Axonal motor protein KIF5A and associated cargo deficits in multiple sclerosis lesional and normal-appearing white matter

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Aims: Understanding the causes of axonal pathology remains a key goal in the pursuit of new therapies to target disease progression in multiple sclerosis (MS). Anterograde axonal transport of many proteins vital for axonal viability is mediated by the motor protein KIF5A, which has been linked to several neurological diseases. This study aimed to investigate the expression of KIF5A protein and its associated cargoes: amyloid precursor protein (APP) and neurofilament (NF) in post mortem MS and control white matter (WM) and to determine if KIF5A expression is influenced by the presence of MS risk single nucleotide polymorphisms (SNPs) identified in the region of the KIF5A gene.

Methods: Using immunoblotting assays we analysed the expression of KIF5A, APP and NF phospho-isoforms in 23 MS cases and 12 controls. Results: We found a significant reduction in KIF5A and associated cargoes in MS WM and an inverse correlation between KIF5A and APP/NF protein levels. Furthermore, homozygous carriers of MS risk gene SNPs show significantly lower levels of KIF5A protein compared to MS patients with no copies of the risk SNPs. Conclusions: We conclude that reduced expression of axonal motor KIF5A may have important implications in determining axonal transport deficits and ongoing neurodegeneration in MS.

Keywords: amyloid precursor protein, axonal transport, KIF5A, multiple sclerosis, neurofilament, white matter

Introduction

Multiple sclerosis (MS) is characterized pathologically by variable degrees of inflammation and neurodegeneration. Axonal loss in both active and chronic white matter (WM) lesions is a key finding of numerous pathological studies and is widely believed to be the substrate for irreversible disability occurring in progressive MS [1,2]. Recent studies have highlighted the presence of early axonal injury in the brain and spinal cord and the potential consequences on long-term axonal viability and neuronal function [3]. Understanding axonal disease pathology remains a key goal in the pursuit of new therapies to attenuate MS disease progression.

Axons require a highly advanced protein transport system due to their high energy demand and the long distances they cover in the CNS. Anterograde axonal transport is largely mediated by kinesin superfamily motor (KIF) proteins which hydrolyse ATP to generate motile forces to shift associated cargoes along the axon via microtubule tracks [4]. There are 45 known members of the KIF family, 38 of which are neuronally enriched [5]. KIFs exist as homo- or heterodimers and can dimerise to form heterotetramers. All KIFs contain a microtubule binding domain and an ATP-
hydrolysing motor domain, which is well conserved between family members. Protein cargo specificity is determined by the KIF carboxy tail domain [6]. KIF function is integral to the entire CNS transport system and several axonal KIFs have been linked to MS susceptibility [7–9]. We have previously reported reduced KIF, (KIF1B, KIF5A and KIF21B), mRNA and protein expression in MS non-lesional grey matter. Notably, our results suggested reduced expression of KIF5A may have particular relevance to MS axonal pathology [10].

Conventional kinesin I was first discovered in 1985 in the squid axon [11]. Kinesin I exists as a heterotrameric structure where protein cargoes generally bind indirectly through association with kinesin light chains (KLCs) and adaptor proteins, bound to the carboxy terminal [12]. Conventional kinesin I, now more commonly known as KIF5, comprises three family members; KIF5A, KIF5B and KIF5C [13]. In humans, KIF5B is ubiquitously expressed, whereas KIF5A and KIF5C are neuronally enriched [14]. Mutations in KIF5A have been linked to several neurological diseases such as hereditary spastic paraplegia (HSP) type 10 (SPG10) [15] and Charcot-Marie-Tooth disease type 2A (CMT-2A) [16] and it is the most extensively studied member of the KIF5 family.

More recently, single-nucleotide polymorphisms (SNPs) in the region of the KIF5A gene (rs12368653 and rs703842), have been linked to MS susceptibility [7,17]. Interestingly, our previous studies found a significant reduction in KIF5A protein expression in non-lesional grey matter in MS patients carrying the rs12368653 SNP [10]. KIF5A is responsible for the transport of several cargoes implicated in neurodegenerative conditions including: phosphorylated neurofilaments (NFs), mitochondria and amyloid precursor protein (APP) [18–20]. In MS, aggregates of APP and various NF phosphoepitopes have been identified pathologically, in the cell body and diffusely along the course of the axon, or as large aggregates in transected axons, commonly termed axonal ‘spheroids’ [21–23].

To better understand disease mechanisms resulting in axonopathy, particularly the abnormal accumulation of intracellular proteins, this study aimed to measure KIF5A expression in MS and control WM and its relationship to associated protein cargoes: APP and phosphorylated NF. This study also aimed to analyse potential differences between KIF5A protein expression in MS patients who are carriers of MS risk gene SNPs (rs12368653 and rs703842), located in the region of the KIF5A gene, which have been linked to MS susceptibility.

Materials and methods

Study cohort

Tissue samples for this study were obtained from The MS Tissue Bank, Imperial College London, UK. The study was carried out according to national ethical guidelines and legal regulations. Experiments were performed on snap-frozen and paraffin-embedded tissue samples from 23 cases of neuropathologically confirmed MS and 12 controls (the deaths of whom were not linked to neurological disease; Table 1). Analysis of MS WM tissue encompassed demyelinated areas, subcategorized into active, chronic active and silent lesions. Normal appearing WM was also studied. The extent of demyelination and lesion classification was performed prior to obtaining tissue by neuropathologists within the MS Tissue Bank.

