developing rat brain and **(B)** myelinating aggregate cultures. Data from two gels was combined to make graph B. Errors are expressed as one standard error of the mean.

II. Western Blot Analysis

Additional Western blot analysis was done to verify the pattern of MBP isoform expression in control and macrophage enriched cultures during development and remyelination and to verify the consistent presence of the putative 16 kDa MBP isoform at early time points.

Figure 20, below, shows a comparison of the accumulation of different MBP isoforms in developing control and macrophage enriched cultures. Although macrophage enriched cultures tended to accumulate more MBP than controls (enriched samples accumulated 11.4 +/- 2.8 μg MBP per mg total protein by DIV48 while controls accumulated only 8.87 +/- 4.2 μg per mg total protein) , the pattern and timing of expression were similar.

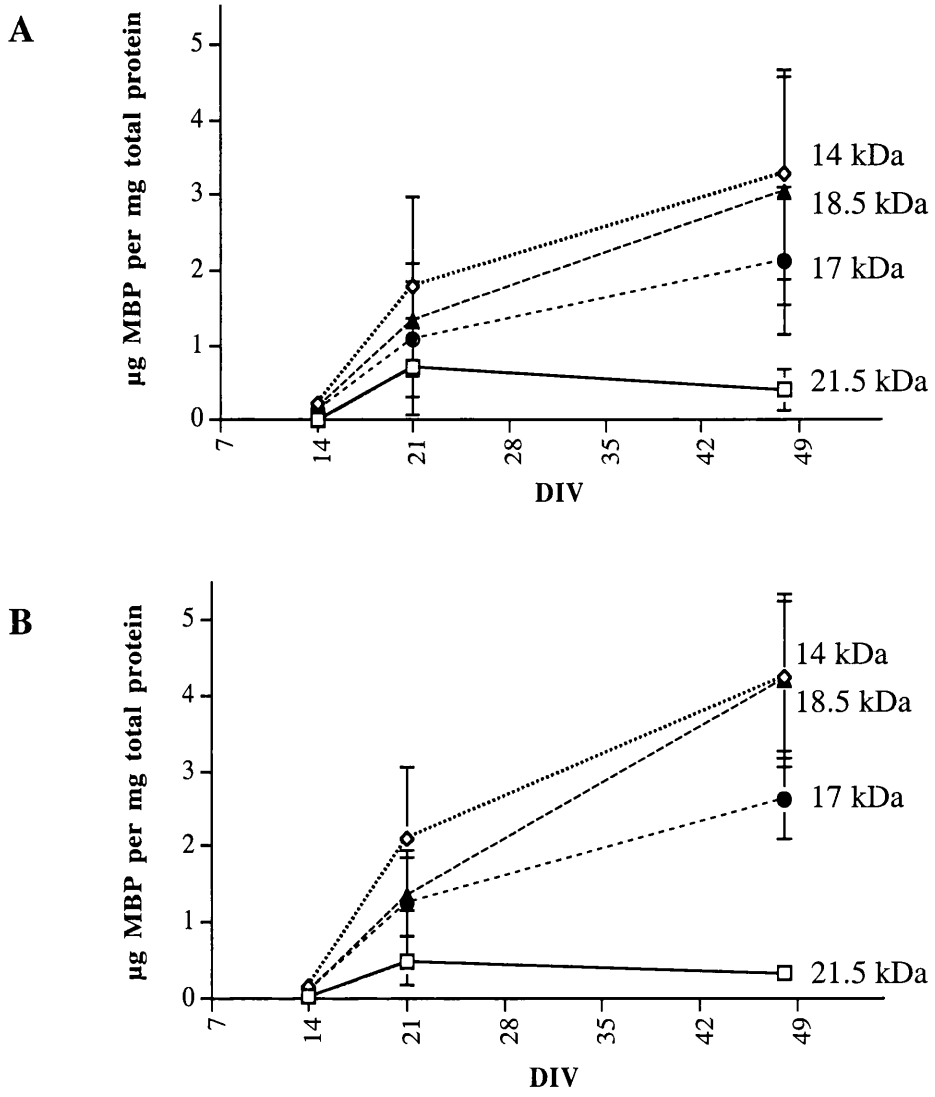

Figure 20. Data from preparation 26 showing similar patterns of MBP isoform expression in control **(A)** and macrophage enriched **(B)** cultures. These graphs reinforce the pattern seen in Figure 15B where data was taken from preparation 24 for early time points and preparation 21 for later time points. The 14 kDa isoform was the predominant isoform by DIV 48, but was not present in significantly greater amounts than the 18.5 kDa isoform in this preparation. Total MBP ranged from 0.67 +/- 0.291 µg/mg protein at DIV 14 to 8.87 +/-4.2 µg/mg protein at DIV 48 for control samples, and from 0.41 +/-0.095 µg/mg protein at DIV 14 to 11.4 +/-2.8 µg/mg protein at DIV 48 for macrophage enriched samples. Values are expressed as means +/- standard error of the mean, and N was between 3 and 6 for all samples.

