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The role of kinesin superfamily proteins (KIFs) in neurodegeneration

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ABSTRACT

Intracellular transport is fundamental for neuronal function and survival. The majority of proteins are synthesized in the neuron cell body and transported along axons and dendrites through molecular motors that move along microtubules. The Kinesin superfamily proteins (KIFs) are a large gene family of molecular motors that selectively transport membranous organelles, protein complexes and mRNA throughout the cell.

Two specific KIFs that have been associated strongly with neurodegenerative processes in humans and in rodents are KIF5A and KIF21B. In fact, KIF5A down regulation has been associated with axonal transport defects in models of multiple sclerosis (MS) and a genome wide association screen for MS correlated single nucleotide polymorphisms located in the KIF21B intron with the disease, establishing this kinesin as a susceptibility locus for MS. Nitric oxide (NO) has a key role in mediating inflammatory axonopathy in MS promoting protein mis-folding, disruption of mitochondrial respiratory chain that cause reducing ATP production, organelle fragmentation, lipid peroxidation and matrix metalloproteinases activation leading to both myelin and axon damage. The first aim of the present study was therefore to determine the effect of NO exposure on the expression of KIF5A and KIF21B in rodent cortical neurons and to evaluate whether KIFs expression correlates with axon pathology. Results demonstrated that NO causes a time dependent decrease of gene and protein expression for both KIF proteins. Furthermore, dot blot analysis of SMI312 (pan-phosphorylated neurofilament marker), NF200 (phosphorylated and non phosphorylated neurofilament marker), KIF5A and KIF21B show that NO causes a time dependent decrease in axon phosphorylation and that KIFs reduction precedes the loss of neurofilament.

Human bone marrow mesenchymal stem cells (MSCs) represent a promising candidate for neuronal repair due to paracrine mechanisms that allow these cells to have anti-inflammatory properties and to secrete neurotrophic and antioxidant factors. The second part of this study was therefore to investigate the capacity of MSC to protect neurons and axonal transport mechanisms in rodent cortical neurons exposed to NO. The results show that MSC are able to preserve axonal length and increase survival in cortical neurons exposed to NO, furthermore MSCs have the ability to preserve both KIF5A and KIF21B protein expression from nitric oxide damage.

Finally in this study, it was evaluated if there were any changes in KIFs gene and protein expression in cerebellum of MS patients in relation to appropriate control patients. Results demonstrated significant changes in KIF5A and KIF21B expression and the presence of KIF positive spheroids aggregates in sections derived from MS patients.

In conclusion, the results of this study allow a better understanding of the mechanisms involved in the abnormal accumulation of proteins in axons during oxidative insult, that represent a hallmark of several neurodegenerative disorders. Moreover, the ability of MSCs to protect KIF expression from NO damage provides further evidence of their significant therapeutic potential in multiple sclerosis.

1. INTRODUCTION

1.1 Axonal transport

Intracellular transport is fundamental for neuronal function, morphology and survival. Compared with other cell types in which only a short distance is required to reach the destination, neurons are highly polarized cells that require very efficient machinery for long-range transport (Hirokawa & Noda, 2008).

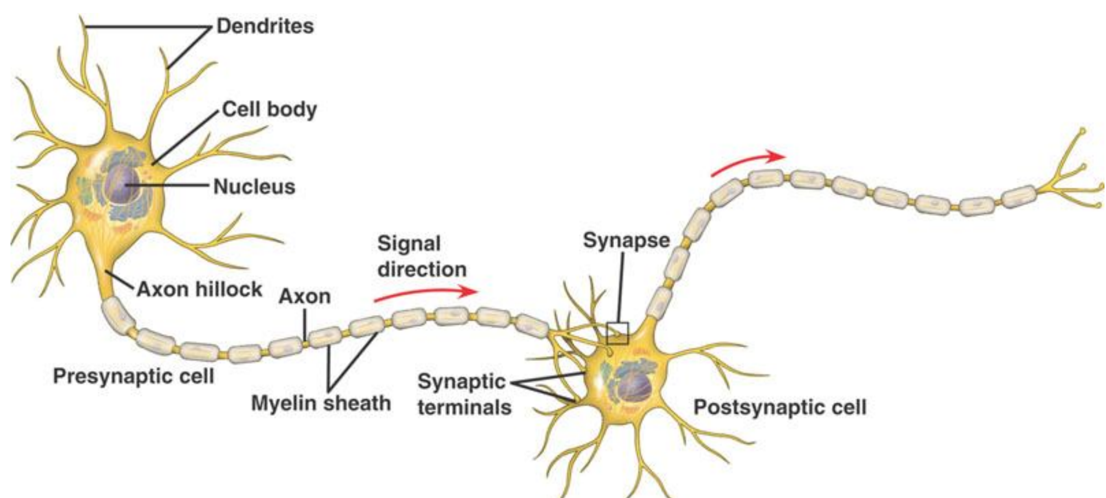
For most of the nineteenth century, there was an on-going debate among researchers about the organization of the nervous system. It was Santiago Ramon y Cajal (1852-1934) who suggested that the neuron was the anatomical and functional unit of the nervous system, leading the “Neuron Doctrine” to be accepted. He also formulated the law of dynamic polarization, according to which a neuron receives signals from its dendrites and transmits them in one direction: away from the cell body.

A typical neuron possesses a cell body, dendrites, axon and axon terminal (W.H. Freeman, 2000) (Figure 1.1):

- The cell body is the metabolic center of the neuron, it contains the nucleus and organelles and is where protein synthesis occurs. It is also the primary site for degradative functions, such as the degradation of misfolded or aggregated proteins.
- Dendrites are cellular extensions with many branches that arise from the cell body. They increase the receptive surface area of the cell body and are specialized to receive chemical signals from other neurons.

- Axons are long processes specialized for the conduction of action potentials away from the cell body toward the axon terminal. A single axon arises from a specialized region of the cell body, the axon hillock, which has the greatest density of voltage-dependent sodium channels and is where the action potential is initiated once a critical threshold is reached. The majority of axons are covered by a myelin sheath that is composed by water, lipids and proteins and increases the speed at which impulses propagate along the fiber. Schwann cells supply the myelin for peripheral neurons, whereas oligodendrocytes myelinate the axons of the central nervous system (CNS).
- The axon terminal contains synapses, specialized junctions that receive the action potential and release neurotransmitters into the synaptic cleft to communicate with target neurons.

Figure 1.1. Structure of a typical neuron



Metabolic labelling of axons with radioactive amino acids has supported the idea that the majority of proteins are synthesized in the cell body; in fact less than 1% of the radioactivity was incorporated in distal axons (Campenot and Eng, 2000). In agreement with this, Mohr and Richter detected no mRNA in axons of mature hippocampal and cortical neurons (Mohr and Richter, 2000).

In mature mammalian nerve cells just the cell body, dendrites and the initial unmyelinated axonal segment have ribosomes and the components required for translation (Steward and Levy, 1982; Steward and Ribak, 1986). Therefore, newly synthesized proteins needed in axon and synaptic terminals are made in the cell body from mRNA that are transcribed and spliced in the nucleus, assembled into organelles by the Golgi and selectively transported to the appropriate destinations through specialized proteins, known as molecular motors that drive axonal transport.

Axonal transport flows in two directions: anterograde transport selectively transports molecules from the cell body to axons and dendrites, while retrograde transport moves molecules from axonal and dendritic terminals to the cell body (Hirokawa and Noda, 2008).

Membrane compartments carrying synaptic vesicles precursors and components of the axonal membrane move in the anterograde direction, while trophic factors and recycling membrane proteins move in the retrograde direction (Dahlstrom et al., 1992; Distefano et al., 1992). All anterogradely-transported cargoes follow the same route in the axon, but they have different destinations (Muresan 2000). For example, synaptic vesicles, vesicles carrying proteins of the synaptic membrane and the machinery required for the retrograde transport have the axon terminal as the final destination. Cargoes such as presynaptic components and cytoskeletal proteins are needed along the axon (Morris et al., 1993); while organelles such as mitochondria

are distributed preferentially to areas with high metabolic demands, such as growth cones, nodes of Ranvier and synapses (Muresan 2000).

Axonal transport is divided in two categories based on the speed at which cargoes moves: fast (100 to 400 mm/day) and slow (0.1 to 4mm/day) axonal transport (Grafstein and Forman, 1980). Membranous organelles such as synaptic vesicle precursors, mitochondria, channel proteins, lysosomes and endosomes are transported by the fast axonal transport system; while cytoskeletal proteins (examples; neurofilament and tubulin) and cytosolic proteins (examples; enzymes of metabolism) are moved by the slow axonal transport (Perrot et al., 2008).

Recent observations have raised the possibility that motors used for slow and fast axonal transport could be identical and the differential transport rates could be due to duration of association between cargoes and motors (Trivedi et al., 2007). This suggests that components moving with the fast axonal transport are attached to the motor for a long period, while slow axonal transport could consist of a fast transport interrupted by prolonged pauses. Indeed, direct observation and modelling studies predict that neurofilament proteins spend the majority of their time detached from the motor, remaining in a stationary state for more than 90% of their time (Brown et al., 2005).

1.2 Neural cytoskeleton

Axons are composed of three cytoskeletal components: actin microfilaments, microtubules (MTs) and intermediate filaments (Ifs). They provide shape and strength to the neuron's cytoplasm, have important functions in axon stability and elongation and are essential for guiding motor proteins.

Actin microfilaments are the thinnest filaments of the cytoskeleton. They are formed from actin monomers that are arranged like two strings of pearls intertwined into fibrils of 4 to 6 nm in diameter. Actin microfilaments are found throughout neurons and glia, but they are particularly concentrated in presynaptic terminals, dendritic spines and growth cones (Kuczmarski and Rosenbaum, 1979). The neuronal cytoskeleton plays a role in maintaining axonal and dendritic proteins into their respective compartments, establishing cell morphology, cell migration, growth cone motility and myelination (Beck et al., 1996).

Microtubules, the thickest cytoskeletal fibers with a diameter of approximately 25 nm, are made up of α and β tubulin heterodimers that line up head to tail to form protofilaments, which are the building blocks for the microtubule structure. Thirteen protofilaments associate laterally to generate the hollow cylindrical structure of a microtubule.

Microtubules are present in mature neurons in the cell body, axons and dendrites (Conde and Caceres, 2009). They have an intrinsic polarity, which is very important for their biological function, with positive (+) and negative (-) ends. The elongation of microtubules occurs from the "fast growing positive-end", while the negative-end is a "slow growing end". MTs have multiple roles in neurons and are necessary for the

extension of neuritis during development, they provide the scaffolding for maintaining neuritis after extension and they help to maintain the definition and integrity of intracellular compartments (Brady, 1996).

Microtubule-based motor proteins use the polarity of microtubules to drive transport. In axons microtubules are unipolar and uniformly oriented with their positive-end pointing away from the cell body, while microtubules in mature dendrites are shorter and have mixed orientation (Bass and Brantley, 1988). Thus, anterograde motors are positive-end-directed motors, retrograde motors are negative-end-directed and in the proximal dendrites both motors can work.

Various microtubule-associated proteins (MAPs) interact with microtubules and control their function, dynamics and stability (Gache et al., 2010). A large variety of MAPs have been identified in many different cell types and they carry out a wide range of functions such as stabilizing and destabilizing microtubules, guiding microtubules towards specific cellular locations and mediating the interactions of microtubules with other proteins in the cell. Axons and dendrites express different MAPs that determine the spatial organization and stabilize the neuronal microtubules; for example MAP2 is exclusively located in dendrites, while tau is enriched in distal axons (Kapitein and Hoogenraad, 2011).

Intermediate filaments are a large family of proteins of various sizes and structure, expressed differentially according to cell types (Perrot et al., 2008).

There are 5 different categories of Ifs, four are localized in the cytoplasm (types I-IV) and one resides in the nucleus (type V): classes I and II represent the cytokeratins; class III is composed by Vimentin, Desmin, Glial Fibrillary Acid Protein (GFAP), Peripherin, Synemin and Nestin; Neurofilament subunits (NF) represent the class IV

and class V is composed by Laminin (Herrmann and Aebi, 2000). The diameter of Ifs are approximately 10 nm and the Ifs expressed by mammalian neurons consist of NF proteins, peripherin, synemin and nestin. Neurofilament is the most abundant cytoskeleton component of large myelinated axons from adult central and peripheral nervous system (Perrot et al., 2008). The three NF subunits, NF-L (light,68 kDa), NF-M (medium, 160 kDa) and NF-H (heavy, 190-210 kDa) share a highly conserved central α-helical domain of approximately 310 amino acids (Rod domain) that is flanked by non-α-helical amino and carboxy-terminal end domains called, respectively, heads and tails.

The main role for NF is to increase the axonal calibre of myelinated axons and their conduction velocity. They are crucial for the correct conduction of the nerve impulse and therefore defects in NFs can contribute to neurodegenerative processes. NF also contributes to axon stability and to the dynamic properties of the axonal cytoskeleton during neuronal differentiation, axonal radial growth and regeneration (Perrot et al., 2008).

The expression of the three NF subunits is finely regulated during nervous system development and coincides with neuron differentiation (Nixon & Shea, 1992). NF-L is the first subunit to be expressed and it is rapidly followed by NF-M expression (Carden et al., 1987). At this early stage, the axon skeleton is composed mainly by microtubules while NF-L and NF-M are expressed only at low levels. The appearance of NF-H occurs after synaptogenesis and is accompanied by an up-regulation of NF-L and NF-M expression that may enhance axon stability and calibre (Carden et al., 1987).

NF subunits are the most extensively phosphorylated proteins in neurons: the head domain is short and rich in Serine and Threonine, while the tail domain of NF-M and

NF-H contain numerous repeats of phosphorylation sites Lysine-Serine-Proline (KSP repeats), which are heavily phosphorylated (Perrot et al., 2008). This phosphorylation is highly regulated, with an intense phosphorylation in axons and little or no phosphorylation in cell bodies and dendrites (Nixon et al., 1992). Phosphorylation of the head domain occurs mainly in cell body soon after the synthesis of NF proteins, while the phosphorylation of the tails domains coincides with their entry into the axon (Nixon et al., 1987). NF-M and NF-H tail domains are the most extensively phosphorylated region; for example 51 sites of phosphorylation are located on the C terminal domain of NF-H (Julien et al., 1982).

NF are involved in defining the structural and the functional integrity of myelinated axons. In fact, abnormal accumulations of NF and neurofilament dephosphorylation are pathological hallmarks of many neurodegenerative disorders. Indeed, many key physiological roles have been attributed to the phosphorylation of NF (Perrot et al., 2008):

- Prevents NF assembly or leads to their disassembly protecting the neuron from abnormal accumulation of NF in cell body.
- Formation of cross-bridges between NF or with MTs that promotes NF alignment.
- Protect NF from proteolysis.
- Increases the total negative charges on side arms and thus causes their lateral extension by repulsive interaction that increase NF spacing and axonal calibre.
- Regulates NF transport rate; the velocity of transport is inversely proportional do the degree of phosphorylation.

1.3 Kinesin superfamily motor

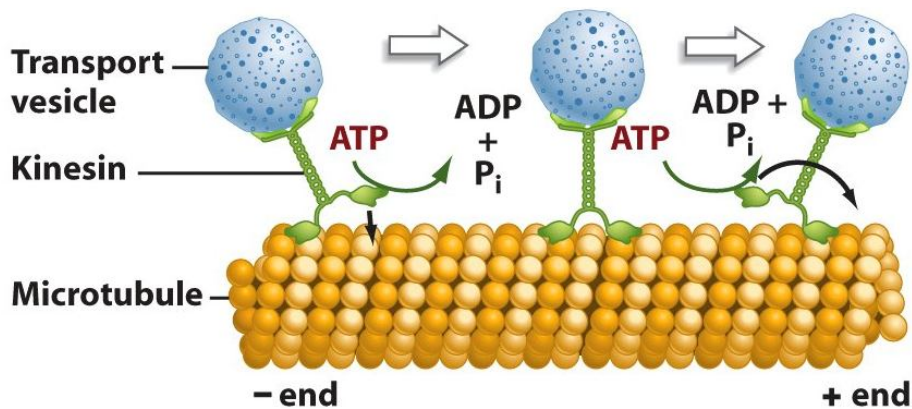
In 1985 kinesin was identified and purified as a novel microtubule-based anterograde motor protein with an ATPase activity (Vale et al. 1985). About 10 years later, the structure of the active domain was determined by x-ray (Kozielski et al. 1997; Kull et al. 1996; Sack et al. 1997) and since then the number of kinesin structures purified has increased substantially. Today, the kinesin superfamily proteins (KIFs) represent a large gene family with 45 members in mice and humans and they are divided in 15 families according to the result of phylogenic analyses (Miki et al., 2001).

KIFs are molecular motors that have a key role in axonal and dendritic transport. Using microtubules as rails to transport cargoes along and ATP to generate motile force, they selectively transport molecules such as membranous organelles, protein complexes and mRNA through the cell (Hirokawa and Takemura, 2005) (Fig. 1.2). They also participate in chromosomal and spindle movements during mitosis and meiosis (Vale et al., 1997) and play fundamental roles in various important physiological processes such as learning and memory, brain wiring, development of central and peripheral nervous systems, neuronal survival, development of the early embryo, left-right determination and tumorigenesis (Hirokawa et al, 2009).

KIFs are composed of a motor domain, a stalk domain and a tail region. All KIFs contain a highly conserved motor domain, a globular head, which is composed by an ATP binding sequence and a microtubule binding sequence. This domain shows an amino acid sequence homology of 30-60% among various different KIFs, while the other regions are quite variable (Hirokawa, 1998). A short and flexible neck linker element connects the motor domain to the stalk domain. The stalk domain, a long alpha-helical coiled coil domain interrupted by non-helical regions that are believed to

act as “hinges”, is responsible for dimerization (Wade and Kozielski, 2000). The stalk domain ends in the tail region that represents the cargo-binding domain (Hirokawa, 1998). The cargo binding domain determines the cargo specificity and the diversity of this region explains how KIFs can transport so many different cargoes.

Figure 1.2. Kinesin “walks” along a microtubule track powered by the hydrolysis of ATP



Pearson Prentice Hall, INC 2005

Depending on the position of the motor domain, KIFs can be classified in three groups (Hirokawa and Takemura, 2005): N-kinesins have the motor domain in the amino-terminal region, M-kinesins have a motor domain the middle and C-kinesins in the carboxy-terminal region. Interestingly, the position of the globular motor domain is indicative of the direction of movement along microtubules (Endow, 1999): N-KIFs generally move towards microtubule positive-ends and drive anterograde transport, whereas C-KIFs move towards negative-ends. M-KIFs have a positive-end-directed motility and a unique microtubule depolymerising activity (Hirokawa and Takemura, 2005).

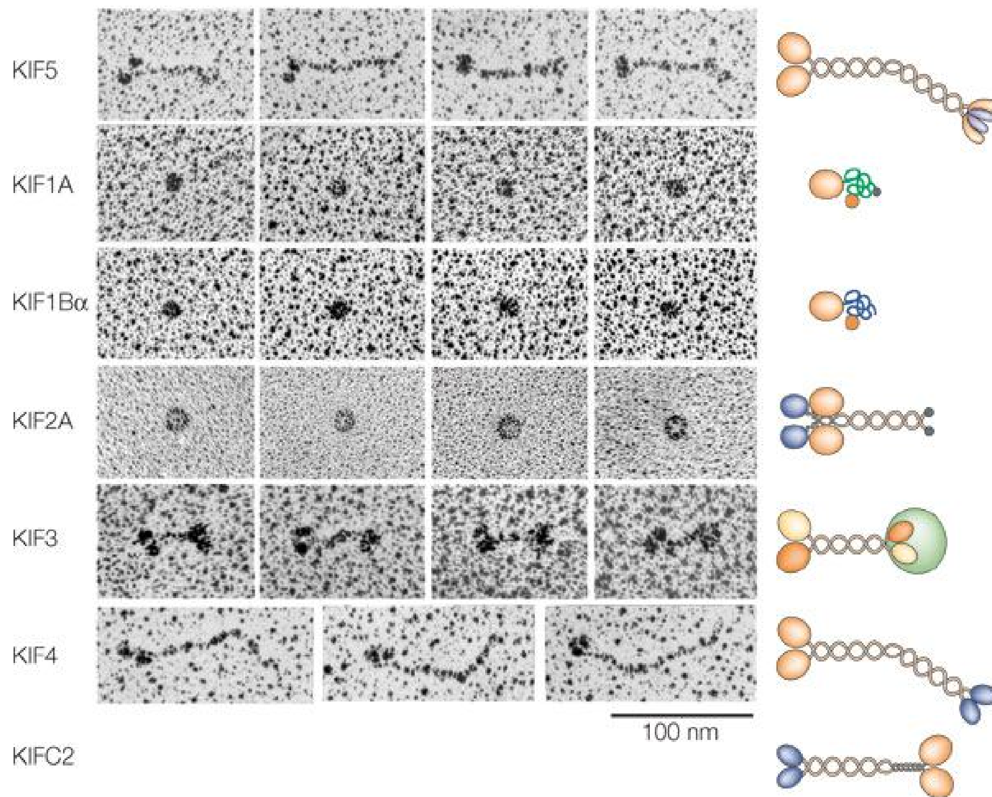
Of the 15 families only one contains M-Kinesin, two contain C-Kinesin and the remaining 12 families are composed by N-kinesin, therefore KIF usually drives anterograde transport.

Most kinesins have two motor domains that work co-ordinately, they are able to move over long distances and their processivity is due to the alternate binding of the two heads to the microtubule (Hackney,1994). Processivity is defined as the distance travelled or number of step made by the motor before it diffuses away from the microtubule and is a prerequisite for efficient transport of cargoes to their destination (Muresan, 2000).

Kinesins have various molecular shapes (Hirokawa and Takemura, 2005): KIF1A and KIF1B are monomeric, many form homodimers (KIF2A and KIF4) or heterodimers binding with other KIFs or with kinesin light chains (KLCs in blue)(see figure 1.3). In some cases, the molecular motors require an adaptor protein and the binding of a cargo protein is not direct, but is mediated by scaffold proteins.

Conventional kinesin (KIF5), the first kinesin identified, is a tetramer consisting of a homodimer of two kinesin heavy chains (KHCs in orange) and two kinesin light chains (blue). The heavy chains are responsible for the motor activity while the light chains connect with cargoes. The kinesin 3 family motor KIF3 form a trimer: heterodimer of KIF3A and KIF3B associated with a kinesin-associated protein 3 (KAP3 in green) (see figure 1.3)

Figure 1.3. Diagrams of the principal members of kinesin superfamily proteins on the basis of electron microscopy or predicted from the analysis of their primary structure



Hirokawa and Takemura, 2005

1.3.1 Axonal and dendritic cargoes and motors

In the axon and dendrites, several KIFs are normally involved in the transport of various different cargoes, however sometimes the function of a specific KIF in a cell can be redundant.

In table 1.1 the known function or cargo transported by each KIF is summarised.

Table 1.1 Family, members and cargo transported or cellular function of each Kinesin

Family name	Members	Cargo transported or function	Reference
N-1 Kinesin	KIF5A, KIF5B, KIF5C	Mitochondria, lysosomes, synaptic vesicles precursors, tubulin dimer, neurofilament proteins, APP, β-secretase and presenilin 1 and APOE receptor 2 in axons; AMPA receptor and mRNA in dendrites	Hirokawa et al.,2005
N-2 Kinesin	KIF3A, KIF3B, KIF3C	Vesicles containing fodrin in neurons; intraflagellar transport to form cilia; essential for proper left-rigth determination in embryos and suppression of tumorigenesis	Hirokawa et al., 2009
	KIF17	NMDA receptor (N-methyl-D-aspartate) in dendrites; important for memory and learning	Hirokawa et al., 2005
N-3 Kinesin	KIF1A, KIF1B, KIF1C	Mitochondria (KIF1Bα), endoplasmatic reticulum (KIF1C) and synaptic vesicles; role in neuronal function and survival (KIF1A, KIF1Bβ)	Hirokawa et al., 2005
	KIF13A, KIF13B	Mannose-6-phosphate receptor (M6PR)	Hirokawa et al., 2005
	KIF16, KIF14	Unknown	
N-4 Kinesin	KIF4A, KIF4B	Membranous organelles; mitotic motor	Miki et al., 2001
	KIF21A, KIF21B	KIF21B may drive transport to the distal region of dendrites;	Marszalek et al., 1999
	KIF 7, KIF27	Unknown	
N-5 Kinesin	KIF11	Spindle formation	Miki et al., 2001
N-6 Kinesin	KIF20A, KIF20B,	Golgi-derived vesicle transport (KIF20A); required for central spindle pole organization and cytokinesis	Miki et al., 2001
	KIF23	Mitosis	Miki et al., 2001
N-7 Kinesin	KIF10	Chromosome segregation	Miki et al., 2001
N-8 Kinesin	KIF18A, KIF18B	Unknown	
	KIF19A, KIF19B	Unknown	
N-9 Kinesin	KIF6, KIF9	Orphans (they have no counterpart in *Droshophila* or *C. Elegans*), function unknown	Miki et al., 2001
N-10 Kinesin	KIF22	Co-localizes with mitotic chromosomes and may bind DNA	Miki et al., 2001

N-11 Kinesin	KIF26	Unknown	
N-12 Kinesin	KIF12	Role in kidney cells	Miki et al., 2001
	KIF15	Expressed in spleen and testis, is reported to associate with a cell proliferation marker	Miki et al., 2001
M Kinesin	KIF2A, KIF2B, KIF2C	Insulin like growth factor receptor; microtubule depolymerising activity that is used to suppress axon collateral elongation	Hirokawa et al., 2009
C-1 Kinesin	KIFC1	Motor for mitosis and meiosis	Miki et al., 2001
C-2 Kinesin	KIFC2	Dendritic transport of multivesicular body-like organelles	Hirokawa et al., 2005
	KIFC3	Organelles containing annexin in polarized epithelial cells	

Two specific KIFs that have been associated strongly with neurodegenerative processes in both humans and in rodents are KIF5A and KIF21B.

KIF5 was the first kinesin to be identified and is the most abundant motor protein (Hirokawa et al., 1989). Mammals have 3 genes encoding for the heavy chains: KIF5A, KIF5B and KIF5C (Miki et al., 2001). KIF5A and KIF5C are neuron specific, while KIF5B is expressed ubiquitously.

KIF5 proteins form heterodimers by recruiting two light chains molecules, therefore they are able to bind their cargoes through two regions: directly through a specific cargo binding region in the tale domain of KHC or indirectly through the amino-terminal region of KLC. KIF5 is able to transport cargoes to axons and dendrites. It seems that cargoes binding to KLC tend to be used for transporting cargoes to axons, whereas binding to the C-terminal region of KHC is used for directing cargoes to the dendrites (Setou et al., 2002).

KIF5 molecular motors play essential roles in axonal transport, in fact they transport several cargoes such the amyloid precursor protein (APP), neurofilament proteins,

tubulin oligomers, apolipoprotein E receptor 2 (APOER2) and mitochondria. KIF5 is also responsible for the transport of retrograde motor proteins. Since cytoplasmic dynein transports cargoes from the axon terminals to the cell bodies, all the components of the dynein complex need to be first transported to axon terminals (Hirokawa et al., 1990).

In dendrites the transport of neurotransmitter receptors, ion channels and mRNAs is essential for synaptic transmission and plasticity. Much less is known about the role of kinesins in dendrites than in axons, but several kinesins that drive transport in dendrites have been identified. In contrast to axons in which microtubules run unidirectionally, the polarity of microtubules in proximal dendrites is mixed.

Although KIF5 molecular motors preferentially move into axons, some dendritic cargos are transported by this motor. For example it transports AMPA glutamate receptor vesicles and GABA glutamate receptor vesicles (Hirokawa et al., 2009). KIF5 also transports a large multisubunit complex of 42 proteins that includes mRNA exclusively in dendrites (Kanai et al., 2004).

KIF21B expression is detected in brain, eye and spleen, but in neurons it is enriched in dendrites. The function of KIF21B is still unknown but could be to deliver cargoes to the distal regions of dendrites (Marszalek et al., 1999).

Recently a genome wide association screen for multiple sclerosis (International Multiple Sclerosis Genetics Consortium, 2010) correlated a single nucleotide polymorphism (SNP) located in the KIF21B intron with the disease, establishing this kinesin as a susceptibility locus for multiple sclerosis. KIF21B is also established as a susceptibility locus for inflammatory bowel disease, this gene has therefore been highly associated with autoimmune disorders (Goris et al., 2010). Since the

expression of KIF21B is detected also in cells of the immune system including T-cells, NK cells and B-cells (T1Dbase), it may also have a yet unknown regulatory function in the immune system.

1.3.2 Regulation of cargo-motor complexes, activation of kinesin and sorting

For efficient transport, a motor has to discriminate the specific cargo to bind, remain attached to the cargo until the correct destination is reached and unload it at the appropriate time and place. How these regulatory processes are achieved is still unknown, but recent studies have started to reveal some regulatory mechanisms that may be involved.

When KIF5 proteins do not bind to cargoes they are in an inhibitory folded state, with two flexible domains in the stalk region that enable an interaction between the tail region and the neck region near the motor domain (Hirokawa and Noda, 2008). In this conformation KIF5s are in a low binding state for microtubules and in a low ATPase activity state and it seems that auto-inhibition is overcome by the binding of cargo to the tail region.

Most cargoes to be transported into the axon are synthesized and assembled in the cell body, therefore it is expected that the interaction between motor and cargo takes place in the cell body. Since the vast majority of membranous cargoes are post-Golgi vesicles, kinesins are probably recruited at the time of its formation in the trans-Golgi compartment (Muresan, 2000). Similarly non vesicular cargoes, such as protein complexes or RNA-protein complexes, are expected to recruit the kinesin motor once they are fully assembled. What triggers the recruitment of the specific kinesin to the cargo is not known. One possibility is that modifications of the cargo such as phosphorylation of a vesicle protein or a modification in the lipid composition may

expose binding sites for the motor. Alternatively, post-translational modification or a shape change of the kinesin molecule may induce both its binding to a component of the cargo and its activation (Muresan, 2000).

With regard to axonal transport, there are indications that phosphorylation, Ca^{2+} signalling, regulation by small GTPases and microtubule tracks may modulate the rate and direction of transport (Muresan, 2000).

Kinesins are phosphoproteins, both heavy and light chains of KIF5 are phosphorylated and their phosphorylation state regulates their function (Sato et al., 1992). Two mechanisms might be controlled by phosphorylation: the association and dissociation of motor to their cargos and the binding of KIFs to microtubules.

The function of KIF5 can be regulated by several kinases. Protein kinase A (PKA)-dependent phosphorylation of KIF5-KLC complexes inhibits the association of this motor with synaptic vesicles (Sato et al., 1992). In addition, glycogen synthase kinase 3 (GSK3) phosphorylates KLC and inhibits the association of KIF5-KLC complexes with membrane organelles (Morfini et al., 2002). So phosphorylation of the motor complex could be used to release the cargo from the motor and unload it at the final destination. It has also been suggested that JNK can phosphorylate KIF5 motors and when phosphorylated the binding of these motors to the microtubules becomes weaker (Morfini et al., 2006).