Tissue dissection and protein extraction

Slices approximately 1 mm in width were cut from each MS and control snap-frozen tissue block using a cryostat blade. Whilst on ice, WM was dissected away from grey matter tissue. Protein was extracted from each WM sample using the Paris Kit (Ambion™; Life Technologies Ltd, Paisley, UK) according to manufacturers’ instructions. Samples were homogenized using Precellys® automated homogenizer (Stretton Scientific, Derbyshire, UK) and stored at −80°C until required.

Antibody specificity

Primary antibodies used for all protein and histological analysis in this study were as follows: rabbit anti-KIF5A (HPA004469; Sigma-Aldrich Ltd, Dorset, UK), mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (AB9484; Abcam, Cambridge, UK); rabbit anti-APP (13-0200; Zymed; Life Technologies Ltd, Paisley, UK), mouse anti-NF-70 (MAB1615; Millipore, Hertfordshire, UK).
pore), mouse anti-SMI32 (SMI32-R; Cambridge Biosciences; Cambridge, UK), mouse anti-SMI31 (SMI31-R; Cambridge Biosciences), mouse anti-SMI34 (SMI34-R; Cambridge Biosciences) and mouse anti-SMI36 (SMI36-R; Cambridge Biosciences). All primary antibodies used for immuno dot-blotting were tested for specificity against chosen antigens using western blotting techniques, as previously documented [10,22]. All antibodies displayed specific bands as described on manufacturer data sheets and/or relative to their reported molecular weights.

**Immuno dot-blotting**

Immuno dot-blotting using the following primary antibodies: KIF5A (1:15 000), GAPDH (1:15 000), NSE (1:2000), APP (1:500), SMI32 (1:3000), SMI31 (1:5000), SMI34 (1:5000), SMI36 (1:5000) and NF-70.

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**Table 1.** Clinical characteristics of MS and control patient cohort

| MS patient no. | Age (years) | Sex (M/F) | Disease duration (years) | Disease course | Lesion types and NAWM | Post mortem delay (h) |
|----------------|-------------|-----------|--------------------------|----------------|-----------------------|-----------------------|
| MS 1           | 49          | F         | 18                       | SP             | CA, S, NAWM           | 31                    |
| MS 2           | 54          | F         | 17                       | SP             | S, NAWM               | 22                    |
| MS 3           | 46          | M         | 8                        | SP             | CA, S, NAWM           | 7                     |
| MS 4           | 39          | F         | 21                       | SP             | A, S, NAWM            | 18                    |
| MS 5           | 38          | M         | 16                       | SP             | A, CA, S, NAWM        | 19                    |
| MS 6           | 50          | F         | 26                       | SP             | CA, S, NAWM           | 12                    |
| MS 7           | 44          | F         | 14                       | SP             | A, CA, S, NAWM        | 18                    |
| MS 8           | 48          | F         | 30                       | SP             | S, NAWM               | 28                    |
| MS 9           | 51          | F         | 21                       | SP             | A, CA, S, NAWM        | 10                    |
| MS 10          | 44          | F         | 19                       | SP             | A, CA, S, NAWM        | 20                    |
| MS 11          | 53          | M         | 16                       | SP             | A, CA, S, NAWM        | 17                    |
| MS 12          | 53          | M         | 11                       | SP             | A, CA, S, NAWM        | 12                    |
| MS 13          | 49          | F         | 14                       | SP             | A, CA, S, NAWM        | 24                    |
| MS 14          | 44          | M         | 10                       | SP             | A, CA, S, NAWM        | 16                    |
| MS 15          | 88          | F         | 30                       | PP             | A, CA, S, NAWM        | 22                    |
| MS 16          | 70          | F         | 12                       | SP             | A, CA, S, NAWM        | 13                    |
| MS 17          | 42          | F         | 19                       | SP             | A, CA, S, NAWM        | 31                    |
| MS 18          | 39          | F         | 15                       | SP             | A, CA, S, NAWM        | 15                    |
| MS 19          | 57          | F         | 19                       | SP             | A, CA, S, NAWM        | 12                    |
| MS 20          | 45          | M         | 16                       | SP             | A, CA, S, NAWM        | 7                     |
| MS 21          | 45          | M         | 18                       | SP             | A, CA, S, NAWM        | 9                     |
| MS 22          | 51          | F         | 21                       | SP             | A, CA, S, NAWM        | 15                    |
| MS 23          | 64          | M         | –                        | Cardiac failure| –                     | 18                    |
| MS 24          | 92          | M         | –                        | Cardiac failure| –                     | 13                    |
| MS 25          | 84          | F         | –                        | Cardiac failure| –                     | 24                    |
| MS 26          | 69          | F         | –                        | Lung cancer    | –                     | 33                    |
| MS 27          | 35          | M         | –                        | Tongue carcinoma| –                    | 22                    |
| MS 28          | 78          | F         | –                        | Myeloid leukaemia| –                    | 33                    |
| MS 29          | 60          | F         | –                        | Ovarian cancer | –                     | 13                    |
| MS 30          | 88          | M         | –                        | Prostate cancer| –                     | 22                    |
| MS 31          | 68          | M         | –                        | Cardiac failure| –                     | 30                    |
| MS 32          | 84          | M         | –                        | Bladder cancer | –                     | 5                     |
| MS 33          | 82          | M         | –                        | Myelodysplastic syndrome| –        | 21                    |
| MS 34          | 54          | M         | –                        | Lung cancer    | –                     | 20                    |
| Mean           | 50          | 18        | –                        | –              | –                     | 17                    |
| SD             | 11          | 6         | –                        | –              | –                     | 7                     |

