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Effects of ALS-related SOD1 mutants on dynein- and KIF5-mediated retrograde and anterograde axonal transport

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Abstract

Transport of material and signals between extensive neuronal processes and the cell body is essential to neuronal physiology and survival. Slowing of axonal transport has been shown to occur before the onset of symptoms in amyotrophic lateral sclerosis (ALS). We have previously shown that several familial ALS-linked copper-zinc superoxide dismutase (SOD1) mutants (A4V, G85R and G93A) interacted and co-localized with the retrograde dynein-dynactin motor complex in cultured cells and affected tissues of ALS mice. We also found that the interaction between mutant SOD1 and the dynein motor played a critical role in the formation of large inclusions containing mutant SOD1. In this study, we showed that, in contrast to the dynein situation, mutant SOD1 did not interact with anterograde transport motors of the kinesin-1 family (KIF5A, B and C). Using dynein and kinesin accumulation at the sciatic nerve ligation sites as a surrogate measurement of axonal transport, we also showed that dynein mediated retrograde transport was slower in G93A than in WT mice at an early pre-symptomatic stage. While no decrease in KIF5A-mediated anterograde transport was detected, the anterograde transport of dynein heavy chain as a cargo was observed in the pre-symptomatic G93A mice. The results from this study along with other recently published work support that mutant SOD1 might only interact with and interfere with some kinesin members which in turn could result in the impairment of a selective subset of cargos. Although it remains to be further investigated how mutant SOD1 affect different axonal transport motor proteins and various cargos, it is evident that mutant SOD1 can induce defects in axonal transport, which subsequently contribute to the propagation of toxic effects and ultimately motor neuron death in ALS.

Keywords

amyotrophic lateral sclerosis; copper-zinc superoxide dismutase; axonal transport; kinesin; dynein

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Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by motor neuron death. Mutations in the Cu/Zn superoxide dismutase (*SOD1*) gene have been shown to cause 10 to 20% of familial ALS (fALS) cases [1,2]. Mutations scattered in the *SOD1* gene in a seemingly random fashion can still specifically and exclusively lead to ALS [3]. Evidence shows that ALS is not caused by the loss of the SOD1 activity, but by toxic properties gained by mutant SOD1 [4,5]. However, the mechanism(s) of mutant SOD1-mediated toxicity remains unclear.

Transport of material and signals between extensive neuronal processes and the cell body is essential to neuronal function and survival. Members of the kinesin motor family transport cargos in the anterograde direction (toward the synapse), whereas cytoplasmic dynein is the major motor protein driving retrograde transport (toward the cell body) in neurons. Many proteins needed in the axon and synaptic terminal must be transported from the cell body where they are synthesized. Both anterograde and retrograde transport are needed to maintain homeostasis and correct localization of mitochondria in neurons [6]. Furthermore, retrograde transport of signals induced by neurotrophic factors are believed to be important for neuronal survival [7,8]. Mutations in the axonal transport motor proteins can cause neurological disorders. For instance, mutations in the anterograde transport motor protein KIF5A can cause spastic paraplegia [9]. Several mutations in the retrograde motor complex dynein-dynactin cause motor neuron degeneration in humans and mice [10,11].

One characteristic of ALS disease is reduced axonal transport activity [12–18]. Interestingly, some ALS-causing SOD1 mutant proteins can differentially affect the axonal transport of distinct cargos. While fast transport of vesicles was observed to be suppressed in both anterograde and retrograde directions in ALS mice [19], transport of cytoskeletal elements including neurofilaments was reported to be slowed only in the anterograde direction [20,21]. Moreover, different studies show that mitochondrial movement could be suppressed in anterograde [19] and retrograde [22] direction. We and others have previously shown that several familial ALS-linked SOD1 mutants co-localize with the dynein-dynactin complex in cultured cells and affected tissues of ALS mice [15,23]. We also showed that mutant SOD1 interacted with the dynein-dynactin complex and that this interaction contributed to the formation of large SOD1 inclusions [23,24].

While the dynein-dynactin complex is the only retrograde transport motor, a total of 45 kinesin superfamily (KIF) genes have been identified in the mouse and human genomes, and of these 38 are expressed in the brain. The KIF genes are classified into 14 families (kinesin 1–14) based on phylogenetic analysis [25]. A recent study showed that mutant SOD1 could interact with KAP3 (kinesin-associated protein 3), a subunit of kinesin-2 complexes. Furthermore the sequestering of KAP3 by mutant SOD1 was suggested to cause reduced transport of ChAT (a known KAP3-kinesin-2 cargo) in an ALS model [26]. A large genome-wide analysis of single nucleotide polymorphisms (SNPs) in a set of 1,821 sporadic ALS cases and 2,258 controls revealed that a variant within the KAP3 gene was associated with decreased KAP3 expression and increased survival in sporadic ALS [27]. These new findings provide an emerging concept that axonal transport might be involved in both familial and sporadic ALS.

The aim of this study was to investigate whether mutant SOD1s can also interact with and affect the transport mediated by three closely related members (KIF5A, KIF5B and KIF5C) of the kinesin-1 family. KIF5A and KIF5C are neuron specific and play essential roles in axonal transport, while KIF5B is also expressed in non-neuronal cells [25]. The KIF5 subtypes can form homo- or hetero-tetramers and about half of the KIF5s are believed to

form tetrameric complexes by recruiting two kinesin light chain (KLC) molecules. The KIF5 motor proteins have been suggested to transport many cargos important for neurons including neurofilaments, mitochondria and SNARE proteins essential for synaptic vesicle docking [25]. In *Drosophila*, mutations in the KIF5 homologue called KHC inhibit neuronal sodium channel activity, action potentials and neurotransmitter secretion [28,29]. Furthermore, these *Drosophila* mutants also develop a motor neuron disease phenotype due to disruption of fast axonal transport [30]. In mice, inhibition of KIF5B dramatically changed the distribution of mitochondria towards the cell center in neurons including motor neurons [31].

In this study we compared the ability of mutant SOD1 to interact with and affect KIF5 transport with the interaction and effect on the retrograde motor complex dynein-dynactin. Using co-precipitations we could show that, unlike with dynein-dynactin, there is no detectable interaction between mutant SOD1s and KIF5s. Immunostaining experiment also showed no change in the expression pattern or sequestering of KIF5 into mutant SOD1 inclusions in G93A ALS mice. Furthermore, sciatic nerve ligation experiments performed on 60 days old pre-symptomatic G93A SOD1 ALS transgenic mice revealed reduced retrograde transport of dynein. In contrast, no decrease in anterograde transport as indicated by KIF5 accumulation could be observed in G93A mice. Interestingly, the anterograde transport of dynein heavy chain as a cargo was observed in the pre-symptomatic G93A mice. Based on the results from this study, we suggest that disruption of dynein-mediated transport is an early event in G93A mice starting before disease onset and could contribute to the neuronal death in ALS. Concerning anterograde transport, mutant SOD1 might only interact with and interfere with some kinesin members which in turn could result in the impairment of a selective subset of cargos.