Patterns and MBP isoform expression before and after demyelinative treatment were also investigated in more than one culture preparation as shown in Figure 21 below. Data in Chapter IV was taken from preparation 21, while the data below was taken from preparation 28 (see the appendix for a complete list of all aggregate culture preparations used for this thesis). The pattern of MBP isoform expression in macrophage enriched cultures following demyelinative treatment is also shown. The graphs are all on the same scale which clearly demonstrates the increased accumulation of MBP in macrophage enriched cultures (Figure 21 A, B) and stronger recovery of those cultures following demyelinative treatment (Figure 21 C, D). However, the patterns of MBP expression are very similar in the control and macrophage enriched cultures during myelination and remyelination.

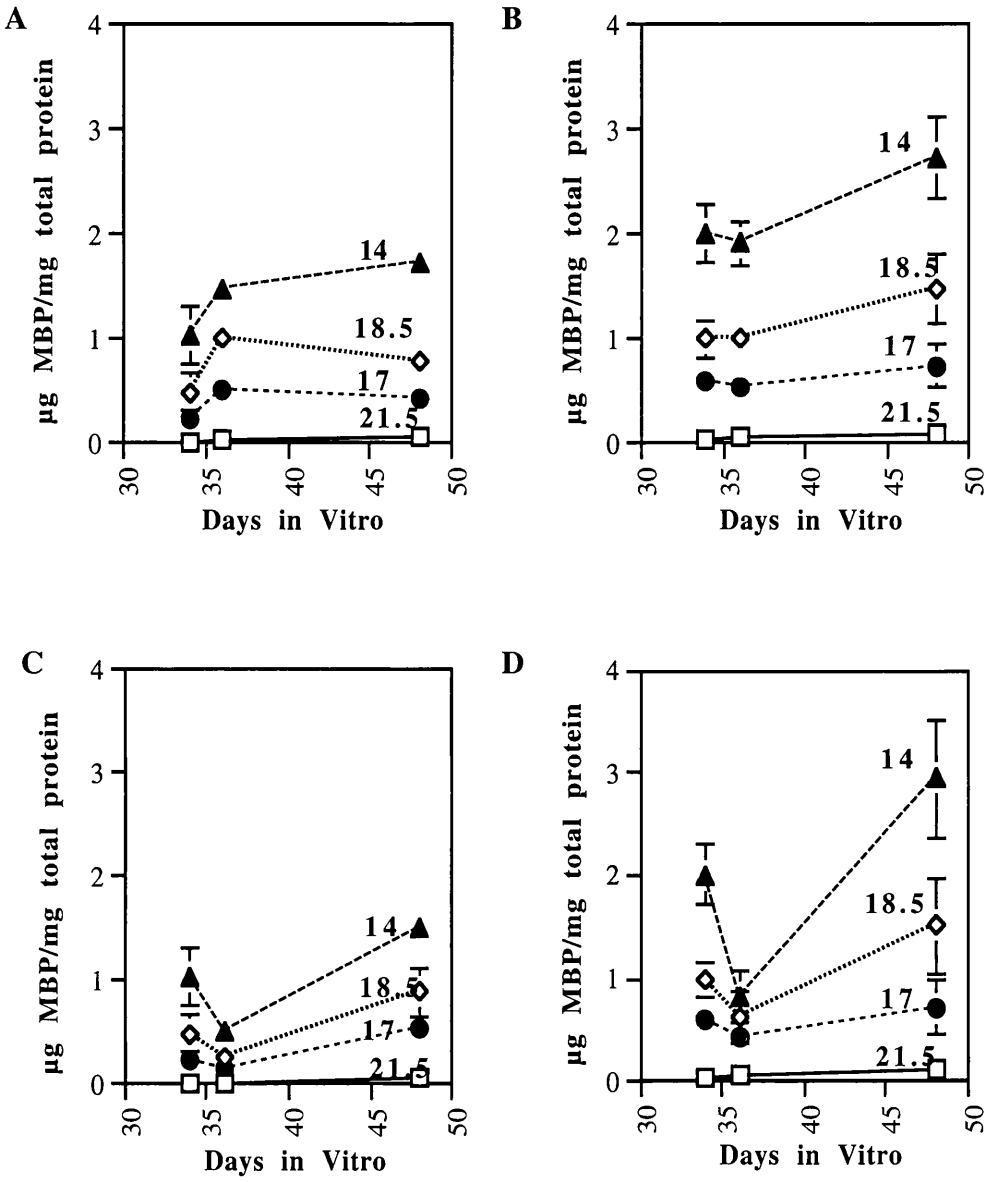

Figure 21. Developing control(**A**) and macrophage enriched(**B**) aggregate cultures. In both cases, the 14 kDa became increasingly the predominant isoform as the cultures matured. Similar patterns of remyelination were also seen in control(**C**) and macrophage enriched(**D**) cultures following a 48 hour demyelinative treatment between DIV 34 and 36. Note that macrophage enrichment in both the developing and the remyelinating cultures resulted in significantly more MBP accumulation by DIV 48. Total MBP levels were 3.02 (no standard error of the mean because only 2 samples were available) and 5.07 +/- 0.47 µg/mg total protein for developing control and macrophage enriched cultures at DIV 48, respectively. N was between 2 and 8 for all samples.

A 16 kDa isoform that cross reacted with the MBP antibody was seen consistently in both control and macrophage enriched samples at DIV 14 which became barely visible by DIV 21. This isoform was not further characterised; but it may, as discussed in Chapter IV, be the protein corresponding to a newly described MBP mRNA with the same developmental pattern of expression (Nakajima et al., 1993). Figure 22 below shows that the single lane shown in Figure 15A was representative of the samples tested from two separate preparations, and that the control and macrophage enriched samples have essentially identical isoform distributions at DIV 14 and 21. Because the putative 16 kDa MBP isoform was only present over a short time period, the fact that the expression is the same in the control and macrophage enriched samples lends support to the theory that macrophage enrichment does not alter the timing or pattern of myelin gene expression. However, nothing can be said definitely without further characterising this protein.