| Control no.   | Age (years) | Sex (M/F) | Cause of death | Post mortem delay (h) |
|---------------|-------------|-----------|-----------------|-----------------------|
| C 1           | 64          | M         | Cardiac failure | –                     | 18                    |
| C 2           | 92          | M         | Cardiac failure | –                     | 13                    |
| C 3           | 84          | F         | Cardiac failure | –                     | 24                    |
| C 4           | 69          | F         | Lung cancer     | –                     | 33                    |
| C 5           | 35          | M         | Tongue carcinoma| –                     | 22                    |
| C 6           | 78          | F         | Myeloid leukaemia| –                     | 33                    |
| C 7           | 60          | F         | Ovarian cancer  | –                     | 13                    |
| C 8           | 88          | M         | Prostate cancer | –                     | 22                    |
| C 9           | 68          | M         | Cardiac failure | –                     | 30                    |
| C 10          | 84          | M         | Bladder cancer  | –                     | 5                     |
| C 11          | 82          | M         | Myelodysplastic syndrome| –        | 21                    |
| C 12          | 54          | M         | Lung cancer     | –                     | 20                    |
| Mean          | 72          |           |                 | –                     | 21                    |
| SD            | 17          |           |                 | –                     | 8                     |

A, active; CA, chronic active; F, female; M, male; MS, multiple sclerosis; NAWM, normal-appearing white matter; PP, primary progressive; S, silent; SP, secondary progressive.
(1:10 000), was performed as previously described [10]. Protein expression was visualized using a chemiluminescence EZ-ECL kit (1:1; Geneflow, Staffordshire, UK) in conjunction with a Biorad Universal III Bioplex imager. Densitometric analysis of protein dots was performed using Image Lab™ 5.0 software (Biorad, Hertfordshire, UK). Values were expressed relative to appropriate loading control proteins; GAPDH, NF 70 kDa (NF-70) and neuron-specific enolase (NSE).

Genotyping

Genotype data for all 23 MS patients whose tissue was used in this study were available from the International MS Genetics Consortium (IMSGC) and Wellcome Trust Case Control Consortium 2 (WTCCC2) genome-wide association studies (GWAS) [17]. Protein data was separated according to patient genotype, based upon those who were homozygous or heterozygous carriers of adenine SNPs located in the region of the KIF5A gene (rs12368653 and rs703842).

Immunoperoxidase staining of paraffin sections

Seven micrometre sections taken from blocks of the frontal and temporal lobes were stained with rabbit primary antibody KIF5A (1:500). Sections were dewaxed, hydrated and immersed in 3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. Sections were subsequently rinsed in phosphate-buffered saline (PBS) and pretreated with sodium citrate buffer in the microwave (0.01 M; pH 6.0, 5 min) to unmask antigenic sites, followed by additional PBS rinses. Immunostaining was performed using the Vectastain® Universal Elite® ABC kit (Vector Laboratories, Peterborough, UK). Sections were blocked in universal blocking serum for 30 min at room temperature before incubation overnight at 4°C with primary antibody diluted in 3% BSA/PBS. Sections were then rinsed in PBS and incubated for 30 min with a secondary biotinylated universal antibody and 30 min with Vecta elite ABC complex, followed by a 10 min incubation with 3,3’-diaminobenzidine (DAB) and 0.01% H2O2. Sections were washed in water, immersed in copper sulphate DAB enhancer (4 min), counterstained with Gills haematoxylin (Sigma-Aldrich Ltd), dehydrated, cleared and mounted. Controls in each run included sections incubated overnight in PBS instead of the primary antibody.

Immunofluorescence staining of paraffin sections

Seven micrometre sections taken from blocks of the frontal and temporal lobes were co-stained with primary antibody rabbit KIF5A (1:200) combined with mouse APP, CNPase and GFAP (all 1:200). Sections were dewaxed, hydrated and immersed in 5 mM copper sulphate/50 mM ammonium acetate for 1 h to block auto-fluorescence. Sections underwent pretreatment with sodium citrate buffer in the microwave (0.01 M; pH 6.0, 5 min) to unmask antigenic sites, followed by PBS rinses. Section were blocked using 10% normal goat serum/0.1% triton/PBS for 1 h at R/T. Sections were subsequently incubated overnight in primary antibodies diluted in blocking solution. After PBS rinses sections were incubated in Alexa fluor® secondary antibodies (Invitrogen, Life Technologies Ltd, Paisley, UK); goat anti-rabbit 555 (IgG) and goat anti-mouse 488 (IgG) (both 1:500) for 45 min at room temperature in the absence of light, before mounting in Vectashield with DAPI (Vector Laboratories) for nuclei identification. Images were acquired using an inverted Leica CTR 6000 fluorescence microscope (Leica Microsystems Ltd, Milton Keynes, UK) and merged with Leica Application Suite Advanced Fluorescence (LAS AF) software.

Statistical analysis

Statistical analysis of data employed a regression model using Stata v12 (StataCorp LP, College Station, TX, USA), which allowed for any correlation among multiple sections coming from the same brain (cluster option). Non-parametric bootstrap analysis was used to estimate standard errors and confidence intervals to account for possible non-normality of the parameter’s distribution. All graphs were generated using GraphPad Prism5™ (GraphPad Software Inc., San Diego, CA, USA). Linear regression, correlation and Student’s t-test were also performed on PRISM 5 for patient cohort analysis of age and post mortem delay of the tissue. For all tests, values of P < 0.05 were considered statistically significant.

