Selective and Compartmentalised Myelin Expression of HspB5.

Shmma Quraishe[(a)], Andreas Wyttenbach[(a)], Nyasha Matinyarare[(a)], V. Hugh Perry[(b)], Robert Fern[(c)], Vincent O'Connor[(a)]

(a) Centre for Biological Sciences, Faculty of Natural and Environmental Sciences, Building 85, University of Southampton, Southampton, SO17 1BJ, UK; (b) Centre for Biological Sciences, Mailpoint 840 (room LD80b), Level D Laboratories and Pathology Block, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, UK; (c) Peninsula School of Medicine and Dentistry, University of Plymouth, Devon, PL4 8AA, UK.

Corresponding Author:

Shmma Quraishe

Faculty of Medicine, Clinical and Experimental Sciences,

Life Sciences Building 85, Highfield Campus

University of Southampton

Southampton

SO171BJ

Email: s.quraishe@soton.ac.uk

Abstract

In the present study, we reveal myelin specific expression and targeting of mRNA and biochemical pools of HspB5 in the mouse CNS. Our observations are based on *in-situ* hybridisation, electron microscopy and co-localisation with 2',3'-Cyclin-Nucleotide 3'-Phosphodiesterase (CNPase), reinforcing this myelin selective expression. HspB5 mRNA might be targeted to these structures based on its presence in discrete clusters resembling RNA granules and the presence of a putative RNA transport signal. Further, sub-cellular fractionation of myelin membranes reveals a distinct sub-compartment specific association and detergent solubility of HspB5. This is akin to other abundant myelin proteins and is consistent with HspB5's association with cytoskeletal/membrane assemblies. Oligodendrocytes have a pivotal role in supporting axonal function via generating and segregating the ensheathing myelin. This specialisation places extreme structural and metabolic demands on this glial cell type. Our observations place HspB5 in oligodendrocytes which may require selective and specific chaperone capabilities to maintain normal function and neuronal support.

Key Words: myelin; small heat shock protein; alphaB-crystallin; oligodendrocyte; CNS; chaperone

Abbreviations: sHsps, small heat shock proteins; CNS, central nervous system; CNPase, 2',3'-Cyclin-Nucleotide 3'-Phosphodiesterase; MS, multiple sclerosis; RTS, RNA transport signal; PBS, phosphate-buffered saline; PI, protease inhibitor; ABC, avidin-biotin complex; DAB, 3,3'-diaminobenzidine; MBP, myelin basic protein; Spy, stratum pyramidale; LMol, lacunosum molecular; DIGCEMs, detergent insoluble glycosphingolipids/cholesterol microdomains; NG2, neuron-glial antigen 2; OPC,

oligodendrocyte precursor cells; MAP, microtubule-associated protein; MT, microtubule; ROS, reactive oxygen species.

Introduction

The sHsps are evolutionarily conserved, ubiquitous proteins with diverse functions. They are grouped together based on their structural homology and low molecular weight (Taylor and Benjamin, 2005). They form large, dynamic, oligomeric complexes of variable size and are able to bind several non-native proteins per complex (Haslbeck et al., 2008). As their name suggests, the sHsps are classically defined as a class of protein that are induced by heat shock and other cellular stressors/insults. However, they are not exclusively induced by stress, but are established proteins that exert their function under physiological conditions as part of normal homeostatic cell regulation (Mymrikov et al., 2011, Bakthisaran et al., 2015). They regulate protein folding and (dis)aggregation, influence pathways that can modulate ageing, inflammatory processes, the cytoskeleton and cell death (Beere, 2004, Haslbeck et al., 2005, Arrigo et al., 2007, Chen and Brown, 2007). The diverse and important homeostatic cellular roles of the sHsps is well illustrated by the finding that mutations in certain members of this protein family result in myopathies, neuropathies and cataract (Beere, 2004, Benndorf and Welsh, 2004, Haslbeck et al., 2005, Arrigo et al., 2007, Chen and Brown, 2007, Mymrikov et al., 2011, Simon et al., 2013, Benndorf et al., 2014).

We previously characterised the brain expression of ten mammalian sHsps. Four sHsps (HspB1/Hsp27, HspB5/αB-crystallin, HspB6/Hsp20 and HspB8/Hsp22) were unequivocally expressed at the protein level in the mouse CNS under physiological conditions (Quraishe et al., 2008). Interestingly, the expression pattern of HspB5 was distinct amongst the CNS selective sHsps since it displayed selective white matter/myelin expression. Indeed, two systematic proteomics studies previously identified HspB5 as part of the CNS myelin proteome (Dumont et al., 2007, Jahn et al.,

2009) and HspB5 expression has also been reported in Schwann cells and oligodendrocytes under physiological conditions (Iwaki et al., 1992, Klemenz et al., 1993).

Oligodendrocytes are highly specialised cells that send out many projections (~60) from a single cell body, each of which is able to wrap extensively around a single axon forming myelin internodes (Fanarraga et al., 1998, Chong et al., 2012). They synthesise a vast amount of myelin membrane and specific sub-sets of proteins and lipids that are associated with biochemically distinct compact and non-compact cellular sub-domains (Simons et al., 2000, Baron and Hoekstra, 2010). Myelin biogenesis requires the selective targeting, transport and integration of proteins and lipids into the emerging myelin sheath, which may lie a significant distance from the cell body. This is achieved by an orchestrated interplay of cytoskeletal elements that co-ordinate outgrowth during oligodendrocyte differentiation and regulate vesicular transport within the cytoplasmic channels of myelin. Microtubules (MTs) enable the polarised and directed transport of mRNA granules, vesicles and organelles within oligodendrocyte processes akin to axonal transport (Carson et al., 1997, Lunn et al., 1997, Richter-Landsberg, 2000). Multiple membrane trafficking pathways, including lipid rafts, direct material to compact sub-domains and function as signalling platforms (Kramer et al., 2001, Poliak and Peles, 2003, Baron and Hoekstra, 2010, Simons et al., 2012).