Several kinesins bind calmodulin in a calcium dependent manner and it seems that calcium negatively regulates microtubule binding and ATPase activity (Rogers et al., 1999). Mitochondria are transported in neurons and concentrated in regions with high energy demands, such as nodes of Ranvier, presynaptic and postsynaptic terminals. In these regions high Ca^{2+} influx occurs and several studies have established that Ca^{2+} arrests mitochondrial motility (Yi et al., 2004; Chang et al., 2006).

Members of the Rab family of small GTPases control organelle localization in a GTP-GDP dependent manner (Zerial and Mcbride, 2001). The GTP-bound forms of Rab proteins specifically bind to 'Rab effector' proteins, whereas the GDP-bound forms do not. If Rab proteins on organelles recruit motor proteins in the GTP-bound form and release them in the GDP-bound form, this could explain why the nucleotide switch of Rab GTPases can control the distribution of organelles (Hirokawa et al., 2009). Therefore, Rab GTPases might have a role in regulating the association and dissociation between kinesins and organelles.

Membrane proteins are transported to axons and dendrites, so how do motors differentiate axons from dendrites? Differences between microtubules are present in neurons, generated by post-translational modification in the tubulin subunits (Laferriere et al., 1997) or by association of microtubules with different microtubule associated proteins (Hirokawa, 1994). In fact MAPs have been shown to affect microtubule-dependent vesicle trafficking (Sato-Harada et al., 1996) and to interfere with the motility of kinesin motors (Ebneth et al., 1998). Since different sets of MAPs are localized to axons versus dendrites, it is likely that motors recognize the intrinsic and specific modifications of microtubules (Hirokawa, 1994).

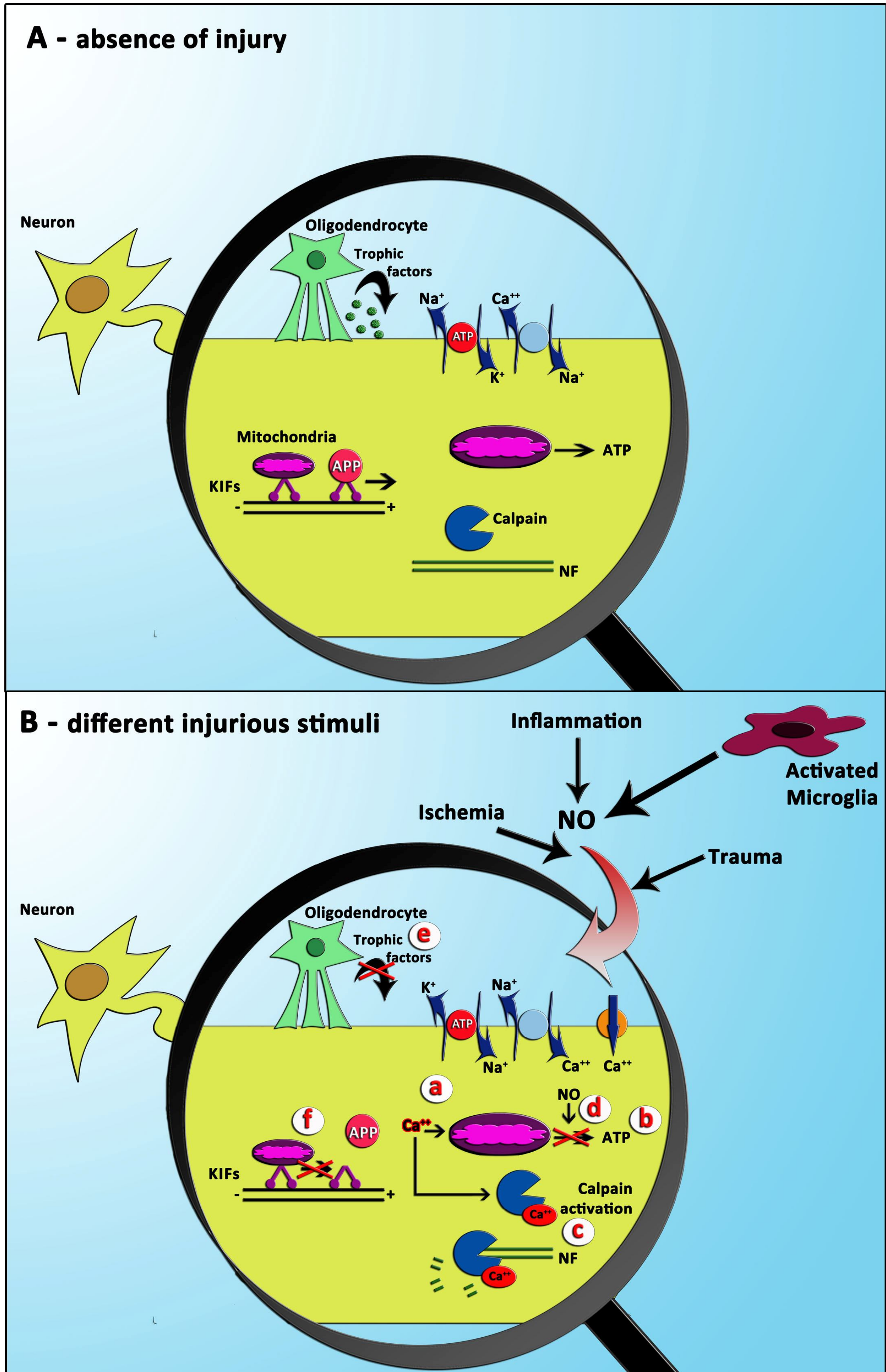
A typical neuron has several dendrites and their proximal segments have large diameters. By contrast, each neuron has only one axon, and the diameter of a typical axon from the initial segment is very small. If the diameter of an axon is one-tenth of that of the cell body, axonally transported materials need to be propelled from the cell body in only about 0.25% of all possible directions to enter the axon (Nakata and Hirokawa, 2003). So structural components, rather than a diffusible signal, must provide a directional cue for efficient sorting (Hirokawa and Takemura, 2005). It has

been shown that microtubules at the initial segment have a high density and serve as the cue for KIF5 to enter the axon (Nakata and Hirokawa, 2003). In fact changes in the dynamics of microtubules by treatment with a low concentration of taxol, induce KIF5 to lose its ability to bind to microtubules in the initial segment and to mediate directional transport to the axon.

1.4 Axonal damage

Axonal dysfunction has a central role in determining the pathology, symptoms and the long-term clinical outcome of many neurodegenerative disorders and acute injuries. Regardless of injury mode, different diseases share a common axonal disruption pattern that is associated with an increased axonal membrane permeability, cytoskeletal breakdown, mitochondrial dysfunction, oxidative and nitrative stress, axonal transport defects and axonal swellings (Medana and Esiri, 2003; Coleman, 2005), as summarized in the figure 1.4.

Figure 1.4. Axonal dysfunction pathway



Brain trauma, ATP deficit and neuroinflammatory disorders can cause changes in the activity of ion channels leading to abnormal physiological increases of intra-axonal Na^+ and Ca^{2+} (Medana and Esiri, 2003). Normally, as soon as Na^+ enters the axon it is pumped out by the Na^+/K^+ ATPase in an energy dependent manner. The dysfunction of Na^+/K^+ ATPase prevents the exchange of the axoplasmic Na^+ for extracellular K^+ leading to the accumulation of Na^+ in axons. The Na^+ influx depolarizes the membrane to open voltage-gated Ca^{2+} channels as well reversing the normal direction of Na^+/Ca^{2+} exchanger activity, leading to an intra-axonal rise in Ca^{2+} levels (Fig. 1.4 a) that will cause a vicious cycle promoting activation of degradative enzymes, impaired mitochondrial respiratory chain, reduced energy production and more axoplasmatic calcium (Trapp and Nave, 2008).

Calcium signaling is critical to trigger the destruction of the cytoskeleton by activation of caspases and a ubiquitous Ca^{2+}-dependent cysteine protease Calpain, which is capable of cleaving several substrates such as neurofilament (Fig. 1.4 c), microtubule-associated components and spectrin (Wang et al., 2012).

Mitochondria are the most efficient producers of ATP and play an important role in calcium homeostasis and apoptosis (DiMauro and Schon, 2003). High levels of Ca^{2+} can open the mitochondrial membrane permeability transition (MPT) pore that allows antioxidant molecules such as glutathione to exit mitochondria. This reduces the ability of the organelle to neutralize reactive oxygen species and causes mitochondria to become permeable to molecules smaller than 1.5 kDa leading to an uptake of water, swelling and the membrane to rupture (Luetjens et al., 2000). The rupture of the mitochondrial membrane can inhibit the respiratory chain with a decrease of ATP production and causes the release of cytochrome c thus activating pro-apoptotic factors (Fig. 1.4 b).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products produced during normal cellular metabolism. However, disturbances in the normal redox state of cells, an overproduction of ROS and RNS and a failure of antioxidant mechanisms, can have toxic effects through the production of free radicals that damage all components of the cell including proteins, lipids, and DNA.

Neurons are constantly exposed to low levels of oxidative and nitrative species but detoxifying enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) have important role in protecting cells against these molecules.

In an inflammatory state, these antioxidant defences can become overwhelmed and the CNS becomes particularly vulnerable to oxidative stress and cellular damage due to several reasons (Merrill and Scolding, 1999; Witherick et al., 2010):

- Brain tissue is very active in oxidative metabolism and large O_2 stores are required for maintenance of electrical activity;
- There are elevated levels of enzymes generating ROS but low levels of antioxidant defences;
- Cells in the CNS, such as neurons and oligodendrocytes, are very sensitive to free radical injury;
- Free divalent iron is elevated in the brain;
- The composition of myelin is a preferential target of lipid peroxidation due to high protein:lipid ratio.

Cells generate energy aerobically by reducing molecular oxygen (O_2) to water. Electrons are transported, along the complexes I, II and III of mitochondrial respiratory chain, to the complex IV where they are donated to oxygen to produce water. At the same time protons are pumped across the mitochondrial inner

membrane to generate membrane potential that drives ATP synthase to generate ATP (Mahad et al., 2008). Inevitably, oxygen radicals and peroxides such as O_2^{*-} and H_2O_2 are produced during this process. If these radical species are not converted to non toxic molecules or are produced in excess, they can damage DNA, RNA, proteins and cause lipid peroxidation.

Nitric oxide (NO) is a gas synthesized from the amino acid L-arginine by enzymes known as nitric oxide synthases (NOS) (Knowles and Moncada, 1994). The NOS family includes 3 isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS and nNOS are constitutively expressed and require Ca^{2+}/calmodulin complexes for their activation. Both eNOS and nNOS generate low levels of NO for short period of time: eNOS produces NO in the endothelial tissue of blood vessels and nNOS is active in central and peripheral neurons. In contrast, iNOS, which is found in immune cells and glial cells (astrocytes and microglia), is activated in response of pathogen recognition and cytokine release and may generate high NO levels for prolonged period of time (Benarroch, 2011). In normal conditions levels of iNOS in the nervous system are low, but this enzyme can be induced following inflammation, viral infection, hypoxia or trauma.

Nitric oxide is involved in several important functions in the CNS, including modulation of synaptic neurotransmission and plasticity, regulation of cerebral blood flow, a toxic agent in the immune cell response to pathogens and even as a neuroprotective molecule (Bennaroch, 2011). However, when produced in excess and in the setting of oxidative stress, NO reacts with oxidant molecules to form RNS: NO reacts with superoxide anion O_2^{*-} to form the highly reactive neurotoxin peroxynitrite ($ONOO^-$) and NO also combines with O_2 to produce nitrosonium ion (NO^+). Peroxynitrite reacts with tyrosine residues of proteins resulting in a tyrosine

nitration that can block protein phosphorylation interfering with cell signaling pathways; while NO^+ interacts with thiols (-SH), such as cysteine residues of proteins, to form S-nitrosylation. Some of the proteins that can be impaired by nitrosylation are enzymes involved in oxidative defences such as glutathione, catalase, the heat shock protein 90 (Hsp90) which has a chaperone function that if altered can promotes β-amyloid and tau aggregates, matrix metalloproteinase-9 (MMP-9) involved in degradation of extracellular matrix proteins and the glycolysis' enzyme GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) (Knott and Bossy-Wetzel, 2009). NO therefore plays a key role in mediating inflammatory axonopathy promoting misfolding of critical proteins and also in promoting disruption of mitochondrial respiratory chain. Nitric oxide in fact competes with oxygen for the oxygen binding sites and may irreversibly inhibit complex IV that lead to the release of cytochrome c and a decreased ATP production (Fig.1.4 d). Furthermore, NO can cause mitochondrial and Golgi fragmentation, liberation of Zn^{2+} from its intracellular ligands increasing free radical production, deaminate DNA, inhibit repair mechanisms and promote synaptic injury and apoptosis (Knott and Bossy-Wetzel, 2009).

Another common feature of axonal damage is the blockage of axonal transport that can lead to accumulation of proteins and organelles. In many CNS neurodegenerative disorders, proteins such as NF and APP can accumulate in axonal spheroids (Fig. 1.4 f) and in some disorders the impaired axonal transport is not just a consequence of axon damage, but part of the cause (Coleman, 2005). This aspect will be described more in detail below.

1.5 Role of axonal transport defects in neurodegenerative disorders

Intact axonal transport is required to maintain the integrity of axons and the overall metabolic balance of the cell. Abnormal axonal and cell body aggregates of organelles and proteins are hallmarks of several neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and Alzheimer's diseases (AD). Multiple factors can induce protein aggregations and it is probably a complex multi-step process that involves deregulation of gene expression, mutations, abnormal post-translational modifications, proteolysis and defects in transport (Perrot et al., 2008).

There are several mechanisms by which axonal transport is disrupted in disease including damage to the molecular motor, to neuronal cytoskeleton, to the cargoes or to the motor-cargo interaction (De Vos et al., 2008).

Damage to molecular motor

Mutations in kinesins can inhibit their activity and have a role in several disease processes. The most common causes of Hereditary spastic paraplegia (HSP) are mutations in the spastin gene, but three distinct autosomal dominant missense mutations have been mapped to the gene that encodes the heavy chain of KIF5A (Reid et al., 2002; Lo Giudice et al., 2006). Two of these mutations alter a highly conserved residue in the motor domain sequence blocking the microtubule activation of ATP hydrolysis; while the third mutation occurs in the neck and may inhibit dimerization. These mutations are predicted to severely impair transport and affected patients show distal axonal degeneration, consistent with an inhibition of anterograde axonal transport.

Mutant mouse models for KIF5A display loss of large calibre axons and neurofilament accumulation in neuronal cell bodies (Xia et al., 2003). The KIF5-mediated transport of APP is likely to be deeply involved in the pathogenesis of Alzheimer diseases (Stokin et al., 2005). Moreover, KIF5A down regulation has been associated with axonal transport defects in models of multiple sclerosis (Kreutzer et al., 2011). In these studies after TMEV infection (Theiler's murine encephalomyelitis virus), mice display axonopathy and accumulation of neurofilament and APP that is correlated with a decrease in KIF5A expression. The appearance of KIF5A-positive spheroids and a decrease of phosphorylated NF are also observed in the TMEV-infected mice.

KIF1A and KIF1Bβ transport synaptic vesicle precursors such as synaptophysin, synaptotagmin and the small GTPase RAB3A from the cell bodies to the synapse. (Okada et al., 1995; Zhao et al., 2001). Mice that lack either KIF1A or KIF1Bβ have reduced synaptic vesicle density at synaptic terminals and impaired sensory and motor nerve function (Yonekawa et al.,1998; Zhao et al., 2001). Although KIF1A and KIF1Bβ redundantly transport the same cargoes, haploinsufficiency of KIF1Bβ results in abnormal neuronal phenotypes, which suggests that the expression level of these motors is crucial for the transport of synaptic vesicle precursors towards axon terminals (Hirokawa et al., 2009). In fact, mutation in the gene encoding KIF1Bβ has been identified as the cause of disease in patients with Charcot Marie Tooth Disease Type 2A (Zhao et al., 2001). CMT2A patients have an autosomal dominant neuropathy, leading to weakness and atrophy of distal muscles.

Phosphorylation has also a very important role in the regulation of axonal transport and can inhibit the activity of kinesin at multiple levels. Mutations, inflammation, oxidative or nitrosative stress can induce an abnormal phosphorylation of KLC which

in turn inhibits the attachment of kinesin to the cargo or disrupts the interaction of kinesin with microtubules (De Vos et al., 2008).

Damage to neuronal cytoskeleton

Mutation, abnormal phosphorylation and deregulation of dynamic properties that affect the integrity and stability of the cytoskeleton can perturb the efficiency of axonal transport.

Abnormal tau can disrupt the transport interfering with the binding of kinesin to microtubules (Seitz et al., 2002). Tau is involved in stabilizing microtubules but hyperphosphorylated tau has a reduced affinity for microtubules and is less efficient at stabilizing them (Wagner et al., 1996), thus leading to loss of microtubule rails required for axonal transport.

Mutations in the spastin gene are responsible for 40% of hereditary spastic paraplegia cases. The spastin gene encodes an AAA family ATPase that functions to sever and bundle microtubules (Evans et al., 2005) and the HSP mutant spastin is defective in this process. How mutant spastin might perturb axonal transport is not fully clear. One possibility is that the mutants alter the microtubule rails to disrupt movement of cargo-carrying motors. Another possibility is that loss-of-function mutant spastin induce abnormal bundling of microtubules and disrupts the transport of microtubules themselves (Perrot et al., 2008). In fact, the length of microtubules is related to their potential to be transported through axons: short microtubules are preferentially transported, while long microtubules are essentially stationary (Ahmad et al. 2006).

Damage to the cargoes and alteration of the motor-cargo interaction

Disruption of motor-cargo interaction may disrupt axonal transport in a number of neurodegenerative diseases.

Abnormal aggregations of neurofilament are hallmark of several neurodegenerative diseases and can be associated with defects of NF transport. Phosphorylation of neurofilaments slows their transport and several studies support the fact that the rate of NF transport is inversely correlated to their phosphorylation state (Perrot et al., 2008). Furthermore, aberrant hyperphosphorylation of NF-M or NF-H side arms domains may therefore promote their release from the molecular motor (Jung et al., 2005) and their accumulation.

Mitochondria are essential for neuronal function and survival and defects in mitochondrial transport are implicated in the pathogenesis of several major neurological disorders. Mitochondrial ATP production supports many important functions, including the mobilization of synaptic vesicles for exocytosis and recycling, the assembly of the actin cytoskeleton for presynaptic development and the generation of axonal and synaptic membrane potential (Sheng et al., 2012). Therefore factors that inhibit mitochondrial transport result in the loss of mitochondria from synaptic terminals that inhibit synaptic transmission, a decrease of ATP supply that affect the whole metabolism of the cell, toxic changes in Ca^{2+} concentration that may trigger synaptic dysfunction and the release of apoptotic cell death signals. Moreover, the resulting diminution of mitochondria in axons will decrease ATP supply to the molecular motors, leading to a decrease in both anterograde and retrograde transport of others axoplasmatic cargoes resulting in a vicious circle degenerative mechanism.

Mitochondria are produced in the cell body and distributed preferentially to areas with high metabolic demands (Morris et al., 1993). The interaction between motor proteins and mitochondria are mediated by adaptor and scaffolding proteins. The Milton-Miro complex and syntabulin are implicated as adaptor between KIF5 and mitochondria (Stowers et al., 2002). When expression of these proteins is reduced or the adaptor contains a mutation, mitochondria are abnormally distributed as expected due to disrupted anterograde transport.

1.6 Multiple sclerosis

Multiple sclerosis is an inflammatory demyelinating and neurodegenerative disease of the CNS (Dutta and Trapp, 2007). The pathologic hallmarks of MS lesions include breakdown of the blood brain barrier, multifocal inflammation, demyelination, oligodendrocyte (OGCs) loss, gliosis and axon degeneration (Prineas, 2001).

There are 4 subtypes of MS (Dutta and Trapp., 2007; Siffrin et al., 2010):

- Relapsing-remitting multiple sclerosis (RRMS) is characterized by alternating episodes of neurologic disability and recovery. The neurologic deficits last for six to eight weeks and then remit partially or completely; the phase between the two relapses are characterized by lack of clinical progression. The majority (80-85%) of patients with MS have a relapsing-remitting disease; females are twice as likely to have RRMS compared to males.
- After 10 years of RRMS, about 50% of patients exhibit a secondary progressive multiple sclerosis (SPMS) characterized by an increasing of permanent neurologic disability. Progressive deterioration primarily affects

motor function of the lower limbs; the relapses are fewer and patients recover incompletely.

- Primary progressive multiple sclerosis (PPMS) is characterized by a decline in neurologic function from disease onset without recovery. Approximately 10% of patients suffer from PPMS and their neurological status deteriorates continuously. The incidence of PPMS is similar in males and females.
- About 5% of patient experienced progressive-relapsing multiple sclerosis (PRMS) characterized by a steady progressive neurologic decline with some acute attacks with or without recovery.

Historically, MS is considered a disease in which the immune system recognises the CNS myelin as a foreign body and therefore activates inflammatory and immune processes to destroy and remove it. The disability is caused by focal areas of inflammatory demyelination (plaques in the white matter) in which the conduction of electrical impulses is blocked. The myelin destruction is mediated by T-cells and much of the inflammation consists of blood-derived monocytes and activated microglia (Siffrin et al., 2010). The main function of the myelin sheath is to increase the speed at which impulses propagate along the axon fibers. In fact myelin allows for rapid saltatory conduction, which is the propagation of action potentials along myelinated axons from one node of Ranvier (small unmyelinated axonal segment) to the next node. At such nodes there is a high density of voltage-gated Na^+ channels that generate action potentials. Furthermore myelin decreases capacitance across the cell membrane and increases electrical resistance, which improves saltatory conduction and prevents the electrical current from leaving the axon. Following demyelination, axonal conduction is severely

impaired and conduction fails due to myelin loss and to a redistribution of Na^+ channels along the entire length of the axon (Trapp and Nave, 2008). Demyelination increases the energy demand of nerve conduction and leads to an ionic imbalance that compromises ATP production and renders the axon more vulnerable to inflammatory stress and degeneration.

In the early stages of the disease there is some degree of remyelination but this process fails with the progression of MS, leading to extensive areas of demyelination. In MS active lesions there is a reduction of oligodendrocyte density with a rapid reappearance of OGCs, which are likely to be recruited from progenitor cells in inactive plaques areas. Repeated demyelination of previously remyelinated areas may lead to an exhaustion of the progenitor pool, which results in persistently demyelinated plaques (Lassmann, 1999).

The immune mediated attack of myelin has been considered to be the primary event in MS pathogenesis and traditionally, it has been assumed that inflammation is the precursor to the entire course of the disease. However, multiple sclerosis has been found to be a much more complex disease than originally thought: the major cause of irreversible neurologic disability is axon loss (Dutta and Trapp, 2007), but the correlation between inflammation and axonal pathology is controversial and research and investigation is currently ongoing into this neurodegenerative process in MS.

Inflammation in MS is not restricted to active demyelinated plaques, but affects the CNS in a more global way. Infiltrates of T cells, B cells and macrophages are found even in inactive plaques (Lassmann, 1999). Inflammatory infiltrates are present not just in the periplaque white matter, but also far from the established demyelinated lesion (Lassmann, 1999). In a study of the staging of plaques in early MS, Gay et al.,

(1997) concluded that T-cell infiltration into the lesions follows the initial step of myelin destruction. Furthermore, MS is not considered a disease affecting only the white matter of the CNS but there can also be an involvement of the grey matter, in particular the cortex, in advanced stages of the disease (Kidd et al., 1999). A diffuse axonal injury and axonal swelling is seen also in the normal appearing white matter (NAWM) alongside demyelination (Kutzelnigg et al., 2005). A moderate degree of neuronal loss and some axonal spheroids are also present in cerebellar cortical lesions of patients with primary and secondary progressive MS (Kutzelnigg et al., 2007).

Collectively these observations suggest that the degeneration of axons can occur independently to the inflammatory plaque and it is yet to be determined if the inflammatory response is a primary event or a secondary reaction to the degeneration of axons (Wilkins and Scolding, 2008). As disease progresses, the MS brain undergoes continuous atrophy in the absence of inflammation and studies have focused on the role of axonal pathology and neurodegeneration as the cause of permanent neurological disability in MS patients (Trapp and Nave, 2008). The idea that two processes, inflammatory and neurodegenerative, may run in parallel and may be independent of each other has emerged, therefore challenging existing concepts and paving the way to new therapies (Wilkins and Scolding, 2008).

1.6.1 Mechanisms of axonal pathology in MS

Axon loss is considered an early event and the major cause of permanent neurologic disability in MS patients. There are several non-inflammatory and inflammatory mechanisms that contribute to axon damage.

Axon damage has been identified in MS by the presence, in both acute active and chronic lesions, of abnormal accumulations of APP (Ferguson et al., 1997) and dephosphorylated neurofilaments (Trapp et al., 1998). Impaired axonal transport can lead to accumulation of organelles, APP and disorganized cytoskeleton in axonal varicosities. The swellings increase in size to form axonal spheroids that frequently appear not singly, but as tandemly-repeated swellings causing a generalize arrest of transport (Coleman, 2005).

Neurofilament dephosphorylation has been used as a marker for axonal pathology in multiple sclerosis adopting the antibody SMI32 that labels non-phosphorylated neurofilaments. SMI32 staining has also been used to mark terminal spheroids, which represent transected axons and irreversible axon destruction (Wilkins and Scolding, 2008). Quantification of terminal axonal spheroids identified a strong correlation between axonal transection and the inflammatory activity of the lesion, therefore axonopathy in the disease may occur early and be related to inflammatory mediators (Trapp et al., 1998).

Microglia and macrophages are the main effector cells of the innate immune response involved in axon damage. Activated microglia and peripherally derived macrophages shifted towards a strongly pro-inflammatory phenotype release toxic substances including pro-inflammatory cytokines (TNF-α and IL 1-β), matrix metalloproteases, oxygen radicals and nitric oxide that play a key role in axonal damage (Ransohoff and Perry, 2009).

Nitric oxide and markers of its production (nitrate and nitrite) are raised in the cerebrospinal fluid, blood and urine of patients with MS and have a role in several features of the disease including: disruption of the blood-brain barrier, oligodendrocyte injury, inhibition of mitochondrial respiration affecting energy

metabolism and ATP synthesis, changes in sodium channels distribution causing axonal conduction block and impairment in the function of ion channels and ions exchangers with an accumulation of intra-axonal calcium that activates proteases resulting in the disruption of cytoskeleton (Smith and Lassmann, 2002).

Inflammation can also induce aberrant glutamate homeostasis, in fact elevated glutamate levels have been reported in MS lesions and in NAWM (Srinivasan et al., 2005). When released in excess glutamate activates ionotropic and metabotropic receptors resulting in toxic cytoplasmatic Ca^{2+} accumulation that can damage oligodendrocytes, myelin and axons (Trapp and Nave, 2008).

Another mechanism that could determine axon damage is the loss of myelin and oligodendrocyte derived trophic support. Myelin is highly trophic to the axon and it is required for the maintenance of axons during post-natal life. The loss of myelin that occurs in MS may lead to a slow degeneration of axons because of the loss of specific interactions between myelin components and the axons themselves that determine their long-term survival (Wilkins and Scolding, 2008). In a similar way, oligodendrocytes secrete soluble factors that influence axonal survival, phenotype and function. The close contact between OGCs and axons allows cross talk: axons have been shown to exert a proliferative effect on oligodendrocyte precursors cells (Bogler et al., 1990) and promote OGC survival (Barres and Raff, 1999), while oligodendrocyte derived factors improve the survival of neurons, increase levels of phosphorylated neurofilament (Wilkins et al., 2003) and influence axonal structure by inducing sodium channels clustering (Kaplan et al., 1997).

Indeed, all these functional and structural changes described can destabilize the axon and predispose it to degeneration. Once the threshold of axon loss is reached

and the compensatory capacity of the CNS is exceeded, irreversible neurologic disability becomes clinically evident (Dutta and Trapp, 2007).

1.7 Mesenchymal stem cell for the treatment of neurological diseases

Cell-based therapies as new therapeutic approach for tissue regeneration and repair have generated a great deal of interest. Specifically, human bone marrow mesenchymal stem cells (MSCs) represent a promising candidate for cell therapy and are currently being tested in clinical trials for several reasons:

- MSCs are adult stem cells and their use is not limited by ethical issues and national legislation limits as for embryonic stem cells;
- MSCs are easily isolated and expanded in culture;
- MSCs have a low immunogenicity that allow allogeneic transplantation;
- MSCs have the ability to migrate into injured tissue and exert their anti-inflammatory and anti-apoptotic features, as well as secrete trophic factors.

Mesenchymal stem cells are multipotent stromal cells that are able to self-renewal and to differentiate into cells of the mesengenic lineage as adipocytes, osteoblasts and chondrocytes (Caplan and Bruder, 2001). Several studies have also shown that MSCs are able to transdifferentiate also in myocytes, muscle cells, hepatic cells and into neuronal-like cells, although there is doubt whether the MSC-derived neurons are truly functional.

MSCs are found in the bone marrow microenviroment within the haematopoietic niche, supporting haematopoiesis and tissue maintenance within the human body.

Through direct cell-cell interactions and secretion of growth factors and cytokines, MSCs support the maintenance and self-renewal of hematopoietic stem cells (HSC) and provide signals for their differentiation and proliferation.

The most accessible source of MSCs is through bone marrow harvests obtained from the posterior iliac crest of the pelvis, tibial and femoral marrow compartments; although MSC have been isolated from several tissues including the liver, adipose tissue, dental pulp, cord blood, Wharton's jelly and amniotic fluid. In the bone marrow MSCs are a very rare population representing less than 0.1% of the total nucleated cells. Therefore an ex-vivo expansion is required to generate sufficient numbers of these cells for clinical treatments.

MSCs are selected from bone marrow mononuclear cells by their adherence to plastic in culture and are expanded in medium consisting of Dulbecco's Modified Eagles Medium (DMEM) low glucose, supplemented with 10% foetal bovine serum (FBS), and L-glutamine at 37˚C with 5% CO2 at densities from $1x10^4$ - $0.4x10^6$ cells/cm^2 (Colter et al., 2000). In vitro MSCs can be extensively expanded as an adherent confluent monolayer and have a spindle-shaped morphology and a large round nucleus containing a prominent nucleolus.

Cultured MSCs express a number of adhesion molecules (CD44, CD29, CD90), stromal cell markers (SH-2, SH-3, SH-4), cytokine receptors (IL-1R, TNF-αR) and are recognized by the monoclonal antibody STRO-1, but none of these markers are entirely specific to the MSC population. To date, no marker has been identified that uniquely defines the MSC population. To address this problem, the International Society for Cellular Therapy (ISCT) have proposed a minimal criteria for defining human multipotent mesenchymal stem/stromal cells for both laboratory-based scientific investigations and for pre-clinical studies (Dominici et al., 2006).

