

Reduced protein O-glycosylation in the nervous system of the mutant SOD1 transgenic mouse model of amyotrophic lateral sclerosis

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A B S T R A C T

In the neurodegenerative disease amyotrophic lateral sclerosis (ALS), a number of proteins have been found to be hyperphosphorylated, including neurofilament proteins (NFs). In addition to protein phosphorylation, another important post-translational modification is O-glycosylation with β -N-acetylglucosamine residues (O-GlcNAc) and it has been found that O-GlcNAc can modify proteins competitively with protein phosphorylation, so that increased O-GlcNAc can reduce phosphorylation at specific sites. We evaluated a transgenic mouse model of ALS that overexpresses mutant superoxide dismutase (mSOD) and found that O-GlcNAc immunoreactivity levels are decreased in spinal cord tissue from mSOD mice, compared to controls. This reduction in O-GlcNAc levels is prominent in the motor neurons of spinal cord. We find that inhibition of O-GlcNAcase (OGA), the enzyme catalyzing removal of O-GlcNAc, using the inhibitor NButGT for 3 days, resulted in increased O-GlcNAc levels in spinal cord, both in mSOD and control mice. Furthermore, NButGT increased levels of O-GlcNAc modified NF-medium in spinal cords of control mice, but not in mSOD mice. These observations suggest that the neurodegeneration found in mSOD mice is associated with a reduction of O-GlcNAc levels in neurons, including motor neurons.

Key words:

O-glycosylation
Phosphorylation
O-GlcNAc
Neurofilament
NButGT
Amyotrophic lateral sclerosis

1. Introduction

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease characterized by neuron and motor neuron death in the spinal cord and brain. A number of studies have demonstrated aberrant protein kinase function and elevated levels of many phosphoproteins in tissue from ALS patients and murine models of ALS [11,12]. For example, hyperphosphorylated neurofilament (NF) proteins have been found in ALS tissue and since the phosphorylation state of NFs regulates slow axonal transport [1], it is thought that disorganized and hyperphosphorylated NFs in the neurons of ALS patients impairs axonal transport and thereby contributes to NF-induced pathology in ALS [26].

Recently, it has been shown that many proteins, including NFs can also be post-translationally modified by a form of

O-glycosylation, O-linked β -N-acetylglucosamine, known as O-GlcNAc [24]. This modification occurs on amino acid residues that are also known to be phosphorylated in some proteins [4,24] and conversely increased phosphorylation of specific proteins results in decreased O-GlcNAc levels, and increased O-GlcNAc levels correlate with decreased protein phosphorylation [24]. The reciprocal relationship between O-GlcNAcylation and phosphorylation has been termed the Yin–Yang hypothesis [24], and has gained support from the recent discovery that the enzyme installing the O-GlcNAc residue, termed O-GlcNAc transferase (OGT) [14], may form a functional complex with certain protein phosphatases [25]. Like phosphorylation, O-GlcNAcylation is a dynamic modification that can be removed and installed several times during the lifespan of a protein [21]. The enzyme catalyzing the removal of O-GlcNAc from modified proteins is a β -N-acetylglucosaminidase known as O-GlcNAcase (OGA) [9]. A potent and highly selective small molecule inhibitor of OGA, termed NButGT, has been described and shown to increase global levels of O-GlcNAc modified proteins in a wide range of tissues including those of the central nervous system (CNS) of rodents [16–18].

Phosphorylation of the NF-medium subunit (NF-M) is regulated by O-GlcNAc modification both in vitro and in vivo and it has been proposed that O-GlcNAc and phosphorylation levels on NF-M are reciprocal, and O-GlcNAc-modified NF-M levels are markedly

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decreased in spinal cords of rats having an ALS-like disorder, while phosphorylation of NF-M is increased [15]. However, it is unknown where O-GlcNAc modified proteins are distributed and whether levels of O-GlcNAc modified proteins are altered in motor neurons in ALS.