The importance of functional oligodendrocytes and appropriate myelination becomes clear when considering diseases such as multiple sclerosis (MS). MS is an inflammatory demyelinating disease, where abnormalities in development, maintenance and damage to the myelin sheath results in a devastating and incapacitating disease with severe neurological symptoms (Lassmann and Lucchinetti,

2008). HspB5 is the most abundant transcript that is unique to early active MS lesions (Chabas et al., 2001). Despite being a negative regulator of inflammation, it has been reported to produce one of the strongest T cell responses in MS patients (van Noort et al., 1995), thus exacerbating inflammation and demyelination in MS (Ousman et al., 2007).

Furthermore, it is increasingly apparent that the neuronal cell population is not the only cell type adversely affected during various age-related neurodegenerative diseases. Associated structures, such as myelin sheaths and glial cells are also affected (Ciarmiello et al., 2006, Mitew et al., 2010). Given the emerging importance of white matter dysfunction during neurodegenerative diseases (Lu et al., 2014, Phillips et al., 2014), the early changes to white matter structural integrity in normal ageing (Barrick et al., 2010, Sullivan et al., 2010, Bennett and Madden, 2014) and in view of the rather selective expression of HspB5, we wanted to better define and characterise the physiological expression profile of HspB5 within the CNS. Expression and localisation of HspB5 mRNA and protein was assessed histologically by *in-situ* hybridisation (emulsion radiography), immunohistochemistry and electron microscopy in rodent brain, spinal cord and optic nerve tissue. Biochemical analysis of HspB5 in myelin enriched samples was conducted by sub-cellular fractionation, followed by detergent, pH and salt extraction. Such biochemical analysis enables the separation and identification of sub-membranous, membrane bound and scaffold-proteins within myelin sub-domains (Kim and Pfeiffer, 1999). This has been used to gain a better understanding of the function/role of abundant myelin proteins such as myelin basic protein (MBP) and CNPase in myelin biogenesis and has revealed distinct biochemical characteristics of these proteins (Kim and Pfeiffer, 1999, Debruin and Harauz, 2007). Our analysis suggests that HspB5, with its intrinsic chaperone

capabilities is well placed to modulate oligodendroglial function in physiological and pathological conditions.

Experimental procedures:

***In-situ* hybridisation**

Adult, p60, C57BL/6J mice were sacrificed by cervical dislocation. Their brains and spinal cords were removed and immersed in Tissue Tek OCT (Bayer diagnostics), on 2-methylbutane over dry ice. 12µM sections were cut on a cryostat, mounted onto Superfrost slides (Menzel-Glaser) and stored at -80˚C prior to use. *In-situ* hybridisation was performed as previously described (French et al., 2001), with an exposure time of 2 weeks. For emulsion autoradiography, slides were dipped in Ilford K-5 emulsion (exposure was extended to 3-fold the standard exposure time), developed and counterstained with Cresyl violet. 45mer oligonucleotides specific to HspB5 were evaluated using the BLAST sequence database comparison software http://blast.ncbi.nlm.nih.gov/Blast.cgi. Antisense and sense oligonucleotides with perfectly matched nucleic acid sequence to HspB5 (100% homology) and low levels of homology to any other known mRNA were identified and supplied by Eurogentec:

HspB5 antisense: AAA CTC AAT GAG GAA AGG GGA TCT ACT TCT TAG GGG CTG CGG CGA

HspB5 sense: TCG CCG CAG CCC CTA AGA AGT AGA TCC CCT TTC CTC ATT GAG TTT

Immunohistochemistry

Coronal sections (10µm) were cut from formalin-fixed, paraffin wax-embedded brains. Antigen retrieval was performed by boiling in citrate buffer, sections were then incubated in a humid chamber overnight at 4˚C with one of the following primary

antibodies: anti-HspB5 polyclonal antibody (1:400; SPA223, Stressgen), anti-CNPase monoclonal antibody (1:400; AB6319, Abcam) and anti-MBP monoclonal antibody (1:1000; Upstate signalling). Negative control (primary antibody omission) sections were incubated in blocking serum. Sections were incubated in appropriate biotinylated secondary antibody (Vector Laboratories, UK), diluted 1:200 in 0.25% BSA/PBS, and followed by incubation in the avidin-biotin complex (ABC) Kit (Vector Laboratories). Location of antibody binding was determined by incubation of sections in a 3,3'-diaminobenzidine (DAB) solution for 1-3 mins. Sections were counterstained with Harris haematoxylin (BDH). Alexa-conjugated secondary antibodies (Molecular Probes, UK) were used in place of biotinylated secondary antibodies for fluorescent labelling and sections were counterstained with DAPI (Vector Shield, UK). Images were taken using a Leica CM500 microscope. LAS-AF software was used for fluorescence microscopy and Q-Win image analysis for brightfield images.

Enrichment of crude myelin

Myelin membrane preparation and differential extraction was carried out using modified protocols (Kim and Pfeiffer, 1999, Phillips et al., 2001). All steps were carried out at 4°C unless otherwise stated. Two adult mice brains (~1g) were homogenised in buffer (0.32M Sucrose, 0.1mM $CaCl_2$, 1mM $MgCl_2$, protease inhibitor (PI) cocktail (Complete, Roche) using a glass Teflon homogeniser. The homogenate was brought to a final sucrose concentration of 1.25M/0.1mM $CaCl_2$ and then overlaid with 1M sucrose/0.1mM $CaCl_2$. The 1M sucrose/0.1mM $CaCl_2$ was overlaid with homogenisation buffer and the resulting sucrose gradient was ultra-centrifuged at 24,000 rpm for 3 hours (SW-28, 141 000g; Beckman). Floating myelin was collected at the 0.32/1M sucrose interface. Excess sucrose was removed from the myelin fraction by re-suspension in phosphate-buffered saline (PBS) and

centrifugation at 19,000 rpm for 30 minutes (SW-28, 30 000g; Beckman). The resultant crude myelin pellet was re-suspended in PBS and stored at -20°C.