Materials and Methods

Plasmid Construction

Mouse kinesin light chain 2 (KLC2) gene was amplified from the pcDNA3-rKLC2 construct (a generous gift from Dr. Xiaojiang Li at Emory University) by PCR using two primers with the sequence 5’-GACTGCGGCCGCTATGGCCACGATGGTGCTTC-3’ and 5’-GACTGCGGCCGCTTAGCCCACGAGGGAGCTT-3’. The amplified DNA fragment was inserted into the pEBG vector that can express glutathione S-transferase (GST) fusion proteins in mammalian cells [23]. The SOD1-FLAG and SOD1-GFP constructs were described in a previous study [23]. The fidelity of all the constructs was confirmed by DNA sequencing.

GST Pulldown and Immunoprecipitation of DHC and KIF5

HEK293 cells transfected with both SOD1-FLAG and KLC2-GST constructs were harvested and lysed in 1 ml of RIPA buffer. The protein concentration was determined by Bradford assay (BioRad). Four hundred micrograms of lysate was incubated with 40 μl of 50% slurry of glutathione Sepharose beads (GE Healthcare) in a total volume of 1 ml for 3h at 4 °C to allow isolation of the GST fusion proteins. The GST beads were then collected, washed, and boiled in 2XSDS sample loading buffer. The proteins released from the beads were subjected to 10% SDS-PAGE followed by Western blotting. The reciprocal FLAG immunoprecipitation was performed using anti-FLAG M2 beads (Sigma).

KIF5 immunoprecipitations were carried out for the dissected mice spinal cords and sciatic nerves using a mouse monoclonal antibody (MAB1614, Chemicon) and protein G-Sepharose (GE Healthcare). The immunoprecipitated proteins were subjected to 10% SDS-PAGE followed by Western blotting. Antibodies used in the Western blotting are rabbit anti-

SOD1 (sc-11407, Santa Cruz), mouse anti-KIF5, mouse anti-KLC (MAB1617, Chemicon) and mouse anti-Myc (sc-40, Santa Cruz). Intensities of Western blotting bands were quantified using the Kodak 1D software (version 3.6.1).

Animals

Transgenic mice strains over-expressing WT and G93A mutant SOD1 were generously provided by Dr. Zuoshang Xu (University of Massachusetts Medical School). The mice were generated from B6.Cg-Tg(SOD1)2Gur/J and B6.Cg-Tg(SOD1-G93A)1Gur/J stains [32] and bred and maintained as hemizygotes at the University of Kentucky animal facility. Transgenic mice were identified using PCR. G93A SOD1 transgenic mice were sacrificed at ages of 35, 60, 90, and 125±5 days. Age-matched WT SOD1 transgenic mice were sacrificed as controls. Mice were anesthetized with an intraperitoneal injection of 0.1 ml pentobarbital (50 mg/ml, Abbott Laboratories) and transcardically perfused with 0.1 M phosphate-buffered saline (PBS), pH 7.5, before spinal cords and other tissues were dissected. All animal procedures were approved by the university IACUC committee.

Mice sciatic nerve ligation

Mice 60 ±5 days old (pre-symptomatic as they were clean of any observable clinical signs) were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylaxine. All surgical instruments were sterilized before surgery and then washed and heat treated. Sciatic nerves on both hind limbs were exposed, the left nerve was ligated and the right nerve was used as sham control. 7-0 silk sutures were used to ligate the left nerve at mid-thigh level. To ensure complete block of transport, two ligations were done 1–2 mm apart. On the sham control side, a small piece of suture was left untied under the nerve. After checking hemostasis, the muscle and the adjacent fascia were closed with sutures and the skin was closed with clips. After 24 or 48 hours, the mice were sacrificed by CO_2 asphyxiation and both sciatic nerves were dissected, fixed, cryo embedded and sectioned.

Kinesin and dynein motor proteins were characterized using immunofluorescence. KIF5A and dynein heavy chain antibodies were used for motor protein accumulation detection. For quantifications, the average signal intensity for a size defined area (0.0625 mm^2) just adjacent to the ligation site on the proximal (P1) or the distal side (D1) was divided by the average signal intensity from a similar sized control area on the proximal (P2) or distal side (D2). Longitudinal sections from ligated and sham nerves from 4–6 G93A and 4–6 WT mice were analyzed at each time point. Two-way Analysis of Variance (ANOVA) followed by Turkey test was performed since our study had two factors: factor A (WT versus G93A mice), and factor B (proximal versus distal sites).

Fluorescence Microscopy

Immunohistological analyses were performed on lumbar spinal cords and sciatic nerves of ALS animals at various ages as indicated. Spinal cords were dissected, post-fixed overnight in 4% paraformaldehyde in 0.1M PBS, dehydrated, and embedded in Paraplast X-tra (Tyco Healthcare). Sections (6 μm) were deparaffinized, rehydrated, and boiled in 0.01 M citrate buffer (pH 6.0) for 15 min to retrieve antigens. Sciatic nerves were post-fixed for 2 h in 4% paraformaldehyde, cryo preserved in 30% sucrose overnight, embedded in tissue-Tek OCT compound [26] and 10 μm sections were cut.

Paraffin and cryo sections were blocked in 10% heat-inactivated fetal bovine serum in 0.1M PBS with 0.1% Triton X-100 (PBST) for 30 min before being incubated with primary antibodies diluted in 1% fetal bovine serum-PBST overnight at room temperature. The sheep SOD1 antibody (PC077, The Binding Site), rabbit KIF5A antibody (K0889, Sigma) and rabbit DHC antibody (sc-9115, Santa Cruz) were used at 1/400, 1/400 and 1/200,

respectively. Following primary antibody incubation, sections were washed with PBST and incubated with 4', 6-diamidino-2-phenylindole dihydrochoride (DAPI, Sigma) at 1:7,500 and incubated with Alexa Fluor 488 anti-sheep (Molecular Probes) at 1:250. Sections were then washed with PBST, incubated with Alexa Fluor 594 anti-rabbit (Molecular Probes) at 1:300 and then mounted using Vectashield mounting medium. Fluorescence microscopy was carried out using a Leica DM IRBE laser scanning confocal microscope with a 100 × objective.

Results

No or very weak interaction between KIF5-KLC2 complexes and wild-type or mutant SOD1 in cultured cells

Decreased axonal transport has been observed in ALS. It has been demonstrated recently that ALS-related mutant SOD1 proteins can interact with both the anterograde motor protein kinesin-2 complex via KAP3 [26] and the retrograde motor protein complex dynein-dynactin [23]. To investigate whether mutant SOD1s can also directly or via a kinesin light chain (KLC) interact with three members of the kinesin-1 family (KIF5A, B or C), the plasmid encoding GST tagged kinesin light chain 2 (GST-KLC2) was co-transfected with SOD1-FLAG constructs into HEK293 cells. Forty-eight hours after the transfection, cell extracts were prepared and GST pull-down performed to precipitate GST-KLC2. As shown in Figure 1A, blotting with an antibody able to recognize all three subtypes of KIF5, showed that KIF5s were co-precipitated with KLC2 consistent with previous studies showing that KLC2 and KIF5s form kinesin complexes (Lanes 1–4 in the lower panel). However, no detectable wild-type (WT) or mutant FLAG-SOD1 could be observed in the GST-KLC2 pull-down (Lanes 1–4 of the top panel). In contrast, Western blotting of cell extracts showed that GST-KLC2 and SOD1-FLAG were expressed in all samples (Figure 1A). These results suggest that, while KIF5-KLC2 complex was pulled-down by GST-KLC2, no significant amount of WT or mutant SOD1 was co-precipitated with the kinesin-1 complexes.