About 20% of patients with familial ALS have mutations in the Cu/Zn superoxide dismutase (*SOD1*) gene (mSOD) and transgenic mice that over-express mSOD (G93A) develop a disorder resembling ALS in humans and are commonly used as a model for the study of ALS [10]. In G93A mSOD mice, aberrant accumulation of hyperphosphorylated NFs occurs in spinal motor neurons. Depletion of the heavily phosphorylated side-arms of NF-M and the heavy subunit of NF (NF-H), increases survival of motor neurons in G37R mSOD mice [13]. Accordingly, mechanisms that can regulate the phosphorylation of NF or other proteins are of interest since it may be possible to exploit them to intervene in the progression of disease.

In this work, we describe the distribution of O-GlcNAc modified proteins in spinal cords of G93A mSOD mice and controls, and explore whether modulation of O-GlcNAc using small molecule inhibitors of OGA will alter levels of O-GlcNAc modified proteins and NF phosphorylation.

2. Methods

Transgenic mice expressing human G93A mutant SOD1 were purchased from Jackson Laboratories (B6.Cg-Tg(SOD1-G93A)1Gur/J) or bred locally. Wild-type (wt) littermates were used as controls in these studies. Protocols governing the use of animals were approved by the Animal Care Review Committee of Simon Fraser University.

Western blot, immunocytochemistry and densitometric analysis were performed as described previously [22] (detailed description in [Supplementary Methods](#)). The CTD110.6 anti-O-GlcNAc and the SMI31 anti-phosphorylated NF-H and NF-M were obtained from Covance; whereas the NL6 anti-O-glycosylated NF-M was obtained from Sigma; the anti-NeuN and anti-CNPase were obtained from Chemicon; the anti-GFAP and anti-Iba1 were obtained from Calbiochem and Wako, respectively. The NF-09 against total NF-M was obtained from Santa Cruz. Images were collected using a Leica fluorescence microscope (DM4000B). In control experiments, sections were performed in parallel but without primary antibody. When pre-treating spinal cord sections, transverse slices were incubated with either wtOGA [3,8] at 105 $\mu\text{g/ml}$ or mutant OGA [3,8] at 86 $\mu\text{g/ml}$ for 3 h in a 25 °C water bath, rinsed, and immunostained.

Motor neuron counts were performed using modified methods previously used by others [26]. Motor neurons were defined and counted as: (1) size >25 μm ; (2) possession of at least one thick process; (3) location in ventral gray matter regions below a horizontal level through the center of central canal. Data were collected by averaging five sections per animal and a total of 30 sections were counted.

A selective OGA inhibitor, 1,2-dideoxy-2'-propyl- α -D-glucopyranoside-[2,1-d]- Δ 2'-thiazoline (NButGT) [17], was used to treat mSOD with advanced disease and wt mice. mSOD and wt mice were fed chow with or without NButGT (100 mg/kg/day) for three days ($n=6$ for each group).

Results were expressed as mean \pm SD. A p -value of less than 0.05 was considered significant.

3. Results

Immunoreactivity against O-GlcNAc-modified proteins was evaluated in transverse sections of spinal cord from wt mice

using CTD110.6 antibody that detects O-GlcNAc modified residues on many different proteins [5,8]. As shown in [Fig. 1A](#), O-GlcNAc immunolabeling was evident in cells of the spinal cord, largely within the gray matter and in both dorsal and ventral horns. To confirm that the observed pattern of O-GlcNAc immunoreactivity was specific for O-GlcNAc modified proteins, sections were treated with a bacterial β -N-acetylglucosaminidase having OGA activity (*Bacteroides thetaiotamicron* GH84, BtGH84) in order to cleave this moiety off from modified proteins [3,8]. As shown in [Fig. 1B](#), following treatment with wtOGA, immunoreactivity was absent, confirming that CTD110.6 antibody detects only O-GlcNAc modified proteins. When sections were treated in an identical manner using an inactive mutant of BtGH84 (D174A, D175A) without enzymatic activity [3], O-GlcNAc immunoreactivity was essentially identical to that seen in control tissues ([Fig. 1C](#)). Under high magnification, O-GlcNAc immunoreactivity was observed in large motor neurons, interneurons, and other cells ([Fig. 1A](#) and [D](#)), generally in the perinuclear region, but also within the cytoplasm and nucleus ([1D](#)). Immunoreactivity against O-GlcNAc was also seen in oligodendroglia, as demonstrated by CNPase, largely within the nucleus ([Fig. 1E](#)), as well as in astrocytes ([Fig. 1F](#)). Immunoreactivity against O-GlcNAc was not seen in microglia.