Alkaline and high salt membrane protein extraction

Crude myelin was thawed and split into three parts: one aliquot was retained as a representation of 'total protein'. The other two aliquots were extracted in either 100mM Na_2CO_3/PI (pH 11.5) or 1M NaCl/PI (pH 7.4) at 4°C for 1 hour. To investigate access of membrane associated HspB5 to detergent and ensure non-extraction is not due to encasement of the protein, myelin extracted in 1M NaCl/PI (pH 7.4) was subjected to treatment with or without proteinase K (2ug/ml) at 37°C for 1 hour. Samples were centrifuged at 100,000 x g (TLA-55, Beckman) for 1 hour to produce an insoluble pellet and a soluble fraction. The detergent insoluble pellet was re-suspended in equal volume PBS to that of the soluble fraction, and all samples were solubilised in sample buffer (62.5mM Tris-HCl [ph6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.000625% Bromophenol Blue).

pH partitioning of myelin

Crude myelin was thawed and split into three parts: one aliquot was retained as a representation of "total protein" and was re-suspended in 1% Triton-TX100 TNE (25mM Tris HCl, 0.15M NaCl, 5mM EDTA, PI) buffer at pH7.2. The other two aliquots were extracted in TNE buffered either at pH6 or at pH8 and incubated at 4°C for 1 hour. Samples were centrifuged at 100,000 x g (TLA-55, Beckman) for 1 hour to produce a detergent insoluble pellet and a detergent soluble fraction. The detergent insoluble pellet was re-suspended in equal volume PBS to that of the soluble fraction and all samples were solubilised in sample buffer.

Detergent extraction of myelin

Crude myelin was thawed and extracted in 1% Triton-TX100 TNE buffer. The mixture was split into three parts: one aliquot was retained as a representation of "total protein" whilst the other two were incubated at either 4°C or 37°C for 30 minutes. Samples were centrifuged at 13,000 x g (Heraeus 7593, Sorvall) at 4°C for 10 minutes to produce a detergent insoluble pellet and a detergent soluble fraction. The detergent insoluble pellet was re-suspended in equal volume PBS to that of the soluble fraction and all samples were solubilised in sample buffer.

SDS-PAGE and western blotting

Total, soluble and insoluble protein fractions were loaded and resolved through 12.5% polyacrylamide gels (Protean-II-mini cell gel apparatus, BioRad) as previously described (Quraishe et al., 2008). Briefly, proteins were wet-transferred to nitrocellulose membranes overnight. Membranes were blocked in 4% non-fat milk for 1h at room temperature and then incubated overnight at 4°C in PBS containing 0.5% Tween-20 and one of the following primary antibodies: anti-HspB5 (1:500; Enzolifesciences), anti-CNPase (1:1000; Abcam) and anti-MBP (1:5000; Abcam). Membranes were washed and incubated for 1h with fluorescently labelled (IR-DYE 680 and IR-DYE 800; 1:10,000) goat anti-mouse and goat anti-rabbit secondary antibodies at room temperature. Protein band intensities were analysed using a LiCor Odyssey infrared detection system (LI-COR Biosciences, UK) following the manufacturer's guidelines.

Electron Microscopy

As previously described (Alix et al., 2012), P10 rat optic nerves were washed in Sorenson's buffer, and post-fixed in 3% glutaraldehyde/Sorenson's buffer. Nerves

were then fixed with 2% osmium tetroxide and de-hydrated prior to infiltration in epoxy resin. Ultrathin sections were counterstained with uranyl acetate and lead. Sections were incubated with HspB5 (1:500; SPA-222 Stressgen) overnight for post-embedding immuno-labelling. Sections were washed and incubated in secondary antibody conjugated to 20nm gold-particles. Primary antibody omission controls were blank. Sections were examined with a Jeol 100CX (Jeol, Tokyo, Japan) electron microscope.

Results

HspB5 mRNA localisation in myelin enriched white and gray matter

In-situ hybridisation of sagittal brain sections generated a signal supporting an abundant myelin specific expression pattern of HspB5 in both white and gray matter (Figure 1A). When we extended this to spinal cord section, the signal intensity from the film was very strong and present throughout both white and gray matter regions, indicating clear expression in the spinal cord (Figure 1B). In order to resolve the cellular basis of the *in-situ* hybridisation signal we used emulsion autoradiography. HspB1 expression was also investigated in comparison to HspB5 (Figure 2A and B) as this sHsp is known to be expressed in motor neurons within the spinal cord. Indeed, HspB1 mRNA expression was localised to the large motor neurons in the ventral horn (Figure 2A) and was restricted to the gray matter (Figure 2B). Motor neurons were identified by their localisation in the ventral horn and characteristic morphology consisting of a large cell body with abundant cytoplasmic tygroid Nissl substance and a large nucleus with a prominent nucleolus (Havert et al., 2000, Maatkamp et al., 2004, Srivastava et al., 2012).