The criteria states MSCs must:

1. Show plastic adherence under standard culture conditions.

2. Express CD105, CD73, and CD90 and lack the expression of CD45 (pan-leukocyte marker), CD34 (primitive hematopoietic progenitors and endothelial cells), markers for monocytes and macrophages (CD14 or CD11b), markers of B-cells (CD79 or CD19) and HLA-DR (unless MSC is stimulated).

3. Differentiate into adipocytes, osteoblasts, and chondrocytes under standard culture conditions.

1.7.1 Neuroprotective mechanisms of MSC

The therapeutic effect of MSCs relies greatly on their capacity to home into injured tissue, to modulate the immune response and on the paracrine release of molecules. In fact, these cells are able to produce cytokines and a variety of soluble factors that can regulate several biological activities.

The use of stem cells for tissue repair may require the cells to access the target to exert their therapeutic effect. Systemically administered MSCs can extravagate from the blood vessels and migrate, in response to several chemokines released after tissue damage, preferentially to the site on injury (Fox et al., 2007). However it is believed that after intravenous infusion, MSC are scarcely able to pass the lung filter and they remain trapped in the lungs, hampering their engraftment to the CNS (Lee et al., 2009). MSCs can also migrate to the lymph nodes, where they have several interactions with immune cells. Despite little engraftment to the CNS, the infusion of MSCs in animal models of neurological diseases is efficacious. Therefore the therapeutic effect of MSCs may not be mediated solely through cell-cell contact

mechanisms and require long-term engraftment, but occurs through a paracrine release of soluble factors (Uccelli et al., 2011).

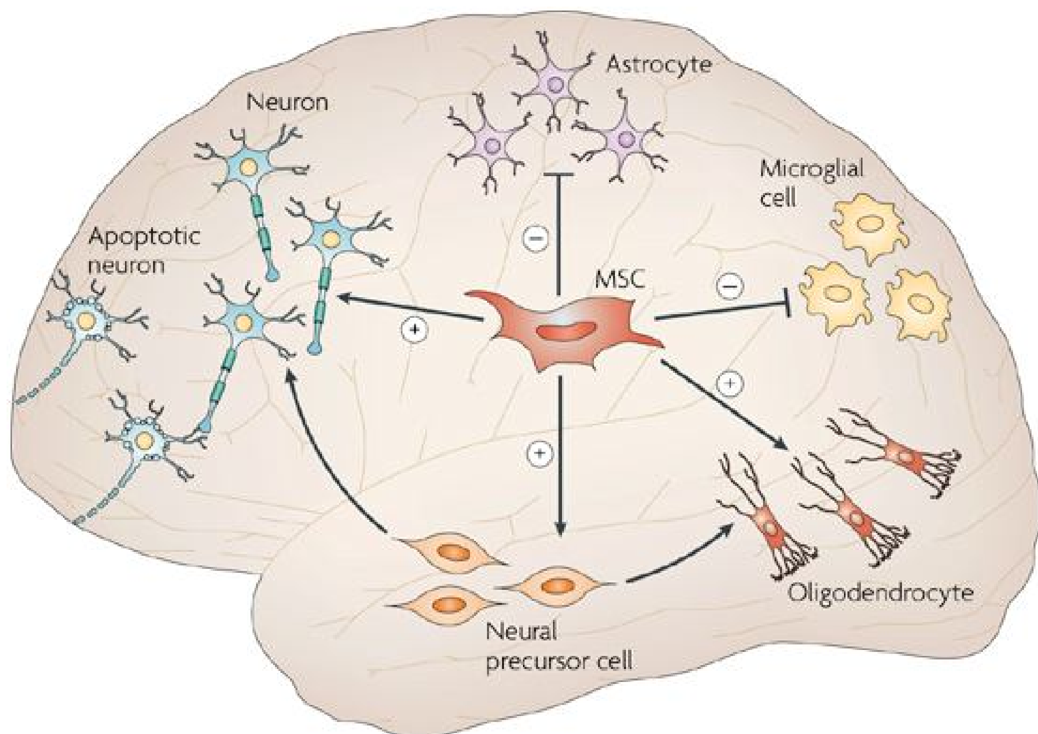
Several studies have provided evidence that MSC could ameliorate neurological deficits through an immunomodulatory effect, in fact these cells can:

- Decrease the pro-inflammatory potential of dendritic cells (DCs) by inhibiting their production of tumor necrosis factor-α (TNF-α) and increasing the DCs secretion of anti-inflammatory interleukin 10 (IL-10) (Aggarwal and Pittenger, 2005);
- Inhibit the cytotoxic activity of NK cells abrogating the NK proliferation and Interferon-γ (IFN-γ) secretion (Spaggiari et al., 2008);
- Inhibit proliferation and differentiation of B-cells (Corcione et al., 2006);
- Suppress T lymphocyte proliferation (Di Nicola et al., 2002);
- Induce a shift in T-cells from a pro-inflammatory state to an anti-inflammatory state decreasing T helper 1 secretion of IFN-γ and increasing the T Helper 2 secretion of IL-4 (Aggarwal and Pittenger, 2005);
- Increase the generation of regulatory T-cells that suppress the activation of the immune system and help to maintain homeostasis and tolerance to self antigens (Maccario et al., 2005).

In addition to their immunomodulatory properties, MSCs can also support tissue repair in the CNS and directly protect neural tissue. In fact, these cell can rescue neural cells from apoptosis (Chen et al., 2003; Zhang et al., 2009), stimulate the proliferation, migration and differentiation of endogenous neural stem cells (Munoz et al., 2005), recruit oligodendrocyte precursors and induce oligodendrogenesis (Zhang et al., 2005), inhibit proliferation of astrocytes involving in gliotic scarring (Aharonowiz et al., 2008) and modulate microglia activation and the production of inflammatory

factors inducing a switch towards a protective phenotype (Uccelli et al., 2011) (Fig. 1.5).

Figure 1.5. The bystander effect of MSC on neural cells



Uccelli et al., 2008

All these neuroprotective properties rely greatly on the paracrine release of molecules, in fact MSC are able to modify the microenvironment of injured tissue and to secrete anti-inflammatory cytokines (Uccelli et al., 2008), neurotrophic (Wilkins et al., 2009) and antioxidant (Lanza et al., 2009, Kemp et al., 2010 a) factors.

In many inflammatory neurodegenerative disorders, including multiple sclerosis, anti-inflammatory and ROS detoxification pathways are postulated to be important in neuroprotection and the demonstration that MSCs secrete antioxidant molecules may explain their therapeutic effectiveness of MSCs (Kemp et al., 2010 b).

MSCs can exert a protective effect against oxidative stress through the modulation/inhibition of signaling pathways involved in cellular damage, inducing cell survival pathways and through the production of antioxidant molecules (Witherick et al., 2011). In fact these cell are able protect rodent cerebellar neurons from NO damage through the secretion of the extracellular antioxidant molecule superoxide dismutase 3 (SOD3) (Kemp et al., 2010 a). SOD3 is a member of the SOD protein family and is the only enzyme that catalyses the conversion of superoxide anion (O_2^-) to hydrogren peroxide (H_2O_2) in the extracellular compartment, which can be detoxified to form water and oxygen by the enzymes catalase and glutathione (Harris et al., 1991). Therefore SOD3 secretion limits the formation of strong neurotoxic oxidants and attenuates tissue damage and inflammation. Furthermore it has been shown that SOD3 secretion by MSCs is increased by activated microglia and regulated synergistically in response to the inflammatory cytokines IFN-γ and TNF-α (Kemp et al., 2010 b). Therefore the secretion of active antioxidant molecules by MSCs provide direct neuroprotection against nitric oxide and activated microglia-mediated stress, representing a valuable therapeutic strategy for several inflammatory diseases of the CNS.

1.7.2 MSC therapy for multiple sclerosis

The rationale for the use of mesenchymal stem cells as a treatment for multiple sclerosis arise from several *in-vitro* and preclinical studies in animal models of MS, in which infused MSCs improved the clinical course and pathology scores of the disease.

In experimental autoimmune encephalomyelitis (EAE), a model for human MS, intravenous infusion of MSCs ameliorated the clinical course of EAE, in fact MSC-

treated mice showed a significantly milder disease, fewer relapses and decreased number of inflammatory infiltrates (Gerdoni et at., 2007). Furthermore, MSCs induced peripheral T-cell tolerance to myelin proteins and homed into the CNS where they preserved axons and reduced demyelination (Zappia et al., 2005; Gerdoni et at., 2007). In others studies it has been observed that MSCs released neurotrophins that had an effect on oligodendrocytes enhancing remyelination (Zhang et al., 2005), soluble factors that reduced axonal loss in mice with EAE (Kassis et al., 2008) and that administration of MSCs reduced EAE-induced levels of tissue damage markers such as as PARP1 (nuclear enzyme functioning as a DNA-damage sensor that is activated by DNA strand breaks) and the tumor suppressor protein P53 that triggers apoptosis (Lanza et al., 2009).

To date, the current hypothesis does not support the possibility that MSCs trans-differentiate into neural cells and shows that limited numbers of injected MSC engraft to the CNS (Gordon et al., 2008). The beneficial effect observed in EAE models after MSC transplantation is mainly due to their paracrine mechanisms, including anti-inflammatory capacity, the ability to protect axons and improve neuronal survival, the direct release of anti-apoptotic, anti-oxidant and neurotrophic factors and the induction of endogenous neurogenesis and oligodendrogenesis (Uccelli et al., 2011).

Furthermore, in addition to these mechanisms of neuroprotection, evidence of fusion between bone marrow-derived stem cells and neuronal cells has recently emerged suggesting a new potential mechanism of neural repair (Weimann et al., 2003; Kemp et al., 2011). Fusion appears to be infrequent under normal biological conditions (Weimann et al., 2003) but is increased by inflammation (Johansson et al., 2008) and could represents a mechanism of neuroprotection through the introduction of functional genes into aged or degenerating cells (Magrassi et al., 2007). In fact, EAE

mice intravenously injected with human MSCs showed an increased number of heterokaryons in the cerebellum compared with control mice and fusion between MSCs and Purkinje cells occurred also *in vitro* and was increased by the addition of IFN-γ and TNF-α (Kemp et al., 2011). In a recent study, Kemp et al. (2012), has shown Purkinje cell fusion also occurs in multiple sclerosis patients and that the formation of binucleate heterokarions were evident and increased in patients with MS compared to normal control patients, suggesting that fusion events in humans could have a large clinical significance.

Some trials to assess the safety and efficacy of MSCs as a potential treatment for multiple sclerosis have started (Karussis et al., 2010; Yamout et al., 2010; Rice et al., 2010; Connick et al., 2012). The mode of delivery, timing of the treatment, ideal cell number for the transplantation, efficacy and above all the long-term safety are still under scrutiny and need to be addressed. Transplanted-related serious adverse effects are likely to be an uncommon event; the main concern is that MSCs might induce tumour cell growth favouring tumour escape from immune system control or increase cell motility and invasion (Uccelli et al., 2011). Malignant transformation upon culture has been described for rat MSCs (Foudah et al., 2009) but not for human MSCs (Bentivegna et al., submitted), but the risk of carcinogenic transformation and infection after in-vitro expansion remains a concern. As a safety measure, minimal expansion may be recommended.
An “International MSCT Study Group” have formed to find a consensus on the preparation and dosage of autologous MSC, to develop a treatment protocol and experimental program that will attest the efficacy and understand the mechanisms that underlie the efficacy of MSCs (Freedman et al., 2010).

Multiple sclerosis affects more than 2 million people worldwide and is the most common non-traumatic cause of disability in young (<50 years) European adults (Pugliatti et al., 2006). Although important advances have been made, no specific therapies are available for patients who have failed to respond to conventional treatments. Innovative approaches are required to control the immune response and to promote tissue repair in MS and mesenchymal stem cells may provide a potential source of cells for reparative therapies due to their multiplicity of neuroprotective and regenerative effects.

2. MATERIALS AND METHODS

CELL CULTURE

2.1 Neuronal cell culture

Neuronal cultures were prepared from cortices of E18 rat embryos. In brief, the pregnant female (time mated), was sacrificed and embryos removed. Each embryo was decapitated, cortices were isolated and the meninges removed. Rounded scalpel was used to cut up the cortex into small pieces and the suspension was centrifuge at 1300rpm for 3 minutes on soft set. Enzymatic and mechanical dissociation were used to obtain a clear cell suspension, in fact cells were treated with accutase for 20 minutes at 37°, DnaseI and cell suspension was triturated using 1ml needle. Cells were then centrifuged at 1600rpm for 3 minutes, counted and plated onto poly-L-lysine coated 13 mm coverslips at $2,5 \times 10^5$/coverslip in 24-well plates or in poly-L-lysine coated 6 well-plate at 2×10^6/well. Cortical cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2% B27 (Gibco, Paisley, UK) and 1% Penicillin and Streptomycin (Sigma-Aldrich, UK) (B27 medium). At 5 days *in vitro* culture, cortical neurons were exposed to experimental conditions. The base medium for all experiments was "minimal medium" (MIN), which consisted of DMEM supplemented with 1% insulin-free Sato (containing 100 µg/mL bovine serum albumin, 100 µg/mL transferrin, 0.06 µg/mL progesterone, 16 µg/mL putrescine, 0.04 µg/mL selenite, 0.04 µg/mL thyroxine, 0.04 µg/mL triiodothryonine), 1% Penicillin and Streptomycin and 0.5% L-glutamine.

2.2 Nitric oxide insult to neurons

A stock solution (50 mM in 10 mM NaOH) of (Z)-1-[2-(aminoethyl)-*N*-(2-ammonioethyl)amino]daizen-1-ium-1,2-diolate (DETANONOate;Alexis Biochemicals, Nottingham, UK) was prepared immediately before use. After 5 days *in vitro* culture, media was removed from all wells, cells were washed in DMEM and minimal media (MIN) or minimal media with 0.1 mM of DETANONOate (MIN+NO) was added to neurons for 1, 2, 4, 6 or 24h.

2.3 Immunocytochemistry

Neuronal cultures were stained after fixation with 4% paraformalaldehyde, permeabilization with100% ice cold methanol at -20˚C for 10 min and blocked with PBS/5% NGS (normal goat serum)/0.1% triton for 30 minutes. Primary antibodies used were phosphorylated axon marker SMI312 (1:600; Sternberger-Cambridge Biosciences, UK), neuronal marker βIII tubulin (1:400; Promega, UK), KIF5A (1:250; Sigma-Aldrich, UK) and KIF21B (1:250; Sigma-Aldrich, UK). Species specific Alexa Fluor® 488 and 555 conjugated secondary antibodies (1:500; Invitrogen, UK) were used to visualize primary antibody staining. DAPI Vectasheid™ (H-1200, Vector Laboratories) was used for nuclear identification.

2.4 Axon length and cell survival assay

After 5 days *in vitro* culture, cortical neurons were exposed to minimal media (MIN), minimal media with NO (MIN+NO) and minimal media with NO and MSCs in a transwell co-culture system (MIN+NO+MSC). Evaluation of axon length and neuronal cell survival was carried out using immunocytochemistry for the phosphorylated axon marker SMI312, neuronal marker βIII tubulin and DAPI for nuclear identification (as

described in section 2.3). Average axon length per field was calculated by measuring the length of SMI312 positive axons per field and co-staining of βIII tubulin/DAPI was used to identify live nuclei for cell survival counts. Cells with fragmented nuclei or with an abnormal shape and size were exclude from the counting. Image J software was used to measure axon length and count cell nuclei. Nine random fields per culture treatment and at least three culture repeats per treatment were analyzed.

2.5 Western blot

Neurons were cultured at 2×10^6/well in a 6-well plate for 5 days before exposure to NO as previously described. At set time points cells were washed in phosphate-buffered saline (PBS) and lysed using Beadlyte cell signalling universal lysis buffer (Upstate™, UK). All protein samples were quantified using a Qubit ® Fluorometer and Quant-iT™ Protein assay kit (Invitrogen, UK) according to manufacturers' instructions to ensure equal loading of samples. Lysates were heated to 95˚C for five minutes with Laemmli 2x sample buffer (Invitrogen, UK) and run on Tris-HCl 10-20% ready gels (Bio-Rad). After transfer to nitrocellulose membrane (Bio-Rad, UK) and blocked in 5% bovine serum albumin (BSA) (Sigma-Aldrich)/Tris-buffered saline-Tween (TBS-T) (Biorad) for 1h, membranes were incubated overnight in primary antibody at 4˚C (in 5% BSA/TBS-T). Antibodies used were rabbit KIF5A (1:15.000; Sigma-Aldrich, UK), rabbit KIF21B (1:15.000; Sigma-Aldrich, UK) and mouse GAPDH (1:15.000; Abcam, UK). Immunoreactivity was detected using secondary anti-rabbit (1:3000) or mouse (1:5000) horseradish peroxidase conjugated antibodies (Abcam, UK) in 5% BSA/TBS-T and specific protein expression patterns were visualized by chemiluminescence using an Amersham ECL Plus™ Western Blotting Detection

System (Amersham, UK). After developing, Image J software (NIH, USA) was used to measure the integrated density of the western blot bands.

2.6 Real-time Polymerase Chain Reaction (RT-PCR)

RNA was extracted and cDNA produced using the Taqman gene expression cells-Ct-kit (Applied Biosystems, UK) according to the manufacturer's instructions. At set time points cells were washed in PBS, lysis solution plus DNAse were added and cells were detached using a scraper. After 5 minutes, stop solution was added for 2 minutes and samples were stored at -80˚C prior to use. All RNA samples were quantified using a Qubit ® Fluorometer and Quant-iT™ RNA assay kit (Invitrogen, UK) according to manufacturers' instructions to ensure equal loading of RNA samples. To synthesize cDNA, 100 ng of extracted RNA was added to the reverse transcription buffer and RT enzyme mix, placed in a thermal cycler, and incubated at 37 °C for 1 hour and 95°C for 5 minutes. RT-PCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, UK) with Assay-on-demand Gene Expression Products for KIF5A, KIF21B, neuron specific enolase (ENO2) and 18S rRNA (Taqman MGB probe, FAM dye-labelled, Applied Biosystems) gene expression master mix using 10 ng of cDNA in a total volume of 20 µl. Reactions were run at 50 °C for 2 minutes; 95 °C for 10 minutes; and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. All samples were analyzed in triplicate. The relative gene expression (RQ value) of KIF5A and KIF21B was calculated using the $2^{-\Delta\Delta Ct}$ method, and the mean taken for each group. ENO2 and 18S rRNA was used as the reference 'housekeeping' gene.

2.7 Dot blot

Neurons were cultured at 2 × 10^6/well in a 6-well plate for 5 days before exposure to NO as previously described. At set time points cells were washed in PBS and lysed using Beadlyte cell signalling universal lysis buffer (Upstate™, UK). All protein samples were quantified using a Qubit ® Fluorometer and Quant-iT™ Protein assay kit (Invitrogen, UK) according to manufacturers' instructions to ensure equal loading of samples. Prior to use, lysates were diluted 1:200 in Tris-buffered saline (TBS) and 100μl of sample added to the Bio-Dot Microfiltration apparatus containing a pre-wet nitrocellulose membrane (Bio-Rad, UK). The samples were then transfered to the membrane for 1 hour using gravity filtration. After transfer to nitrocellulose membrane and blocking in 5%BSA/TBS-T, membranes were incubated overnight in primary antibody at 4˚C (in 5%BSA/TBS-T). Antibodies used were rabbit anti-KIF5A (1:15.000; Sigma-Aldrich, UK), rabbit anti-KIF21B (1:15.000; Sigma-Aldrich, UK), mouse anti NF200 (1:10.000; Sigma-Aldrich, UK) and mouse anti SMI312 (1:3000; Sternberger-Cambridge Biosciences, UK). Immunoreactivity was detected using secondary anti-rabbit or anti-mouse horseradish peroxidase conjugated antibodies (Abcam, UK) in 5%BSA/TBS-T and specific protein expression patterns were visualized by chemiluminescence using an Amersham ECL Plus Western Blotting Detection System (GE Healthcare, UK). Densitometic analysis of protein dots was performed using using ImageJ software.

2.8 Establishment of mesenchymal stem cells cultures

Bone marrow samples were obtained by an Orthopaedic surgeon at Southmead Hospital, Bristol with informed written consent and hospital ethic committee approval. Bone marrow was taken at the time of total hip replacement surgery from the femoral

shaft and placed into a sterile 50 mL tubes containing 1000 IU heparin. Patients with a history of malignancy, immune disorders or rheumatoid arthritis were excluded from the study. Femoral shaft bone marrow donors were healthy apart from osteoarthritis, and were not receiving drugs known to be associated with myelo suppression or bone-marrow failure.

Femoral shaft marrow samples were broken up with a scalpel and washed with Dulbecco's Modified Eagles Medium (Sigma-Aldrich, Gillingham, UK) until remaining material (bone) looked white at the bottom of the 50 mL tube. All washings were pipetted into a new 50 mL tube and kept for centrifugation. The suspension was centrifuged and re-suspended in DMEM and overlaid onto an equal volume of Lymphoprep™ (Axis-Shield, Dundee, UK; density 1.077 ± 0.001 g/mL) and centrifuged at 600 *g* for 35 min at 24°C to separate the mononuclear cells (MNCs) from neutrophils and red cells. The MNC layer was harvested and washed twice in DMEM.

2.9 Mesenchymal stem cells culture

Isolated MSCs were centrifuged and re-suspended in MSCs medium consisting of DMEM with 10% foetal bovine serum which selected for the growth of MSCs (StemCell Technologies, London, UK), and 1% Penicillin and Streptomycin (Sigma-Aldrich). Vented flasks (25 cm^2) containing 10 mL of MSC medium were seeded with 1×10^7 cells for primary culture. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and fed every week with MSCs medium to remove non-adherent hematopoietic cells until the adherent fibroblast-like MSCs reached approximately 70% confluence. On reaching confluence the adherent cells were re-suspended using 0.25% trypsin (Sigma-Aldrich) and re-seeded at 2.25×10^5 cells per

(75 cm^2) flask into first passage. Cultures were then incubated, fed every week with MSC medium, and again trypsinized, a cell count taken and re-seeded at 2.25×10^5 cells per flask (75 cm^2).

2.10 Mesenchymal stem cells immunophenotype

Cells harvested from femoral shaft marrows displayed all the typical characteristics of MSCs in culture. To ensure a homogenous population of MSCs had been cultured, immunophenotyping of surface markers was carried out using flow cytometry. Cells at third passage were detached by trypsinization, collected in tubes (10^6 cells in each tube) and centrifuged at 1,000 rpm for 10 min. Then, cells were decanted, washed with dilution buffer containing 3% FBS and 0.01% sodium azide diluted in PBS, and centrifuged twice at 1,000 rpm for 10 min. Incubation with the following monoclonal antibodies was performed for 30 min at 4°C: PE-conjugated anti-CD73, fluorescein isothiocyanate (FITC)-conjugated anti-CD105, PE-conjugated anti-CD34 and FITC-conjugated anti CD-45 (eBioscience, USA).

In other tubes, cells were incubated with the following appropriate isotype control reagents to estimate the nonspecific binding of target primary antibodies to cell surface antigens: isotype-matching IgG-FITC (Biolegend, San Diego, CA, USA) and IgG-PE (Biolegend, San Diego, USA). After incubation, cells were centrifuged, decanted and resuspended in dilution buffer three times. Analysis was performed on a flow cytometer (BD FACScanto™ FlowCytometer, BD Biosciences, San Jose, USA) after the establishment of gating windows for forward light scatter and side scatter and nonspecific bindings. Data were analyzed using FACS Diva software.

2.11 Mesenchymal stem cells differentiation

MSCs were analyzed for their capacity to differentiate towards osteogenic, adipogenic and chondrogenic lineages at third passage using specific protocols. MSCs grown in culture medium without any differentiating agent were used as a control.

Osteogenic differentiation. Cells were seeded at approximately 4,000 cells/cm^2 on dishes in a culture medium composed of DMEM supplemented with 10% defined FBS until subconfluence occurred. After this period, cells were grown in culture medium alone or in osteogenic medium (OS medium) consisting of the same culture medium with the addition of the following supplements (Sigma-Aldrich, St. Louis, MO): 100 nM dexamethasone, 10 mM β-glycerophosphate and 0.05 mM ascorbic-2-phosphate acid. Osteogenic differentiation was evaluated by Alizarin red S staining which visualizes calcium deposits.

Adipogenic differentiation. Cells were seeded at approximately 20,000 cells/cm^2 onto dishes containing a culture medium composed of DMEM supplemented with 10% defined FBS. After 24 hours cells were induced by treatment with adipogenic induction medium (AIM), consisting of DMEM High Glucose (glucose 4.5 g/L) plus 10% defined FBS supplemented with 10 μg/ml insulin, 500 μM isobutylmethylxanthine, 100 μM indomethacin and 1 μM dexamethasone (Sigma-Aldrich, St. Louis, MO). After 12 days of treatment with AIM, MSCs were treated with adipogenic maintainance medium (AMM) consisting of DMEM (4.5 g/L glucose) plus 10% defined FBS supplemented with 10 μg/ml insulin. Adipogenic differentiation was evaluated by examining the accumulation of lipid vacuoles using Oil Red O staining.

Chondrogenic differentiation. Chondrogenic differentiation was induced by growing cells as a pellet in 15ml tubes, at approximately 250,000 cells/tube, in chondrogenic

medium for about 6 weeks. MSCs chondrogenic medium consisted of serum-free DMEM (4.5g/L glucose) with the addition of ITS+premix (BD Pharmigen, Germany; 1:100), 1 mM piruvate (Lonza, Verviers, Belgium), 100 nM dexamethasone, 50 μg/ml ascorbic acid 2-phosphate and 10 ng/ml TGF-β_3 (PeproTech, London, UK). ITS+premix was used as a serum substitute and consisted of 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenic acid, 5.35 μg/ml linoleic acid and 1.25 μg/ml BSA. Sections of paraffin-embedded pellets were stained with hematoxylin-eosin and Safranin O staining, in order to evaluate the formation of cartilaginous structures and the presence of proteoglycans and glycosaminoglycans.

2.12 Mesenchymal stem cells conditioned medium

Confluent mesenchymal stem cell cultures, at third passage, were washed in DMEM and cultured for 24h in minimal base medium. Media was then removed, added to neuronal cultures for 3 hours and then cells were exposed to DETANONOate.

2.13 Mesenchymal stem cells transwell cultures

Confluent mesenchymal stem cell cultures, at third passage, were washed in DMEM, trypsinised and cultured in transwell inserts (Millipore, Watford, UK) at 100.000 cell per well (24 well plate) or 400.000 cell per well (6 well plate) for 72h in DMEM with 10% FBS and 1% Penicillin and Streptomycin. Media was then removed, minimal media was added and bone marrow-derived stem cells in transwells were added to cortical neurons cultures 3 hours prior to the addition of DETANONOate.

HUMAN TISSUE

2.14 Patients

Post-mortem cerebellum samples from 8 patients with multiple sclerosis and 6 control patients were obtained through collaboration with The UK Multiple Sclerosis Tissue Bank at the Imperial College, London, UK (Table 1). All patients had been clinically diagnosed as having multiple sclerosis and this diagnosis had been confirmed during neuropathological autopsy examination. Control cerebellum samples were derived from patients who had died from causes other than neurological disease. All tissues were collected with the donors' fully informed consent via a prospective donor scheme. At death, brains were removed; and either snap frozen or fixed in neutral buffered formalin and tissue blocks embedded in paraffin. Sections were cut from cerebellar tissue of each patient and mounted onto glass slides.

Table 1. Characteristics of both control and multiple sclerosis patients

Patient	Control/ MS	Age (yrs)	Sex (M/F)	Cerebella lesion	Duration of disease (yrs)	Classification of MS
1	Control	82	M	non present	0	n/a
2	Control	35	M	non present	0	n/a
3	Control	88	M	non present	0	n/a
4	Control	68	M	non present	0	n/a
5	Control	84	M	non present	0	n/a
6	Control	82	M	non present	0	n/a
1	MS	75	M	non present	38	RR
2	MS	72	F	non present	41	RR/SP
3	MS	78	F	CI	42	RR/SP
4	MS	64	F	CA	36	SP
5	MS	49	F	CI	18	SP
6	MS	49	F	CI	23	SP
7	MS	42	F	active	6	PP
8	MS	44	M	CA/active	10	SP

MS = multiple sclerosis

n/a = not applicable

CA = Chronic active

CI = Chronic inactive

RR = Relapsing-remitting

PP = Primary progressive

SP = Secondary progressive

2.15 DAB staining on paraffin sections

To characterize MS lesions, immunostaining was performed to identify the centres and borders of the lesions and lesion activity. Cerebellar sections 10 µm in thickness were immunostained with antibodies to myelin basic protein (MBP) (1:3200; Serotec, Oxford, UK), HLA-DR (1:800; Dako, Cambridgeshire, UK), KIF5A and KIF21B (1:500; Sigma-Aldrich, UK). Sections were deparaffinised in Clearene, dehydrated in 100% ethanol, hydrated in distilled water, and immersed in 3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity, rinsed and microwaved in sodium citrate buffer (0.01 M, pH 6.0, 5 minutes) and rinsed in PBS. Non-specific binding was blocked with Vectastain blocking serum (20 minutes). After addition of the primary antibody in 3% BSA/0.1% azide, sections were incubated overnight at 4°C. The sections were then rinsed in PBS before incubation for 20 minutes with secondary antibody (Vectastain Biotinylated Universal antibody) and 20 minutes with VectaElite ABC Complex (PK-6200, Vector Laboratories, Peterborough, UK) followed by a 10-minute incubation with 3,3′-diaminobenzidine (DAB) and 0.01% H_2O_2. Sections were washed in water, immersed in copper sulphate DAB enhancer (4 minutes), counterstained with hematoxylin, dehydrated, cleared and mounted. For light imaging, images were acquired using an Olympus IX70 microscope coupled with Image-Pro Plus software.

2.16 Immunofluorescence staining on paraffin sections

Sections (10 μm) were deparaffinized, hydrated and washed, as above. To reduce auto-fluorescence, sections were incubated in 5mM copper sulphate and 50mM ammonium acetate for 1 h at room temperature prior to microwaving in sodium citrate buffer (0.01 M, pH 6.0, 5 min). Cells were labelled by double immunofluorescence using mouse anti-APP (1:200; Chemicon) and rabbit anti-KIF5A or KIF21B (1:200; Sigma-Aldrich, UK).

Non-specific binding was blocked with 10% normal goat serum diluted in PBS containing 0.1% Triton. Sections were incubated at 4°C overnight with primary antibodies. Sections were then washed in PBS and incubated for 30 min in the dark with Alexa Fluor 488 goat anti-mouse (1:500) or Alexa Fluor 555 goat anti-rabbit (1:500; Invitrogen) before being washed in PBS and mounted in Vectasheid™ medium containing the nuclear dye DAPI (H-1200, Vector Laboratories).