In comparison, the mutant SOD1 proteins (A4V, G85R and G93A) were readily co-precipitated with the dynein-dynactin complex in GST-DIC (dynein intermediate chain) pull-downs (Lanes 7–9), whereas WT SOD1 was not (Lane 6). A dynactin subunit p150 was visualized to show co-precipitation of dynactin with GST-DIC (Lanes 6–9 in the middle panel). The GST control vector was co-transfected with A4V (Lane 5) and G93A SOD1 (Lane 10) as negative controls.

The reciprocal FLAG immunoprecipitation was performed to verify the above results. As shown in Figure 1B, no or very little GST-KLC2 could be detected in either WT or mutant SOD1 immunoprecipitations. Western blotting of cell extracts again showed that expression levels of GST-KLC2 (Lanes 1–5) and SOD1-FLAG (Lane 1–4 and 6–9) were comparable in all samples. The data again suggest that there is no specific interaction between SOD1 and the KLC2-containing kinesin-1 complexes (Lanes 1–4). For comparison, GST-DIC was co-precipitated with A4V, G85R and G93A mutants (Lanes 7–9) but not with WT SOD1 (Lane 6) or FLAG control (Lane 10). The results concerning the interaction between mutant SOD1 and the dynein complex in both Figure 1A and 1B are consistent with our previously published data [23].

No or very weak interaction between KIF5 and wild-type or G93A SOD1 in transgenic mice

We further tested whether SOD1 could interact with KIF5s in the WT and G93A SOD1 transgenic mice. Spinal cord homogenates from 125 days old WT or G93A SOD1 transgenic mice were subjected to immunoprecipitation using an antibody that can recognize all three KIF5 subtypes (KIF5, Figures 2A and 2B). For comparisons, co-precipitations of SOD1 and

dynein-dynactin complex were also performed in parallel using a DIC antibody (Figure 2C). Consistent with the previously published data [23], G93A mutant SOD1 was co-precipitated with DIC whereas WT SOD1 was not (Figure 2C). However, although we could immunoprecipitate KIF5 (Figure 2A, lanes 2 and 3) and kinesin light chains KLC1 and KLC2 (Figure 2A, middle panel) using the KIF5 antibody, neither WT nor G93A SOD1 was co-precipitated with the KIF5s (Figure 2A, lower panel). Western blotting of the spinal cord extracts showed that WT and G93A SOD1, KIF5, KLC1 and KLC2 were all expressed (Lanes 4 and 5). Furthermore, the immunoprecipitation experiment was also performed with 35, 60 and 90 days old mice. As shown in Figure 2B, neither WT nor G93A SOD1 was co-precipitated with KIF5 in mouse samples at any time point. Western blotting of the spinal cord extracts showed that KIF5 levels in the protein extracts where similar in all samples. These data suggest that, in contrast to the situation with the dynein-dynactin, there is no detectable interaction between mutant SOD1 and KIF5 or KIF5-KLC complexes.

No co-localization between KIF5A and SOD1 inclusions in G93A transgenic mice

We next tested whether any co-localization of SOD1 and KIF5A could be observed in the WT and G93A SOD1 transgenic mice. Spinal cord and sciatic nerve sections from 60, 90 and 125 days old WT and G93A SOD1 transgenic mice were co-immunostained with SOD1 and KIF5A antibodies to visualize possible co-localization in neurons (Figure 3A and 3B). No indication of sequestering of KIF5A into mutant SOD1 inclusions was observed in either spinal cord motor neurons or the sciatic nerve axons in SOD1 transgenic mice at any time point. The co-immunostaining data are consistent with our *in vitro* and *in vivo* co-precipitation results, suggesting that KIF5A and KIF5A-KLC complexes have no or little interaction with mutant SOD1. The data are also consistent with the findings in a recent study also showing no co-localization of mutant SOD1 and kinesin [33]. The results suggest that KIF5A is different from the kinesin-2 subunit KAP3 [26] or various dynein-dynactin subunits [15, 23] that have been reported to co-localize with mutant SOD1 inclusions.

In addition, the stainings of the neuronal specific KIF5A revealed a punctuated pattern in anterior horn motor neurons, consistent with the role of KIF5A in trafficking. Moreover, the KIF5A levels appeared similar in the remaining motor neurons in G93A mice to those found in motor neurons of age-matched WT SOD1 transgenic mice (Figure 3). The results are consistent with the Western blot results in Figure 2, suggesting that KIF5A levels remained largely unchanged in motor neurons.

Suppression of dynein-dynactin retrograde transport but not KIF5A anterograde transport in pre-symptomatic G93A mice

To examine the effect of mutant SOD1 on bidirectional axonal transport in neurons of G93A mice, sciatic nerve double ligation surgery and subsequent immunostaining were performed. The pre-symptomatic 60 days old G93A mice were used to investigate early effects on axonal transport and to prevent axonal loss in the G93A mice from interfering with the analysis. The age-matched WT SOD1 transgenic mice were included for comparison. Molecules transported in the anterograde direction will accumulate on the proximal side of the ligation while retrograde molecules will accumulate on the distal side of the ligation (see scheme in Figure 6A). Accumulation of kinesin and dynein motor proteins was hence used as a surrogate measurement of anterograde and retrograde axonal transport, respectively. The mice were euthanized 24 or 48 hours after ligations, the sciatic nerves from both sides (one ligated and one sham) were dissected, sectioned and stained with an antibody against neurofilament-M and the dynein heavy chain (DHC) or neuronal KIF5A. Figure 4 and 5 show the representative images of DHC and KIF5A immunostaining in the sciatic nerve sections, respectively. The quantifications of dynein and kinesin accumulation are shown in Figure 6B and 6C. The intensity of dynein or kinesin immunofluorescence close to the

ligation on the proximal side (area P1) or the distal side (area D1) was normalized by division of the background intensity of kinesin or dynein measured at proximal or distal control areas (P2 or D2) in the same section (see Figure 6A). The accumulation of DHC and KIF5A is presented as the normalized intensity, i.e. the ratios of P1/P2 or D1/D2 in both G93A and WT mice. Statistical analysis was performed using two-way ANOVA followed by Turkey test.

Analysis of dynein staining revealed that, compared to the sham nerve control, increased dynein staining could be observed on the distal side of the ligation after 24 and 48 hours in both WT and G93A mice (Figure 4A, 4B and 6B). In nerves 24 hours after ligation, the distal accumulation of dynein in G93A mice was significantly less compared to the accumulation in WT mice ($p = 0.031$). However, after 48 hours there was no statistically significant difference in the distal accumulation of dynein in WT and G93A mice ($p = 0.820$). These result suggested that retrograde transport as inferred by the accumulation of dynein motor protein is not completely blocked, but slowed down in the presence of mutant SOD1.

Dynein also accumulated on the proximal side of the ligation compared to sham control in both WT and G93A mice (Figure 4A, 4B and 6B). Dynein accumulation at the proximal side after nerve crush or ligation has been previously reported by other laboratories and is most likely due to kinesin-mediated anterograde transport of dynein toward the synapse [21, 34, 35]. The proximal accumulation of dynein at 24 hrs was also decreased in G93A mice compared to WT mice ($p=0.013$). This suggests that the anterograde transport of dynein could be impaired in G93A mice.