In contrast, HspB5 expression by emulsion autoradiography was localised to myelin enriched brain regions, predominately within white matter tracts (Figure 2C and D) but also within cortical gray matter regions (Figure 1A). Intense staining over and diffuse staining around glial cell bodies was observed in both the spinal cord and myelin rich regions of the brain, such as the corpus callosum (Figure 2C and D). These glial cells were identified as oligodendrocytes based on their small round morphology, compact, darker-stained nuclei in Nissl-stained sections, and characteristic arrangement in chains (Hof et al., 2003, Hamidi et al., 2004, Peters and Sethares, 2004, Saher et al., 2005, Garman, 2011). Higher magnification images of the HspB5 emulsion autoradiography *in-situ* hybridisation signal in the corpus callosum and spinal cord showed localisation of HspB5 mRNA over cell bodies (Figure 3). However, non-somatic staining was seen to extend away from the cell bodies, forming discrete clusters/clumps that were present along these processes, resembling 'RNA granules' as described for MBP mRNA (Bessert and Skoff, 1999). This phenomenon was observed in many areas of the CNS including the corpus callosum (Figure 3A) and the spinal cord (Figure 3B-D). Excess unlabelled anti-sense oligonucleotides and labelled sense oligonucleotides confirmed the specificity of the abundant HspB5 signal (Figure 1A.ii, Bii and data not shown).

Bioinformatics sequence analysis of transport elements within HspB5 mRNA

To gain insight into the potential mechanism by which HspB5 is targeted and localised within oligodendrocyte processes, we used a bioinformatics approach. Conserved elements have previously been identified for a number of RNA species that are specifically targeted or have selective signals for distinct cellular regions in different cell types (Ainger et al., 1997). These mRNAs contain a 21-nucleotide

sequence, termed the RNA transport signal (RTS). The RTS consensus sequence (GCCAAGGAGCCAGAGAGCAUG) contains two partially overlapping, homologous decanucleotide sequences: GCCAAGGAGC (RTS 1) and GCCAGAGAGC (RTS 2) (Ainger et al., 1997). A perfect or almost perfect match to either of these sequences is observed in many-transported mRNA's (Figure 4A – Adapted from Ainger et al., 1997). The complete mouse HspB5 mRNA sequence obtained from the NCBI database (accession no: NM_009964.3) was used to determine homology with either RTS 1 or 2 decanucleotide sequences (Figure 4A/B). Sequences were processed in EMBOSS Needle (Nucleotide Alignment) (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html) which revealed an almost perfect match to the second of the decanucleotide sequences (9/10) in the 5'UTR (Figure 4A, also highlighted in red in Figure 4B). The human HspB5 mRNA sequence (NM_001289807.1) also revealed sequences similar to both RTS 1 (8/10) and RTS 2 (8/10) in the 5'UTR (data not shown).

HspB5 co-localisation with CNPase, a non-compact myelin protein

We next investigated the cellular localisation of HspB5 by fluorescence immunohistochemistry using an anti-αB-Crystallin polyclonal antibody (SPA-223). We determined specificity of labelling by the morphology and distribution of immunopositive cells and by the absence of signal in appropriate controls. An alternative, monoclonal primary antibody (SPA-222) against HspB5 also validated the labelling pattern (data not shown). Additionally, western blotting of Hela cell lysates transfected with HspB1 (Hsp25), HspB5 (αB-crystallin), HspB6 (Hsp20) and HspB8 (Hsp22) confirmed the specificity of the HspB5 antibody signal, in terms of immuno-detection and expected molecular weight within HspB5 transfected samples only (data not shown).

The immunofluorescence staining of HspB5 displayed a myelin specific expression pattern (Figure 5), consistent with its mRNA profile (Figure 1A.i, 2C and 2D). Immunostaining was observed in white matter specific tracts such as the corpus callosum (cc), cingulate gyrus (cg) (Figure 5) and the fimbria (data not shown). HspB5 protein expression co-localised with CNPase (a robust marker for mature oligodendrocytes) staining in both cell bodies and myelinating fibers. However, HspB5 immunofluorescence was more pronounced in oligodendrocyte cell bodies than CNPase in both fiber tracts of the corpus callosum (Figure 5 - arrowheads) and fiber bundles in the cingulate gyrus (Figure 5 - arrows). This suggests that despite non-somatic targeting of HspB5 mRNA, the relative protein abundance in oligodendrocyte processes and fibres is not enhanced compared to somatic staining. Unlike HspB5/CNPase staining evidencing the cell body and processes, MBP staining only revealed myelinated fibers.

To confirm co-localisation of HspB5 with CNPase, brain tissue from the dorsal hippocampus was analysed (Figure 6). The hippocampus is a distinct gray matter region that is sparsely myelinated in comparison to white matter. However, it contains defined sub-populations of oligodendrocytes (Vinet et al., 2010). As expected, the hippocampus showed weak HspB5 immunoreactivity in comparison to intense staining in cell bodies and fiber tracts in the corpus callosum (Figure 6A-C). The CA3 hippocampal region displayed an intricate pattern of staining. Cell bodies were intensely staining with delicate processes extending away into the CA3 pyramidal cell layer and surrounding area (Figure 6D). This pattern was also observed in the CA1 pyramidal cell layer with intense staining of the cell bodies and a meshwork of processes extending into the stratum pyramidale (Spy) (Figure 6E). Staining of the lacunosum molecular (LMol) region of the CA1 also highlighted the elaborate nature

of HspB5 expression, with immunopositive cells forming characteristic tandem arrays, typical of oligodendrocytes (Figure 6F). HspB5 immunoreactivity co-localised with CNPase staining in oligodendrocyte cell bodies (Figure 6G.i-iii). It has been observed that oligodendrocyte cell bodies forming linear arrays akin to 'pearls on a string' also contain astrocytes amongst them (Lu et al., 2000). This raises the possibility that some of the HspB5 signal observed may arise from other (non-oligodendrocyte) cell types. To resolve the nature of the selective HspB5 expression we performed post-embedded immuno-gold electron microscopy in optic nerve sections (Figure 7). Immuno-gold labelling, apparent as symmetrical circulate bead-profiles of the appropriate diameter (e.g., Figure 7, arrows), was observed in oligodendrocyte somata and processes (Figure 7A-G). In this oligodendrocyte-rich preparation, the cell type has been extensively characterized and the ultrastructural features include the presence of embedded axon profiles, homogenous chromatin with frequent nucleoli, occasional microtubules, a wide-bore endoplasmic reticulum and a high mitochondrial fraction. In addition to the absence of clear astrocyte features such as glial filaments and glycogen particles (astrocytes are the only other major cell type present in the optic nerve) (Alix et al., 2012, Alghamdi and Fern, 2015). Myelin sheaths surrounding axons were labelled but no staining in neighbouring astrocyte processes was observed (Figure 7A, D & E). Primary antibody omission controls were blank with no immuno-gold labelling observed.