2.17 Real-time Polymerase Chain Reaction (RT-PCR)

Frozen tissue blocks were thawed on ice and the grey matter carefully dissected away from white matter layers. The grey matter was then homogenized on ice by use of the PARIS kit (Ambion, UK) and a protease and phosphatase inhibitor cocktail (Thermo Scientific, UK) added at a dilution 1:100. RNA was then further extracted from 300μl of tissue lysate using the PARIS kit (Ambion, UK) according to the manufacturer's instructions. All RNA samples were quantified using a Qubit Fluorometer and Quant-iT RNA assay kit (Invitrogen, Paisley, UK) according to manufacturers' instructions to ensure equal loading of RNA samples. Samples were stored at -80˚C prior to use. To synthesize cDNA, 100 ng of extracted RNA was

added to the reverse transcription buffer and RT enzyme mix, placed in a thermal cycler, and incubated at 37 °C for 1 hour and 95°C for 5 minutes. RT-PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Paisley, UK) with Assay-on-demand Gene Expression Products for KIF5A, KIF21B, GAPDH, neuron specific enolase (ENO2) and 18s rRNA (Taqman MGB probe, FAM dye-labelled, Applied Biosystems, Paisley, UK) using 10 ng of cDNA in a total volume of 20 µl. Reactions were run at 50 °C for 2 minutes; 95 °C for 10 minutes; and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. All samples were analyzed in triplicate. The relative gene expression (RQ value) of a specific gene was calculated using the $2^{-\Delta\Delta Ct}$ method, and the mean taken for each group. GAPDH, ENO2 or 18S was used as the reference 'housekeeping' genes.

2.18 Dot blot

Frozen tissue blocks were thawed on ice and the grey matter carefully dissected away from white matter layers. The grey matter was then homogenized on ice by use of the PARIS kit (Ambion, UK) and a protease and phosphatase inhibitor cocktail (Thermo Scientific, UK) added at a dilution 1:100. The supernatants were removed and stored at −80°C until required. Prior to use, lysates were diluted 1:200 in Tris-buffered saline (TBS) and 100µl of sample added to the Bio-Dot Microfiltration apparatus containing a pre-wet nitrocellulose membrane (Bio-Rad, UK). The samples were then transfered to the membrane for 1 hour using gravity filtration. After transfer to nitrocellulose membrane and blocking in 5%BSA/TBS-T, membranes were incubated overnight in primary antibody at 4˚C (in 5%BSA/TBS-T). Antibodies used were rabbit anti-KIF5A (1:15.000; Sigma-Aldrich, UK), rabbit anti-KIF21B (1:15.000; Sigma-Aldrich, UK), mouse anti-GAPDH (1:10.000; Abcam, UK), rabbit anti-ENO2

(1:3000; Abgent, UK), mouse anti-microtubule associated protein 2 (MAP2) (1:5000; Sigma-Aldrich, UK) and mouse anti-calbindin D28K (1:5000; Sigma-Aldrich, UK). Immunoreactivity was detected using secondary anti-rabbit or anti-mouse horseradish peroxidase conjugated antibodies (Abcam, UK) (in 5%BSA/TBS-T) and specific protein expression patterns were visualized by chemiluminescence using an Amersham ECL Plus Western Blotting Detection System (GE Healthcare, UK). Densitometic analysis of protein dots was performed using using ImageJ software. GAPDH, ENO2, MAP2 and calbindin was used as the reference loading control proteins.

3. RESULTS

3.1 The effect of nitric oxide exposure on both the expression of KIF5A and KIF21B in rodent cortical neurons and subsequently the correlation between KIF expression and axon pathology

3.1.1 The effect of nitric oxide exposure on the neuronal expression of KIF5A and KIF21B

Cortical neurons were maintained in B27 supplemented DMEM for 5 days before exposure to minimal media (MIN), which consisted of DMEM (without serum) supplemented with chemically defined factors, with or without the presence of the nitric oxide donor DETANONOate. After 24h cultures were fixed and immunolabelled for KIF5A and KIF21B with the addition of the nuclear stain DAPI Vectashield.

KIF5A expression was localised in both the cell body and axon of cortical neurons; no differences in KIF5A expression were evident between neurons cultured either in B27 or MIN media. KIF5A expression was decreased when neurons were exposed to NO. This loss was especially evident in the axons of cells (with nearly a complete absence), whereas a small number of cell bodies still presented a low KIF5A expression post nitric oxide exposure (Fig. 3.1.1 A). KIF21B is considered to be enriched in dendrites (Marszalek et al., 1999), in this study KIF21B expression was detected in both the cell body and in what appears to be a network of dendrites. As with KIF5A, KIF21B expression in neurons exposed to B27 and MIN media were seemingly identical. Furthermore, NO exposure to neuronal cultures resulted in an extensive loss of KIF21B protein (Fig. 3.1.1 B).

Fig. 3.1.1 Nitric oxide causes a reduction in both KIF5A (A) and KIF21B (B) expression.

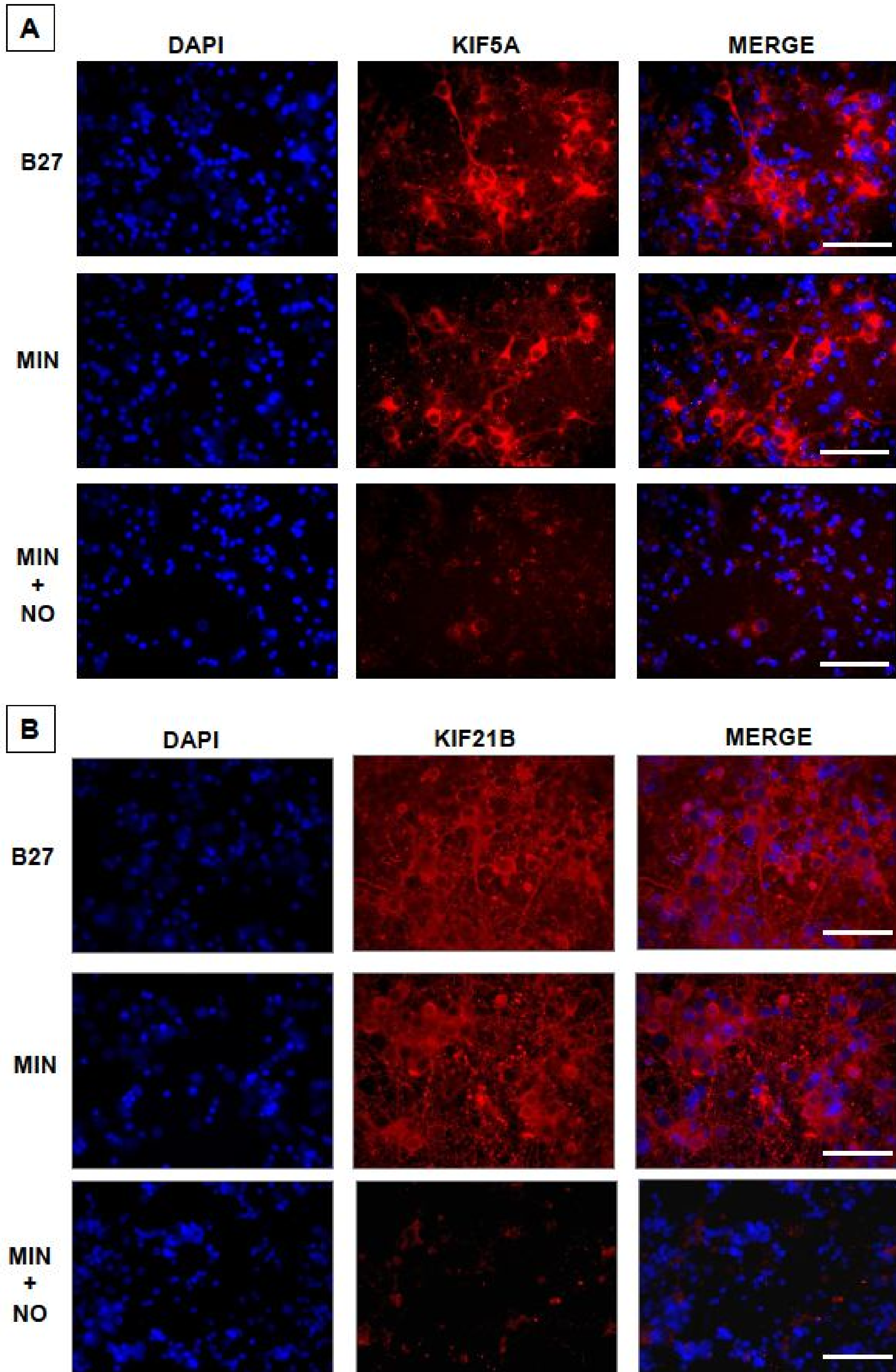


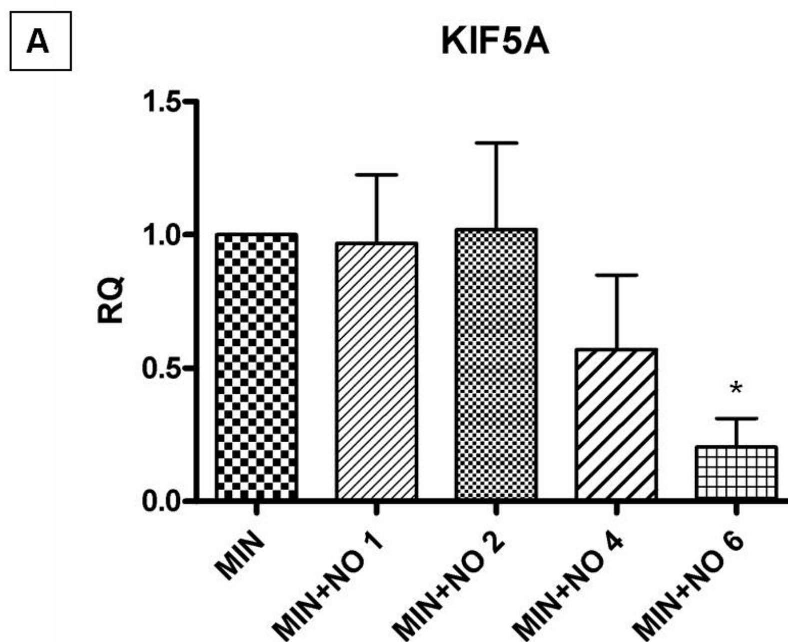
Fig. 3.1.1 Cortical neurons were exposed to B27 media, MIN media and MIN media with the addition of NO for 24h. Cultures were labeled for KIFs proteins (red) and the nuclear marker DAPI (blue). Bars: 100µm.

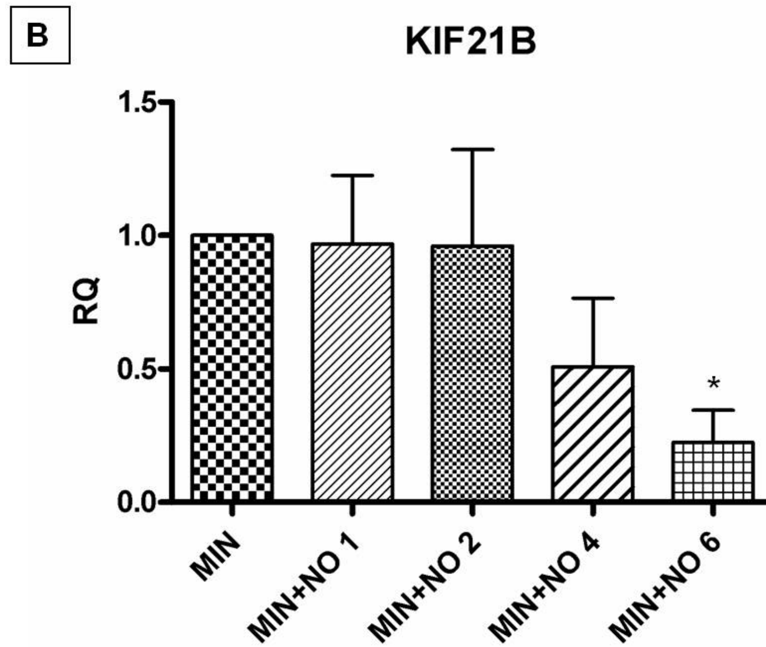
3.1.2 Nitric oxide reduces KIF5A and KIF21B gene expression in a time dependent manner

To investigate the effect of NO exposure on KIF gene expression, KIF expression at the genomic level was investigated using RT-PCR. Cortical neurons were maintained in B27 supplemented DMEM for 5 days and then neurons were exposed to minimal medium with the addition of nitric oxide for 1, 2, 4 and 6h. After each time point mRNA was extracted and RT-PCR performed. Cortical neurons cultured in minimal media alone represent control levels of KIF gene expression.

Analysing the KIF5A and KIF21B mRNA over a 6 hour period, revealed that NO exposure resulted in an approximate 75% decrease in both KIF5A and KIF21B mRNA expression in cortical neurons when compared to exposure to minimal medium alone ($p<0.05$) (Fig. 3.1.2).

Fig. 3.1.2 KIF5A (A) and KIF21B (B) gene expression in cortical neurons at different time points post NO exposure. Results are expressed as the mean +/- standard error (n=5; *p<0.05).



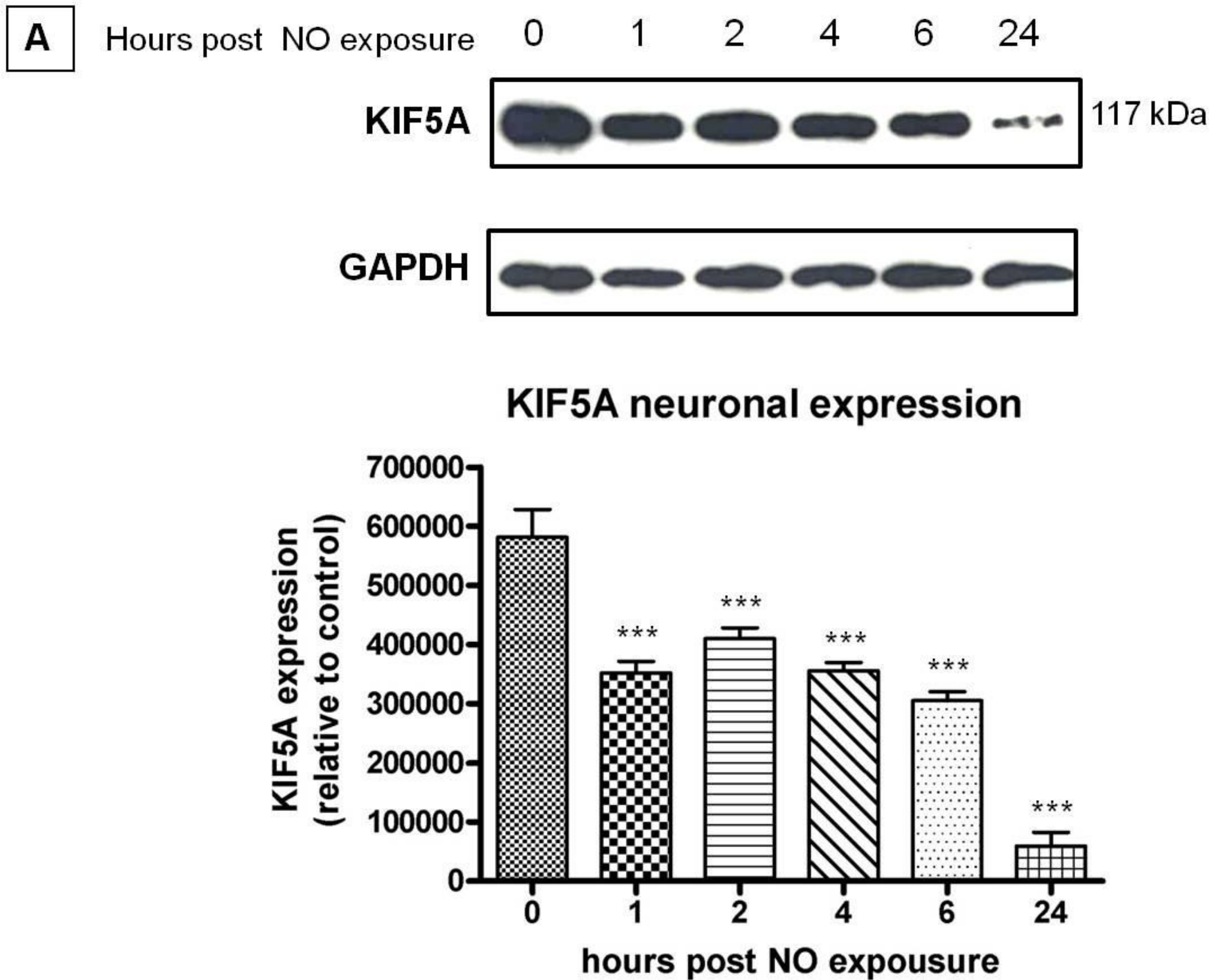


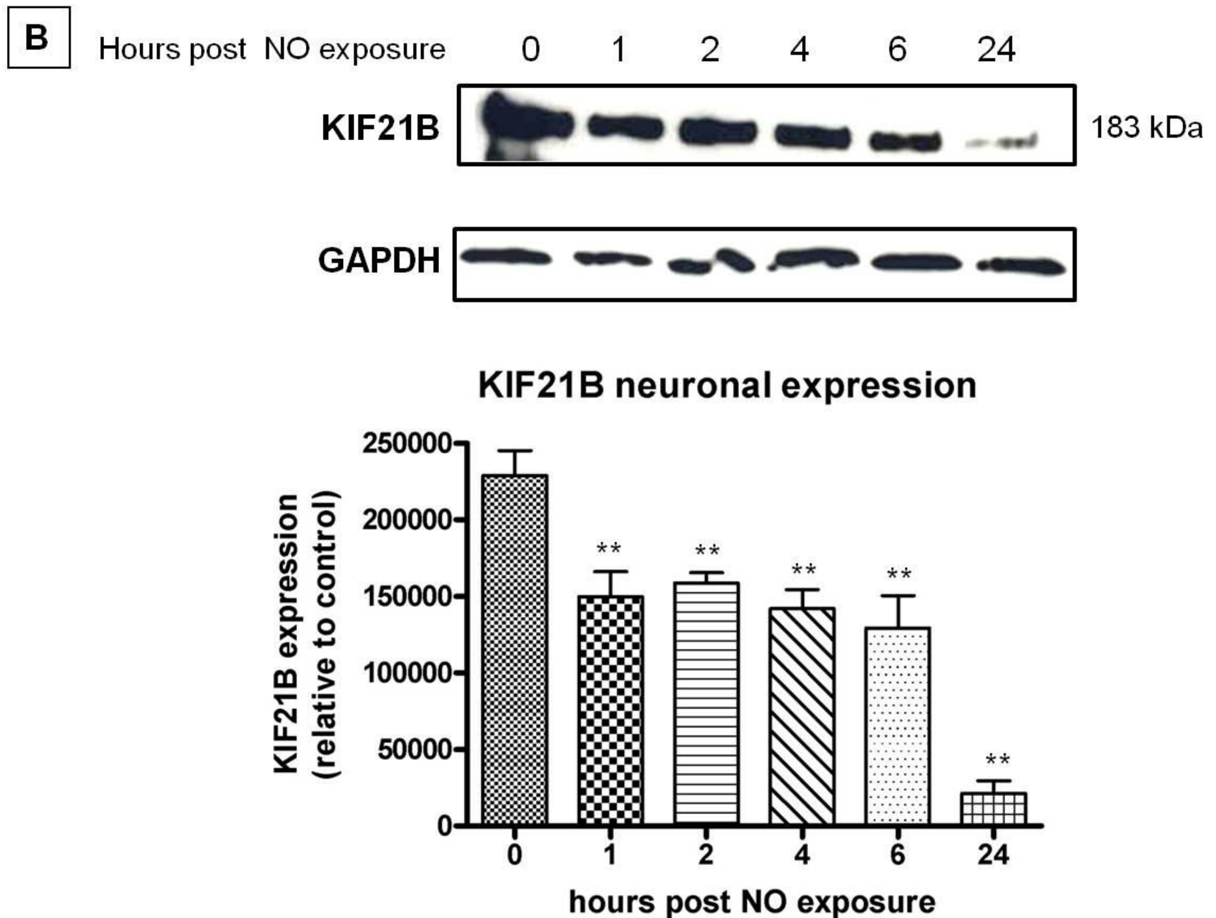
3.1.3 Nitric oxide reduces both KIF5A and KIF21B protein expression in a time dependent manner

To further investigate the effect of NO exposure on KIF protein expression, cortical neurons were exposed to minimal medium with the addition of nitric oxide for 1, 2, 4, 6 and 24h and after each time point proteins were extracted and western blot analysis performed. Cortical neurons cultured in minimal media alone represent control levels of KIF protein expression.

Using densitometric analysis of the western blots bands, results showed that NO caused a significant reduction in both KIF5A ($p<0.001$) (Fig. 3.1.3 A) and KIF21B ($p<0.01$) (Fig. 3.1.3 B) protein expression in a time dependent manner over a 24h period. After the first hour post NO exposure there was a significant decrease in protein expression of both KIF5A (approx 40%) and KIF21B (approx 35%), which after 24h reached a final reduction of approximately 85% for both proteins.

Fig.3.1.3 KIF5A (A) and KIF21B (B) western blots and corresponding imaging analysis of cortical neurons exposed to NO for different time periods. Results are expressed as the mean +/- standard error (n=3; ***p<0.001; **p<0.01).





3.1.4 Both axonal length and neuronal survival decrease in rodent cortical neurons exposed to nitric oxide

Cortical neurons were maintained in minimal media alone or with the addition nitric oxide for 24h, cells were then fixed and stained for the neuronal marker βIII tubulin, pan-phosphorylated axonal marker SMI312 and the nuclear marker DAPI Vectashield (Fig. 3.1.4 A). Axonal length was measured using SMI312 labelling per culture field while the number of viable neurons were counted using DAPI nuclear morphology and βIII tubulin positivity per field.

Cortical neurons when cultured with NO showed a significant reduction in phosphorylated axonal length compared to control ($p<0.001$) (Fig. 3.1.4 B). The

number of neurons expressing DAPI and βIII tubulin were counted, excluding cells with fragmented nuclei or with nuclei with an abnormal shape, to determine the neuronal survival in each condition. Neurons exposed to MIN showed a small reduction in survival when compared to B27 and total live cells were significantly decreased in the presence of NO ($p<0.001$) (Fig. 3.1.4 C).

Fig. 3.1.4 NO causes reduction in both axonal length and neuronal survival

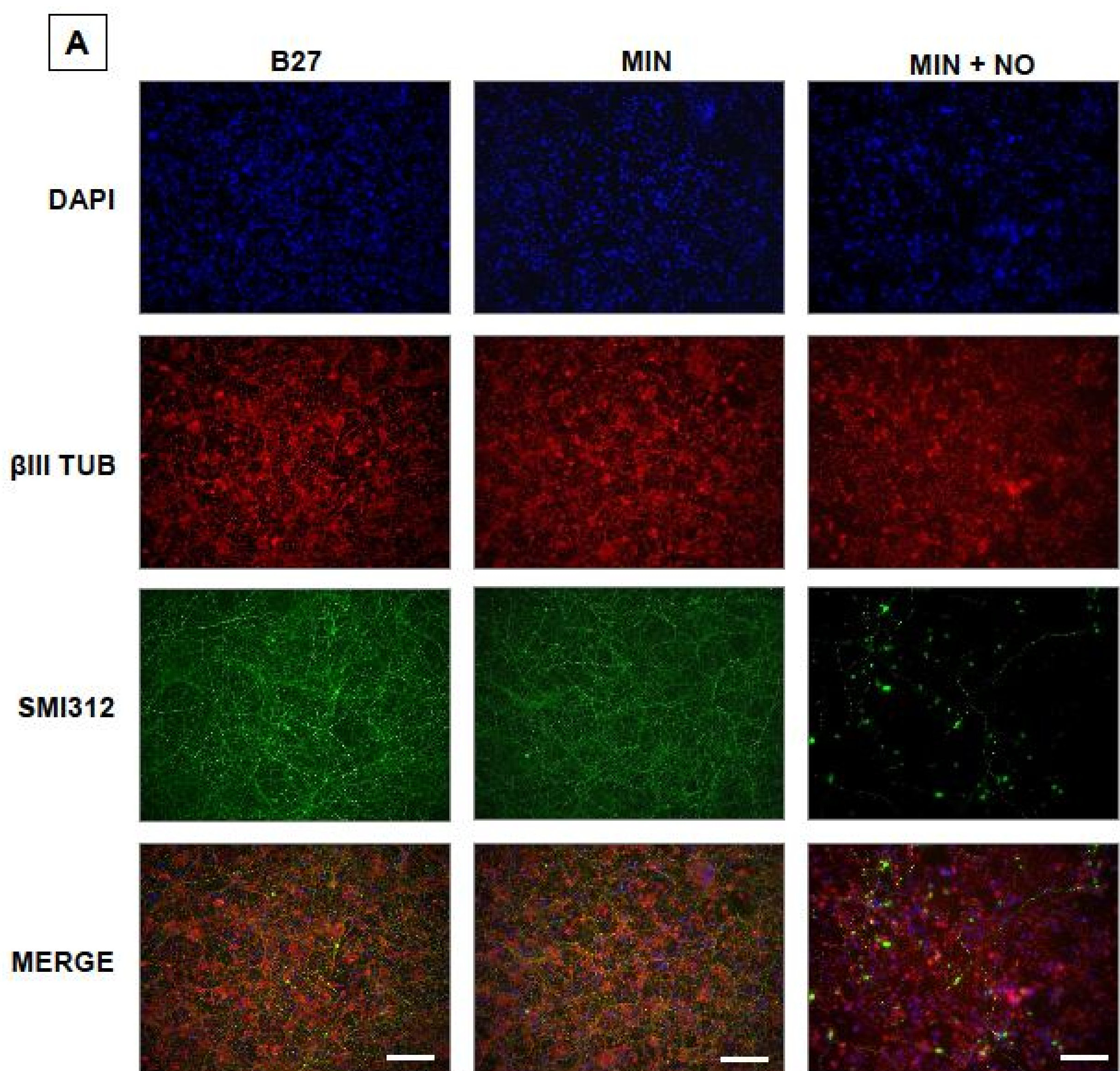


Fig 3.1.4 Cortical neurons were exposed to B27 media, MIN media and MIN media with the addition of NO for 24h. Cultures were stained for the nuclear marker DAPI (blue), neuronal marker βIII tubulin (red) and phosphorylated axonal marker SMI312 (green). Bar: 100µm.

B

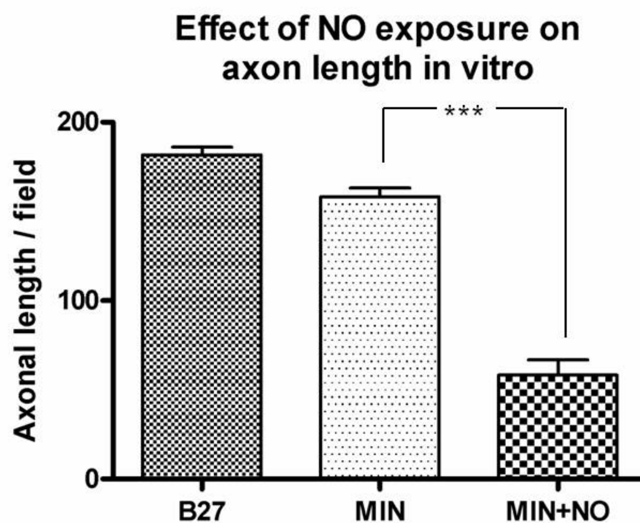


Fig. 3.1.4 Exposure of cortical neurons to NO for 24h causes a reduction in axonal length per culture field compared to minimal media (MIN) alone. Results are expressed as the mean +/- standard error (n=4; ***p<0.001).

C

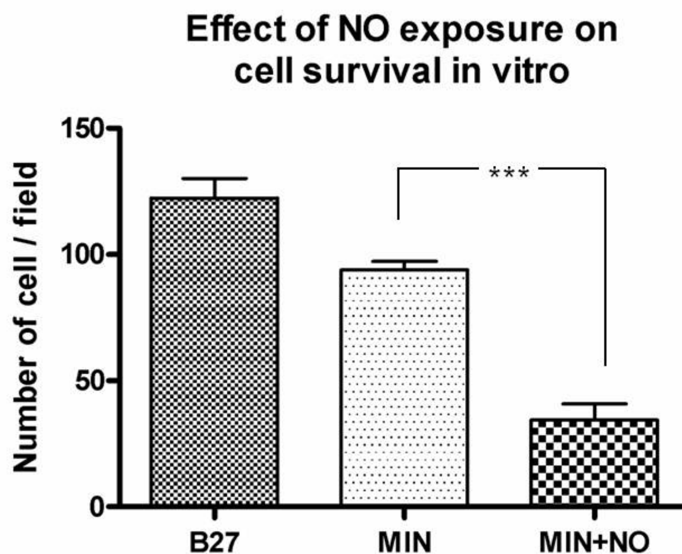


Fig. 3.1.4 Exposure of cortical neurons to NO for 24h causes a reduction in total neuronal survival per culture field compared to minimal media (MIN) alone. Results are expressed as the mean +/- standard error (n=4; ***p<0.001).

3.1.5 Nitric oxide causes a time dependent loss of axon phosphorylation

In order to analyze the effect of NO exposure on the axon phosphorylation, dot blot protein expression analysis of SMI312 (pan-phosphorylated neurofilament marker) and NF200 (phosphorylated and non phosphorylated neurofilament marker) was performed. Cortical neurons were maintained in B27 supplemented DMEM for 5 days before exposure to NO for 1, 2, 4, 6 and 24h and at each time point proteins were extracted and dot blot performed.

Results show that NO caused a time dependent decrease in SMI312 and NF200 expression over a 24h period ($p<0.001$), but the reduction of SMI312 was greater and occurred earlier than that of NF200 (Fig. 3.1.5.1). In order to calculate an index of total phosphorylated neurofilament in axons after NO exposure, the ratio SMI312/NF200 (phosphorylated neurofilament/total neurofilament) was determined. Using this analysis it was demonstrated that NO caused a significant time dependent decrease ($p< 0.01$) in axon phosphorylation over a 24h period (Fig. 3.1.5.2).

Fig. 3.1.5.1 NF200 and SMI312 dot blots and corresponding densitometric analysis of cortical neurons exposed to NO over a 24h period. Results are expressed as the mean +/- standard error (n=3; ***p<0.001).