KIF5A immunostaining showed stronger accumulation on the proximal ligation side than the distal side in both WT and G93A (Figure 5 and 6C). Furthermore, no decreased accumulation of KIF5A could be observed in G93A mice compared to control mice. Instead, although not statistically significant a trend toward a slight increase in KIF5A proximal accumulation could be observed in G93A at both 24 and 48 hours.

Taken together, these data show that reduced axonal transport could be observed in both anterograde and retrograde directions at an early pre-symptomatic stage of the ALS mice using the immunostaining intensity of DHC as a marker at the proximal and distal sides of the ligation sites. In comparison, using KIF5A as a marker, we could not observe any change in KIF5A-mediated anterograde transport in G93A mice at this pre-symptomatic stage.

Discussion

We have previously shown that several ALS mutants of SOD1 can interact with the dynein-dynactin complex which is responsible for retrograde axonal transport [23]. We have also demonstrated that the interaction between mutant SOD1 and dynein-dynactin plays a functional role in the formation of mutant SOD1 inclusions [24]. In this study, we used the sciatic nerve double ligation technique, which has long been used to evaluate the rate of bi-directional transport under physiological and pathological conditions [36–38], to determine whether the bi-directional axonal transport was impaired. In addition, we investigated whether mutant SOD1 could interact with and disrupt the anterograde axonal transport mediated by the kinesin-1 family members. Kinesin-1 proteins are abundant in neurons and have been shown to transport several cargos vital for neuronal function and survival. The goal of the study was to compare the interaction of mutant SOD1 with the dynein-dynactin and the kinesin-1 members, and to compare the effect of mutant SOD1 on the corresponding retrograde and anterograde axonal transport.

The interaction between mutant SOD1 and dynein-dynactin was again demonstrated in cultured cells and ALS mouse spinal cords in this study. Based on quantification of motor protein DHC accumulation at the distal side of the sciatic nerve ligation site 24 hour post-ligation, we showed that dynein-mediated axonal transport was significantly suppressed in 60 days old pre-symptomatic G93A mice compared to WT SOD1 mice (Figure 4 and 6B). Interestingly, the data from the 48 hour post-ligation samples, showed no difference in dynein accumulation between G93A and WT SOD1 mice, suggesting that retrograde transport is not completely blocked but rather slowed down. These data together with our previous studies [23,24] support the hypothesis that there is an early impairment of axonal retrograde transport in the G93A SOD1 transgenic mice, presumably due to the interaction between mutant SOD1 and the dynein-dynactin. This hypothesis is further supported by other studies that mutant SOD1 could alter the sub-cellular localization of dynein-dynactin [15]. The same study also showed a decrease of retrograde transport in 50 days old G93A mice using a neurotracer approach.

The reduced retrograde transport could contribute to the motor neuron death by decreasing the removal of damaged mitochondria from the synapse, or by reducing the level of survival signals induced by neurotrophic factors secreted by the muscle [39]. Since dynein-dynactin is the only major motor protein responsible for retrograde transport, the alterations in the retrogradely transported cargos may be an alternative mechanism contributing to motor neuron death. In fact a recent study reported a switch in retrograde signaling from pro-survival to stress in ALS mice [38].

Besides its role in retrograde axonal transport, the dynein-dynactin complex also have many other functions, including participation in mitosis, endoplasmic reticulum to Golgi vesicular trafficking, neuronal migration, neurite outgrowth, synapse formation, formation of aggresomes and protein degradation by autophagy (for review see [40]). The interaction between mutant SOD1 and dynein-dynactin could hence have various consequences. In two other recent studies from our lab we found that the amount of mutant SOD1 interacting with dynein-dynactin increases with disease progression and that this interaction between mutant SOD1 and dynein-dynactin plays a role in formation of large aggresome-like SOD1 inclusions [23,24]. Increasing levels of dynein-dynactin interacting with mutant SOD1 and sequestration of dynein into aggresome-like inclusions could also lead to depletion of the pool of dynein available for axonal transport. Since dynein is also important for autophagic degradation of protein aggregates [41], disruption of dynein function might also slow down the autophagic degradation of misfolded SOD1. This would further increase the cellular load of mutant SOD1 and the subsequent sequestration of dynein-dynactin.

In contrast to the interaction with and reduction of dynein-dynactin retrograde transport, we could not find any evidence for an interaction between mutant SOD1 and the members of the kinesin-1 family (KIF5A, 5B, or 5C) in cell culture or in mice spinal cords (Figures 1 and 2). Immunostaining of the neuron specific KIF5A in both spinal cords and sciatic nerves of transgenic ALS mice failed to reveal any co-localization of KIF5A and mutant SOD1 aggregates in motor neurons (Figure 3). Furthermore, we could see no decrease in KIF5A-mediated anterograde transport in 60 days old G93A mice using KIF5A accumulation as a surrogate measurement. This was unexpected since reduced anterograde transport has previously been shown to be an early event in ALS. In fact, a study using an antibody recognizing all three KIF5 subtypes showed reduced proximal accumulation of KIF5 after sciatic nerve ligations in pre-symptomatic low copy version of G93A [21]. This difference could be due to usage of the different G93A lines or different antibodies recognizing all KIF5 subtypes versus the antibody specific for neuronal KIF5A used in our study. Perlson *et al* recently showed that both retrograde and anterograde transport were suppressed in 85 days old G93A mice by measuring accumulation of DIC and kinesin heavy chain at the

sciatic nerve ligation sites [38]. The age of mice used in this study was 60 days, younger than those used in [38]. It is possible that the anterograde transport is not altered in 60 days old mice while impairment of transport becomes detectable in 85 days old mice that are at the verge of developing clinical symptoms. In addition, the antibodies used in the two studies were different, particularly the kinesin antibodies. We used an antibody specific for neuronal isoform KIF5A. It is possible that the anterograde transport mediated by KIF5A motor protein is not changed in G93A mice while the transport mediated by other kinesin family members is changed.

A recent study showed that misfolded SOD1 interacted with the kinesin-2 motor complex via the KAP3 subunit. Furthermore, sequestering of KAP3 by mutant SOD1 was suggested to result in inhibition of ChAT transport [26]. In addition, a genome-wide SNP analysis in a large set of sporadic ALS cases in U.S. and Europe revealed that a variant within the KAP3 gene was associated with decreased KAP3 expression and increased survival in sporadic ALS [27]. The above two independent studies suggest that interference with kinesin-2 might contribute to both familial and sporadic ALS.