Biochemical partitioning of HspB5 in myelin sub-compartments

The myelin sheath is uniquely organised, containing a high lipid to protein ratio. It has distinct compact and non-compact myelin subdomains, with a specific selection of lipids and proteins that accumulate from highly regulated sorting and trafficking

mechanisms (Kim and Pfeiffer, 1999). Biochemical sub-fractionation of myelin has been used to gain a better understanding of the function of associated proteins in myelin biogenesis and maintenance (Kim and Pfeiffer, 1999, Debruin and Harauz, 2007). We therefore subjected myelin-enriched fractions to salt, pH and detergent induced biochemical extraction before fractionating into soluble and insoluble material. We compared the distribution of HspB5 to CNPase and MBP as biochemical determinants of the distinct non-compact and compact myelin domains respectively.

Exposing myelin membranes to low ionic carbonate (Na_2CO_3) or high salt (1M NaCl) concentrations allows the separation and solubilisation of peripheral and associated membrane proteins (soluble fraction) by disruption of non-covalent, electrostatic interactions (Fujiki et al., 1982). Cytoskeletal components and proteins not attached to membrane-associated proteins should not be disrupted and solubilised with this method (pellet fraction). HspB5, CNPase and MBP partitioned into both the pellet and soluble supernatant fractions after Na_2CO_3 treatment (Figure 8A). The more stringent, 1M NaCl extraction resulted in the preferential partitioning of HspB5 and MBP into the peripheral membrane associated protein fraction (soluble) whereas CNPase was present equally in both fractions (Figure 8A). Comparable results were obtained for myelin extracted in 1M NaCl with the addition of proteinase K, confirming that all proteins under investigation were exposed to treatments and not encapsulated in resealed membranes (data not shown).

pH dependent detergent extraction is also used to define sub-membranous and scaffold/cytoskeletal-bound proteins. At pH6, the detergent (TX-100) soluble fraction consists of proteins not associated with the cytoskeleton and the detergent insoluble fraction contains cytoskeletal and membrane bound proteins. At pH8, cytoskeletal

associated proteins are solubilised and membrane associated proteins remain insoluble (Phillips et al., 2001). Both CNPase and HspB5 partitioned equally in the soluble and insoluble pools (Figure 8B). However, the low molecular weight (MW) MBP isoforms displayed intrinsic solubility properties and high MW isoforms remained insoluble, consistent with hydrophobic membrane association (Figure 8B).

We next determined the association of HspB5 with detergent insoluble, glycosphingolipids/cholesterol microdomains (DIGCEMs) or lipid rafts. These lipid rafts are thought to provide platforms for transport and initiating signalling cascades (Kim and Pfeiffer, 1999, Debruin and Harauz, 2007). At 4°C, lipid raft associated proteins are insoluble and at 37°C, become detergent soluble. HspB5 and CNPase partitioned in both soluble and insoluble fractions. However, both proteins fractionated more strongly in the soluble fraction at 4°C and this was only modestly increased at 37°C, reflective of some association with lipid rafts (Figure 8C). Interestingly, the high molecular weight MBP isoforms partitioned predominantly in the insoluble fractions at both 4°C and 37°C reflective of an association with the lipid rich, cytoplasmic face of the myelin membrane (Figure 8C).

Discussion

HspB5 is an intrinsically soluble protein, expressed mainly in the lens but also in non-lenticular tissue. It is one of 4 sHsps that we have previously reported to be expressed at the protein level in the mammalian CNS (Quraishe et al., 2008). Using a number of different techniques, we have characterised the physiological expression of HspB5 and have established a robust mRNA and protein expression profile in oligodendrocytes and associated myelin in the CNS.

Targeting of HspB5 mRNA in oligodendrocytes

The results of our *in-situ* hybridisation study provide new information on the cellular and topographic distribution of myelin-specific HspB5 mRNA. HspB5 mRNA are clearly localised over oligodendrocyte cell bodies and within cellular processes extending away from the cell body in all myelinated regions analysed (Figure 2 & 3). This mRNA expression profile suggests two potential mechanisms of localisation. The abundant HspB5 mRNA species may diffuse from somatic areas to surrounding regions, however, this is unlikely as translocation of mRNAs within oligodendrocytes is highly regulated by the MT cytoskeleton (Carson et al., 1997). Or, the mRNA may be selectively targeted to sub-cellular compartments within myelin as shown for a number of myelin specific proteins such as MBP (Ainger et al., 1997, Torvund-Jensen et al., 2014). Selective targeting of mRNA has also been described in neuronal dendrites, suggesting similar processes can occur in different systems (Kuhl and Skehel, 1998). HspB5 mRNA distribution is akin to that observed for MBP (Trapp et al., 1987, Amur-Umarjee et al., 1990, Campagnoni et al., 1991). In contrast, proteolipid protein, myelin-associated glycoprotein, and CNPase mRNAs remain associated with the oligodendrocyte cell bodies and their protein products are targeted to myelin by other mechanisms subsequent to translation (Amur-Umarjee et al., 1990).