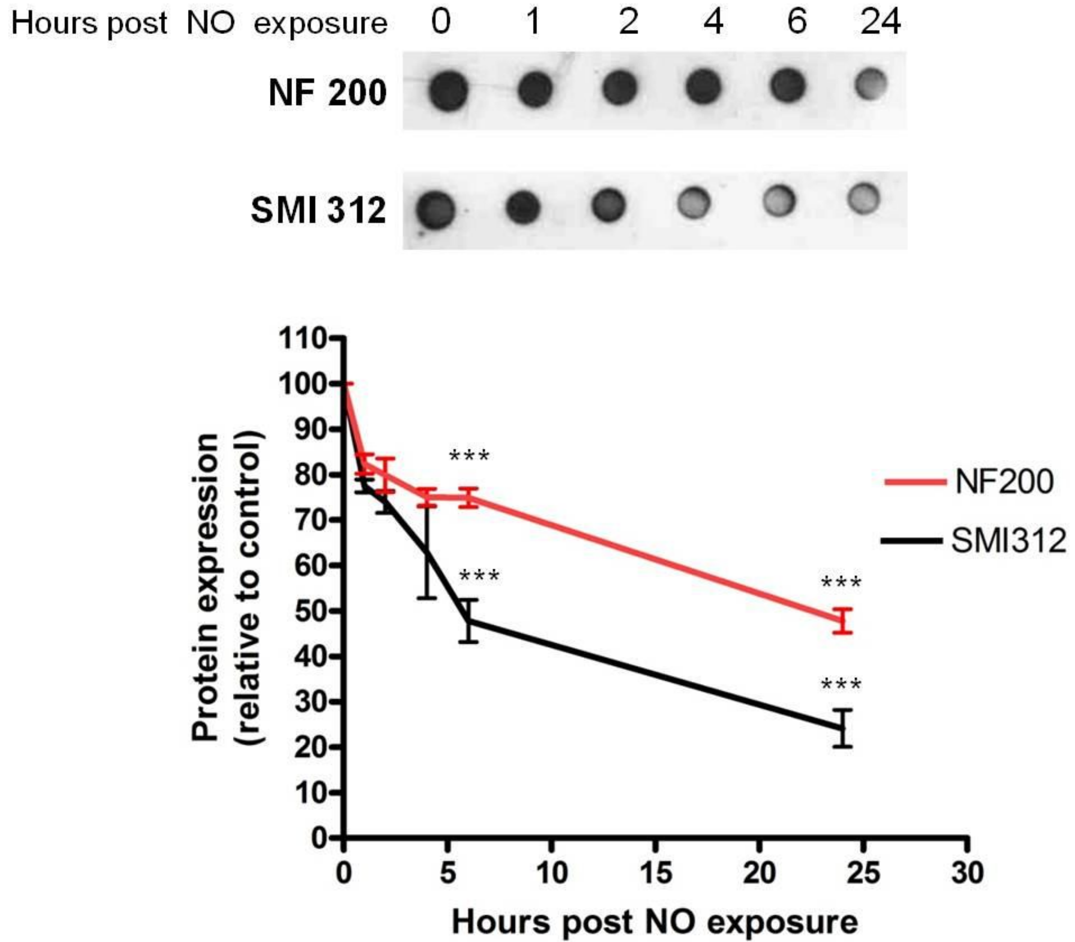
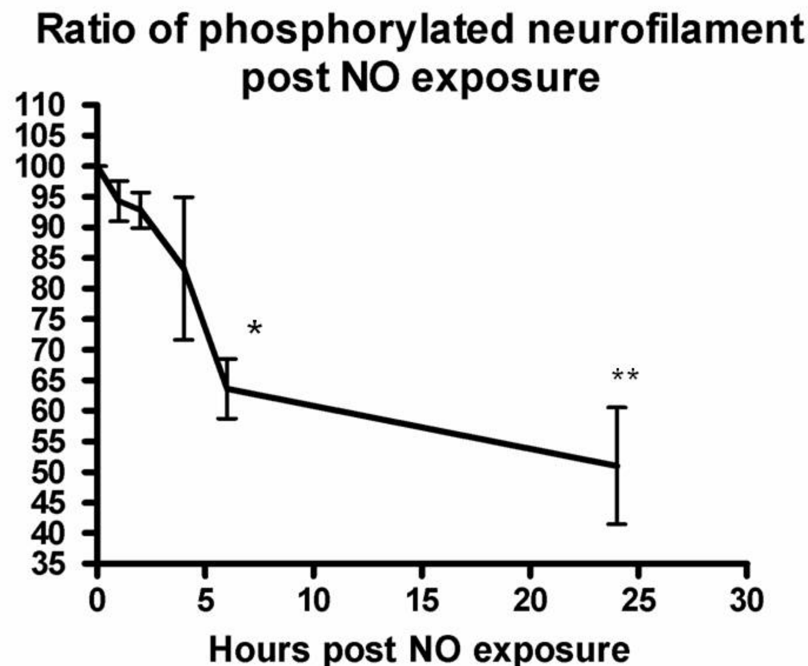


Fig. 3.1.5.2 NO causes a time dependent decrease in axonal phosphorylation calculated using the ratio of phosphorylated neurofilament/total neurofilament (SMI312/NF200). Results are expressed as the mean +/- standard error (n=3; *p<0.05; **p<0.01).



3.1.6. KIFs reduction precedes the loss of neurofilament

In order to determine if KIF expression correlates with axon damage, dot blots for the axon marker NF200 and KIF proteins were perform in cortical neurons after 1, 2, 4, 6 and 24h post NO exposure.

NO caused a time dependent decrease in NF200, KIF5A and KIF21B expression over a 24h period ($p<0.001$) (Fig. 3.1.6.1). To clarify if axon loss follows KIF reduction or if KIF reduction is caused by axon loss, the ratio KIF5A/NF200 and KIF21B/NF200 was calculated at each time point. Results showed that both ratios decreased in a time dependent manner, suggesting that KIF5A ($p<0.05$) and KIF21B ($p<0.01$) reduction precedes the loss of total NF (Fig. 3.1.6.2).

Fig. 3.1.6.1 NF200, KIF5A and KIF21B dot blots and corresponding densitometric analysis of cortical neurons exposed to NO over a 24h period. Results are expressed as the mean +/- standard error (n=3; * p<0.05; **p<0.01 ; ***p<0.001).

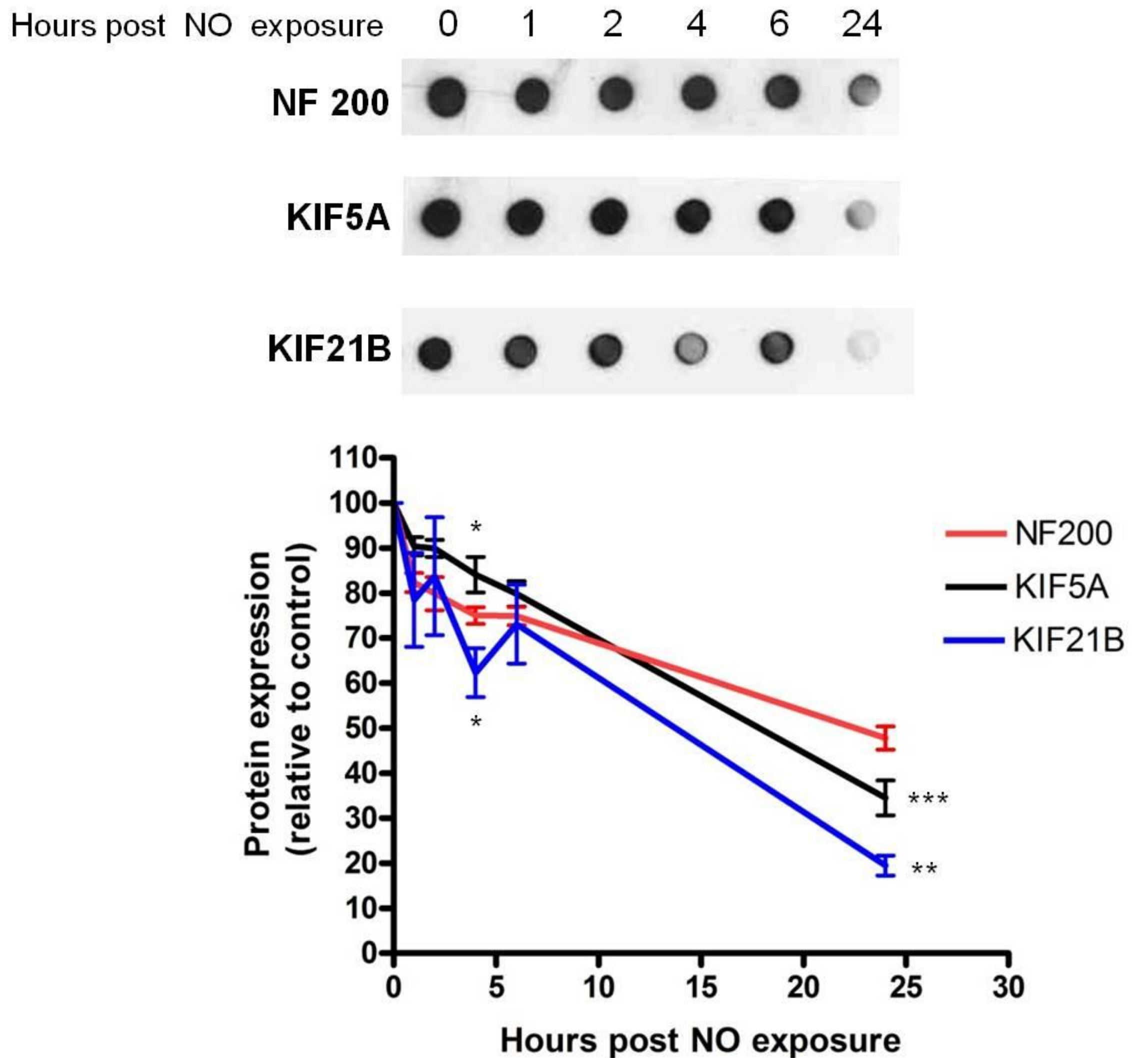
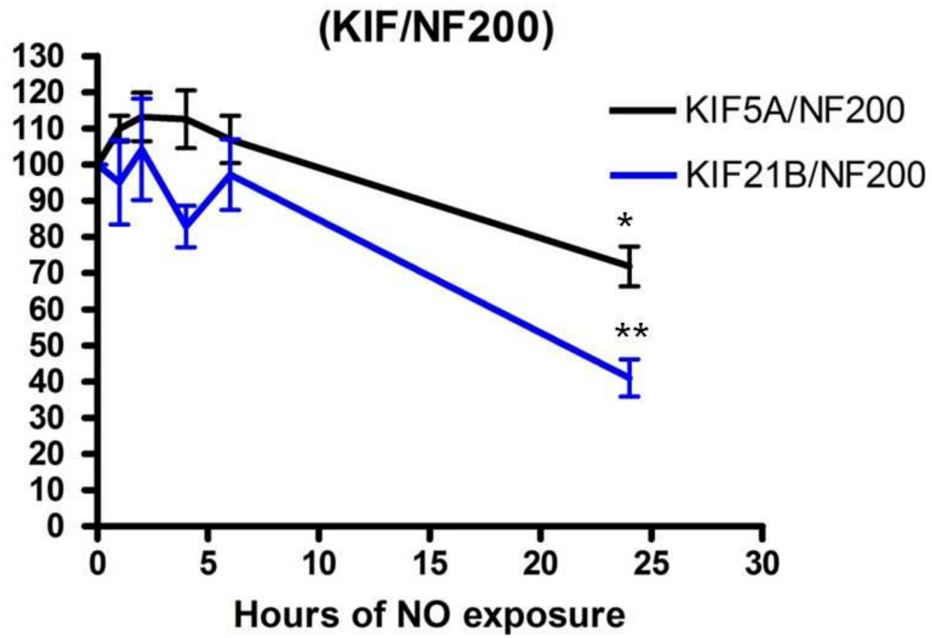


Fig. 3.1.6.2 KIFs reduction precedes the loss of NF calculated using the ratio KIF/NF200. Results are expressed as the mean +/- standard error (n=3; * p<0.05; **p<0.01).



3.2 The capacity of MSCs to protect axons and axonal transport in cortical neurons exposed to nitric oxide

3.2.1 MSC characterization

Femoral shaft-derived human MSCs cultured *in vitro* were plastic-adherent, displayed fibroblastic morphology characterized by their spindle-like shape and capable of extensive proliferation when maintained in standard culture conditions. The immunological characterization of MSCs was performed at third culture passage by flow-cytometric analysis using specific antibodies for the membrane antigens CD73, CD105, CD34 and CD45. Approximately 90% of MSCs were positive for both CD73 and CD105 and negative for CD34 and CD45 (Fig 3.2.1.1).

The differentiation potential of MSC towards adipogenic, osteogenic, and chondrogenic lineages was tested by culturing cells under specific *in vitro* culture conditions. Adipocyte differentiation was demonstrated by the accumulation of vesicles, visible macroscopically, of lipid and triglycerides stained using oil red O (Fig. 3.2.1.2 A). Osteogenic differentiation was observed using alizarin red S staining for basic calcium phosphate crystals (Fig. 3.2.1.2 B) while differentiation of MSCs into chondrocytes was visualized using safranin O, that stains proteoglycans and glycosaminoglycan of the extracellular matrix of cartilage with a bright red color (Fig. 3.2.1.2 C).

Fig. 3.2.1.1 MSCs immunophenotype

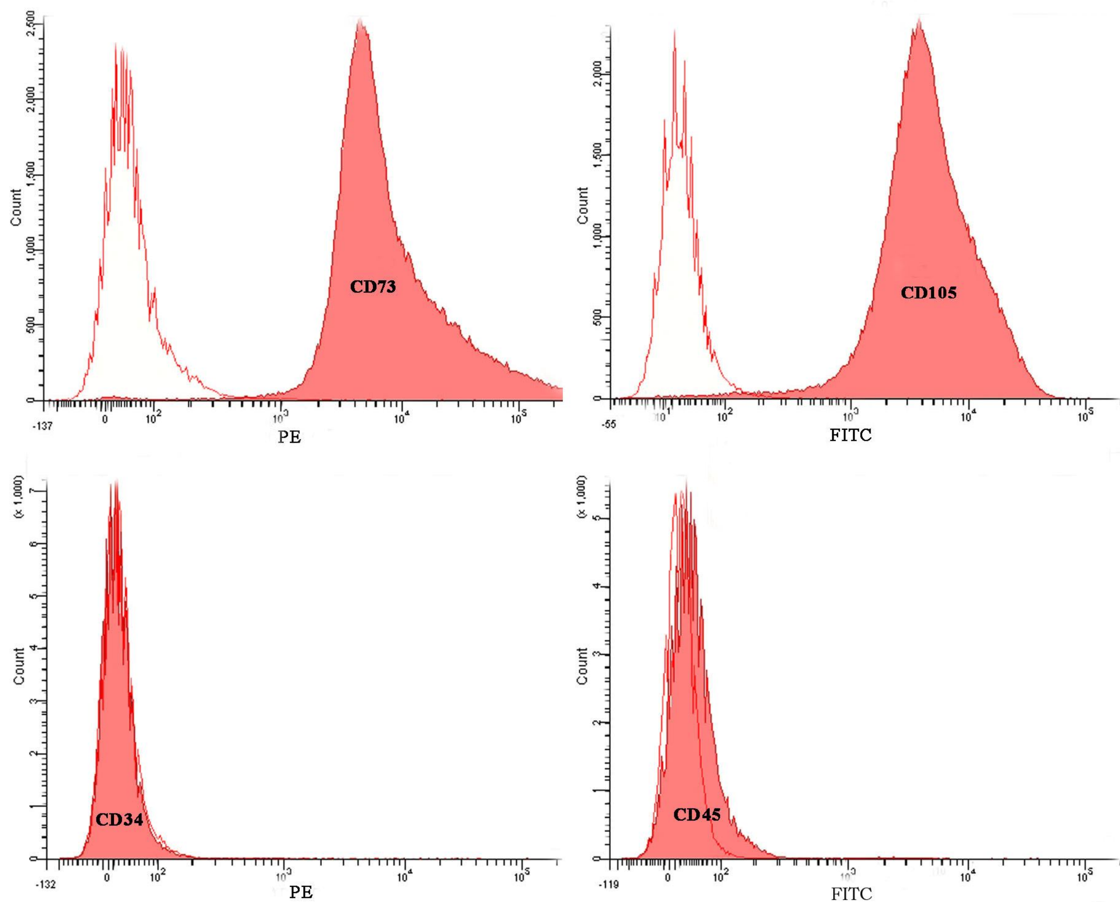


Fig. 3.2.1.1 Human MSCs were characterized by flow cytometric analysis for the expression of the following markers (pink histograms): CD73, CD105, CD34 and CD45. Approximately 90% of MSCs were positive for CD73 and CD105 and negative for CD34 and CD45. Isotype-matching IgGs were used to determine non specific signals (white histograms).

Fig. 3.2.1.2 Mesengenic differentiation of MSCs

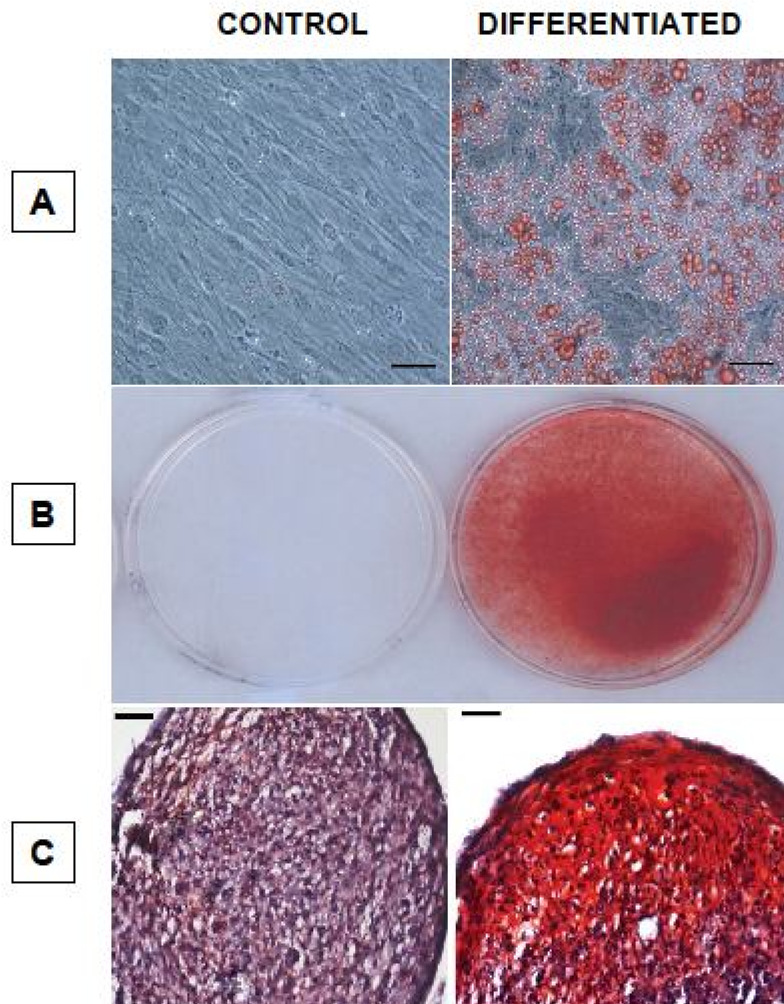


Fig. 3.2.1.2 After treatment with specific induction media, MSC at third passage, were differentiated toward adipogenic, osteogenic and chondrogenic lineages (DIFFERENTIATED). Adipogenic differentiation was evaluated by oil red O staining that labels lipid droplets (A), osteogenic differentiation was evaluated by alizarin red staining that visualizes calcium deposits with a bright red color (B) and chondrogenic differentiation was evaluated by safranin staining of paraffin-embedded sections that labels in red proteoglycans and glycosaminoglycan of the extracellular matrix of cartilage (C). MSCs not treated with specific induction media (CONTROL) were negative for all the staining methods. Bar:100 µm.

3.2.2 MSCs preserve axonal length and increase survival in cortical neurons exposed to nitric oxide

Cortical neurons were maintained in B27 supplemented DMEM for 5 days before exposure to minimal media and nitric oxide with/without the addition of MSCs in a transwell co-culture system, which allows the exchange of soluble factors without any direct contact between the neurons and MSCs. After 24h, neurons were fixed and stained for the neuronal marker βIII tubulin, pan-phosphorylated axonal marker SMI312 and the nuclear marker DAPI Vectashield (Fig. 3.2.2 A).

Axonal length was measured using SMI312 labelling and the number of viable neurons was counted using DAPI nuclear morphology and βIII tubulin positivity. Both axonal length (Fig. 3.2.2 B) and total live cells (Fig. 3.2.2 C) were significantly increased in co-cultures of MSC and cortical neurons exposed to NO compared with cortical neurons exposed to NO alone ($p<0.001$).

Fig. 3.2.2 MSCs increase axonal length and survival of cortical neurons exposed to NO.

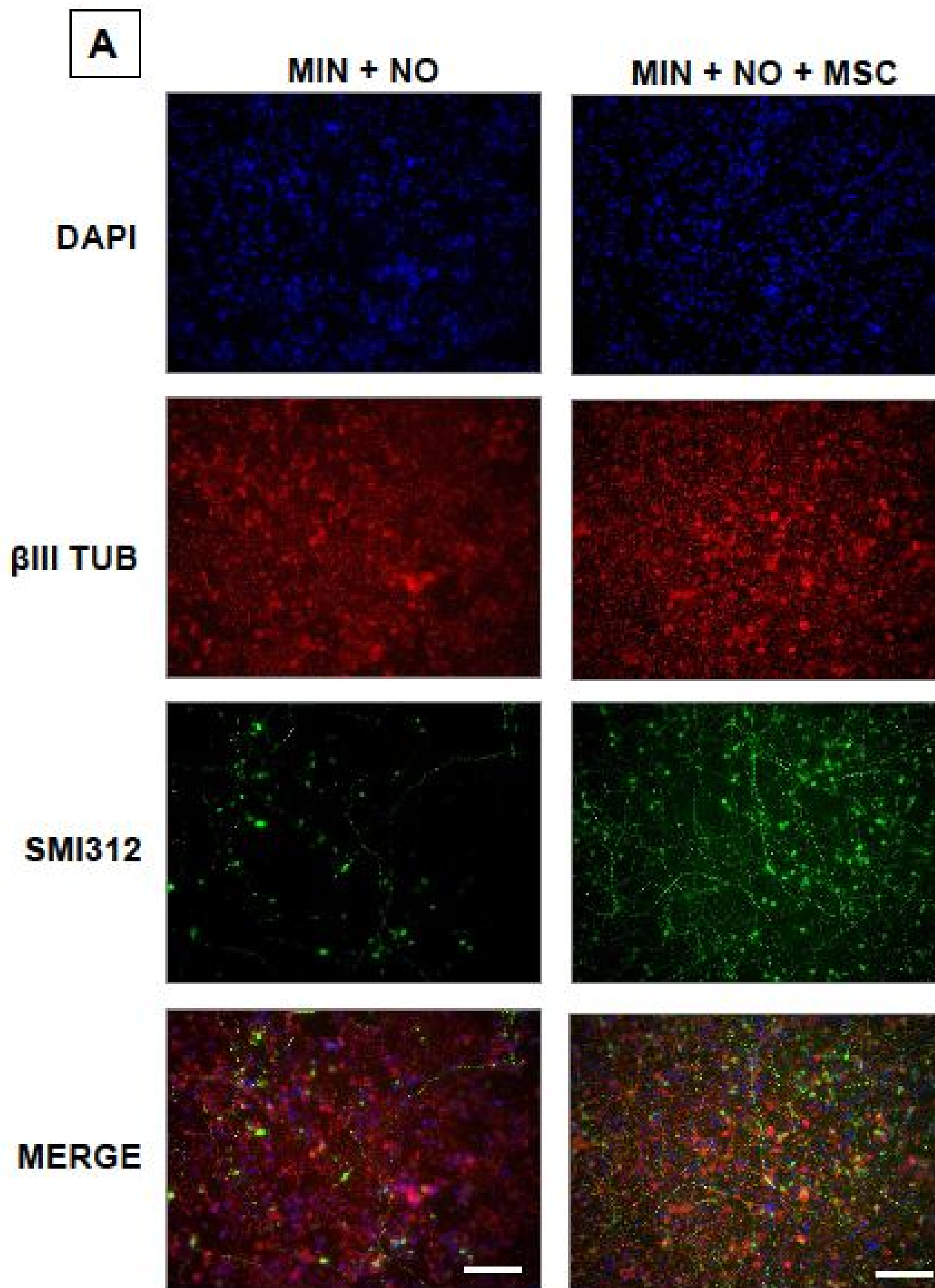


Fig. 3.2.2 Cortical neurons were exposed to MIN media with NO alone or with the addition of MSCs in a transwell co-culture system for 24h. Cultures were stained for the nuclear marker DAPI (blue), neuronal marker βIII tubulin (red) and axonal marker SMI312 (green). Bar: 100μm.

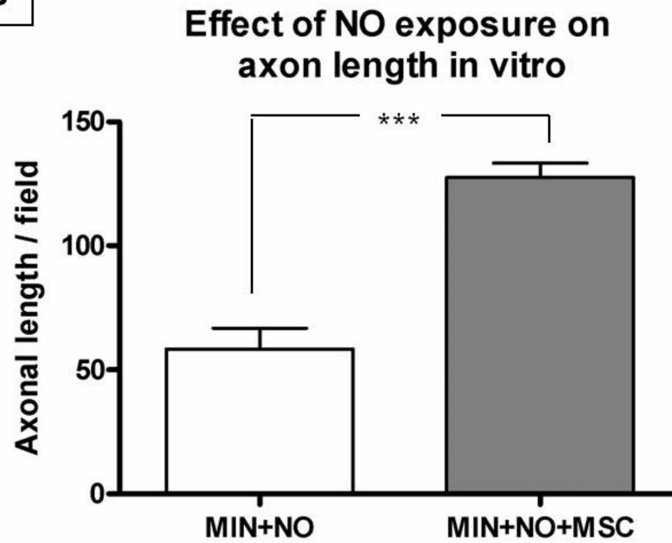


Fig. 3.2.2 Exposure of cortical neurons to NO for 24h with the addition of MSCs causes an increase in axonal length per culture field compared to cortical neurons exposed to NO alone. Results are expressed as the mean +/- standard error (n=4; ***p<0.001).

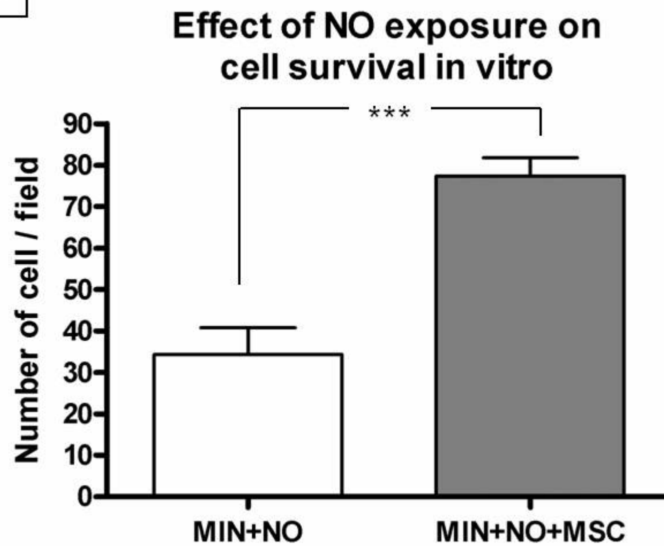
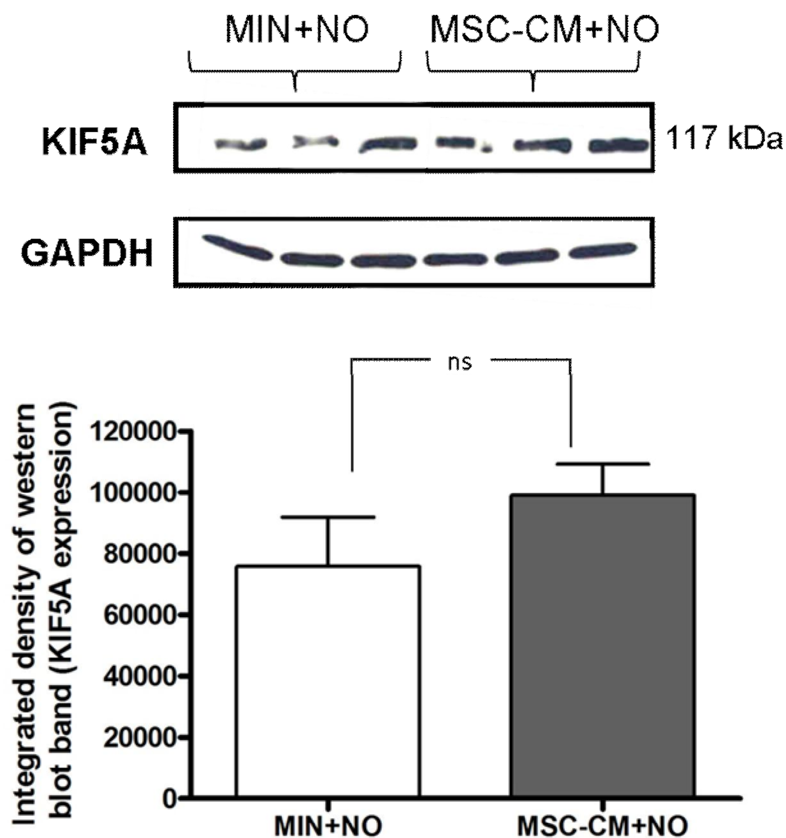


Fig. 3.2.2 Exposure of cortical neurons to NO for 24h with the addition of MSCs causes an increase in total neuronal survival per culture field compared to cortical neurons exposed to NO alone. Results are expressed as the mean +/- standard error (n=4; ***p<0.001).

3.2.3. MSC-conditioned medium does not increase KIF5A protein expression in rodent cortical neurons exposed to nitric oxide

Cortical neurons were maintained in MIN media and exposed to NO or exposed to NO in the presence of MSC-conditioned medium. After 24h proteins were extracted and western blots for KIF5A performed in order to verify if the MSC-conditioned medium was able to preserve/increase KIF5A neuronal expression post NO exposure. Densitometric analysis of the western blot bands showed that although seemingly higher, MSC-conditioned medium did not significantly increase KIF5A expression in cortical neurons exposed to NO (Fig. 3.2.3).

Fig. 3.2.3 KIF5A western blot and corresponding imaging analysis of cortical neurons exposed to NO alone (MIN+NO) or with MSC-conditioned media (MSC-CM+NO). Results are expressed as the mean +/- standard error (n=3).