Results

Sample cohort variances

The study cohort of patient clinical histories (Table 1) was analysed to determine any potential effect of age
or post mortem delay of tissue on protein expression. There was no significant difference in the mean post mortem delay of tissue collection between control (21 ± 8 h) and MS patients (17 ± 7 h) (P = 0.13; Table 1). However, there was a significant difference in the mean age of control (72 years) and MS patients (50 years) (P < 0.001; Table 1), related to the high variability in MS disease course. Further linear regression analysis revealed no significant effect of patient age on KIF5A, APP and SMI31 protein expression in control and MS cases, when normalized to both GAPDH and NSE (data not shown).

Reduced KIF5A expression in MS WM

The immuno dot blot technique was used to collect data on KIF5A protein expression in the cohort and protein levels normalized to respective ubiquitous house-keeping proteins GAPDH and neuronal-specific NSE. The results showed a significant reduction in KIF5A protein expression in MS WM compared to control, when normalized to GAPDH (P < 0.05) and NSE (P < 0.001; Figure 1A). Further segregation of MS WM data into lesion subtypes, predefined by immunohistochemical analysis, revealed a significant reduction in KIF5A protein expression in active (P < 0.001; normalized to GAPDH and NSE), chronic active (P < 0.01; normalized to NSE) and silent (P < 0.001; normalized to NSE), WM lesions compared to control. KIF5A expression was also significantly reduced in nonlesional normal-appearing WM compared to control, when normalized to GAPDH (P < 0.01) and NSE (P < 0.05; Figure 1B).

Altered expression of associated KIF5A protein cargoes in MS WM

In addition to measuring KIF5A expression, we analysed the expression of phosphorylated NF, which is known to be vital in maintaining axonal structure and integrity. We performed immunoblotting using the well-characterized Sternberger monoclonal antibody SMI31, which is directed against phosphorylated epitopes in the NF-H and NF-M subunit. The results showed a significant reduction in phosphorylated NF (SMI31) protein expression in MS WM compared to control, when normalized to GAPDH (P < 0.01) and NSE (P < 0.001; Figure 2A). Furthermore, segregation of the data into MS lesion subtypes showed a significant reduction in phosphorylated NF across all WM lesion types: active (P < 0.05; normalized to GAPDH and NSE), chronic active (P < 0.05; normalized to NSE) and silent (P < 0.001 normalized to GAPDH; P < 0.01 normalized to NSE), when compared with control WM. Phosphorylated NF expression was also reduced in nonlesional normal-appearing WM compared to control.

Figure 1. Reduction in KIF5A protein expression in MS patient WM. (A) Protein levels derived from immunoblotting show a significant reduction in motor protein KIF5A expression in MS WM (n = 23) compared to control WM (n = 12), when normalized to GAPDH and NSE. (B) Data categorized according to lesion types show a significant reduction in KIF5A protein expression in active lesions (n = 15) compared to control, when normalized to GAPDH and NSE; and in chronic active (n = 16) and silent (n = 16) lesions, when normalized to the neuronal protein NSE. KIF5A protein expression is also significantly reduced in MS NAWM (n = 15) compared to control, when normalized to GAPDH and NSE. Results expressed as ±SEM. *P < 0.05, **P < 0.01, ***P < 0.001. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KIF5A, kinesin superfamily protein 5A; MS, multiple sclerosis; NAWM, normal-appearing white matter; NSE, neuron-specific enolase; SEM, standard error of the mean.

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when normalized to GAPDH and NSE ($P < 0.01$; Figure 2B). In addition, we analysed protein expression of APP but found no difference between MS and control, when normalized to GAPDH ($P = 0.55$) and NSE ($P = 0.06$; data not shown). Expression of KIF5A appeared to be almost exclusively axonal; co-staining of KIF5A and CNPase (an oligodendrocyte marker) was absent and co-staining of KIF5A and GFAP (an astrocyte marker) was minimal across control, MS lesional and MS normal-appearing WM (NAWM; data not shown).

Correlation analysis was performed to elucidate any relationship between KIF5A expression and its well-characterized cargoes; phosphorylated NF and APP. Cargo proteins normalized to neuronal NSE were correlated against KIF5A protein normalized to ubiquitous GAPDH to avoid the occurrence of a spurious correlation [24]. Interestingly, the correlation coefficients revealed a moderate negative correlation, both of which were highly significant, between KIF5A and both cargoes: SMI31 (Spearman $r = -0.32$; $P < 0.01$; Figure 3A) and APP (Pearson $r = -0.37$; $P < 0.01$; Figure 3B). This suggests that lower levels of KIF5A are associated with higher levels of SMI31 and APP in the WM, which could be linked to pathological protein accumulations found commonly in MS axons. Indeed, immunoperoxidase and immunofluorescence staining performed on paraffin-embedded sections from active lesions in MS WM revealed the presence of KIF5A positive axonal swellings and co-positive KIF5A/APP spheroids (Figure 3C).