MBP mRNA is specifically targeted to oligodendrocyte processes in 'RNA granules' that are observed as globular round clumps along the processes (Bessert and Skoff, 1999). The transport of MBP mRNA makes it available for local synthesis on free polysomes, primarly in cell processes in close proximity to the myelin forming plasma membrane (Trapp et al., 1987). Targetting and localisation generally requires the presence of specific sequences or secondary structural motifs (RTS; RTS 1 and RTS 2). Interestingly, the HspB5 mRNA sequence contains an almost perfect (9/10) match to the consensus RTS 2 sequence (Figure 4). The similarity of HspB5 mRNA expression with MBP, extending into the peri-somatic stuctures/processes, and the presence of an RTS 2 sequence suggests it is also specifically targetted within oligodendrocytes. Indeed, local synthesis of HspB5 has previously been reported in injury conditioned dorsal root ganglion neuronal cultures (Willis et al., 2005). In myelin this could enable temporal and spatial control of protein expression, allowing localised and rapid synthesis of a chaperone protein involved in cytoskeletal regulation, redox homeostasis with anti-apoptotic function to counteract any changes within the cellular environment and the surrounding mileu in this highly metabolic cell type. Synthesis and maintanence of myelin sheaths makes the metabolic requirements of energy, iron, omega-3 fatty acids and cholesterol 2-3-fold higher in oligodendrocytes compared to other brain cells (Connor and Menzies, 1996, Sanchez-Abarca et al., 2001, Bartzokis, 2004). Oligodendrocytes are therefore exposed to higher levels of physiological metabolic stress, rendering them more vulnerable to other stressors, insults and injury (Bartzokis, 2004, 2011). However, oligodendrocytes have developed some compensatory mechanisms, such as enrichment of glutathione and high specific activities of glutathione peroxidases and reductases to counteract some of these vulnerabilities (Hirrlinger et al., 2002). Thus

the selective expression of a molecular chaperone such as HspB5 within oligodendrocytes, is well placed to regulate physiological metabolic/oxidative stress arising from myelin formation and maintanence.

HspB5 co-localises with CNPase, a non-compact myelin protein

We confirmed expression of HspB5 in oligodendrocytes and associated myelin by double immunoflourescence labelling with CNPase (Figure 5 and 6). HspB5 immunoreactivity was stronger in the cell bodies compared to CNPase staining, which, although present in cell bodies, was masked by intense staining of the surrounding myelin fibers in the corpus callosum and fimbria (Figure 5). Higher HspB5 protein expression in myelin fibre tracts compared to the cell body may have been predicted given the apparent non-somatic targeting of mRNA (Figure 3). However, mRNA expression levels and cellular localisation do not always correlate with protein expression, which depends on the relative translation efficiency of distinct pools of somatic and targeted mRNA. (Gygi et al., 1999, Wang et al., 2004, Ghazalpour et al., 2011). Thus, our observation that non-somatic mRNA expression does not enhance HspB5 immunoreactivity is not surprising.

MBP staining was present in myelinated fiber tracts, but lacking in the cell soma consistant with localised expression within compact myelin (Girolamo et al., 2010). Similar staining has been reported in the developing and adult chicken retina, where MBP is relatively devoid in the cell soma but concentrated in the myelin sheaths. This study also showed that HspB5 expression was temporally consistent with MBP expression which along with CNPase is expressed in mature oligodendrocytes and myelinated nerve fibers (Reynolds and Wilkin, 1988, Kim et al., 2012).

Hippocampal myelination is relatively sparse in comparison to heavily myelinated structures such as the corpus callosum. This hippocampal organisation allows the

cellular basis of protein expression within oligodendrocytes to be better defined. Characterisation of oligodendrocytes within the hippocampus has revealed differentially distributed sub-populations that are thought to reflect different stages of maturation (Vinet et al., 2010). HspB5 immunostaining within the hippocampus displayed morphological characteristics akin to the CNPase-eGFP-expressing stellar and ramified oligodendrocyte (mature) sub-populations described by Vinet et al. (2010). Interestingly, the HspB5 staining pattern also resembles neuron-glial antigen 2 (NG2) progenitor cells. NG2 is expressed by oligodendrocyte precursor cells (OPCs) (Nishiyama et al., 2014, Dimou and Gallo, 2015). These NG2+, OPCs have a stellar morphology with small cell bodies and thin radiating processes but are usually devoid of mature myelin markers, such as MBP and CNPase (Polito and Reynolds, 2005, Karram et al., 2008). Thus, CNPase immunolabeling allows easy discrimination between NG2 positive pre- and mature oligodendrocytes. However, transgenic mice expressing CNPase-eGFP, revealed a population of CNPase positive cells that were also NG2 positive (Mangin et al., 2008, Vinet et al., 2010). NG2 cells in the gray matter generally remain quiescent, although these cells are OPC's they also have the potential to differentiate into interneurons in the hippocampus (Belachew et al., 2003, Aguirre et al., 2004). Despite the similar morphological characteristics of HspB5 expressing cells to NG2-OPCs, it is unlikely that HspB5 is expressed at the same developmental stage, particularly as its appearance coincides with that of MBP (Kim et al., 2012). Thus, HspB5-expressing cells, which we confirmed to be CNPase positive, are likely mature, myelinating oligodendrocytes.