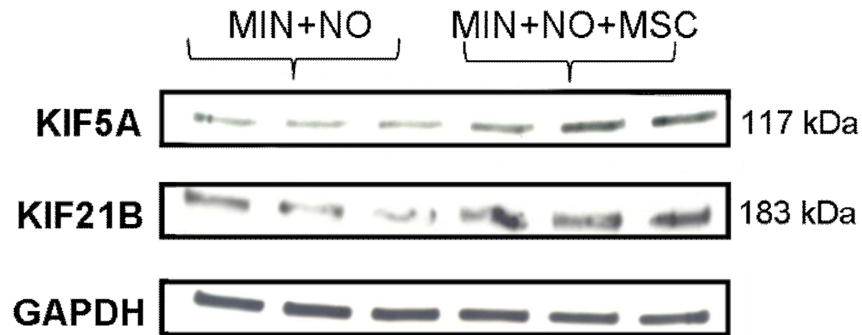
3.2.4. Indirect co-culture of MSCs and rodent cortical neurons preserve KIF5A and KIF21B protein expression from nitric oxide exposure without increasing KIF gene expression.

Since MSC-conditioned medium did not increase the expression of KIF proteins, it was then investigated if co-culture of MSC with cortical neurons was able to preserve KIF5A and KIF21B expression in neurons exposed to NO. Cortical neurons were exposed to NO in the presence of MSCs seeded in a transwell system which allows the exchange of soluble factors without any direct contact between neurons and MSCs. KIF protein expression was analysed after 24 hours and gene expression after 6 hours post NO exposure (previous results have shown a significant reduction in KIF protein expression after 24h and gene expression after 6h, see section 3.12 and 3.13).

For both, KIF5A (Fig.3.2.4.1 A) and KIF21B (Fig.3.2.4.1 B), protein expression, calculated using densitometric analysis of the western blot bands, was significantly increased in cortical neurons exposed to NO and MSCs when compared to NO alone ($p<0.01$).

No significant changes in the gene expression of KIF5A and KIF21B were observed between neurons exposed to NO alone or with the addition of MSCs in transwells (Fig. 3.2.4.2).

Fig. 3.2.4.1 KIF5A (A) and KIF21B (B) western blot and corresponding densitometric analysis of cortical neurons exposed to NO alone (MIN+NO) or with the addition of MSCs in a transwell co-culture system (MIN+NO+MSC). Results are expressed as the mean +/- standard error (n=3; **p<0.01).



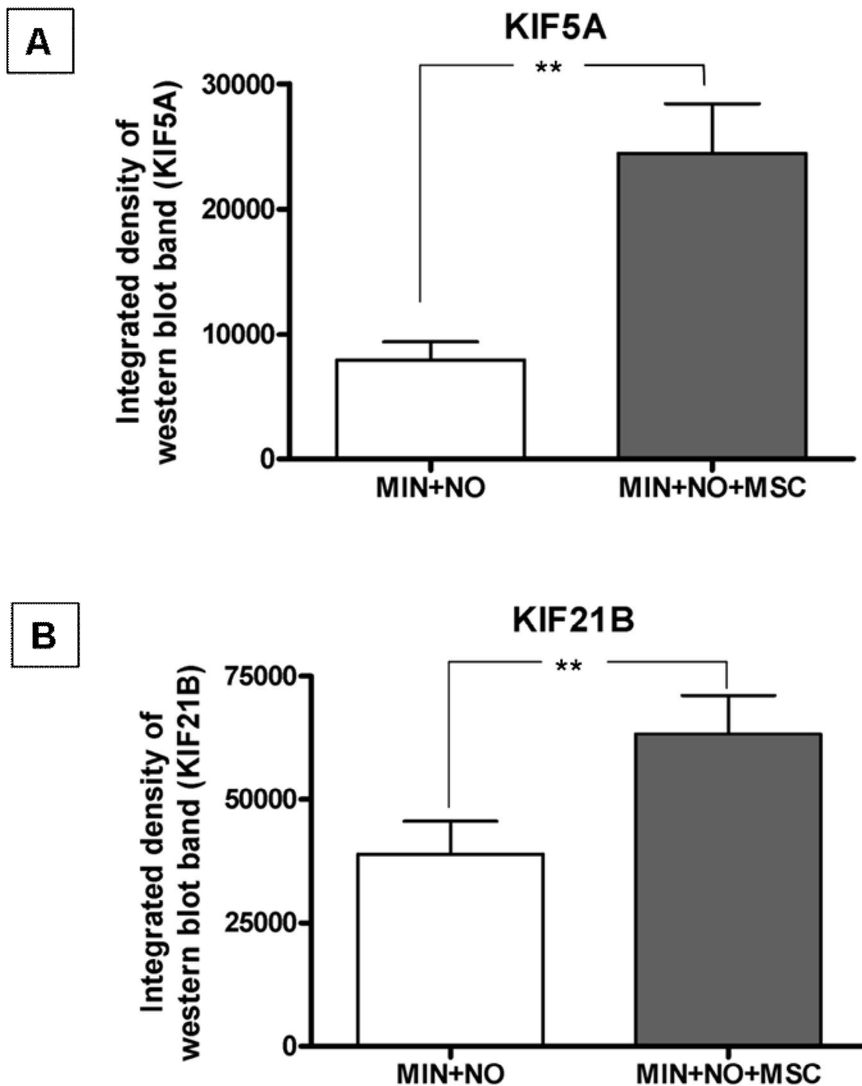
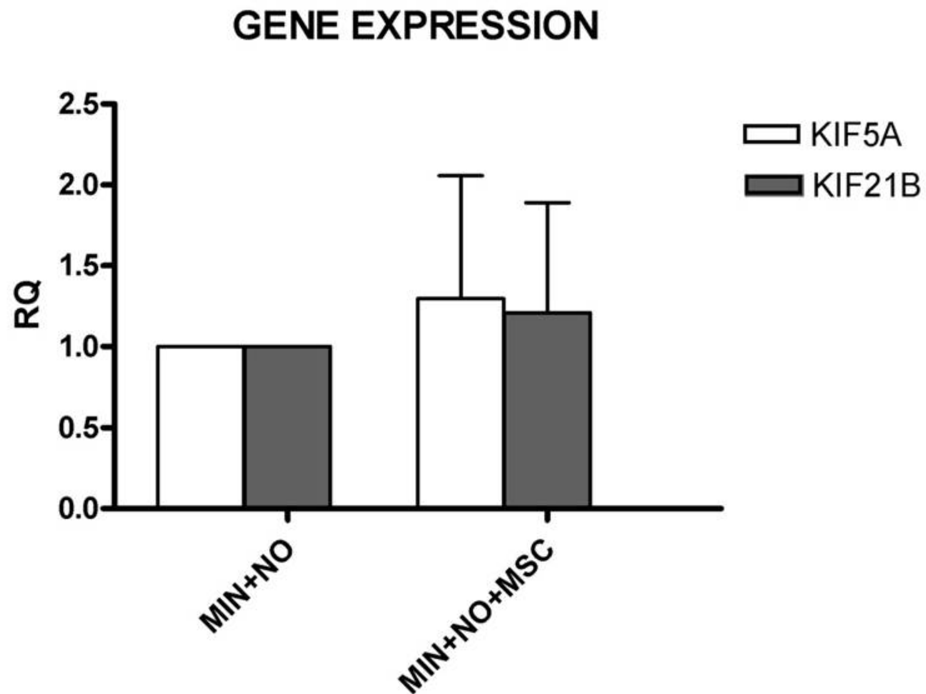


Fig. 3.2.4.2 KIF5A and KIF21B gene expression in cortical neurons exposed to NO alone or with the addition of MSCs in a transwell co-culture system. Results are expressed as the mean +/- standard error (n=5).



3.2.5. Immunofluorescence microscopy analysis of KIF5A and KIF21B proteins in cortical neurons exposed to NO and MSCs in a transwell co-culture system

Cortical neurons in MIN media were exposed to nitric oxide with/without the presence of MSCs in a transwell co-culture system. After 24h of NO exposure cells were fixed and stained for KIF5A, KIF21B and the nuclear marker DAPI Vectashield. Immunofluorescence analysis confirmed that MSCs protect both KIF5A (Fig. 3.2.5 A) and KIF21B cortical neuronal expression from NO damage (Fig. 3.2.5 B). KIF expression was seemingly lost when neurons were exposed to NO, while KIF labelling was preserved in several neuronal cells when exposed to NO in the presence of MSCs.

Fig. 3.2.5 KIF5A (A) and KIF21B (B) expression in cortical neurons exposed to NO alone (MIN+NO) or with the addition of MSCs in a transwell co-culture system (MIN+NO+MSC).

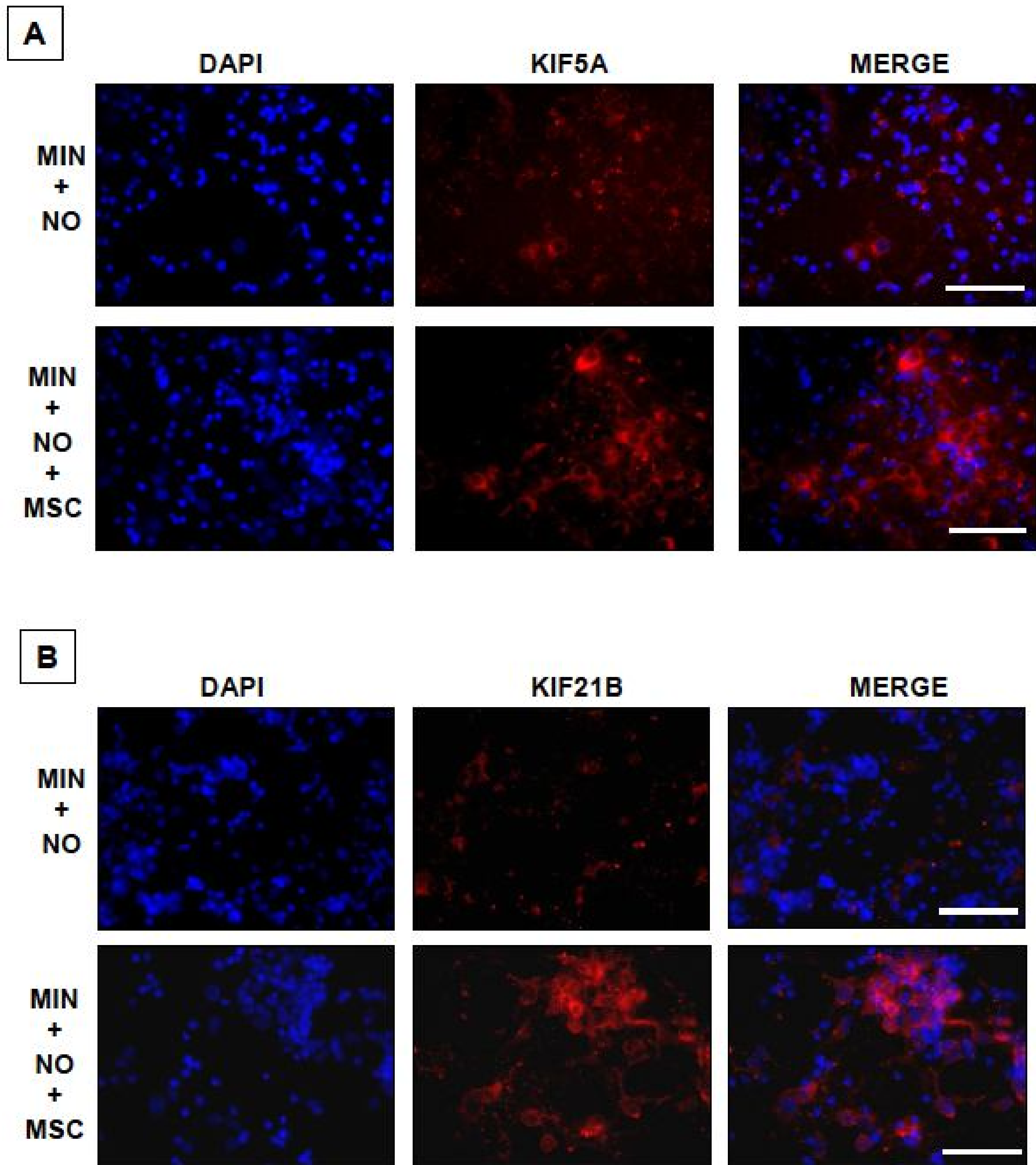


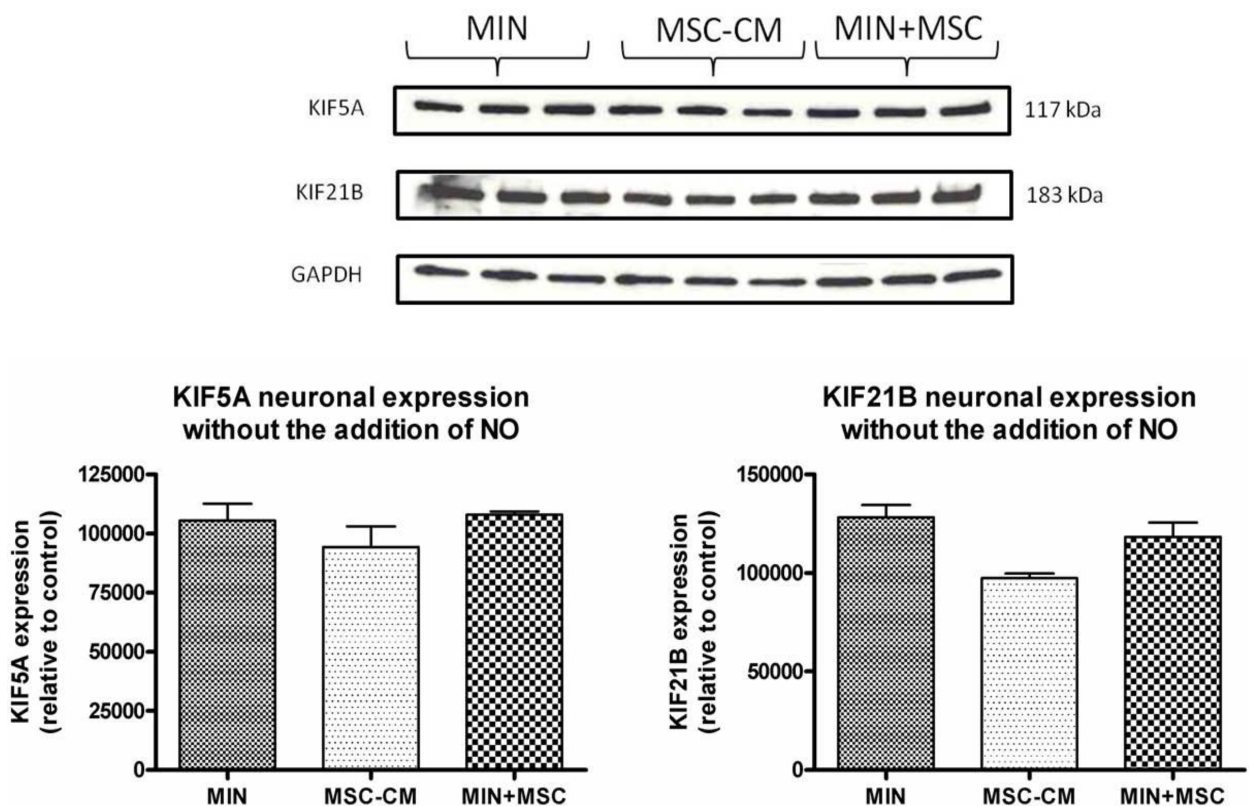
Fig. 3.2.5. Cortical neurons were exposed to MIN media with NO with/without the addition MSCs in a transwell co-culture system for 24h. Cultures were stained for KIFs protein (red) and nuclear marker DAPI (blue). Bar: 100µm.

3.2.6. MSCs do not regulate KIF5A and KIF21B protein expression in standard culture conditions

In order to clarify if MSCs could regulate KIF expression in the absence of toxic insult, cortical neurons were maintained in MIN media, MSC-conditioned media or cultured with MSC in a transwell co-culture system (all without the addition of NO). After 24h proteins were extracted and western blot analysis for KIF5A and KIF21B expression performed.

The densitometric analysis of the western blot bands revealed no changes in KIF expression among the different conditions tested, suggesting that MSCs do not regulate KIF expression under standard culture conditions (Fig. 3.2.6).

Fig. 3.2.6 KIF5A and KIF21B western blot analysis of cortical neurons exposed to minimal media (MIN), MSC-conditioned medium (MSC-CM) or with MSCs in a transwell co-culture system (MIN+MSC) (n=3).



3.3 KIF5A and KIF21B expression in the cerebellum of MS patients

3.3.1 Characterization of control and MS cerebellum tissue

Representative human cerebellar sections derived from controls and patients with multiple sclerosis were characterized by DAB staining using antibodies against myelin binding protein (MBP) and for the reactive macrophage/microglial marker HLA-DR. The sections were also counterstained with haematoxylin for nuclear labelling.

No demyelination (Fig.3.3.1 A) or inflammatory cell infiltration (Fig. 3.3.1 B) were detected in cerebellar sections derived from control patients. In contrast, cerebellar sections from MS patients showed lesions with loss of myelin (Fig. 3.3.1 C) and extensive inflammatory cell infiltration (Fig. 3.3.1 D). As expected, the observed macrophage/microglia infiltration (Fig. 3.3.1 F) corresponded with areas of demyelination (Fig 3.3.1 E).

Fig. 3.3.1 Characterization of human cerebellar sections derived from both a control patient and a patient with multiple sclerosis (MS).

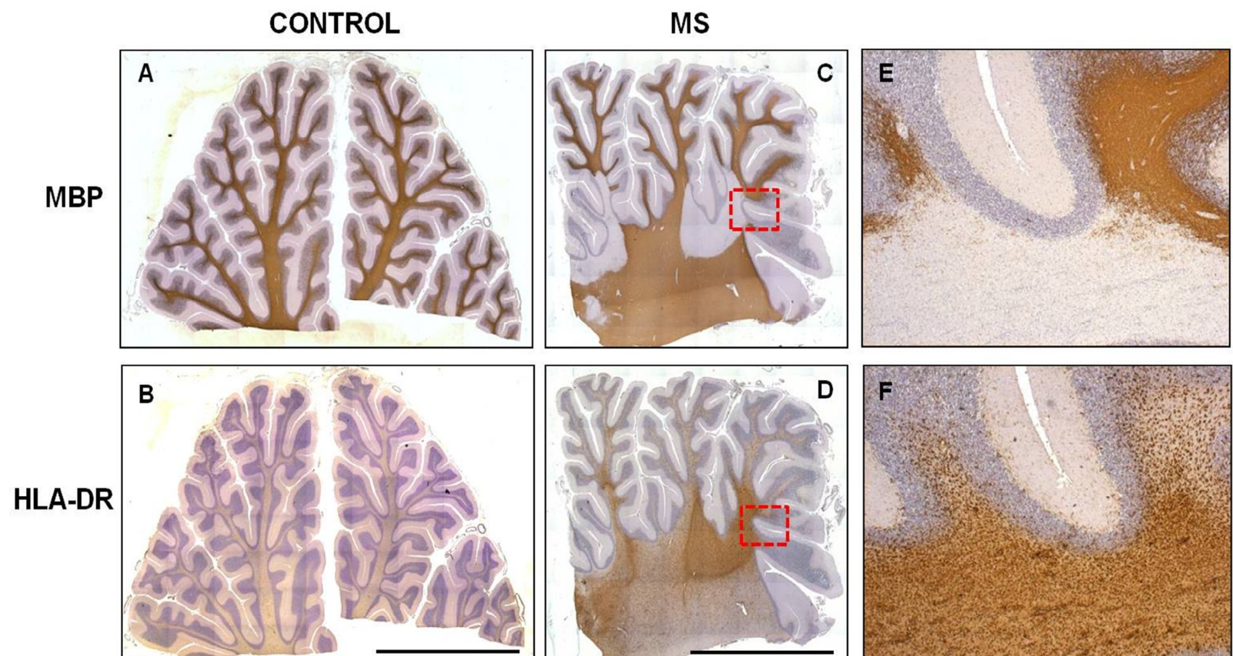
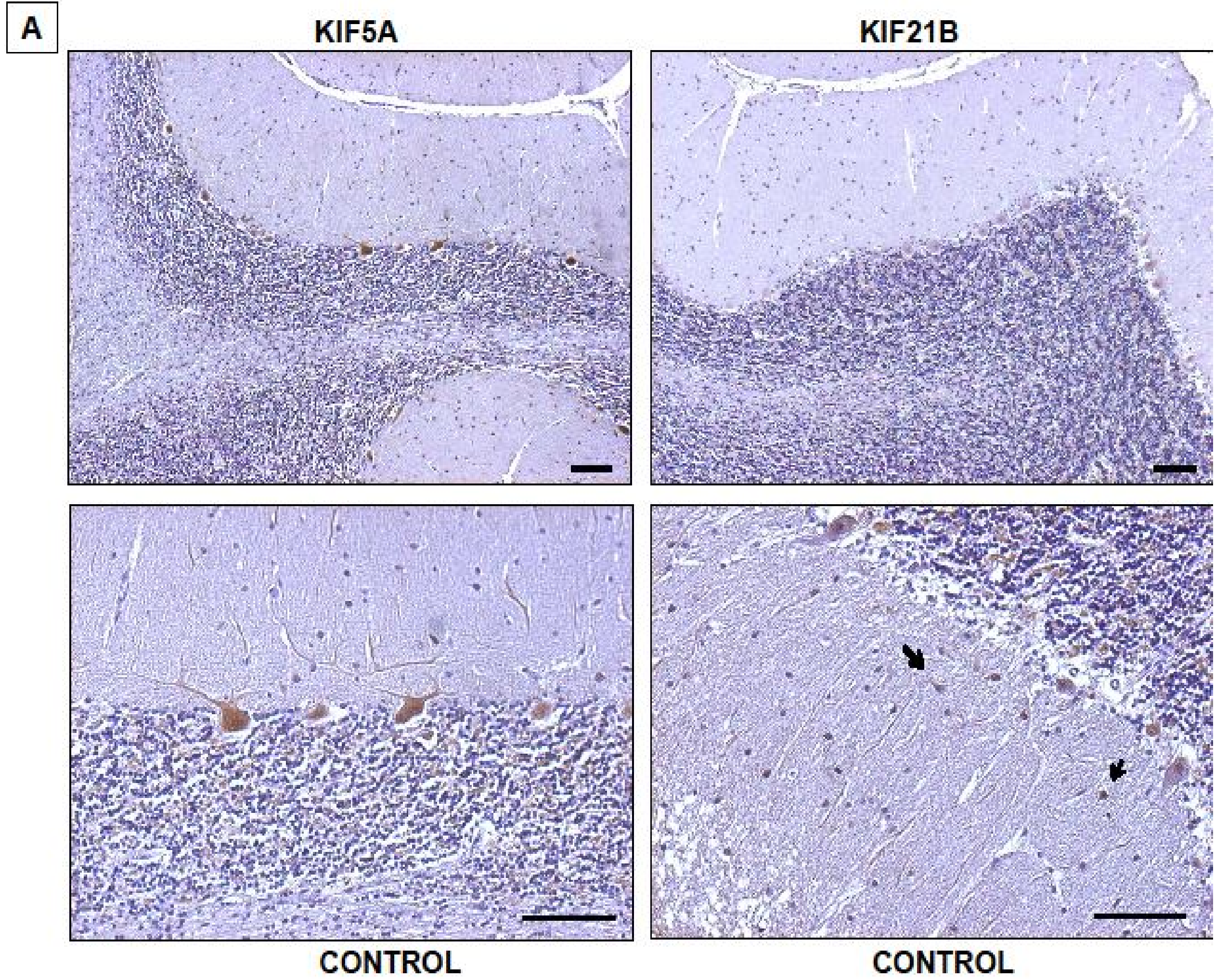


Fig. 3.3.1 Human cerebellar sections DAB immunolabelled with MBP (A, C, E) and HLA-DR (B, D and F) and counterstained with haematoxylin. MS patient show lesions with loss of myelin (C) and inflammatory cell infiltration (D), whilst no demyelination (A) or cell infiltration (B) was detected in control sections. Microglia infiltration (F) was particularly evident in areas of demyelination (E). Bar: 10mm.

3.3.2 Control and MS cerebellum express KIF5A and KIF21B

Control and MS cerebellar sections were immunostained with antibodies to KIF5A and KIF21B. It was shown that in the cerebellum the cell body and dendrites of Purkinje cells were positive for KIF5A and KIF21B in cerebellar sections from both control (Fig. 3.3.2.1 A) and MS patients (Fig. 3.3.2.1 B). KIF21B expression was also detected in other cells populations in the molecular layer, which were possibly basket or stellate cells (Fig.3.3.2.1 A, B arrows). Furthermore, in the cerebellum of MS patients, aggregates of a spheroid nature, positive for both KIF5A and KIF21B, were detected in the granular layer (Fig. 3.3.2.2).

Fig. 3.3.2.1 Human cerebellar sections immunolabelled for KIF5A and KIF21B derived from both a control patient (A) and a patient with multiple sclerosis (B).



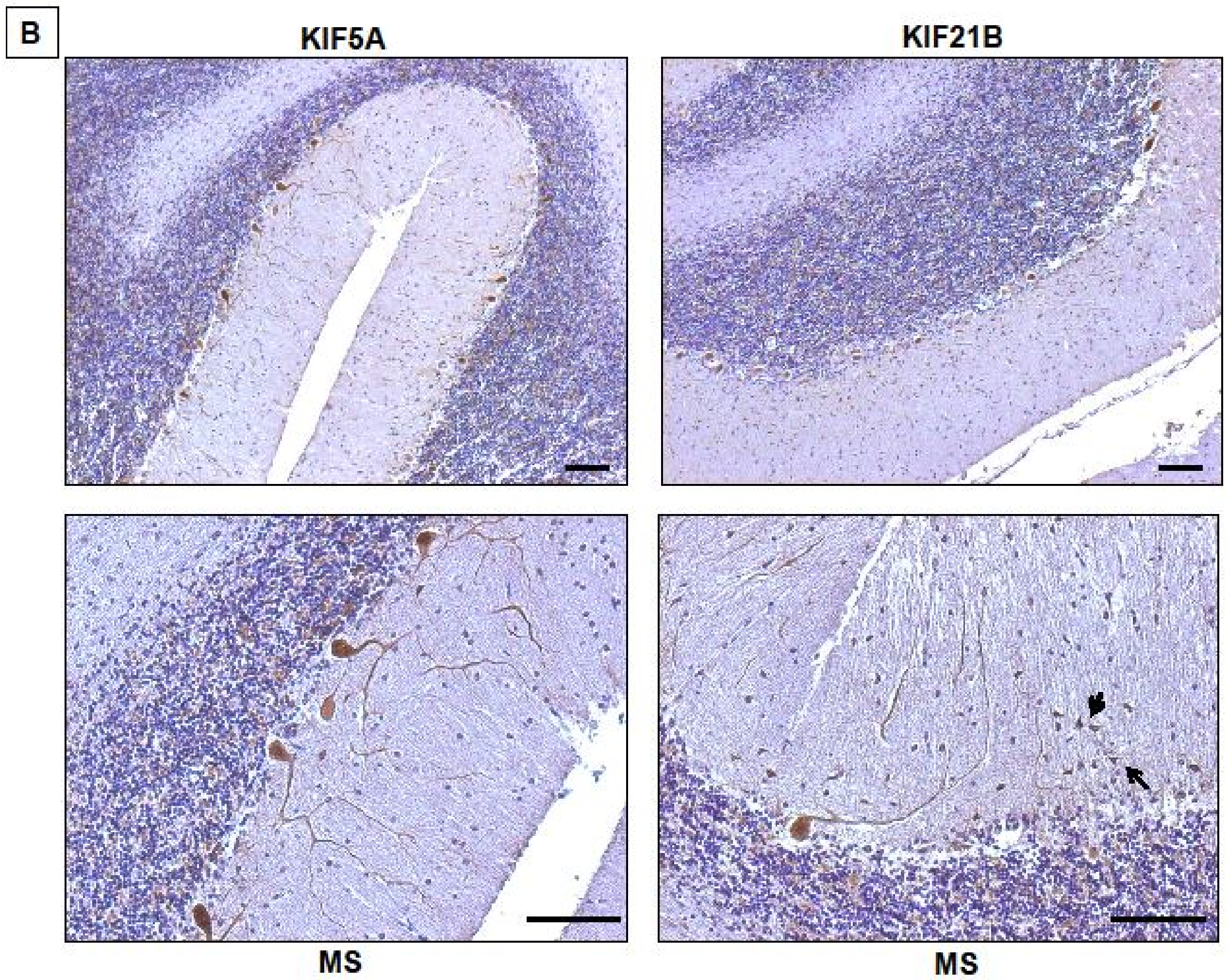


Fig 3.3.2.1 Control (A) and MS patient (B) human cerebellar sections DAB immunolabelled with KIF5 and KIF21B and counterstained with haematoxylin. Cell body and dendrites of Purkinje cells were positive for KIF5A and KIF21B; KIF21B was also detected in other cells of the molecular layer (arrows). Bar: 100μm.

Fig. 3.3.2.2 Human cerebellar sections derived from patients with multiple sclerosis have spheroid aggregates positive for both KIF5A and KIF21B.

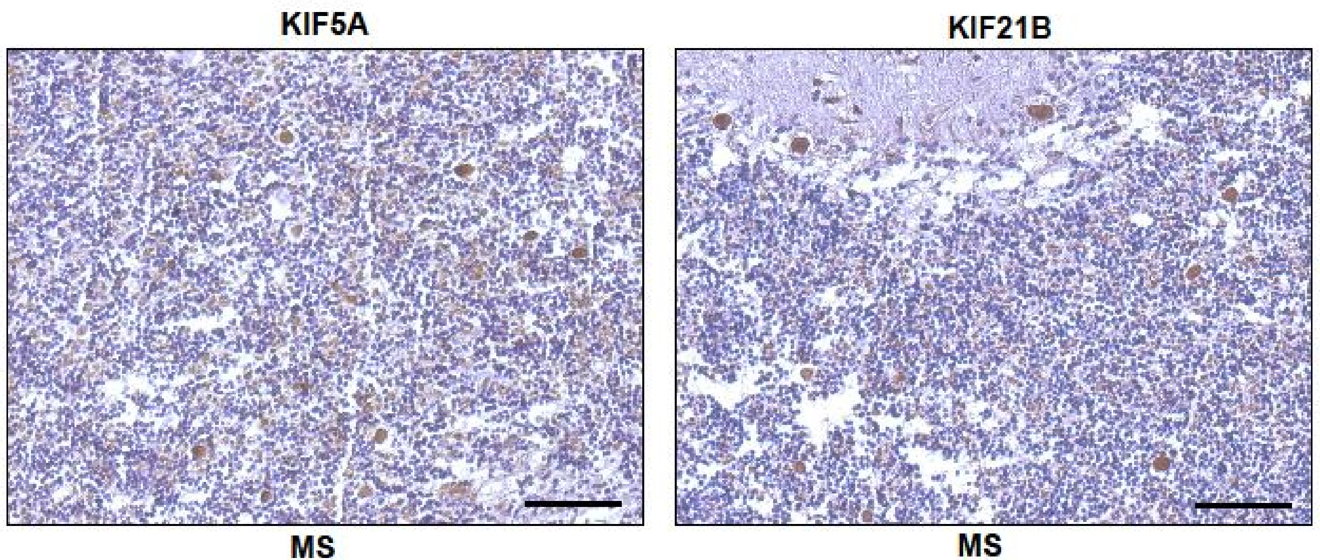


Fig. 3.3.2.2 Human cerebellar sections derived from MS patients DAB immunolabelled with KIF5A and KIF21B and counterstained with haematoxylin. Only in the granular layer of MS patients aggregates positive for KIFs proteins were detected. Bar: 100µm.