**Variation in expression of NF phospho-forms in MS WM**

Neurofilaments exist in several phospho-forms and are commonly found pathologically in MS tissue to be dephosphorylated or hyperphosphorylated. NF-70 is a marker for the NF light chain (NF-L), which is phospho-independent but is required for the assembly of the NF heteropolymer containing NF-M or NF-H and is therefore deemed a suitable marker for total NF expression. NFs are vital for axonal structure and integrity and our results have shown a significant reduction in total NF in MS compared to control when normalized to NSE ($P < 0.01$; Figure 4A). In addition, total NF expression was reduced across MS lesion types (active; $P < 0.01$ and silent; $P < 0.001$) and NAWM ($P < 0.01$), compared to control, when normalized to NSE (Figure 4B). This gives us insight into total axon loss in MS WM. Due to their pathological links with axon pathology and MS, we investigated the expression of several NF phospho-forms using a panel of highly specific Sternberger monoclonal antibodies, normalized to both general neuronal marker NSE and axonal NF marker NF-70. There was no significance difference in phosphorylated NF-M/NF-H expression (SMI31) when
Figure 3. Relationship between transport motor protein KIF5A and associated cargoes: APP and NF in MS patients. (A) Protein results obtained from immunoblotting show a significant negative correlation between KIF5A protein levels normalized to GAPDH and SMI31 (NF-M and NF-H subunit), levels normalized to NSE ($n = 77$). (B) Significant negative correlation between KIF5A protein levels normalized to GAPDH and APP protein levels normalized to NSE ($n = 72$). Values are expressed as the mean ± 95% CI; **$p < 0.01$. (C) Immunoperoxidase staining of frontal lobe active lesion sections from paraffin-embedded MS tissue show KIF5A immunopositive axons in MS WM and the presence of KIF5A-positive axonal swellings (i) and spheroid formation (ii), highlighted by black arrows. Immunofluorescence staining of paraffin-embedded MS sections confirms immunonegative staining of control WM for spheroids (iii) and immunopositive spheroid staining in MS NAWM (iv) and active lesion (v). Enlarged images of MS active lesion spheroids denoted by the white box (v) confirm immunopositive co-staining of KIF5A (red; $v_{a}$) and APP (green; $v_{b}$) and immunonegative staining for the nuclear marker DAPI (blue; $v_{c}$), circled in white. Scale bar 50 μM. APP, amyloid precursor protein; CI, confidence interval; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KIF5A, kinesin superfamily protein 5A; MS, multiple sclerosis; NAWM, normal-appearing white matter; NF-M, neurofilament medium; NF-H, NF heavy; NSE, neuron-specific enolase.
normalized to total NF (NF-70; Figure 5A). SMI36, a maker of phosphorylated NF-H, was significantly reduced in MS compared to control when normalized to NSE \((P < 0.001; \text{Figure 5B})\) but not significantly different from control when normalized to NF-70 (Figure 5C).

Like SMI31, SMI34 is a marker of phosphorylated NF-M and NF-H but has also been implicated as a pathological hallmark of NF-H hyperphosphorylation in MS cell bodies and axons \([22,23]\) and in neurofibrillary tangles in Alzheimer’s disease \([25]\). Interestingly, we found a significant reduction in SMI34 expression in MS compared to control when normalized to NSE \((P < 0.05; \text{Figure 5D})\) and conversely, a significant increase in SMI34 expression in MS compared to control when normalized to total NF \((P < 0.05; \text{Figure 5E})\). Similarly, SMI32, a marker of dephosphorylated NF-H, is significantly reduced in MS compared to control, when normalized to NSE \((P < 0.05; \text{Figure 5F})\) but significantly increased when normalized to total NF \((P < 0.01; \text{Figure 5G})\). These results point towards a general loss of NF in MS WM with remaining axonal NFs more susceptible to hyperphosphorylation and dephosphorylation.

### Differential expression of KIF5A protein and dephosphorylated NF in patient carriers of MS risk SNPs rs12368653 and rs703842

Genotype data for all 23 MS cases studied were made available through IMSGC and WTCCC2 GWAS studies \([17]\); based on the discovery of MS risk SNPs rs12368653 and rs703842, located in the region of the KIF5A gene. These SNPs are found more frequently in MS patients compared with the general population and therefore are believed to confer disease susceptibility. KIF5A data separated according to patient genotype revealed a significant decrease in KIF5A protein expression in patients who are homozygous (AA; \(P < 0.01\)) or heterozygous (AG; \(P < 0.05\)) carriers of the rs12368653 risk gene SNP compared with patients who carry no copies (GG), when normalized to GAPDH (Figure 6A). Similarly, MS carriers of the rs703842 risk gene SNP have significantly lower levels of KIF5A protein compared to patients with no copies (GG), when normalized to GAPDH (AA; \(P < 0.01\), AG; \(P < 0.01\)) and NSE (AA; \(P < 0.001\), AG; \(P < 0.001\)) (Figure 6B). Interestingly, combined rs12368653 and rs703842 risk SNP data show patients with varying frequencies of adenine substitutions \((1–4)\) have decreased KIF5A protein expression compared to homozygous guanine carriers, when normalized to GAPDH (GG/AG; \(P < 0.01\), AG; \(P < 0.01\)) and NSE (AA; \(P < 0.001\), AG; \(P < 0.001\)) (Figure 6C). Interestingly, combined rs12368653 and rs703842 risk SNP data show patients with varying frequencies of adenine substitutions \((1–4)\) have decreased KIF5A protein expression compared to homozygous guanine carriers, when normalized to GAPDH (GG/AG; \(P < 0.01\), AG; \(P < 0.01\)) and NSE (AA; \(P < 0.001\)) (Figure 6D). In addition, we analysed expression of dephosphorylated NF, a pathological hallmark of MS, according to MS genotype. The results showed a significant increase in dephosphorylated NF expression in patients heterozygous (AG/AG; \(P < 0.05\)) or homozygous (AA/AA; \(P < 0.05\)) for both the rs12368653 and

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**Figure 4.** Reduced total NF expression in MS WM. (A) Protein levels derived from immunoblotting show a significant reduction in total NF (NF-70; Figure 5A). SMI36, a maker of phosphorylated NF-H, was significantly reduced in MS compared to control when normalized to NSE \((P < 0.001; \text{Figure 5B})\) but not significantly different from control when normalized to NF-70 (Figure 5C).