CNPase, like HspB5 is expressed in oligodrendrocytes in the CNS and also in Schwann cells in the peripheral nervous system. It is localised to the cytoplasmic membrane consistent with a role in membrane synthesis and maintanence as well as

signal transduction (Trapp et al., 1988, Lee et al., 2005). Up to 40% of CNP is thought to associate with the detergent insoluble membrane fraction containing lipid rafts (Kim and Pfeiffer, 1999). HspB5 has also been shown to bind membrane lipids *in vitro* (Cobb and Petrash, 2000). This is dependent on the lipid composition, with a higher binding capacity associated with a higher amount of sphingolipids and lower amounts of phosphotidylethanolamine-related lipids (Grami et al., 2005). Detergent and pH extraction of myelin revealed partitioning of HspB5 into distinct biochemical compartments in a similar fashion to CNPase. Despite its intrinsic solubility, HspB5 can selectively recruit to insoluble fractions with many features consistent with an ability to behave like a peripheral membrane protein (salt extraction) a cytoskeletal associated protein (detergent/pH extraction) and a lipid raft associated protein (detergent/temperature extraction). In all circumstances, HspB5, CNP and MBP partition into both soluble and insoluble fractions, this is likely reflective of multiple physiological (structural and signalling) roles of these proteins. Interestingly, MBP displayed differential isoform extraction profiles, consistent with a role of the 18.5kDa (top band) isoform in compaction as it utilises ionic interactions to drive association with the cytoplasmic face of the myelin membrane (Staugaitis et al., 1990). This biochemical analysis supports expression of HspB5 within the non-compact myelin compartment similar to CNPase.

HspB5 and CNPase also have analogous cellular functions. They both modulate the actin (microfilament) and tubulin (MT) cytoskeleton (Dyer et al., 1995). CNPase acts as a microtubule-associated protein (MAP) linking tubulin with the plasma membrane (Bifulco et al., 2002), whereas HspB5 binds to MTs via MAP's (Fujita et al., 2004). CNPase is able to induce MT polymerisation, and direct the formation of branched process outgrowth (Lee et al., 2005) and HspB5 stabilises the MTs (Fujita et al.,

2004). The overlapping functions of both proteins suggests that HspB5 may also be involved in signalling events, affecting myelin cytoskeletal components, and/or the organisation of membrane architecture. The abundance of HspB5 may thus reflect the intrinsic vulnerabilies of oligodendrocytes and the need for a molecular chaperone that is able to modulate many cellular responses.

HspB5 knockout animals suffer from muscular dystrophy, kyphosis and die prematurely (Brady et al., 2001). This is consistent with phenotypes observed in CNP-deficient mice (Lappe-Siefke et al., 2003). Interestingly these CNP-deficient mice, as well as mice deficient in myelin proteolipid protein (PLP) and myelin associated glycoprotein (MAG) are all well myelinated, with intact compact myelin and a largely normal ultrastructure. However, when examined carefully, these mice develop axonal swellings and progressive, age-dependant axonal degeneration (Klugmann et al., 1997, Griffiths et al., 1998, Lappe-Siefke et al., 2003, Pan et al., 2005, Edgar et al., 2009). Neither the ultrastructural organisation of myelin nor the impact on axonal function has been explored in HspB5 knockout mice.

In summary, our results show that HspB5 is an integral component of CNS myelin with distinct sub-compartment properties. It is well placed to modulate and maintain oligodendroglial function, sustain the elaborate architecture and high intrinsic molecular demand of this cell type and its associated myelin.

Acknowledgments

Financial support from the Gerald Kerkut Charitable Trust is gratefully acknowledged.

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Figure Legends

Figure 1. Anatomical distribution of HspB5 mRNA in brain and spinal cord.

Mouse brain and spinal cord sections were hybridised with radiolabelled antisense oligonucleotide probes against HspB5. A myelin specific mRNA expression pattern was observed with intense staining in white matter structures within the cerebellum (cb) and the corpus callosum (cc) (sagittal section shown) (A.i). Intense HspB5 mRNA expression was observed in spinal cord sections (thoracic section shown) with signal present in both white and gray matter regions (B.i). Sense oligonucleotide probes (control) did not hybridise in brain and spinal cord sections (A.ii and B.ii) proving specificity of the HspB5 signal observed in A.i. and B.i. All sections were processed in parallel and exposed to radioactive probe for 14 days. Images are representative of sections from 3 animals.

Figure 2. Cellular expression of HspB1 (non-myelin, gray matter) and HspB5 (myelin, white matter) mRNA in the CNS. Emulsion radiography was used to visualise mRNA expression of HspB1 and HspB5 in the mouse CNS. Radiolabelling of coronal spinal cord sections with antisense oligonucleotides against HspB1 showed discrete clusters of silver halide grains over the large motor neurons in the ventral horn (A). HspB1 labelling of transverse spinal cord sections also showed labelling of large motor neurons in the gray matter with no labelling of the white matter (B). HspB5 labelling of coronal spinal cord sections showed mRNA expression in cells characteristic of oligodendrocytes that formed linear arrays (C). Brain sections (horizontal section shown) also showed selective labelling of cells characteristic of oligodendrocytes in white matter tracts such as the corpus callosum (D). Sections

were counterstained with Cresyl violet. Images are representative of sections taken from 3 animals.

Figure 3. Somatic and non-somatic labelling of HspB5 mRNA in the brain and spinal cord. High resolution, emulsion radiography was used to visualise HspB5 expression in mouse brain (A) and spinal cord (B-D) sections. In addition to labelling of white matter glial cell bodies, non-somatic labelling was observed to occur in structures emanating away from the cell body (arrows). Distinct labelling in the form of small clumps (arrowheads) reminiscent of RNA granules was observed. Sections were counterstained with Cresyl violet. Images are representative of sections taken from 3 animals.

Figure 4. RNA transport sequence (RTS) homology in the HspB5 mRNA sequence (NM_009964.3). Many mRNAs that are transported within cells contain RTS 1 and 2 sequences (A). The complete mRNA sequence for HspB5 was used to determine homology with RTS decanucleotide sequences. RTS homology in the 5' untranslated region (5' UTR) is highlighted in red. Start and stop codons are highlighted in green (B). HspB5 mRNA shows an almost perfect match to RTS 2 consensus decanucleotide sequence using EMBOSS Needle (Nucleotide Alignment) software. No other regions in the HspB5 mRNA sequence produced overlapping sequence similarity.