Interestingly, we observed a decrease in proximal dynein accumulation 24 hours post-sciatic nerve ligation in G93A mice compared to the WT mice (Figure 4 and 6B). Accumulation of dynein at the proximal side has also previously been shown and is most likely due to kinesin-mediated anterograde transport of dynein as a cargo toward the synapse [21,34,35]. Thus, our data suggest that dynein might be a cargo whose anterograde transport is affected by mutant SOD1 in ALS. Using live imaging of primary DRG neurons isolated from ALS mice, De Vos *et al* examined axonal transport of other cargos and showed that transport of membrane bound organelles was affected in both anterograde and retrograde directions whereas transport of mitochondria was only affected in the anterograde direction [19]. A separate study however showed that axonal transport of mitochondria in differentiated NSC34 cells was affected in both anterograde and retrograde directions in the presence of mutant SOD1 [22,42]. The specific kinesin motor protein(s) transporting these cargos which showed reduced transport was not investigated in those studies. Furthermore the mechanism(s) underlying the decreased anterograde transport by mutant SOD1 is also unclear. It is possible that the reduced transport of these cargos could depend on reduced binding of these cargos to the motors rather than decreased motor transport/activity. In fact, reduced cargo-binding have been suggested in ALS disease (for review see [43]). Studies have suggested that pathogenic proteins implicated in various neurodegenerative diseases can alter kinase signaling cascades and cause disruption in kinesin-mediated fast axonal transport [44–46]. A hypothesis suggesting changes in the p38 signaling in the presence of mutant SOD1 has also been proposed [18].

In our study looking directly at the KIF5A kinesin motor transport, we could not see decrease in anterograde movement of the motor itself. However, there could still be reduced transport of certain KIF5A cargos if cargo attachments where problematic. The observation of the impaired anterograde transport of DHC as a cargo provides an example for such disruptive events. Taken together, the results of this study and previous studies suggest that mutant SOD1 might only interact with and interfere with specific kinesin members which in turn could result in the impairment of a selective subset of cargos. Thus, it is critically important to examine the effect of mutant SOD1 on specific kinesin subtypes as well as different cargos using both *in vitro* live imaging and *in vivo* approaches to further our understanding of axonal transport defects in ALS disease.

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Abbreviations

ALS	amyotrophic lateral sclerosis
SOD	copper-zinc superoxide dismutase
FBS	fetal bovine serum
PBS	phosphate buffered saline
GST	glutathione *S*-transferase
WT	wild type
DHC	dynein heavy chain
DIC	dynein intermediate chain
KHC	kinesin heavy chain
KLC	kinesin light chain

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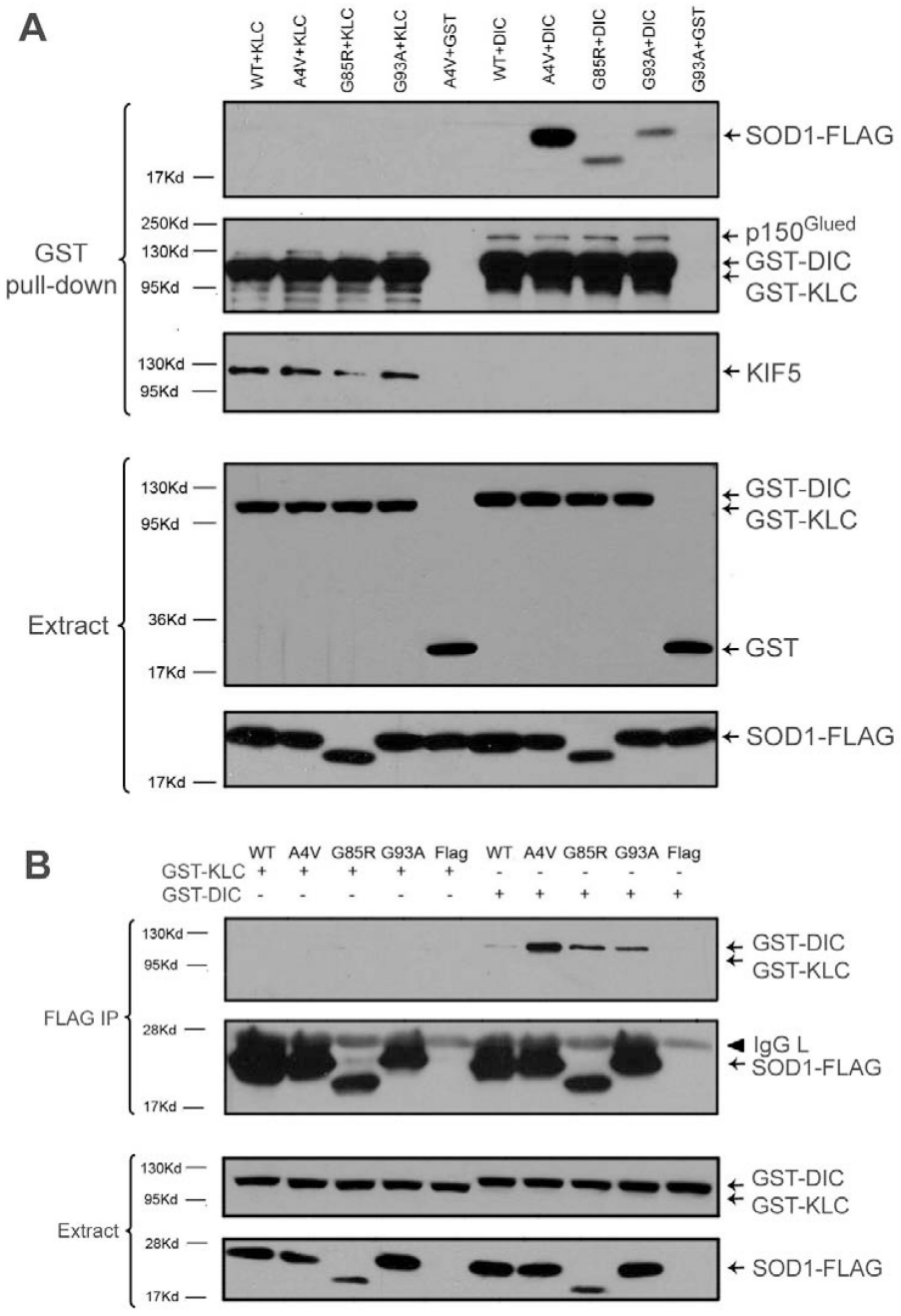


Figure 1.

Mutant SOD1 interacts with dynein-dynactin but not with KIF5-KLC2 in HEK293 cells. **(A)**. HEK293 cells were co-transfected with GST-KLC2 or GST-DIC and SOD1-FLAG (WT, A4V, G85R and G93A). GST pull-down analysis revealed that KIF5 was pulled-down with KLC2-GST, but none of the A4V, G85R nor G93A SOD1 mutant was pulled down with the kinesin complex. In contrast, all SOD1 mutants were pulled down with the dynein complex in GST-DIC pull-downs. Blotting for the dynactin p150^{Glued} subunit showed that the dynactin complex was also co-precipitated with dynein. **(B)**. Reverse FLAG IPs showed that neither GST-KLC2 nor KIF5 was co-precipitated with WT or mutant SOD1. However, GST-DIC was co-precipitated with mutant SOD1 but not with WT SOD1.