To compare the levels of immunoreactivity of O-GlcNAc modified proteins in affected and non affected mice we performed densitometry on sections from the same lower lumbar spinal cord region from end stage mSOD ($n=3$) and wt mice ($n=3$). As shown in [Fig. 1G](#), densitometry of O-GlcNAc immunoreactivities in ventral horn regions of spinal cord demonstrated that O-GlcNAc levels were significantly lower ($p < 0.001$) in mSOD than control spinal cord. As neurons have O-GlcNAc immunoreactivity in spinal cord, the decreased O-GlcNAc immunoreactivity in end stage mSOD spinal cord could stem from a specific reduction in the levels of O-GlcNAc protein modification in neurons or from a reduction in the number of motor neurons. Thus, we conducted densitometry of O-GlcNAc immunoreactivity of individual motor neurons and counted motor neurons in both end stage mSOD mice and controls. We found significantly decreased O-GlcNAc immunoreactivity of individual motor neurons from mSOD mice compared to controls ([Fig. 1H](#)), and, as expected, mSOD mice had significantly lower numbers of remaining motor neurons than the control mice ($p < 0.001$), both factors likely accounting for some of the reduction of O-GlcNAc immunoreactivity ([Fig. 1I](#)).

To evaluate the levels of O-GlcNAc modified proteins in spinal cord tissue from the same lower lumbar region from wt mice ($n=9$) and mice over-expressing mSOD ($n=9$) at early stages (no deficit), mice with mild neurological deficits, and with severe deficits, immunoblotting was performed. O-GlcNAc immunoreactivity was evident for a large number of protein bands in the western blot, both in wt and mSOD tissue ([Fig. 2A](#), left lanes). However, no significant difference in total O-GlcNAc levels was seen as measured by integrating total band densities for both mSOD and wt tissues ([Fig. 2A](#), lower panel, and data not shown).

In an effort to modulate levels of O-GlcNAc modified proteins in spinal cord we treated mSOD ($n=6$) and control mice ($n=6$) with a specific inhibitor of OGA (NButGT) for 3 days. As shown in [Fig. 2B](#), O-GlcNAc immunoreactivity in NButGT treated mice was increased in spinal cord sections as compared to tissue from non-treated mice in both gray and white matter of spinal cord. Enhanced O-GlcNAc immunoreactivity was found in the nuclei of astrocytes and oligodendroglia, and in the cytoplasm and nuclei of neurons, including surviving motoneurons from mSOD mice ([Fig. 2B](#)). [Fig. 2A](#) shows western blot data indicating that levels of O-GlcNAc modified proteins were significantly increased ($p < 0.05$) both in wt and mSOD spinal cord tissue in the NButGT treated groups, as compared to non-treated animals ([Fig. 2A](#), right lanes, and lower