ARC, activity related cytoskeleton-associated protein; Ca-N, N-type calcium channel α1; GABAR(A), gaba amino butyric acid receptor α subunit; GFAP, glial fibrillary acidic protein; MOBP, myelin-associated/oligodendrocytic basic protein; NOS, nitric oxide synthase; RC3, neurogranin (Table A, adapted from Ainger et al., 1997).

Figure 5. HspB5 shows co-localisation with the non-compact myelin specific protein CNPase. HspB5 immunostaining co-localised with CNPase staining in white matter rich structures such as the fiber tracts/bundles of the corpus callosum and cingulate gyrus. HspB5 immunofluorescence was more evident in oligodendrocyte cell bodies than CNPase in both fiber tracts of the corpus callosum (arrowheads) and fiber bundles in the cingulate gyrus (arrows). MBP expression also displayed a similar staining of white matter regions as observed for HspB5 and CNPase. Corpus callosum (cc), cingulate gyrus (cg). Scale Bars: 50µM Images are representative of sections taken from 4 animals.

Figure 6. HspB5 expression in the hippocampus and corpus callosum. Coronal brain sections of the dorsal hippocampus including the overlying corpus callosum were subjected to immunohistochemistry for detection of HspB5 immunoreactivity. The sparse myelination of the hippocampus in comparison to other highly myelinated brain regions such as the cc and fi enabled cell body and process staining to be easily observed (A). HspB5 immunoreactivity was observed in the fiber tracts of the cc as well as in cell bodies in and around this region. The SOr had limited HspB5 staining in comparison to the cc (B). Higher magnification images of the cc revealed distinctive staining of the cell bodies as well as fiber tracts (C). The CA3 hippocampal region showed an intricate pattern of staining (D). Immunoreactivity in the CA1 displayed a similar pattern to that observed in the CA3, with intense staining of the cell bodies and a meshwork of processes extending into the Spy (E). Staining of the LMol highlighted the elaborate nature of HspB5 expression in oligodendrocytes within the hippocampus. Cells bodies and processes were clearly and intensely

stained (F). Co-localisation of HspB5 with CNPase confirmed expression in oligodendrocytes within the hippocampus (G.i-iii). Corpus callosum (cc), fimbria (fi), stratum oriens (SOr), stratum pyramidal (Spy), stratum radiatum (SRad), CA3 pyramidal cell layer (CA3Py), lacunosum molecular (LMol). Scale Bars: A, 200µM; B, 100µM; C, 50µM; D, 50µM; E, 50µM; F, 20µM; G, 50µM. Images are representative of sections taken from 4 animals.

Figure 7. Immunogold EM confirms HspB5 selectively localises to oligodendrocytes. HspB5 immunogold labelling showed staining in oligodendrocyte processes ("Gp") navigating an axon ("Ax"). The staining is apparent as a single spherical profile of the appropriate diameter (e.g., arrow) (A). Triple staining (3 gold particles) in an oligodendrocyte ("Oli"). The oligodendrocyte soma contains typical narrow-bore endoplasmic reticulum and has a typical oligodendrocyte nuclei morphology with a single nucleoli visible (B). Double (2 gold particles) and single (1 gold particle) staining of an immature oligodendrocyte. Note the embedded axon profile (center, top), typical of the cell type (C). Triple (3 gold particles) staining of an oligodendrocyte process myelinating an axon ("Ax"), with no staining in neighbouring astrocyte processes ("Ap"). The oligodendrocyte process has deposited compact myelin and the astrocyte processes contain dense glial filaments; both are cell-type specific features (D). Double (2 gold particles) staining of an oligodendrocyte process myelinating an axon (E). Intense focal staining inside an oligodendrocyte (F). 3x higher magnification image of F (G).

Gold particles indicated by arrows. Acronyms used in figure: Axon (Ax), glial (oligodendrocyte) process (Gp), astrocyte process (Ap), oligodendrocyte (Oli). Scale Bars: A and B, 1µM; C and F, 2µM; D and E, 500nM.

Figure 8. Biochemical analysis of HspB5 localisation within myelin. Myelin enriched samples were subjected to salt, pH and detergent extraction. Extraction in salts (Na_2CO_3/NaCl) enables the separation of peripheral membrane proteins. HspB5, CNPase and MBP partitioned into both the pellet and soluble supernatant fractions after Na_2CO_3 treatment. 1M NaCl extraction resulted in the preferential partitioning of HspB5 and MBP into the peripheral membrane associated protein fraction (soluble) whereas CNPase was present equally in both fractions (A). Detergent extraction at pH6 gives rise to a soluble fraction consisting of cytosolic proteins not associated with the cytoskeleton and an insoluble fraction containing cytoskeletal and membrane bound proteins. At pH8, cytoskeletal associated proteins are solubilised and membrane associated proteins partition into the insoluble fraction. Both CNPase and HspB5 partitioned equally in the soluble and insoluble pools (B). However, the low molecular weight (MW) MBP isoforms displayed intrinsic solubility properties and high MW isoforms remained insoluble, consistent with hydrophobic membrane association (Figure 8B). Myelin was further subjected to detergent (TX-100) extraction at 4°C and 37°C. At 4°C membrane proteins that interact with detergent insoluble, glycosphingolipids / cholesterol microdomains (DIGCEMs) are found in the insoluble fraction. At 37°C the DIGCEMs disintegrate and become detergent soluble. HspB5 and CNPase fractionated preferentially in the soluble fraction at 4°C and this was only modestly increased at 37°C (C). Interestingly, the high molecular weight MBP isoforms partitioned predominantly in the insoluble fractions at both 4°C and 37°C (C). Data is representative of three salt, pH and detergent extractions from three independently produced myelin fractions.