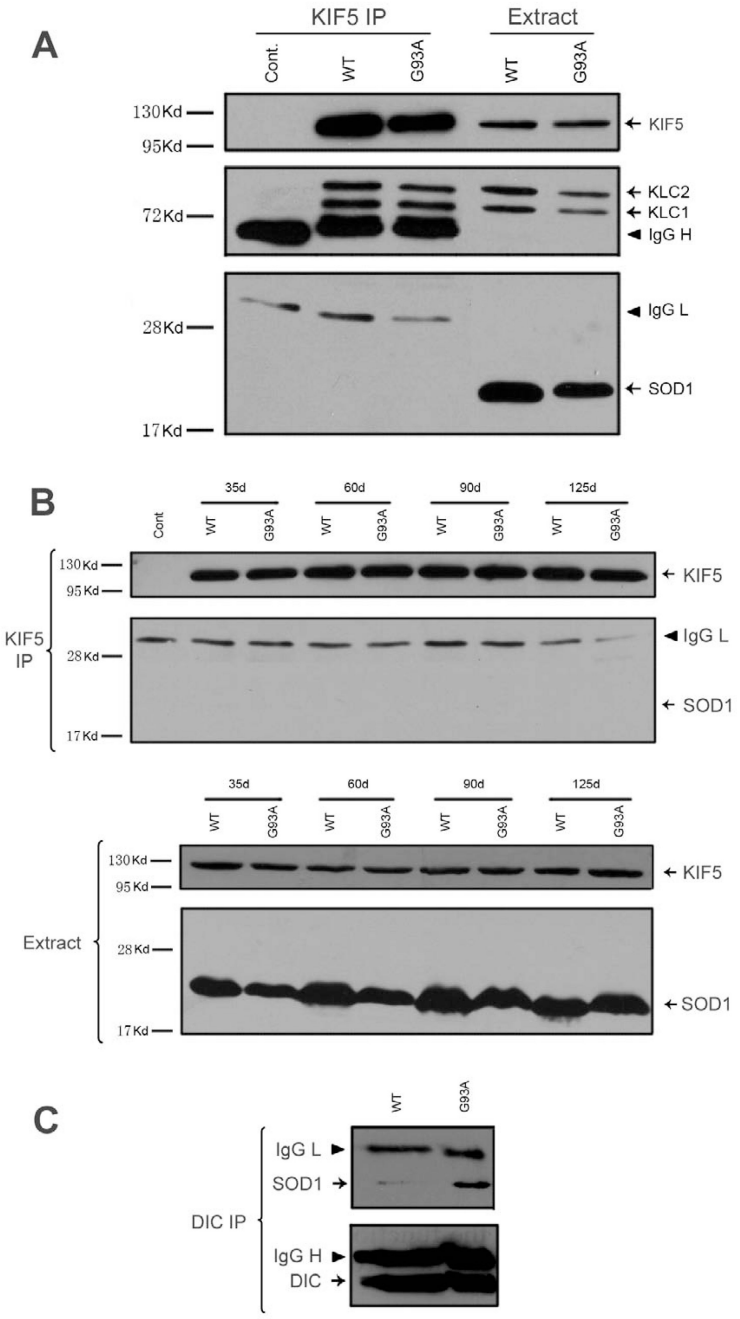
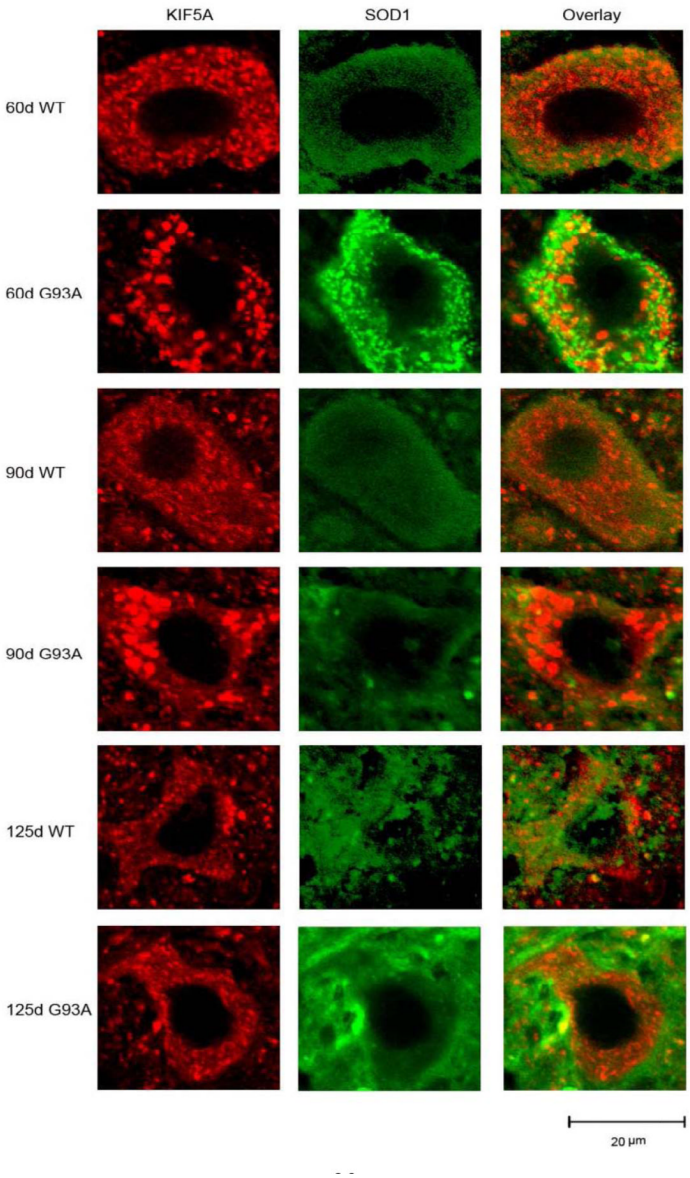


Figure 2.

No detectable interaction between SOD1 and KIF5 motor proteins in G93A ALS mice. **(A)**. All three KIF5 subtypes were immunoprecipitated from spinal cord protein extracts of 125 days old WT and G93A mice using an anti-KIF5 antibody and Protein G Sepharose. Western blotting of IP samples showed that kinesin light chains KLC1 and KLC2 known to form complexes with KIF5s were also pulled down together with the KIF5s. However, neither WT nor G93A SOD1 was co-precipitated with KIF5. **(B)**. KIF5 IPs were performed as described above from spinal cord of ALS mice of different ages (35d, 60d, 90d and 125d). Again, neither WT nor G93A SOD1 could be detected in KIF5 IPs. Input control showed that WT and mutant SOD1 was expressed in all samples. **(C).** DIC

immunoprecipitation showed that mutant SOD1 was co-precipitated with the dynein-dynactin complex while WT SOD1 showed little co-precipitation. The IgG heavy chain and light chain of antibodies used in the IPs are noted.

Figure 3A



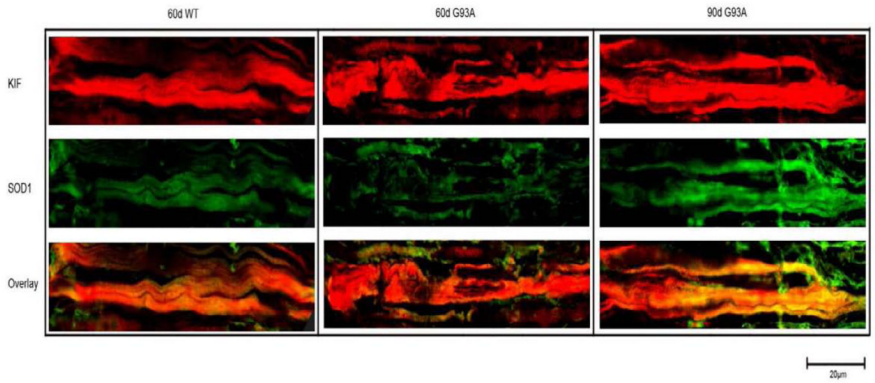


Figure 3.