3.3.3 The correlation between APP and KIFs in MS cerebellum

To further investigate the KIF-positive aggregates observed in the cerebellum of MS patients, double immunofluorescence labelling using APP and KIF5A or KIF21B antibodies was perform. The majority of aggregates were both positive for APP and KIF5A (Fig. 3.3.3 A), while no co-labelling was observed for APP and KIF21B (Fig. 3.3.3 B).

Fig. 3.3.3 Immunohistochemical analysis of KIF5A, KIF21B and APP in human cerebellar sections derived from patients with multiple sclerosis.

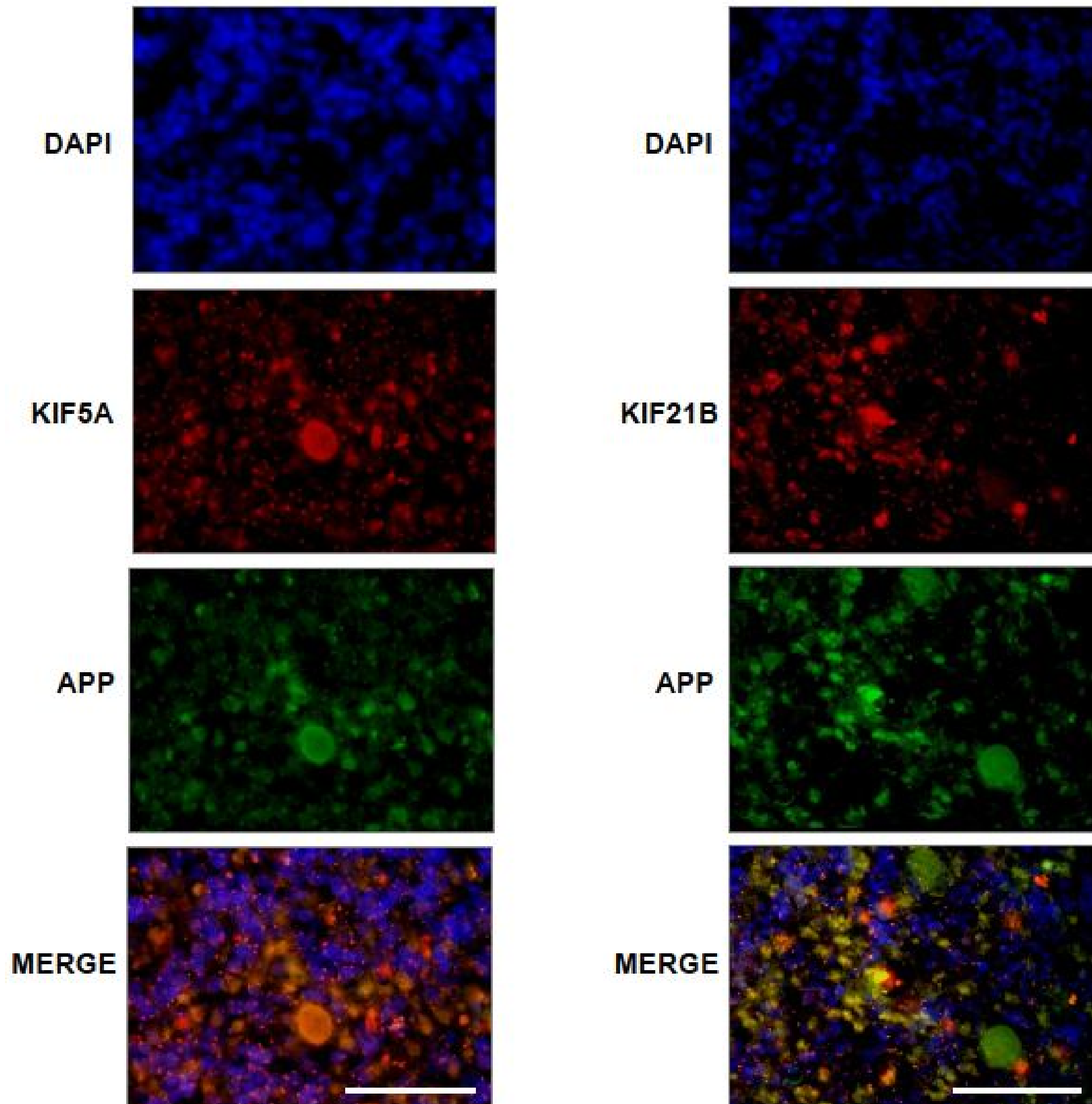


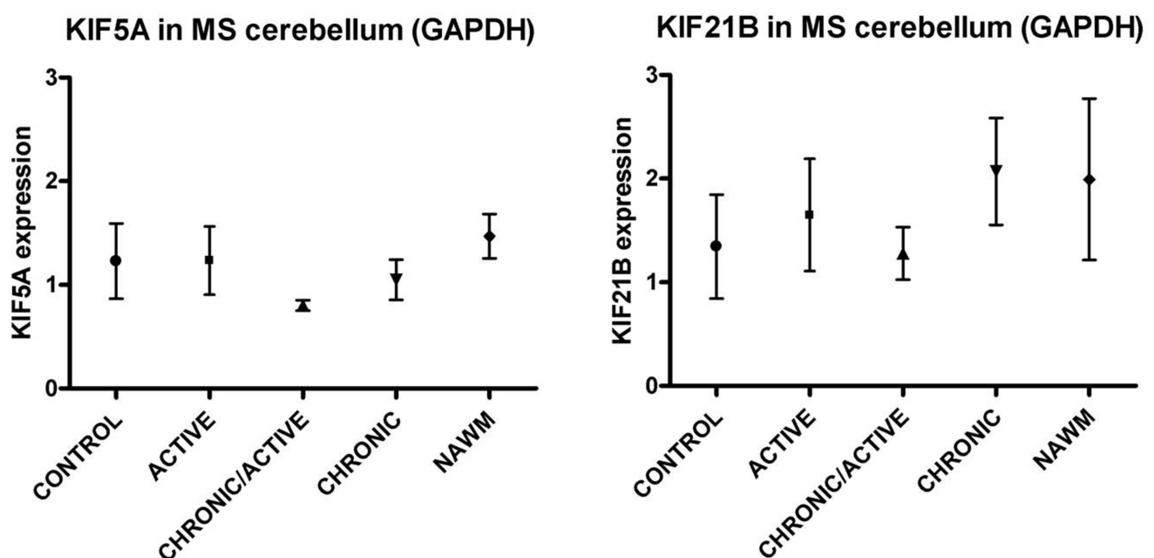
Fig. 3.3.3 Human cerebellar sections derived from patients with multiple sclerosis immunolabelled with the nuclear marker DAPI (blue), KIFs proteins (red) and APP (green). Many aggregates were double positive for KIF5A and APP while no double staining was observed for APP and KIF21B. Bar: 100μm.

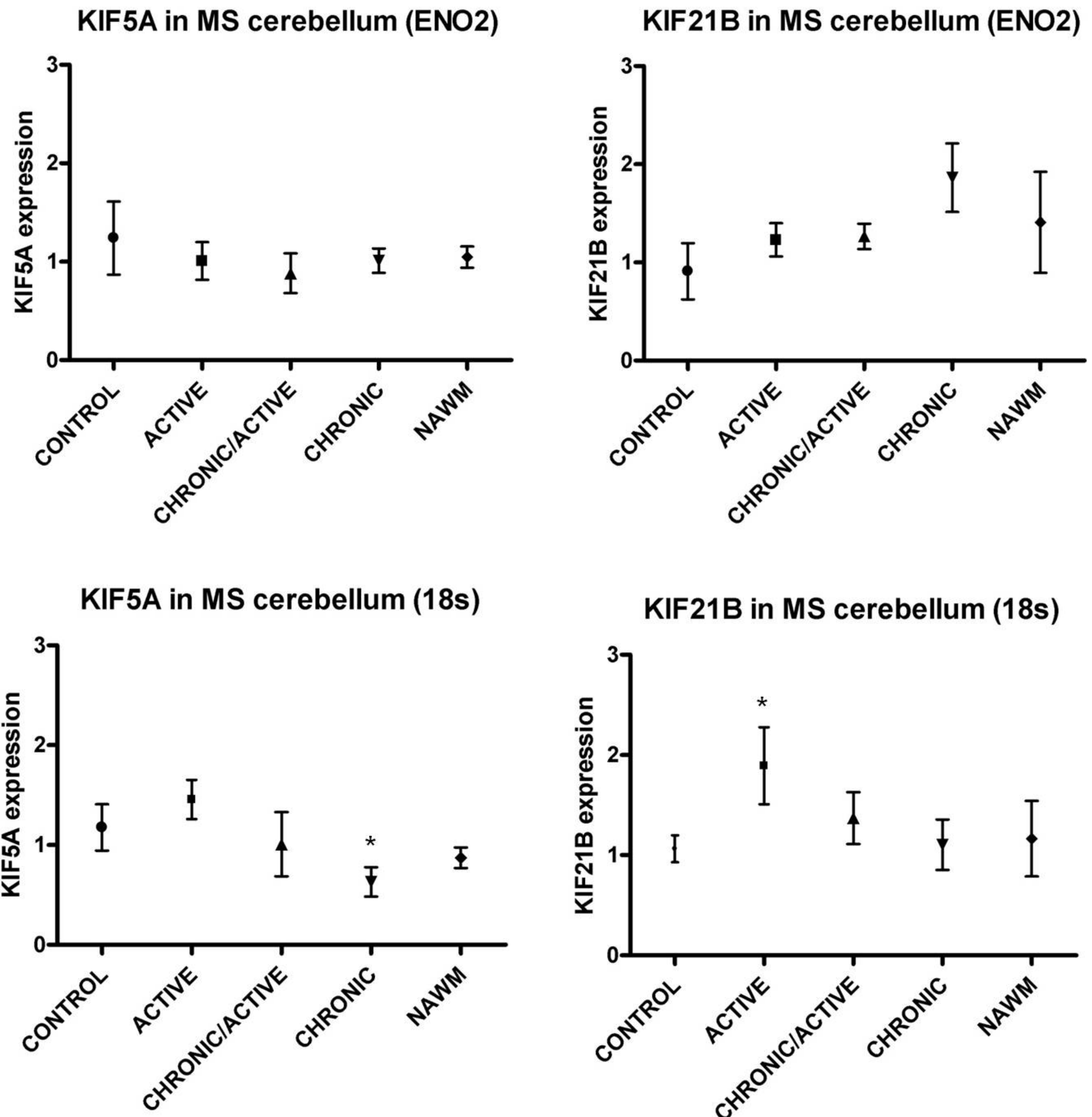
3.3.4 Changes in KIF5A and KIF21B gene expression in MS patients

To investigate the gene expression of KIF5A and KIF21B in MS patients, mRNA was extracted from homogenised tissue of 8 MS patients and 6 controls and RT-PCR performed. A glycolysis' enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), neuronal specific enolase (ENO2) and 18S ribosomal RNA (18S) were used as the reference 'housekeeping' genes.

The RT-PCR results showed a significant decrease in KIF5A expression in grey matter adjacent to chronic lesions in MS cerebellum, while there was an increase of KIF21B in grey matter adjacent to active lesions in MS patients when using 18s as an endogenous control. There were no significant changes in KIF5A and KIF21B when compared to the other selected housekeeping genes (Fig. 3.3.4).

Fig. 3.3.4 The gene expression of KIF5A and KIF21B in the cerebellum of control and MS patients determined using RT-PCR. mRNA samples isolated from the grey matter were grouped according to the adjacent white matter lesion type. Results are expressed as the mean +/- standard error (*p<0.05).



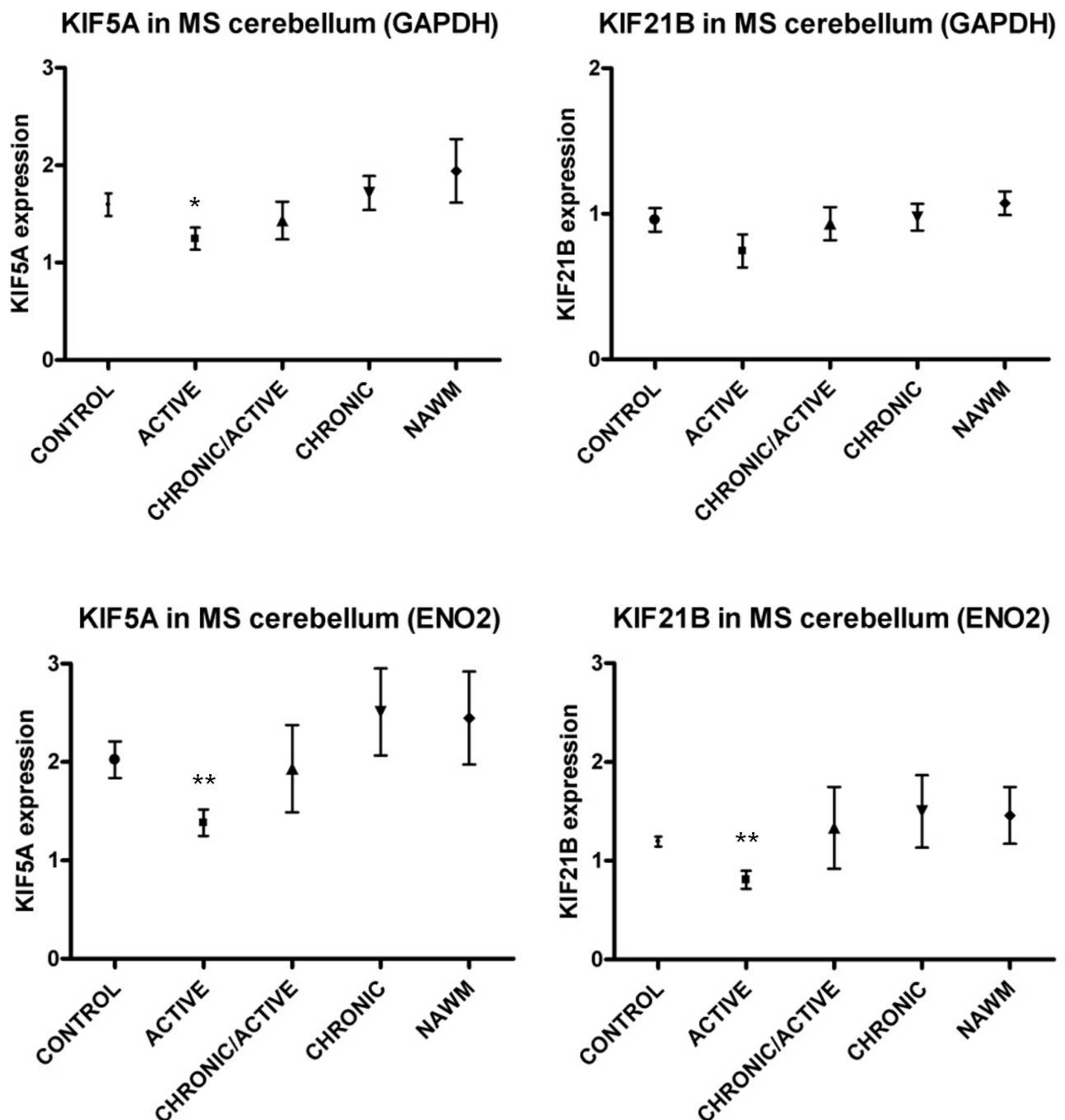


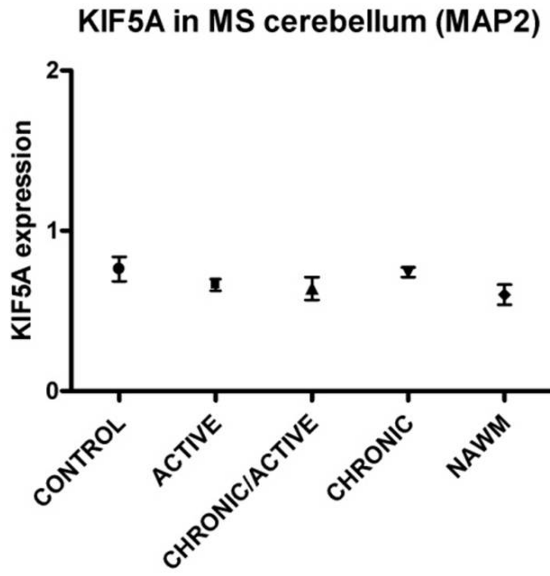
3.3.5 Changes in KIF5A and KIF21B protein expression in MS patients

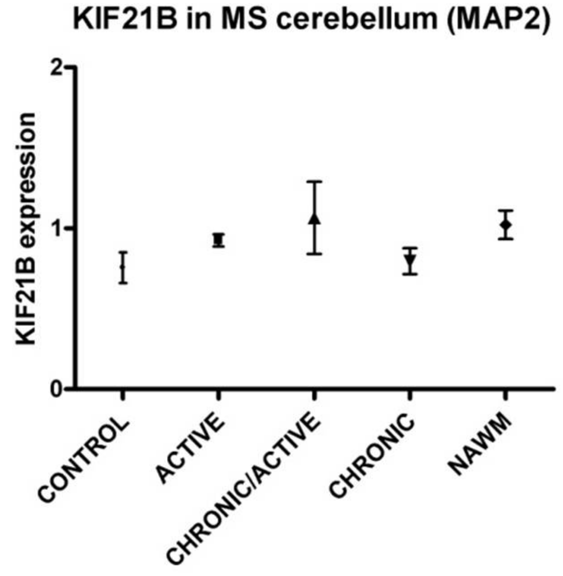
To further investigate whether in MS patients there were any changes in the protein expression of KIFs proteins relative to control samples, dot blots were performed. Proteins were extracted from homogenized tissue and dot blots were perform using antibodies against KIF5A and KIF21B. Dot blots were also carried out against GAPDH, ENO2, microtubule-associated protein 2 (MAP2) that is enriched in dendrites and the calcium-binding protein calbindin to use as reference control

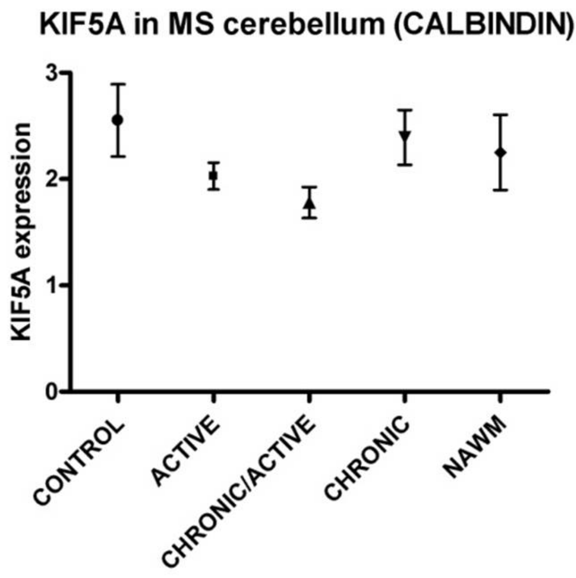
proteins. In the grey matter adjacent to active lesions in MS patients there was a significant decrease in the protein expression of KIF5A when using GAPDH as endogenous control and a decrease of both KIF5A and KIF21B when using ENO2 as control. There were no significant changes in KIF5A and KIF21B when compared to the other selected reference proteins (Fig. 3.3.5).

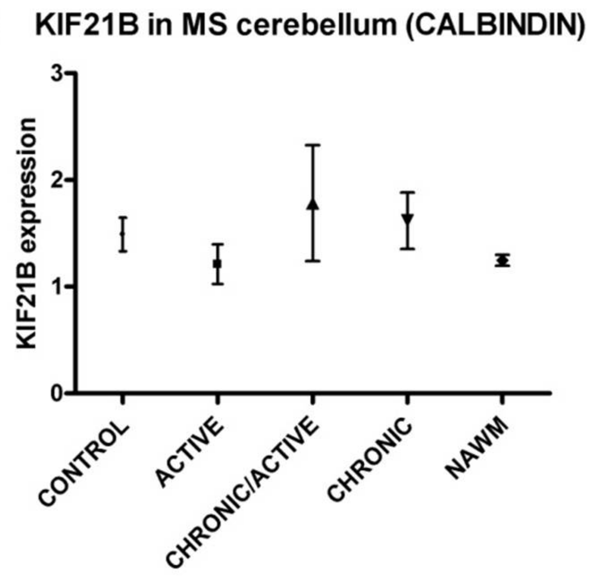
Fig. 3.3.5 The expression of KIF5A and KIF21B protein in the cerebellum of control and MS patients analyzed using dot blot assays. Proteins samples isolated from the grey matter were grouped according to the adjacent white matter lesion type. Results are expressed as the mean +/- standard error (*p<0.05; **p<0.01).




KIF5A in MS cerebellum (MAP2)
KIF5A expression
2
1
0
CONTROL
ACTIVE
CHRONIC/ACTIVE
CHRONIC
NAWM


KIF21B in MS cerebellum (MAP2)
KIF21B expression
2
1
0
CONTROL
ACTIVE
CHRONIC/ACTIVE
CHRONIC
NAWM


KIF5A in MS cerebellum (CALBINDIN)
KIF5A expression
3
2
1
0
CONTROL
ACTIVE
CHRONIC/ACTIVE
CHRONIC
NAWM


KIF21B in MS cerebellum (CALBINDIN)
KIF21B expression
3
2
1
0
CONTROL
ACTIVE
CHRONIC/ACTIVE
CHRONIC
NAWM

4. DISCUSSION

Intact axonal transport is required to maintain the integrity of axons and the overall metabolic balance and function of the neuron. The majority of proteins are synthesized in the neuronal cell body and transported along axons and dendrites through molecular motors that move along microtubules. Abnormal accumulation of intra-axonal components, such as amyloid precursor protein (APP) and neurofilaments (NF), are hallmarks of many neurodegenerative disorders including multiple sclerosis (MS), hereditary spastic paraplegia and Alzheimer's disease. Several studies hypothesize that such accumulations are caused by impaired axonal transport, specifically through abnormalities in KIF protein expression. The Kinesin superfamily proteins (KIFs) are a large gene family of molecular motors that selectively transport membranous organelles, protein complexes and mRNA throughout the cell. Two specific KIFs that have been associated strongly with neurodegenerative processes in humans and in rodents are KIF5A and KIF21B.

KIF5A molecular motors play essential roles in axonal transport. Indeed, they transport several cargoes such as mitochondria, lysosomes, synaptic vesicles precursors, tubulin dimers, neurofilament proteins, APP and APOE receptor 2 along axons; AMPA receptor and mRNA in dendrites (Hirokawa and Takemura, 2005). Because of the large number of cargoes that KIF5A transports, it is a highly important component of the axon; and because of this, changes in its expression are thought to be involved with several human disorders. In fact, three distinct autosomal dominant missense mutations have been mapped to the gene that encodes the heavy chain of KIF5A in hereditary spastic paraplegia patients (Reid et al., 2002; Lo Giudice et al., 2006). Mutant mouse models for KIF5A display loss of large calibre axons and neurofilament accumulation in neuronal cell bodies (Xia et al., 2003). KIF5A has also

found to be essential for viability in neonatal animals. These studies show that constitutive KIF5A knockout (KIF5A$^{-/-}$) mice die early after birth and KIF5A$^{-/-}$ motor neurons have reduced survival rates, axonal and dendritic length, number of axonal branches and cell body area compared with KIF5A$^{+/-}$ and KIF5A$^{+/+}$ (Karle et al., 2012; Xia et al., 2003). Furthermore, absence of KIF5A causes a reduction in the velocity of mitochondrial transport and axonal swellings in motor neurons (Karle et al., 2012). In relation to neurodegenerative disorders, KIF5-mediated transport of APP is also likely to be deeply involved in the pathogenesis of Alzheimer diseases (Stokin et al., 2005) and KIF5A down regulation has been associated with axonal transport defects in models of multiple sclerosis (Kreutzer et al., 2012).

KIF21B expression is detected in the brain, eye and spleen. In neurons it is enriched in dendrites; its function is still unknown but could be to deliver cargoes to the distal regions of dendrites (Marszalek et al., 1999). Recently a genome wide association screen for multiple sclerosis (International Multiple Sclerosis Genetics Consortium, 2010) correlated a single nucleotide polymorphisms (SNPs) located in the KIF21B intron with the disease, establishing this kinesin as a susceptibility locus for multiple sclerosis.

Collectively all this evidence reveals an essential role for KIFs in neuronal morphology, function and survival and support the hypothesis that impaired intracellular transport is strongly associated with neurodegenerative processes. Thus, to get a greater understanding of the association between KIFs and neurodegenerative processes there were three main aims to this study:

- In order to better understand the mechanisms involved in the abnormal accumulation of proteins in axons during oxidative insults associated with neurodegenerative processes, the first part of this study was to determine the

effect of nitric oxide (NO) on the expression of KIF5A and KIF21B in rodent cortical neurons and to evaluate whether changes in KIF expression correlates with axon damage and pathology.

- Human bone marrow mesenchymal stem cells (MSCs) represent a promising candidate for neuronal repair and may improve clinical outcome in several neurodegenerative diseases including MS due to paracrine mechanisms that allow these cells to have anti-inflammatory, neurotrophic and antioxidant properties. The second part of this study was therefore to investigate the capacity of MSCs to protect neurons and axonal transport mechanisms in rodent cortical neurons exposed to NO.
- Finally, it was evaluated if there were any changes in KIF gene and protein expression in cerebellum post-mortem tissue derived from patients who had MS in relation to appropriate controls.

Nitric oxide reduces KIF5A and KIF21B gene and protein expression in a time dependent manner

Nitric oxide is involved in several important functions in the central nervous system, including modulation of synaptic neurotransmission and plasticity, regulation of cerebral blood flow, a toxic agent in the immune cell response to pathogens and even as a neuroprotective molecule (Bennaroch, 2011). However, disturbances in the normal redox state of cells, an overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and a failure of antioxidant mechanisms, can have toxic effects through the production of free radicals that damage all components of the cell including proteins, lipids, and DNA. When produced in excess and in the setting of oxidative stress, NO reacts with oxidant molecules to form toxic species:

NO reacts with superoxide anion O_2^{*-} to form the highly reactive neurotoxin peroxynitrite ($ONOO^-$) and NO also combines with O_2 to produce nitrosonium ion (NO^+) which plays a key role in mediating inflammatory axonopathy in several neurodegenerative diseases including MS, promoting protein misfolding, disruption in the mitochondrial respiratory chain, a reduction in ATP production, organelle fragmentation, lipid peroxidation and activiation of matrix metalloproteinases, all leading to both axon damage and demyelination.

Studies suggest a relationship among ROS/RNS, protein accumulation, mitochondrial dysfunction and neuronal cell injury but the mechanisms of how oxidative and nitrosative stress contribute to these pathological features is still not entirely clear.

In this study immunocytochemical analysis of KIF5A and KIF21B was performed on cortical neurons exposed to NO for 24h. Under culture conditions without NO, KIF5A expression was localized in both the cell body and axon of cortical neurons while KIF21B expression, considered to be enriched in dendrites (Marszalek et al., 1999), was detected in both the cell body of neurons and in what appeared to be their respective dendritic networks. All KIFs proteins are produced in the cell body, motors must therefore differentiate axons from their dendrites for the correct transport of a specific cargo to the appropriate cellular destination. Differences in microtubules are evident in neurons, generated by post-translational modifications in the tubulin subunits (Laferriere et al., 1997) or by association of the microtubules with different microtubule associated proteins (Hirokawa, 1994). For example, MAPs have been shown to affect microtubule-dependent vesicle trafficking (Sato-Harada et al., 1996) and they have the ability to interfere with the motility of kinesin motors (Ebneth et al., 1998). Since different sets of MAPs are localized in axons when compared to those

located in dendrites, it is likely that molecular motors recognize the intrinsic and specific modifications made to microtubules in different cellular locations (Hirokawa, 1994). So structural components, rather than a diffusible signal, must provide a directional cue for efficient sorting (Hirokawa and Takemura, 2005).

Both KIF5A and KIF21B labelling was decreased when neurons were exposed to NO *in vitro*. For KIF5A this loss was especially evident in the axons of cells, with nearly a complete absence post NO exposure, whereas a small number of cell bodies still presented a low KIF5A expression post NO exposure. KIF21B expression in neurons exposed to NO resulted in an extensive and ubiquitous loss of KIF protein, with just few cell bodies still expressing KIF21B.

To investigate further the effect of NO exposure on KIFs, cortical neurons were exposed to NO for different lengths of time and subsequently both KIF gene and protein expression was determined using RT-PCR and western blot analysis. The results in this study have demonstrated for the first time that NO caused a time dependent decrease in gene and protein expression of KIF5A and KIF21B, suggesting a possible mechanism that can cause proteins to accumulate in axons. A possible explanation for this decrease in gene and protein expression could be a process called S-nitrosylation, by which an NO^+ group undergoes a redox reaction with critical cysteine thiols of target proteins. Evidence suggests that S-nitrosylation is similar to phosphorylation in regulating the biological activity of many proteins (Lipton et al., 1993; Uehara et al., 2006) and can mediate either protective or neurotoxic effects depending on the function of the target protein affected. In fact, an overproduction of NO can mediate neurotoxic effects via S- nitrosylation that impairs the function of several proteins such as enzymes involved in oxidative defenses such as glutathione and catalase, matrix metalloproteinase-9 involved in degradation of

extracellular matrix proteins, XIAP (X-linked inhibitor of apoptosis protein) that is a potent antagonist of caspase-3 activity and prevents apoptosis, the fission protein dynamin-related protein 1 (Drp1) resulting in excessive mitochondrial fission leading to its fragmentation and bioenergetic impairment, the chaperone heat shock protein 90 (Hsp90) which has a chaperone function that if altered can promotes β-amyloid and tau aggregates and the ubiquitin-proteasome system (UPS) that in normal condition conjugate a ubiquitin chain to proteins that need to be degradate by the proteasome (Knott and Bossy-Wetzel, 2009; Nakamura et al., 2012).

Taking these facts into consideration, NO could therefore mediate S-nitrosylation reactions that can impair KIFs function in several ways: a) preventing the bound of KIFs to microtubules and their processivity along the rails; b) causing a conformational change that can modify the KIF's cargo binding domain preventing the binding between KIFs and their cargoes; c) abolishing KIF's ATPase activity that generates motile force.