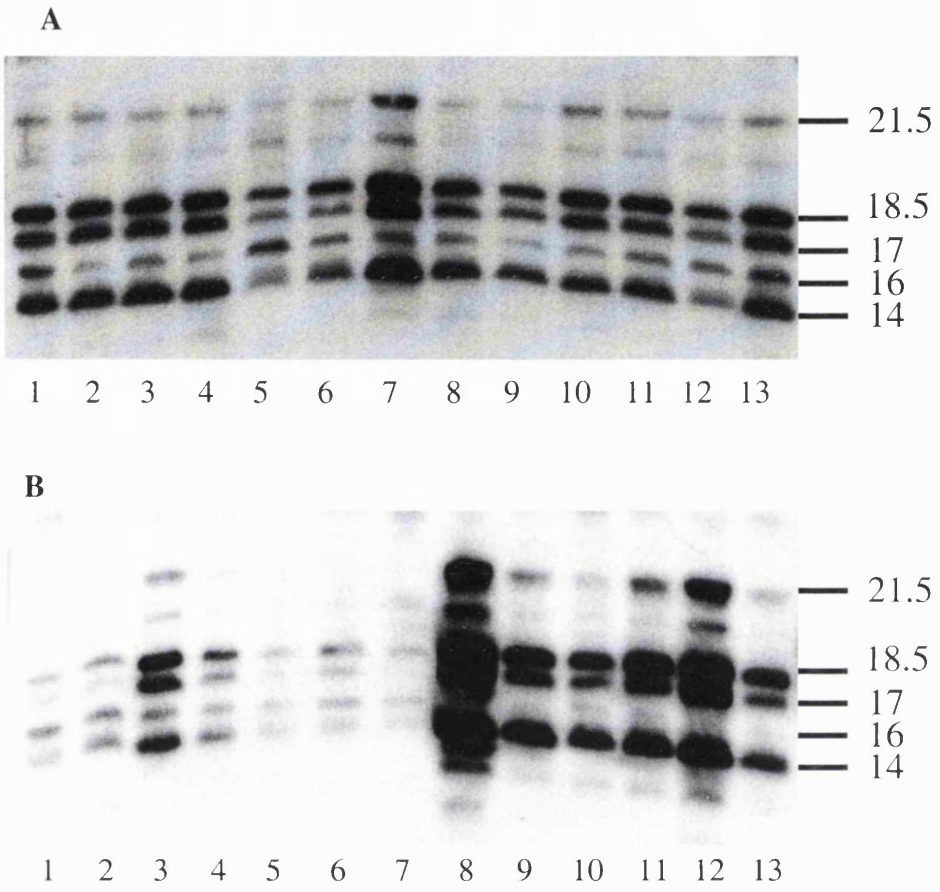

Figure 22. The 16 kDa putative MBP isoform. **(A)** Multiple DIV 14 samples from preparation 24 (the same as shown in Figure 15) showing the presence of a 16 kDa band in all of them. None of these samples were macrophage enriched. **(B)** A typical Western blot from preparation 26 also showing the clear presence of a 16 kDa band. Samples are DIV 14 control (1-3), DIV 14 macrophage enriched (4-6), DIV 21 control (7-10), and DIV 21 macrophage enriched (11-13). Note that the 16 kDa band was barely visible in most samples by DIV 21. Molecular weights of the MBP isoforms are indicated at right in kilodaltons.

Discussion

MBP expression in control and macrophage enriched rat brain aggregate cell cultures was compared by Northern and Western blot analysis in order to learn more about the role of macrophages during myelination and remyelination. Northern blot data from early time points showed that both total and exon-2 specific MBP mRNA could be detected in macrophage enriched samples by day 8 in culture while neither were detectable in control cultures until the next sampling point at DIV 14. This study was not able to resolve whether the macrophage enrichment led to an earlier expression of these genes or whether they were only detectable because they were expressed at a higher level than in control cultures. However, the fact that both control and enriched cultures reached peak myelination (as evidenced by peak total MBP mRNA expression) at DIV 21 suggests that the macrophage enrichment is not altering the timing of MBP expression. This is also suggested by the fact that the expression of the putative 16 kDa MBP isoform was the same in the control and macrophage enriched samples. However, this issue should be further explored with a more sensitive technique of measuring very low levels of mRNA expression such as RT-PCR in order to resolve whether macrophage enrichment results in an earlier onset of myelination in the aggregate cultures.

A second interesting result of the Northern blot data was a difference in pattern of GAPDH expression between developing rat brain and developing aggregate cultures. In developing rat brain there was no significant difference between expression at postnatal day 6 and postnatal day 70, while in developing aggregate cultures, expression significantly increased between DIV 14 and 21. This was unexpected because DIV 11 should correspond approximately to postnatal day 6. It is possible that the GAPDH is