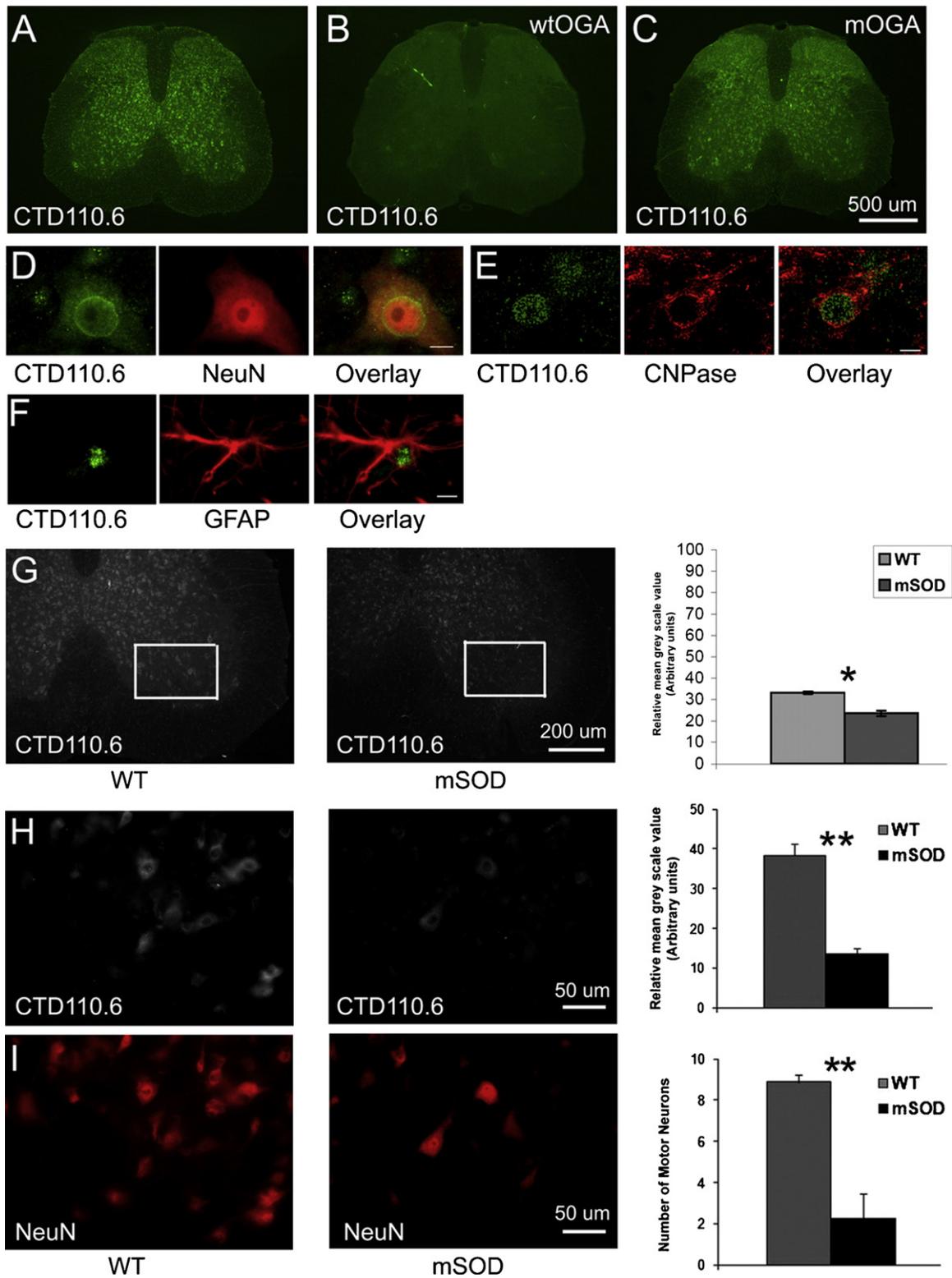


Fig. 1. Distribution and levels of O-GlcNAc modified proteins in spinal cord. Immunoreactivity of O-GlcNAc modified proteins in transverse sections of lumbar spinal cord from wild type (wt) mice detected using CTD110.6 antibody in the absence of O-GlcNAcase (OGA) (A); in the presence of functional OGA (wtOGA) (B); and in the presence of mutant, non-functional OGA (mOGA) (C). Immunofluorescence is observed in cells largely in gray matter and in both ventral and dorsal horns (A, C, and D); immunostaining is absent after wtOGA treatment (B). Immunolabeling of O-GlcNAc (green) in ventral horns of lumbar spinal cord sections from wt mice localizes to cells exhibiting the neuronal marker, NeuN (red) (D); the oligodendrocyte marker CNPase (red) (E); the astrocyte marker GFAP (red) (F). (G) Densitometric analysis of O-GlcNAc immunoreactivity in ventral horn regions of spinal cords shows that immunoreactivity is significantly lower in mSOD than wt ($*p < 0.01$). (H) Densitometric analysis of O-GlcNAc immunoreactivity in motor neurons shows that immunoreactivity is significantly lower in mSOD than wt ($**p < 0.001$). (I) Motor neuron counts show significantly lower survival of NeuN+ (red) neurons in mSOD than wt ($**p < 0.001$). Scale bars: (D) 15 μ m; (E and F) 5 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