KIF5A does not co-localize with mutant SOD1 in inclusions. **(A)**. KIF5A and SOD1 localization was investigated using co-immunoflourescence staining of lumbar spinal cord from 60, 90 and 125 days old G93A mice and age-matched controls. A decrease in the total number of surviving motor neurons could be observed in end-stage (125 days old) G93A mice. KIF5A staining revealed robust KIF5A expression in surviving motor neurons and no sign of co-localization of KIF5A with mutant SOD1 aggregates. **(B)**. KIF5A and SOD1 stainings of sciatic nerve sections from 60 and 90 days old G93A mice. No co-localization of KIF5A and mutant SOD1 aggregates could be observed. The scale bars are 20 μm.

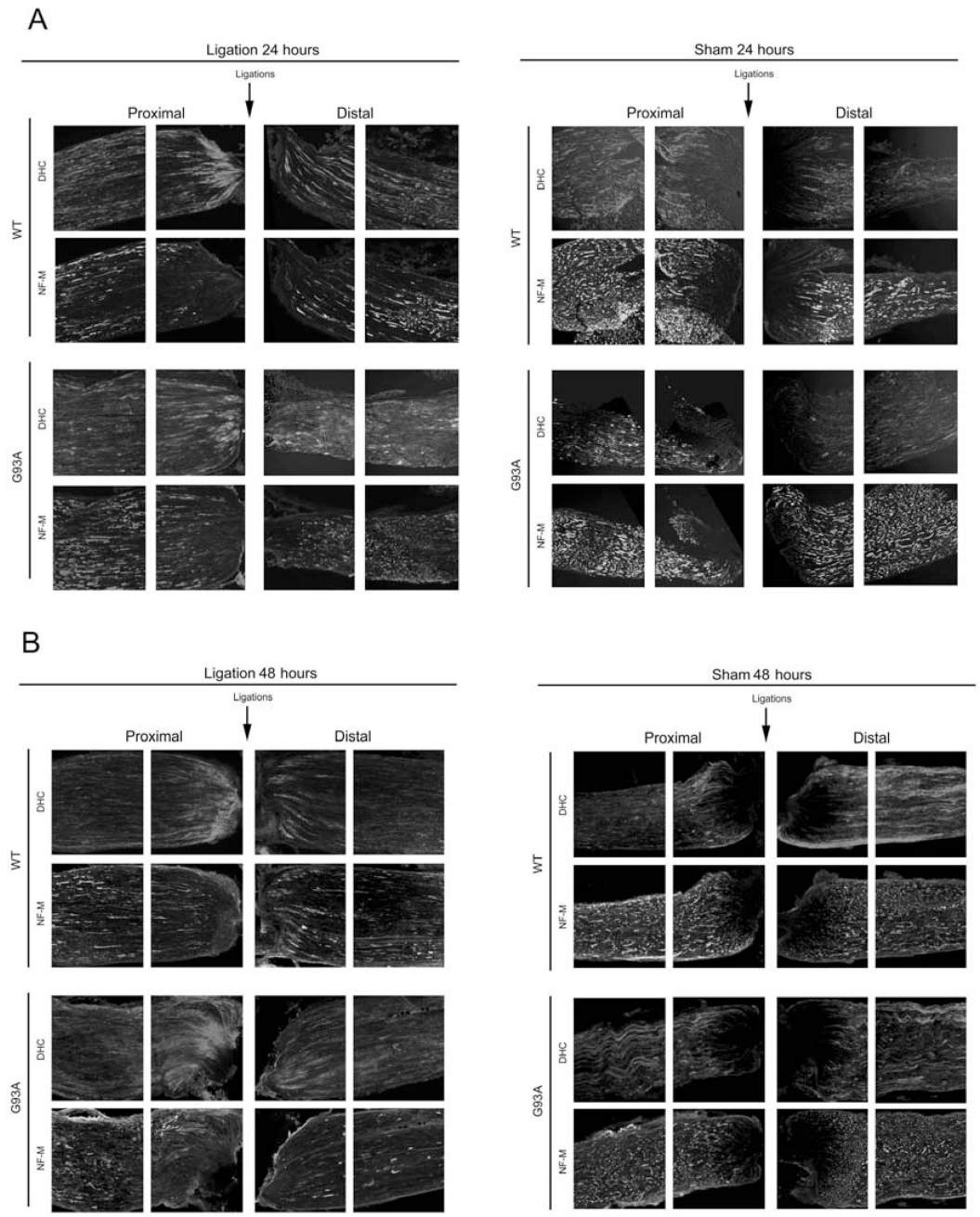


Figure 4.

Representative immunostaining of dynein after sciatic nerve ligation. The left sciatic nerve was exposed and ligated whereas the right nerve was a sham control. Twenty four **(A)** or 48 **(B)** hours after the ligation surgery, the mice were euthanized and the nerve collected, sectioned and stained for dynein using an anti-dynein heavy chain antibody. The nerve was also stained with a neurofilament-M antibody. Dynein stainings revealed accumulation of dynein on both proximal and distal sides of the ligation in WT and G93A mice in the ligated, but not the sham nerve. Compared to WT, reduced accumulation was observed in G93A mice 24 hours after ligation (A). No visible difference in dynein accumulation between WT and G93A mice was observed 48 hours after ligation (B).