lower in rat brain in early development, but increases by postnatal day 6. The aggregates would then just be on a slight delay compared to developing rat brain. This is, however, unlikely because previous studies have found GAPDH expression in rat brain to remain constant over early development. For example, El-Din El-Husseini et al. (1994) found comparable GAPDH mRNA expression in rat cerebrum on postnatal days 1, 3, 7, 14, 21 and 28. Another study has actually found a slight increase in GAPDH mRNA expression

during early development compared to later time points. Lustig et al. (1993) found by slot-blot hybridisation that GAPDH mRNA in the developing cerebral cortex was maximal at birth, fell 23-26% by postnatal day 11 to adult values and then remained constant. In that study, brains were sampled on postnatal days 1, 4, 11, 23, and 60. Another possibility is that the dissociation of the fetal telencephalon cells resulted in a downregulation of GAPDH expression. Cell organisation into aggregates and reformation of normal cell contacts and tissue architecture may have then caused GAPDH levels to return to normal. This area should be further explored before any definite conclusions can be drawn since mRNA data was only available at these early time points from one culture preparation, and absolute levels of GAPDH mRNA expression could not be determined. In addition, relative levels of GAPDH could not be compared between developing rat brain and developing aggregate cultures since they were not run on the same gel (Only a very limited about amount of RNA is obtained from early culture timepoints; therefore, this could not be repeated at the time of the study). This would be necessary in order to determine if GAPDH levels dropped as a result of dissociation of the cells.

The results of this chapter reinforce the conclusions made in Chapter IV by showing that the pattern of expression of different MBP isoforms at the mRNA and protein levels are highly reproducible between aggregate culture preparations. They also show that the system may have some limitations in its ability to mimic the *in vivo* state at very early time points.

Chapter VI. Conclusions

Expression of MBP isoforms was investigated at both transcriptional and translational levels in the rat brain aggregate cell culture model during myelination and remyelination. Results demonstrated that the timing and pattern of MBP isoform expression in the aggregate cultures closely mimics that of developing rat brain.