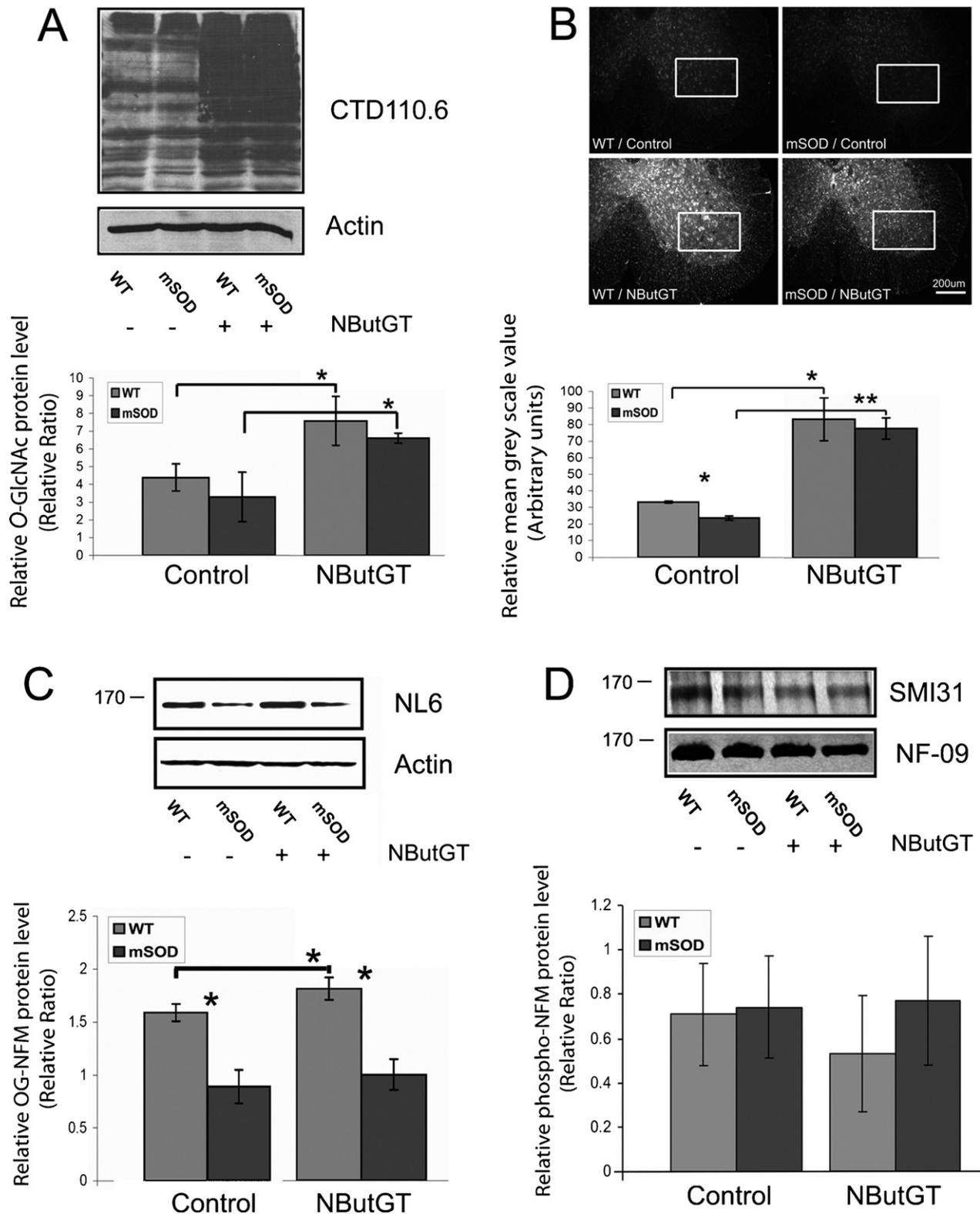


Fig. 2. NButGT significantly enhances O-GlcNAc level in spinal cords. mSOD and wt mice were treated orally with a specific inhibitor of OGA (NButGT) for 3 days. (A) Using CTD110.6 antibody, western blotting shows that levels of O-GlcNAc modified proteins were significantly increased both in wt and mSOD in the NButGT treated groups, compared with non-treated animals ($p < 0.05$). There is no significant difference in total O-GlcNAc band densities between wt and mSOD spinal cords. Values are expressed as a ratio of CTD110.6 versus Actin. (B) This observation is evaluated further by densitometric analysis of O-GlcNAc immunoreactivity in the ventral horn regions of spinal cord ($*p < 0.01$, $**p < 0.001$, respectively). (C) Western blotting shows levels of OG-NF-M proteins in spinal cord of mSOD and wt mice from NButGT treated and non-treated groups using NL6 antibody. Levels of OG-NF-M proteins are significantly reduced ($*p < 0.01$) in mSOD spinal cord in both NButGT treated and non-treated groups, compared to wt mice. There is a significantly increase in OG-NF-M protein in wt spinal cord in the NButGT treated group, compared with non-treated (control) mice ($*p < 0.05$). Values are expressed as a ratio of NL6 versus Actin. (D) Western blotting shows levels of phosphorylation of NF-M proteins from spinal cords using SMI31 antibody. Levels were quantified by densitometry and normalized to total NF-M level (NF-09 immunoreactivity), are not significantly different ($p > 0.05$) between wt and mSOD tissue in either NButGT treated or non-treated (control) groups. Values are expressed as a ratio of SMI31 versus NF-09.

panel). There was no statistically significant difference in total *O*-GlcNAc band densities by western blot between wt and mSOD after NButGT treatment (Fig. 2A). *O*-GlcNAc immunoreactivity was evaluated by densitometric analysis in ventral horn regions of spinal cord where wt spinal cord exhibited significantly higher ($p < 0.01$) immunoreactivity as compared to mSOD spinal cord (Fig. 2B). NButGT treatment significantly increased both wt and mSOD *O*-GlcNAc immunoreactivities ($p < 0.01$ and $p < 0.001$, respectively) as measured using immunohistochemistry (Fig. 2B).