Like SMI31, SMI34 is a marker of phosphorylated NF-M and NF-H but has also been implicated as a pathological hallmark of NF-H hyperphosphorylation in MS cell bodies and axons \([22,23]\) and in neurofibrillary tangles in Alzheimer’s disease \([25]\). Interestingly, we found a significant reduction in SMI34 expression in MS compared to control when normalized to NSE \((P < 0.05; \text{Figure 5D})\) and conversely, a significant increase in SMI34 expression in MS compared to control when normalized to total NF \((P < 0.05; \text{Figure 5E})\). Similarly, SMI32, a marker of dephosphorylated NF-H, is significantly reduced in MS compared to control, when normalized to NSE \((P < 0.05; \text{Figure 5F})\) but significantly increased when normalized to total NF \((P < 0.01; \text{Figure 5G})\). These results point towards a general loss of NF in MS WM with remaining axonal NFs more susceptible to hyperphosphorylation and dephosphorylation.

### Differential expression of KIF5A protein and dephosphorylated NF in patient carriers of MS risk SNPs rs12368653 and rs703842

Genotype data for all 23 MS cases studied were made available through IMSGC and WTCCC2 GWAS studies \([17]\); based on the discovery of MS risk SNPs rs12368653 and rs703842, located in the region of the KIF5A gene. These SNPs are found more frequently in MS patients compared with the general population and therefore are believed to confer disease susceptibility. KIF5A data separated according to patient genotype revealed a significant decrease in KIF5A protein expression in patients who are homozygous (AA; \(P < 0.01\)) or heterozygous (AG; \(P < 0.05\)) carriers of the rs12368653 risk gene SNP compared with patients who carry no copies (GG), when normalized to GAPDH (Figure 6A). Similarly, MS carriers of the rs703842 risk gene SNP have significantly lower levels of KIF5A protein compared to patients with no copies (GG), when normalized to GAPDH (AA; \(P < 0.01\), AG; \(P < 0.01\)) and NSE (AA; \(P < 0.001\), AG; \(P < 0.001\)) (Figure 6B). Interestingly, combined rs12368653 and rs703842 risk SNP data show patients with varying frequencies of adenine substitutions \((1–4)\) have decreased KIF5A protein expression compared to homozygous guanine carriers, when normalized to GAPDH (GG/AG; \(P < 0.01\), AG; \(P < 0.01\)) and NSE (AA; \(P < 0.001\)) (Figure 6C). Interestingly, combined rs12368653 and rs703842 risk SNP data show patients with varying frequencies of adenine substitutions \((1–4)\) have decreased KIF5A protein expression compared to homozygous guanine carriers, when normalized to GAPDH (GG/AG; \(P < 0.01\), AG; \(P < 0.01\)) and NSE (AA; \(P < 0.001\)) (Figure 6D). In addition, we analysed expression of dephosphorylated NF, a pathological hallmark of MS, according to MS genotype. The results showed a significant increase in dephosphorylated NF expression in patients heterozygous (AG/AG; \(P < 0.05\)) or homozygous (AA/AA; \(P < 0.05\)) for both the rs12368653 and

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rs703842 risk SNP, when compared with patients with no copy of either SNP (GG/GG; Figure 7).

**Discussion**

Understanding how axonal transport deficits contribute to MS pathology is an emerging field [26]. In this study, we compared post mortem WM tissue from control patients with no sign of neurodegenerative disease to MS cases; mainly with late-stage secondary progressive disease and including different types of inflammatory lesions and normal-appearing WM. Our results showed a significant decrease in KIF5A protein expression in MS WM compared to control. We also investigated KIF5A protein expression in relation to inflammation by comparing KIF5A expression in lesional- and normal-appearing WM. When compared with control, there is a significant reduction in KIF5A protein expression across several lesions types with varying degrees of inflammatory infiltrates: active, chronic active and silent, and also in normal-appearing WM. This suggests that reduced KIF5A expression in MS not only occurs in acute inflammatory lesions but may be part of a widespread neurodegenerative pathological process.
Moreover, we have previously shown reduced KIF5A expression in non-lesional MS grey matter [10]. The importance of KIF5A-mediated axonal transport has been highlighted in numerous studies. Knock-out of the \textit{kif5a} gene in a mouse model has proven neonatally lethal and targeting of the \textit{kif5a} gene postnatally in the same model, using a synapsin-promoted Cre-recombinase transgene, caused NF accumulation in peripheral sensory neuronal cell bodies, reduced axon calibre, axon loss and hind limb paralysis [18]. More recently, studies in zebrafish have found \textit{kif5a} to be essential for mitochondrial localization in the axon, with zebrafish mutants displaying axonal degeneration [27]. We hypothesize that reduced KIF5A expression affects axon viability through disrupted transport of intracellular cargoes, such as NFs, mitochondria and APP. Our results demonstrated a significant reduction in phosphorylated neurofilament (NF-M and NF-H) expression in MS lesional WM and NAWM, compared to control. NF phosphorylation in the axon is vital for maintaining axonal viability. The three main NF subunits: NF-light (NF-L; 68 kDa), NF-medium (NF-M; 150 kDa) and NF-heavy (NF-H; 190–210 kDa) co-assemble to form type IV intermediate filaments; one of the main cytoskeletal components of the axon. The C-terminal region in the NF-M and NF-H subunit con-