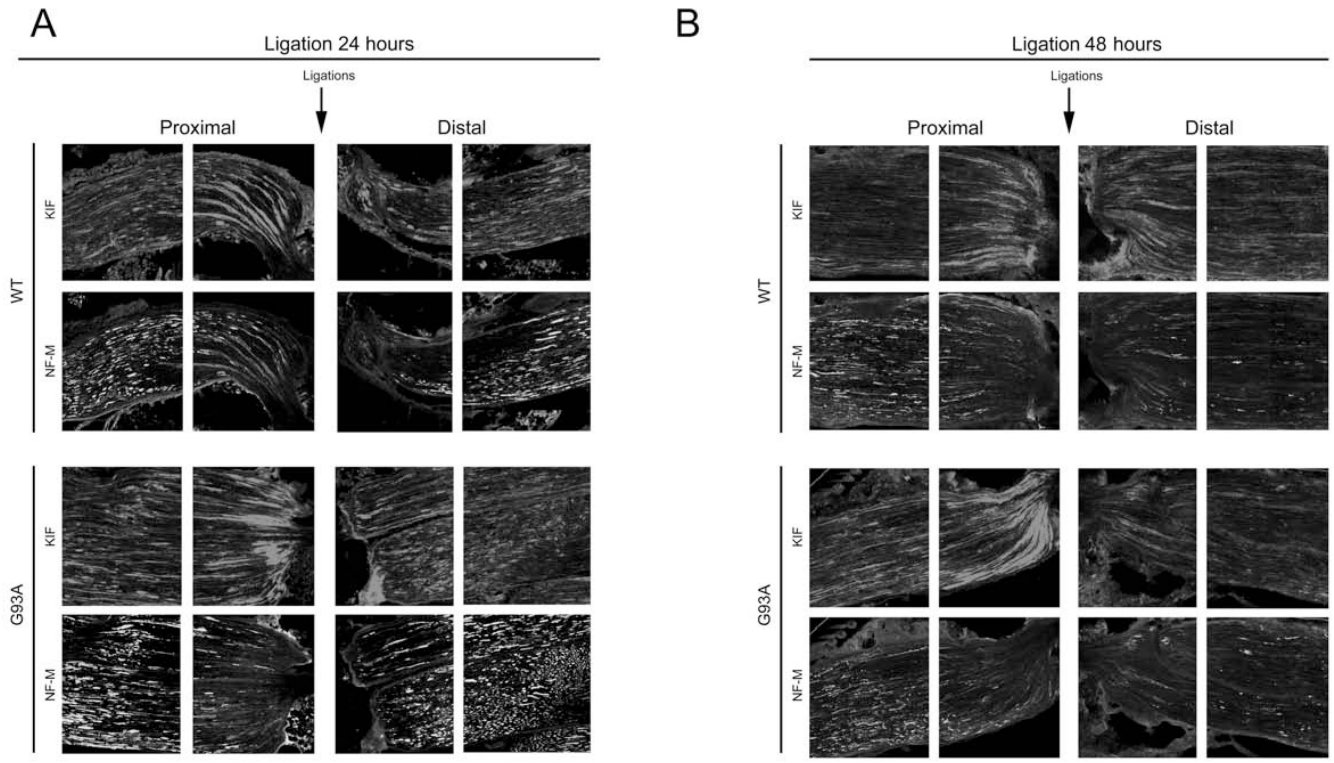


Figure 5.

Immunostainings of KIF5A after sciatic nerve ligations. Nerves were ligated and prepared as in Figure 4 and then stained with a KIF5A specific antibody and a neurofilament-M antibody. KIF5A showed mostly proximal accumulation after ligation for 24 **(A)** or 48 **(B)** hours. No visible difference between WT and G93A could be observed in either panel.

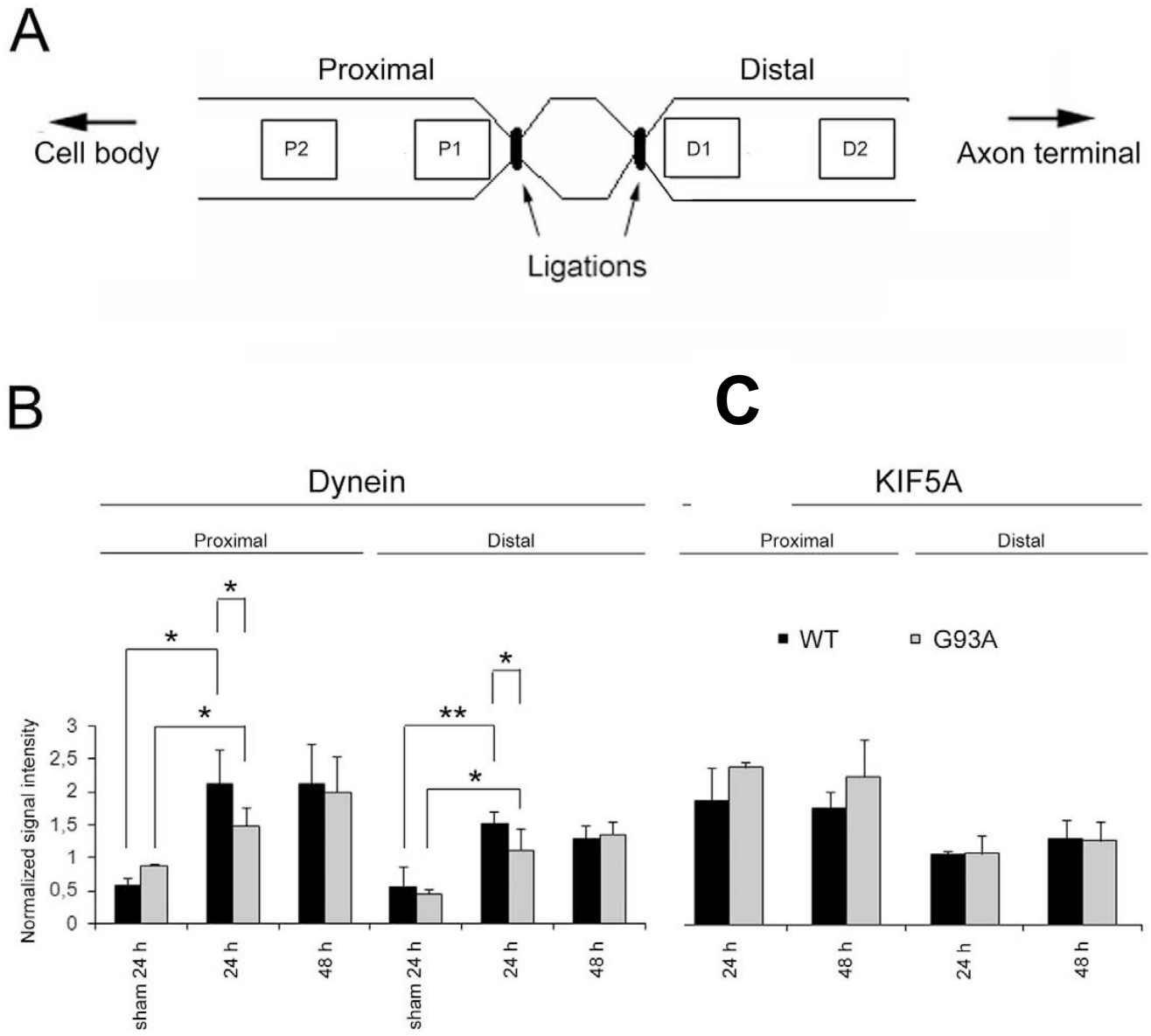


Figure 6.

Quantification of dynein and KIF5A staining after sciatic nerve ligation showed decreased dynein retrograde transport, but no decrease in KIF5A anterograde transport. **(A)**. Schematic drawing of the quantification set up. The average signal intensity for a size defined area (0.0625 mm^2) just adjacent to the ligation site on the proximal (P1) or the distal side (D1) was normalized against the average signal intensity from a similar sized control area on the proximal (P2) or distal side (D2). Longitudinal sections from ligated and sham nerves from 4–6 G93A and 4–6 WT mice were analyzed at each time point. Quantifications of dynein **(B)** and KIF5A **(C)** stainings at the proximal and distal sides of the ligation site. Statistics was performed using two-way ANOVA followed by Turkey test. A statistically significant proximal and distal accumulation of dynein in both WT and G93A mice was observed 24 hours after ligation when compared to the sham control nerve. However, both the proximal and distal accumulation was statistically significantly reduced in G93A mice compared to

WT mice. Forty-eight hours after ligation, there was no longer any difference in dynein accumulation between WT and G93A mice. KIF5A showed predominantly proximal accumulation in both WT and G93A mice and no statisticallt significant difference could be observed between WT and G93A mice at any time point. * $p \leq 0.05$, ** $p \leq 0.005$.