Macrophage enrichment of the cultures had previously been shown to result in an increased accumulation of MBP during development and a stronger recovery following demyelination (Loughlin et al., 1997). This phenomenon was further investigated in the present study, and enrichment was found to likely alter only the level, not timing or pattern of MBP isoform expression. However, this issue has not been completely resolved because MBP expression was detectable earlier in macrophage enriched than control cultures (DIV 8 versus DIV 14) by Northern blot analysis. This was thought to be due to the higher levels of MBP expression in macrophage enriched tissue making it easier to detect since all other data indicated that the control and macrophage enriched cultures were developing on the same time scale including: the timing of peak expression of both total and exon-2 specific MBP, the timing of the presence of the 16 kDa putative MBP isoform, and the patterns of MBP isoform expression. In order to verify that macrophage enrichment is not altering the timing of the onset of myelination, several additional studies could be done. A second culture preparation with very early time points should be studied to verify this result since mRNA data was only available from one such culture preparation. A more sensitive technique to measure specific mRNA levels such as RT-PCR could be used to monitor the onset of myelination, and levels of more than one myelin protein mRNA could be monitored (e.g. PLP or MAG). In addition, if techniques were developed to make it feasible, aggregate cultures depleted of normal brain microglia could be compared to control cultures. This could potentially be done by using fluorescent antibody mediated cell sorting by flow cytometry prior to seeding the flasks. If macrophages do alter the timing of the onset of myelination, complete deprivation of that lineage in the aggregates is likely to have a more profound effect than enrichment with macrophages.

Although not the focus of this study, of particular interest was the detection of a protein of approximately 16 kDa present at early time points only (DIV 14 to 21 in aggregate samples and postnatal days 6 to 11 in rat brain). The 16 kDa putative MBP isoform which was observed both in the developing brain and in myelinating cultures may correspond to a novel MBP transcript identified by RT-PCR (Nakajima et al., 1993) which showed similar patterns of expression and had a predicted molecular weight of 15.6 kDa. Because of the potential importance of this protein in the onset of myelination, further studies should be done to characterise it. First, sequencing or partial sequencing of the detected protein should be done in order to determine what it is and to find the corresponding mRNA. Probes specific for its mRNA sequence could then be used to follow its pattern of expression in control and macrophage enriched cultures by Northern blotting or RT-PCR. Measuring mRNA levels corresponding to this protein would also be a more reliable method of detecting whether or not it is present or activated following a demyelinative insult. It was not reliably detected in remyelinating cultures, but that could be because it was at very low levels compared to remaining MBP proteins. This could yield information about any role that protein may be playing in the onset of myelination, and potentially a way to differentiate myelination from remyelination.

Finally, we should further explore why a difference was seen in the level of GAPDH expression in early time points of developing aggregates versus developing rat brain. In developing brain, GAPDH levels remained nearly constant over the period studied (from postnatal day 6 to adult) while in developing aggregate cultures they significantly increased between DIV 14 and 21 and then remained relatively constant. Disrupting the normal tissue architecture and contacts of CNS cells may disrupt the normal GAPDH expression causing the aggregates to have lower GAPDH at early time points which then normalises once the aggregates have fully formed. One could test this, for example, by correlating the histology of the aggregates with the GAPDH level. It would also be useful to compare the absolute levels of GAPDH expression between developing aggregates with age matched rat brain samples on the same Northern blot. This would rule out the possibility that GAPDH levels are initially similar to those of developing rat brain, and then later increase dramatically due to apoptosis for example (Chen et al., 1999). Although

GAPDH is not an enzyme of particular interest in our studies and was only being used as a marker, this difference in gene expression between developing aggregate cultures and developing brain could be indicative of other changes in the pattern of gene expression or normal development in these systems. Therefore, it should be further investigated to make sure that future studies with the aggregate culture system are yielding a realistic view of myelination and remyelination at those very early time points.

The results of this thesis reinforce the value of the aggregate culture system for investigating the processes of myelination and remyelination in a controlled manner and for identifying features that may distinguish regeneration of myelin.

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Appendix

Prep Number	Demyelinative and Control Treatments	Analysis
21	8–18C5 Ab + GPS IgG1 + GPS GPS only DIV 34-36	Western
24	None	Western
26*	None	Western
28*	Z12 Ab + GPS IgG_{2a} + GPS GPS only DIV 34-36	Western Northern
30*	None	Northern
31*	IL-1α + BSA BSA only DIV 32-36 8–18C5 Ab + GPS GPS only DIV 34-36	Western Northern

Table 2. Index of Rat Brain Aggregate Cell Culture preparations used for analysis. Preparations marked with a '*' indicates that they were used to analyse both control and macrophage enriched cultures. Z12 and 8-18C5 are anti-myelin protein antibodies. Data from preparations 21, 24, 30, and 31 were shown in figures in Chapter IV.