Figure 6. Influence of MS patient genotype and the presence of risk gene SNPs on KIF5A protein levels. Protein data (collected via immunoblotting techniques), was separated according to MS patient genotype (\(n = 23\)). (A) KIF5A protein expression was significantly reduced in patients who are heterozygous (AG; \(n = 9\)) or homozygous (AA; \(n = 7\)) carriers of the MS risk rs12368653 gene SNP located in the region of the KIF5A gene, compared to MS patients with no copies of the SNP (GG; \(n = 7\)), when normalized to GAPDH. (B) KIF5A protein expression was significantly reduced in heterozygous (AG; \(n = 9\)) and homozygous (AA; \(n = 10\)) MS carriers of the MS risk rs703842 gene SNP located in the region of the KIF5A gene, compared to MS patients with no copies of the SNP (GG; \(n = 4\)), when normalized to both GAPDH and NSE. (C) MS patient data categorized according to genotypes containing the presence of both MS risk rs12368653 and rs703842 gene SNPs show significant decreases in KIF5A protein expression in patients with varying copies of the SNPs (GG/AG, \(n = 3\); AG/AG, \(n = 6\); AG/AA, \(n = 3\); AA/AA, \(n = 7\)), compared to patients with no copies of the SNPs (GG/GG; \(n = 4\)), when normalized to GAPDH and NSE (D). Results expressed as ±SEM. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\). AA, homozygous adenine; AG, adenine and guanine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GG, homozygous guanine; KIF5A, kinesin superfamily protein 5A; NSE, neuron-specific enolase; SEM, standard error of the mean; SNP, single-nucleotide polymorphism.
tains 10–15 and 40–50 lysine-serine-proline (KSP) repeats, respectively, which act as phosphorylation sites. These phosphorylation sites are regulated through the activation of proline-directed kinases which are active in both the cell body and proximal axon [28]. Phosphorylation of NF-M and NF-H KSP repeats within the C-terminal side arms increases the charge based repulsion of neighbouring filaments, causing an overall increase in interfilament spacing and axonal calibre, which is important in optimizing axonal saltatory conduction [29]. Reduced NF phosphorylation within the axon may decrease axonal calibre and hence viability in MS. Historically, abnormal NF phosphorylation has been identified and associated with axonal pathology in MS [21,23]. Therefore, in this study we used a panel of well-characterized antibodies to NF phospho-isoforms to determine any changes in NF phosphorylation states in addition to our findings of decreased phosphorylated NF in MS WM. To achieve this, we normalized protein expression of total NF and NF phospho-isoforms to NSE, which is ubiquitously expressed in neurons. The results showed a net loss of total NF and NF phospho-isoforms in MS neurons compared to control, consistent with the process of axonal loss that is known to occur in the disease. In addition, we normalized protein expression of NF phospho-isoforms to NF-70 (NF-L subunit) for analysis of NF-specific changes within the axon. NF-M and NF-H need to be in the presence of the NF-L subunit to form intermediate filaments [29]. NF-L head domain phosphorylation in the cell body inhibits NF assembly and its dephosphorylation is necessary to allow polymerization of NF proteins (NF-L, NF-M and NF-H), prior to their transport into the axon. When normalized to NF-70, phosphorylated NF levels in MS WM were not significantly different from control but levels of NF hyperphosphorylation (SMI34) and dephosphorylation levels (SMI32) were significantly increased in MS. This finding is in keeping with previous documentation of NF dephosphorylation and hyperphosphorylation in MS [21,22,30]. The aberrant phosphorylation status of NFs in MS axons could be attributed to dysregulated transport via KIF5A.

To further investigate this hypothesis we correlated protein expression of phosphorylated NFs with KIF5A expression in MS patients and found a significant negative correlation, suggesting that lower levels of KIF5A leads to an accumulation of phosphorylated NF. We further studied protein expression of APP, another well-characterized cargo of KIF5A. We saw no significant difference in overall APP expression in MS WM compared to control but did find aggregates of APP on immunohistochemistry [31]. Evidence suggests that accumulation of APP within axons relates to impaired axonal transport. In MS there is no suggestion that APP expression is altered and our findings are in keeping with this. We did find a significant inverse correlation between KIF5A and APP expression which supports the hypothesis that reduced KIF5A expression relates to APP accumulation. APP is a type I transmembrane protein with suspected roles in cell adhesion, regulating gene expression and iron export [32]. It is thought in axonal transport that APP acts as a cargo receptor for other binding proteins and that fast anterograde axonal transport of APP towards the synapse via KIF5A is mediated through KLC association with APP-containing vesicles [19]. Historically, APP accumulation has been identified pathologically in MS active and chronic lesions [33]. Immunofluorescence staining performed on paraffin-embedded sections in this study