S-nitrosylation could affect KIF expression in both a direct and indirect fashion. After 24h of NO exposure the protein expression of KIF5A and KIF21B was decreased by approximately 80% and this could be caused by a direct S-nitrosylation on KIF proteins that can increase KIF's susceptibility to degradation by the proteasome system.

As shown in this study, a more upstream explanation for the decrease in KIF protein expression observed post 24h of NO is highly likely due to a decrease in gene expression, in fact after 6h of NO exposure KIF5A and KIF21B gene expression was decreased by 70%. S-nitrosylation could possibly alter cell signal proteins that regulate transcription factors that encode for KIF proteins, indirectly decreasing KIF expression. It's known that NO regulates a number of intracellular signaling

pathways, including mitogen-activated protein kinases (MAP kinases) (Ghatan et al., 2000) and that exposure of neurons to NO activates p38 MAP kinase signaling and that inhibition of this pathway increases neuronal survival (Wilkins and Compston; 2005). NO could also decrease the gene expression of KIF proteins through directly damaging the DNA within the neurons; it is known that NO can mediate DNA breakdown, deaminate DNA and inhibit repair mechanisms (Witherick et al., 2010).

NO causes a time dependent decrease in axon phosphorylation

As described above, decreases in KIF gene and protein expression maybe mediated through oxidative mechanisms, such as S-nitrosylation. However, in order to try and asses if the decrease of KIFs expression post NO exposure could be also due to an alteration of axon structure or alternatively if a decrease in KIF expression could promote abnormal axonal changes, we set about to determine if NO causes a change in axon phosphorylation.

Neurofilaments are very important constituents of axons and are involved in defining the structural and the functional integrity of myelinated axons. NF subunits are the most extensively phosphorylated proteins in neurons and this phosphorylation is highly regulated, with an intense phosphorylation in axons and little or no phosphorylation in cell bodies and dendrites (Nixon et al., 1992). Indeed, many key physiological roles have been attributed to the phosphorylation of NF including the control of axon caliber, promotion of NF alignment, increasing interfilament spacing contributing to axon structure and modulating the axonal conduction (Perrot et al., 2008).

Perturbations of NF metabolism and aberrant NF phosphorylation are associated with axon damage and are frequently observed in neurodegenerative diseases,

including MS. Dephosphorylation of neurofilaments can lead to destabilization of the cytoskeleton and subsequent axon degeneration. Therefore primarily in this study, to show the effects of NO on axonal pathology, both the neuronal survival and axonal length of rodent cortical neurons were examined post NO exposure. Cortical neurons were maintained in base medium alone or with the addition of NO for 24h. Cells were then fixed and stained for the neuronal marker βIII tubulin, pan-phosphorylated axonal marker SMI312 and the nuclear marker DAPI Vectashield. Axonal length was measured using SMI312 labelling while the number of viable neurons were counted using DAPI nuclear morphology and βIII tubulin positivity. Cortical neurons cultured with NO showed a significant reduction in phosphorylated axonal length and total live cells compared to controls. This demonstrates that nitric oxide exposure causes both specific axonal structural changes resulting in dephosphorylation of neurofilaments and has also the ability to mediate neuronal cell death. Several studies demonstrated that nitric oxide is a key mediator of neuronal cell death through several mechanisms including (Brown and Bal-Price, 2003; Yuan et al., 2007): energy-depletion induced necrosis by inhibition of mitochondrial respiration due to the binding of NO to cytochrome oxidase in competition with oxygen, induction of mitochondrial fission, glutamate release and subsequent excitotoxicity, p38 MAP kinase activation followed by Bax (a pro-apoptotic member of the Bcl-2 family) translocation and clustering into foci on the outer mitochondria membrane leading to its permeabilization and release of pro-apoptotic factors including cytochrome c and activation of p53 probably due to NO mediated damage to DNA. Less clear, however, is how nitric oxide mediated axon dephosphorylation. Therefore to further investigate the effect of NO on axon phosphorylation, dot blot analysis of SMI312 (pan-phosphorylated neurofilament marker) and NF200 (phosphorylated and non phosphorylated neurofilament marker)

were performed on cortical neurons exposed to nitric oxide for different lengths of time. The results showed that NO caused a time dependent decrease in SMI312 and NF200 expression over a 24h period, but the reduction of SMI312 was greater and occurred earlier than that of NF200. In order to calculate an index of total phosphorylated neurofilament in axons after NO exposure, the ratio SMI312/NF200 (phosphorylated neurofilament/total neurofilament) was determined. Using this index results demonstrated that NO caused a time dependent decrease in axon phosphorylation over a 24h period. Phosphorylation of NF depends on two kinases: cycline-kinase Cdk5 and MAP kinases; growth factors, calcium influx and myelination trigger signal transduction cascades that activate these kinases. It's known that NO regulates a number of intracellular signaling pathways, including MAP kinases, therefore it is possible that NO could alter the dynamic balance between kinases and phosphatases leading to NF dephosphorylation.

In multiple sclerosis, NF dephosphorylation occurs in white matter lesions and it is associated with destabilization of the cytoskeleton and irreversible axonal transection that leads to subsequent axon degeneration. Transected axons are therefore a consistent feature of multiple sclerosis lesions and the majority of terminal axonal ovoids or end bulbs are positive for SMI32 staining, a marker for non-phosphorylated neurofilament (Trapp et al., 1998). Certainly axon dephosphorylation could represent a possible mechanism by which NO mediates its toxic effects on neurons. In fact, high levels of NF phosphorylation protect axons against proteolysis, therefore loss of NF phosphorylation renders the axons more susceptible to degradation by calpain that leads to cytoskeleton instability and blocks action potential conductivity (Pant, 1988). Furthermore, phosphorylation controls axon caliber and interfilament spacing increasing the total negative charges on NF side arms and their lateral extension by

repulsive interaction. Dephosphorylation could cause irregular cross bridges due to a reduction of the repulsive interaction that could decrease NF stability and render the axon more vulnerable to nitrosative stress. Phosphorylation also regulates NF transport rate promoting their release from kinesin (Yabe et al., 2000) and increases their affinity for the retrograde motor dynein (Motil et al., 2006). Hypophosphorylated NF preferentially associate with kinesin, as NF is progressively phosphorylated it detaches from the motor and forms a macro structure that cannot undergo transport (Shea and Flanafan, 2001). NFs are transported intermittently in axons with fast movement interrupted by prolonged pauses. Modulation of NF phosphorylation could therefore mediate NF to dissociate (pauses) or reassociate (transport) with its motor. So a decrease in NF phosphorylation could prevent the dissociation of NF from kinesin, which then may lead to a dysfunction in axonal transport.

KIFs reduction precedes the loss of neurofilament

Abnormal aggregations of NF and axon damage have been observed in several neurodegenerative disorders, including MS. Since several studies suggest a direct interaction between NF and kinesin (Yabe et al., 2002; Jung et al., 2005) and that disruption of KIF5A induces the accumulation of NF in the cell bodies of peripheral sensory neurons (Xia et al., 2003), impaired axonal transport may turn out to be an important component that fails in several neurodegenerative disorders. Abnormal aggregates could be caused by impaired axonal transport but the mechanisms causing these blockages are still unknown. Therefore, in order to try and determine how KIF expression correlates with axon damage, dot blots for the axonal marker NF200 and KIF proteins were perform on cortical neurons at different time points post NO exposure. NO caused a time dependent decrease in NF200, KIF5A and

KIF21B expression over a 24h period. To clarify if axonal loss follows KIF reduction or if KIF reduction is caused by axon loss, the ratio KIF5A/NF200 and KIF21B/NF200 was calculated at specific time points post NO exposure. Interestingly, the ratio KIF/NF at each time point decreased and therefore it is possible to hypothesize that KIFs reduction precedes the loss of NF. This suggests that the reduction found in KIF expression is not caused by damage to the structural integrity of axons which then prevents kinesins to function, but is probably due to a primary event caused by NO exposure. This loss of KIF expression is seemingly independent, or at least partially, to axonal loss and may further contribute to the severe progression of axonal injury alongside the direct toxic effects of NO. Besides the direct NO-mediated toxic effects including increased axonal membrane permeability, cytoskeletal breakdown, mitochondrial dysfunction and nitrative stress, blockage of axonal transport can both lead to accumulation of proteins in axonal spheroids causing axonal disturbance and prevent critical factors such as mitochondria or synaptic vesicles to reach their destinations. For example, blockage of mitochondrial transport result in the loss of mitochondria from synaptic terminals that inhibit synaptic transmission, cause a decrease of ATP supply that affect the whole metabolism of the cell and may trigger synaptic dysfunction. Moreover, the resulting diminution of mitochondria in axons will decrease ATP supply to the molecular motors, leading to a decrease in both anterograde and retrograde transport of others axoplasmatic cargoes resulting in a degenerative vicious circle mechanism.

Conclusion

Together these data suggest a possible mechanism for the abnormal accumulation of proteins along axons, a common pathological phenomenon evident in

neurodegenerative disorders of the central nervous system. Results showed that NO exposure to cortical neurons caused a reduction in KIF5A and KIF21B expression at both the gene and protein level, and it could therefore be hypothesized that this change in expression could result in blocking the transport of proteins along axons and dendrites. Experiments did demonstrate that KIFs reduction precedes the loss of neurofilament and axonal integrity. Impaired axonal transport seems not just to be a consequence of axon damage, however maybe a major contributory factor that leads to axonopathy during oxidative insult. Dysfunctional kinesin transport is thought to cause proteins to accumulate promoting axon varicosities and swellings to form, leading to further axonal disturbance and blockage of transport. Furthermore, it could be assumed that axonal transport would be impaired in both directions since the retrograde motor protein dynein is synthesized in the cell body and all the machinery required for the retrograde transport has to be transported to the axon terminal by KIF5 motors. Certainly impairment of the anterograde transport would result in a lack of the distribution of mitochondria, synaptic vesicles, NF and APP that are all critical for neuronal function. Furthermore, blocking retrograde transport would prevent the movement of proteins, such as trophic factors, to the cell body that regulate neuronal survival. Unquestionably, all these factors caused by an impaired axonal transport system resulting from NO exposure could promote both the significant axonal and neuronal loss evident within our culture systems.

MSC are able to preserve axonal length, increase neuronal survival and preserve both KIF5A and KIF21B protein expression from nitric oxide induced damage

Cell-based therapies as new therapeutic approaches for tissue regeneration and repair have generated a great deal of interest. Specifically, human bone marrow mesenchymal stem cells represent a promising candidate for cell therapy and are currently being tested in clinical trials due to several beneficial properties: MSCs are adult stem cells and their use is not limited by ethical issues and/or national legislation limits; they are easily isolated and expanded in culture; MSCs have a low immunogenicity (expressing very low levels of major histocompatibility complex class II molecules and co-stimulatory antigens) that allow allogeneic transplantation and these cells are known to have the ability to migrate into injured tissue and exert their anti-inflammatory and anti-apoptotic features, as well as secrete trophic factors. Several studies have shown that MSCs promote neuronal survival *in vitro* (Crigler et al., 2006; Wilkins et al., 2009) and reduce axonal loss and disease severity in experimental autoimmune encephalomyelitis (EAE) mice (Zhang et al., 2005; Gerdoni et al., 2007) through the production of neurotrophic factors that support neuronal survival. Therefore to analyze the capacity of MSCs to promote neuronal cell survival *in vitro*, cortical neurons were exposed to nitric oxide with and without the addition of MSCs in a transwell co-culture system, which allows the exchange of soluble factors without any direct contact between the neurons and MSCs. Results showed that both axonal length and total live cells were significantly increased in co-cultures of MSC and cortical neurons exposed to NO compared with cortical neurons exposed to NO alone. Human MSCs have been shown to secrete neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor

(NGF), glial cell line-derived neurotrophic factor (GDNF) and several neurite-inducing factors, as well as axon guidance and neural cell adhesion molecules (Crigler et al., 2006; Zhang et al., 2005). BDNF has been shown to increase survival of neurons post NO exposure through the activation of a number of cell survival pathways (Wilkins et al., 2009), NGF has been shown to stimulates axonal repair in both acute and chronic CNS injury (Walsh et al., 1999) and GDNF has been shown to protect catecholaminergic and serotonergic neuronal perikarya from oxidative stress (Whone et al., 2012). Therefore MSCs secretion of soluble factors such as BDNF, NGF and GDNF could have contributed to the reduction of axonal injury and the increased cell survival.

Next to investigate if MSCs were able also to preserve KIF5A and KIF21B protein expression in cortical neurons exposed to NO, western blot analysis was performed. Two experimental conditions were evaluated in this study: cortical neurons exposed to NO in the presence of either MSC-conditioned medium or in a co-culture system. Results demonstrated that although seemingly higher, MSC-conditioned medium did not significantly increase/preserve KIF protein expression in cortical neurons exposed to NO. On the contrary, for both KIF5A and KIF21B, protein expression was significantly increased in co-cultures of cortical neurons and MSCs exposed to NO when compared to NO alone. Western blot analysis for KIF proteins was also performed on cortical neurons in the presence of MSC-conditioned media or cultured with MSC in a transwell co-culture system without the addition of NO, in order to determine if MSCs regulate KIF expression in the absence of toxic insult. Densitometric analysis of the western blot bands revealed no changes in KIF expression among the different conditions tested, suggesting that MSCs did not regulate KIF expression under standard culture conditions.

These results demonstrate how communication between MSCs and neurons are critical for protective mechanisms. MSCs did not regulate KIF5A and KIF21B expression in the absence of NO, furthermore MSC increased/protected KIF expression from NO only in the co-cultures system, supporting the hypothesis that MSCs respond to soluble factors that have been secreted from neurons after nitric oxide damage activating the release of neurotrophic and neuroprotective factors by MSCs. In fact Kemp et al. (2010 b), have demonstrated that the antioxidant molecule superoxide dismutase 3 (SOD3) secretion by MSCs is increased by activated microglia, which are in part responsible for excessive production of NO in neuroinflammatory diseases, and regulated synergistically in response to the inflammatory cytokines IFN-γ and TNF-α.

To verify if the increase in KIF protein expression observed during co-culture of neurons and MSCs post NO exposure was due to an increased gene expression, neurons in co-cultures with MSCs were exposed to nitric oxide for 6h (according to the previous results showing that post 6h of NO exposure the neuronal gene expression of KIF5A and KIF21B were significantly decreased by approximately 70%), mRNA was extracted and RT-PCR performed. No significant changes in the gene expression of KIF5A and KIF21B were observed in neurons in the presence of MSCs in transwells compared to neurons exposed to NO alone. From this result it was shown that MSCs did not increase the gene expression of KIF proteins, however as KIF protein expression was increased during identical culture conditions, results support the hypothesis that MSCs have some kind of trophic effect and have prevented the degradation of both KIF5A and KIF21B protein from nitric oxide induced damage. This result supports the current theory that the major protective role of MSCs is their capacity to secrete a range of potentially neuroprotective and

antioxidant factors. To date, studies *in vivo*, in most situations, seemingly do not support the possibility that MSCs trans-differentiate into neural cells and show that limited numbers of injected MSC engraft to the CNS (Gerdoni et al., 2007; Gordon et al., 2008). Despite little or no engraftment to the CNS, MSC transplantation mediates beneficial effects in several models of diseases, such as EAE. This certainly suggests that neuronal repair is mainly due to the paracrine release of soluble factors, including anti-inflammatory, anti-apoptotic, anti-oxidant and neurotrophic molecules and the induction of endogenous neurogenesis and oligodendrogenesis (Uccelli et al., 2011). The recent finding that MSCs secrete the antioxidant molecule superoxide dismutase 3 could be a possible mechanism by which MSCs protect KIFs from NO. As already discussed above, when produced in excess and in the setting of oxidative stress, NO reacts with superoxide anion O_2^{*-} to form the highly reactive neurotoxin peroxynitrite ($ONOO^-$) and NO also combines with O_2 to produce nitrosonium ion (NO^+). SOD3 is a member of the SOD protein family and is the only enzyme that catalyses the conversion of superoxide anion (O_2^-) to hydrogren peroxide (H_2O_2) in the extracellular compartment, which can be detoxified to form water and oxygen by the enzymes catalase and glutathione (Harries et al., 1991). Therefore SOD3 secretion by MSCs could limit the formation of strong neurotoxic oxidants that can promote protein misfolding, preventing damage to KIFs proteins.

Reports have shown p38 MAPkinase activation during NO mediated neuronal death (Wilkins and Compston, 2005) and the importance of PI3kinase/Akt pathway in mediating neuronal survival (Wilkins et al., 2009). In the latter, study MSC-conditioned medium activated the PI3kinase/Akt pathway and reduced p38 signaling in neurons exposed to NO. The addition of anti-BDNF neutralizing antibodies to MSC-conditioned medium attenuated this neuroprotective effect. Therefore BDNF

secretion by MSC could protect neurons from NO-mediated cell death causing a preservation in KIF protein expression.

MSCs have also been shown to reduce EAE-dependent oxidant stress in CNS alongside reducing the expression of nuclear enzyme PARP-1 (poly ADP-ribose polymerase 1) (a marker of cell damage since it is activated by DNA strand breaks) and the pro-apoptosis transcription factor p53 (Lanza et al., 2009). The down-regulation of molecules involved in the cellular response to stress conditions, protecting neurons from death, could therefore be another mechanism by which MSCs preserve KIF protein expression post NO exposure.

Conclusion

This part of the study has demonstrated the ability of MSCs to protect KIF expression from NO damage in a co-culture system, showing the importance of cross-talk between MSCs and neuronal cells that have undergone an injury stimulus. *In vivo* studies demonstrate that systemically administered MSCs can extravagate from the blood vessels and migrate, in response to several chemokines released after tissue damage, preferentially to the site on injury (Fox et al., 2007). MSCs express a number of cytokine and chemokine receptors that allow these cells to respond to the inflammatory environment (Croitoru-Lamoury et al., 2007) and to produce anti-inflammatory and anti-oxidant factors. Some studies in fact support the idea that an *in vitro* “stimulation” of MSCs, before their administration in clinical trials, could improve their properties (Croitoru-Lamoury et al., 2007).

Some trials to assess the safety and efficacy of MSCs as a potential treatment for multiple sclerosis have started (Karussis et al., 2010; Yamout et al., 2010; Rice et al., 2010; Connick et al., 2012). The mode of delivery, timing of the treatment, ideal cell

number for the transplantation, efficacy and above all the long-term safety are still under scrutiny and need to be addressed. Transplanted-related serious adverse effects are likely to be an uncommon event; the main concern is that MSCs ability to suppress immune response could promote a state of immune deficiency leading to infection or activation of benign tumors (Djouad et al., 2003). Another concern is that MSCs might migrate to tumours promoting tumor growth and metastasis increasing cell motility and invasion (Karnoub et al., 2007; Kidd et al., 2009). Malignant transformation upon culture has been described for rat and mouse MSCs (Foudah et al., 2009; Zhou et al., 2006) but not for human derived mesenchymal stem cells (Bernardo et al., 2007; Aguilar et al., 2007) even if the topic is still open and further studies are needed to ensure the long term safety of MSCs. A recent study has monitored alterations in phenotype, telomerase length, senescence state, gene expression patterns and DNA methylation profiles of human MSCs upon prolonged *in vitro* expansion (Redaelli et al., 2012 in press). Telomerase length were markedly shorter at later culture passages (p6, p9, p12) in comparison with early passages (p0, p3) and differences in the methylation profile were observed. But chromosomal stability of MSCs was evident for six out of eight healthy donors even in later passages, moreover for one donor the abnormal karyotype did not persist upon prolonged culturing maybe due to *in vitro* negative selection of the aneuploid clone. However, risk of carcinogenic transformation and infection after *in vitro* expansion remains a concern, therefore as a safety measure minimal expansion may be recommended.

The results obtained in this part of the study provide further evidence of MSCs' significant therapeutic potential for neurological disorders of the CNS involving oxidative injury. MSCs may represent a source of cells for reparative therapies due to

their multiplicity of neuroprotective and regenerative effects, particularly, as evident in this study, through the secretion of soluble factors.

Changes in both KIF5A and KIF21B expression are evident in multiple sclerosis cerebellum and may be linked to pathological changes in the axons

Multiple sclerosis is an inflammatory demyelinating and neurodegenerative disease of the central nervous system (Dutta and Trapp, 2007). Historically, MS is considered a disease in which the immune system recognises the CNS myelin as a foreign body and therefore activates inflammatory and immune processes to destroy and remove it. The disability is caused by focal areas of inflammatory-mediated demyelination of the brain and spinal cord white matter, in which the conduction of electrical impulses is blocked due to myelin loss and to a redistribution of Na^+ channels along the entire length of the axon (Trapp and Nave, 2008). In addition to the commonly described white matter lesions, MS lesions can also involve gray matter (Peterson et al., 2001; Kutzelnigg et al., 2005). Less is known about cortical gray matter lesions because these lesions are difficult to detect macroscopically in autopsy specimens due to the fact that they do not change color and they are not apparent from using routine histological stains (Trapp and Nave, 2008). Furthermore, cortical gray matter demyelination occurs without significant influx of leukocytes (Bo et al., 2003, Trapp and Nave, 2008) and is not detected by conventional MRI modalities (Sharma et al., 2001). Plaques in the white matter only partially explain the clinical deficits in patient’s and several studies have recently demonstrated a strong correlation between gray matter atrophy and clinical disability. Indeed, gray matter atrophy and cortical demyelination are considered to be a relevant marker of MS disease progression (Fisher et al., 2002; Kutzelnigg et al., 2005).

The primary function of the cerebellum is the modulation of movement, coordination and balance. The cerebellar cortex is divided into three layers. At the bottom lies the thick granular layer, densely packed with granule cells and interneurons. In the middle is present the Purkinje cell layer that contains only the cell bodies of Purkinje cells. And at the top lies the molecular layer, which contains two types of inhibitory interneurons, stellate cells and basket cell, the extensive dendritic trees of Purkinje cells and the array of parallel fibers penetrating the Purkinje cell dendritic trees. Purkinje cells are the largest neurons in the human brain, they receive more synaptic inputs than any other neuron and the cerebellum are critical not to initiate movements but to regulate the force, range, direction, velocity and coordination of muscle contractions.

In the cerebellar cortex of EAE mice a decrease in the volume of the whole cerebellum has been observed alongsidecerebellar cortex atrophy and an increase in the number of caspase 3 positive Purkinje cells (Mackenzie-Graham et al., 2009). Clinical MS studies have found that one third of subjects have functionally relevant cerebellar deficits (Alusi et al., 2001) and have demonstrated a decrease in cerebellar volume in MS patients (Edwards et al., 1999). Kutzelnigg et al (2007) suggests that the cerebellar cortex is extensively involved in MS patients, particularly in those with primary and secondary progressive disease in which large cerebellar demyelinated areas are present.

Several researchers within the “Multiple Sclerosis and Stem Cell Group” in Bristol are currently evaluating KIF expression within the cortex of MS patients. Therefore to complement their ongoing research, in this study KIF5A and KIF21B expression was analyzed in the cerebellum of MS patients and in appropriate controls. To characterize cerebellar lesions, sections from MS patients and control patients were

immunostained against myelin basic protein (MBP) and the reactive macrophage/microglial marker HLA-DR. As expected, no demyelination or inflammatory cell infiltration was detected in cerebellar sections derived from control patients. In contrast, cerebellar sections from MS patients showed lesions with loss of myelin and extensive inflammatory cell infiltration. Control and MS cerebellar sections were also immunostained with antibodies to KIF5A and KIF21B. Using both these antibodies, in control and MS patient groups the most prominent staining was evident in the cell body and dendrites of Purkinje cells. KIF21B expression was also detected in other cells populations in the molecular layer, which were possibly basket or stellate cells. In MS sections, Purkinje cells seemed preserved, however aggregates of a spheroid nature, positive for both KIF5A and KIF21B, were detected in the granular layer. Others studies have observed axonal spheroids in MS cerebella, in fact Kutzelnigg et al (2007) have shown cerebellar demyelinated areas with relative neuronal preservation and axonal spheroids in patients with progressive MS. Moreover, KIF5A positive spheroids, axonopathy and accumulation of neurofilament and APP has been associated with KIF5A down regulation and axonal transport defects in models of multiple sclerosis (Kreutzer et al., 2012). Axonal transection and axonal spheroids are a hallmark of MS and APP-positive axonal swellings are common in the disease (Trapp et al., 1998). Kamal et al (2000) demonstrated a direct biochemical interaction between APP and the kinesin light chain (KLC) of KIF5. This study also showed a reduction in axonal transport of APP in a mouse model expressing mutant KLC, showing direct *in vivo* evidence for the role of KIF5 in the transport of APP. To further investigate the KIF-positive aggregates observed in the cerebellum of MS patients, double immunofluorescence labelling using both APP and KIF5A or KIF21B antibodies was perform. Results have shown that the majority of

aggregates were both positive for APP and KIF5A, while no co-labelling was observed for APP and KIF21B. This data seemingly provides more evidence to confirm that KIF5A molecular motors transports APP and that impaired axonal transport may be linked, and be a possible cause, to some of the pathological changes occurring in axons. It is possible that inflammatory mediators may not only reduce the gene and protein expression of KIFs, as demonstrated in the first part of this study, but also impair the unloading of cargoes that could cause APP and KIF5A complexs to accumulate within axons. A primary dysfunction/reduction in axonal molecular motors could impair protein transport along axons, thus causing proteins to accumulate and axon swellings to form, leading to an overall axonal disturbance. At this stage, destabilization of the cytoskeleton may interfere with the interaction of kinesin with microtubules leading also to accumulation of kinesin itself, as suggested by the KIF5A and KIF21B positive aggregates observed in the cerebellum of MS patients.

To investigate whether in the gray matter of MS cerebellum there were any changes in KIF5A and KIF21B gene and protein expression, homogenized tissue derived from 8 MS patients and 6 controls were analyzed. The homogenized tissue from MS cerebellum was harvested from gray matter and characterized according to the adjacent white matter lesion type.

To choose a stable and appropriate housekeeping gene and protein is essential for accurate normalization in order to obtain reliable results. A housekeeping marker should be a gene/protein which expression remains constant in the cells or tissues under investigation. However, studies that have compared the expression of several different housekeeping genes/proteins have shown that their levels of expression differ between different tissue types or between normal and disease states (Tricarico

et al.. 2002; Barber et al., 2005; Caradec et al., 2010). Finding a validated housekeeping marker for a certain tissue or disease is complex, therefore in this study several different housekeeping markers were selected as reference controls. Besides the commonly used GAPDH and ribosomal 18s, the neuronal specific enolase (ENO2), microtubule associated protein 2 (MAP2) and the calcium-binding protein calbindin-D28K were used.

PCR results showed no significant changes in KIF5A gene expression in the gray matter adjacent to active lesions, while there was a significant decrease in the protein expression of KIF5A when using GAPDH as endogenous control and a decrease of both KIF5A and KIF21B when compared to ENO2. Inflammatory cells contain GAPDH, therefore ENO2 maybe a better and more reliable endogenous control because it normalizes to neuronal cell quantity. This KIF protein decrease was significant only in the gray matter adjacent to active lesions, suggesting that the active focal inflammatory microenvironment contains a variety of soluble and mobile substances including cytokines, free radicals, oxidative factors, NO, proteolytic enzymes that all could injure the adjacent gray matter. iNOS, the inducible nitric oxide synthase, is up regulated in acute inflammatory MS lesions generating high levels of NO. This increase in NO could certainly damage KIFs proteins by S-nitrosylation, as mentioned in the first part of the study, even in the gray matter. KIF molecular motors need ATP to generate motile force, so also the reduction in ATP production caused by a NO-mediated disruption of the mitochondrial respiratory chain could be a mechanism that inhibits axonal transport.

As mentioned above, in the gray matter adjacent to active lesion types, KIF5A gene expression was not changed. However, PCR results did show a decrease in KIF5A gene expression in the gray matter adjacent to chronic lesions when using 18s as an

endogenous control. Kutzelnigg et al (2005) suggest that MS starts as a focal inflammatory disease which gives rise to circumscribed demyelinated plaques in the white matter, but with chronically diffuse inflammation accumulates throughout the whole brain and is associated with slowly progressive axonal injury in the NAWM and cortical demyelination. The cerebellum is a major predilection site for cortical demyelination in MS. In most instances, the cerebellar cortex is affected independently from white matter lesions but cortical demyelinations sometimes occurs together with demyelination in the adjacent white matter (Kutzelnigg et al., 2007.) Therefore patients with chronic lesions in the white matter could have also the adjacent gray matter affected. It is not known the influence of myelin and oligodendrocyte-derived factors on axonal transport mechanisms. Demyelination itself could reduce gene expression of axonal transport proteins or alter cell signalling proteins that regulate transcription factors that encode for KIF proteins.

PCR results also showed an increase of KIF21B gene expression in the gray matter adjacent to active lesion types when using 18s as an endogenous control. A possible explanation for the increased KIF21B expression in the gray matter near active lesion could be some compensatory mechanism in response to inflammation. Since there was a decrease of KIF21B protein expression, increased gene expression could try and overcome the evident protein reduction. Why this increase was observed only for KIF21B and not for KIF5A it is not clear. This maybe because Purkinje cells have an extensive dendritic network and KIF21B is enriched in dendrites. However, the expression of KIF21B is detected also in cells of the immune system including T-cells, NK cells and B-cells and may have a yet unknown regulatory function. Therefore another possibility is that the KIF21B increase was due to immune cells present expressing KIF21B. Further promoting this is the fact that the KIF21B

increase was only significant when using 18s as endogenous control and not when normalized to neuronal cell quantity using ENO2.

Conclusion

Multiple sclerosis affects more than 2 million people worldwide and is the most common non-traumatic cause of disability in young (<50 years) European adults (Pugliatti et al., 2006). Axonal loss is considered an early event and the major cause of permanent neurologic disability in MS patients. Therefore, understanding the mechanisms that lead to the axon's functional and structural changes that predispose it to degeneration is a major challenge for MS research. KIF21B has been established as a susceptibility locus for multiple sclerosis and KIF5A down regulation has been associated with axonal transport defects in models of multiple sclerosis. Clarification in the role of axonal transport during disease initiation and progression and the molecular mechanism of APP and NF accumulations are therefore needed in order to understand disease mechanisms and develop new therapies.

Final conclusion

Abnormal accumulations of proteins and organelles within the axons are hallmarks of many neurodegenerative disorders, including MS. Several studies hypothesize that such pathogenic accumulations are caused by impaired axonal transport, specifically through abnormalities in KIFs proteins, but the mechanisms involved are still unknown. The results of this study allow a better understanding of the mechanisms that can lead to axonal disturbance and blockage of transport during oxidative insult and possibly in MS. Impaired axonal transport seems not just to be a consequence of axon damage, however maybe a major contributory factor that leads to axonopathy.

Moreover, the ability of MSCs to protect KIF expression from NO damage provides further evidence of their significant therapeutic potential in multiple sclerosis.

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