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Figure 7. Increased dephosphorylated NF levels associated with axon pathology, in MS patients carrying risk gene SNPs. Protein data (collected via immunoblotting techniques), were separated according to MS patient genotype (n = 23). Dephosphorylated NF (SMI32) expression was significantly increased in patients who are heterozygous (AG; n = 6) or homozygous (AA; n = 7) carriers of the MS risk rs12368653 and rs703842 gene SNPs, compared to patients with no copies of either SNP (GG; n = 4), when normalized to NF-70. Results expressed as ±SEM. *P < 0.05. **P < 0.01. ***P < 0.001. AA, homozygous adenine; AG, adenine and guanine; GG, homozygous guanine; KIF5A, kinesin superfamily protein 5A; NF, neurofilament; NSE, neuron-specific enolase; SEM, standard error of the mean; SNP, single-nucleotide polymorphism.
confirmed the presence of co-stained KIF5A/APP-positive spheroids in MS active lesions and NAWM. The reasons behind APP accumulation in MS remain elusive but we hypothesize accumulation is influenced by deficiencies in anterograde transport, potentially through reduced motor protein KIF5A expression and/or inflammatory mediators affecting KIF5A binding affinity for its cargo [34]. Large protein accumulations in the axon, comprising both NFs and APP, are likely to hamper existing transport processes, likened to an intracellular ‘traffic jam’ [35]. With increasing axonal blockages and reduced protein transport the energy demands of the cell will not be met, resulting in increased reactive oxygen species production; lipid peroxidation, NF cross-linking and eventual axon atrophy and net neuronal loss [36,37].

Taken together, our results suggest that MS WM areas with higher levels of KIF5A are associated with less accumulation of APP and phosphorylated NF. Furthermore, our previous studies in MS non-lesional grey matter have shown a significant positive correlation between KIF5A expression and patient disease duration, suggesting increased survival time that we postulate is partially attributed to maintained axonal cytoskeletal transport mechanisms [10].

Recent GWAS have identified risk SNPs in the region of the KIF5A gene (rs12368653 and rs703842) which confer susceptibility to MS [7,17]. The rs12368653 SNP is an intronic adenine substitution (predicted in a transcription factor binding site), in the AGAP2 gene (ArfGAP with GTPase domain, ankyrin repeat and PH domain 2), upstream of the KIF5A gene by approximately 0.2 Mb, which does not alter the amino acid sequence. The rs12368653 SNP is downstream of the rs703842 SNP by <0.1 Mb. The rs703842 SNP is a missense mutation (predicted in the transcription start site/enhancer region), in the CYP27B1 gene (cytochrome P450, family 27, subfamily B, polypeptide 1), upstream of the KIF5A gene by approximately 0.3 Mb. The rs703842 nonsynonymous SNP is an adenine substitution that causes a change in the amino acid sequence from an isoleucine to threonine. Linkage disequilibrium is relatively strong between certain SNPs and therefore the non-coding regions within several genes close can affect disease susceptibility [38,39]. SNPs that are not in protein-coding regions can still affect gene splicing, transcription factor binding, mRNA degradation, or the sequence of non-coding RNA. The rs12368653 and rs703842 SNPs form part of a locus control region: a group of multiple cis-acting elements, including enhancers, silencers and insulators which are involved in regulating an entire locus or gene cluster [7].

We have previously shown reduced KIF5A protein expression in MS patients with the rs12368653 risk gene SNP in non-lesional grey matter [10]. Our results in WM show patients who are either heterozygous or homozygous carriers of the rs12368653 or rs703842 risk gene SNP have significantly lower levels of KIF5A protein expression when compared with MS patients who carry no copies of the risk gene SNP. Moreover, combining genotype data for both the rs12368653 and rs703842 risk SNPs suggest any number of adenine substitutions can decrease KIF5A protein expression in MS patients. Mutations in KIF5A have previously been linked to both CMT-2A and HSP. A missense mutation in the KIF5A gene (N256S) causes an autosomal dominant form of HSP (SPG10), which has been implicated in disturbed intracellular axonal transport and is characterized by axonal loss in the corticospinal tract [15,40]. Due to similarities in disease phenotype between MS and HSP, one genotype study analysed 68 SNPs across 11 genes linked to HSP in a MS cohort, including SNPs in the region of the KIF5A gene. The data revealed no significant effect of HSP SNPs on MS susceptibility but it did correlate SNPs in the region of KIF5A to MS patient clinical outcomes, with some initial differences found in the frequency of two SNPs (rs2620678 and rs775245) between relapsing-remitting and secondary progressive patients [41]. These findings imply SNPs in the region of the KIF5A gene may have an effect on MS disease course. In light of this study and our current findings we hypothesize that carriers of the rs12368653 and rs703842 MS risk gene SNP may have a more severe axonal pathology due to lower KIF5A protein expression and consequently, reduced and/or dysregulated axonal transport. In support of this hypothesis our study also found a significant increase in dephosphorylated NF expression in MS patients who are heterozygous or homozygous carriers of both the rs12368653 and rs703842 risk SNP. NF dephosphorylation in axons has long been established as a pathological hallmark of dysregulated axonal transport and neuronal damage in MS [21,30].

Overall, this study has shown a significant reduction in KIF5A protein expression in MS WM both in inflam-
imentary lesions and non-inflammatory areas, which correlate with levels of important axonal cargoes implicated in the disease: APP and phosphorylated NF. For future studies it would be interesting to ascertain the functional relevance of reduced KIF5A expression both in vitro and in vivo on axonal transport rates and axonal blockages, to decipher potential differences in KIF5A-mediated axonal pathology in MS: in the presence and absence of inflammation. Our findings that link KIF5A protein levels to patient genotype, based on the occurrence of MS risk SNPs in the region of the KIF5A gene, are interesting and warrant further investigation in larger study cohorts to generate an in-depth picture of the potential impact of MS genotypes on disease prognosis.

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Author contributions

KH, AW and NS conceived and designed the experiments. KH, JR and KK performed the experiments. KH, AW and CR analysed the data. AW and NS contributed reagents, materials and analysis tools. KH and AW wrote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The Author(s) declare(s) that there is no conflict of interest.

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