Spinal cord homogenates were probed with the antibody (NL6), which recognizes an *O*-GlcNAc modified NF-M epitope (OG-NF-M) [15]. As seen in Fig. 2C, western blot revealed that levels of OG-NF-M proteins were significantly reduced ($p < 0.01$) in mSOD mice versus wt mice in both NButGT treated and non-treated groups. We also found that levels of OG-NF-M proteins were significantly increased ($p < 0.05$) in wt spinal cord tissues in the NButGT treated groups, as compared to tissue from non-treated animals. There was, however, no significant difference in OG-NF-M band densities in mSOD spinal cord tissue arising from treatment with NButGT ($p > 0.05$). As we observed that NButGT could modulate global *O*-GlcNAc and specific OG-NF-M levels, we further examined phospho-NF-M levels in these tissues using SMI31 antibody, which is known to recognize phosphorylated epitopes within the repeating KSP motifs of NF-H and NF-M. As shown in Fig. 2D, levels of phospho-NF-M in spinal cord tissues did not show any statistically significant differences between wt or mSOD mice, either with or without NButGT treatment, after densitometry was corrected by normalization using total NF-M levels.

4. Discussion

Previous studies have demonstrated that when compared to appropriate controls, CNS tissues from ALS patients, and murine models of ALS, have increased levels of protein kinases and phosphoproteins [11,12,19]. Notably, increased immunoreactivity of the highly phosphorylated isoform of NF-H has been reported and it has been postulated that NF-H acts as a sink for phosphorylation by hyperactive cyclin-dependant kinase-5 (CDK-5) to hinder uncontrolled phosphorylation of proteins having critical cellular functions [19,23]. However, extensive hyperphosphorylation of the KSP-repeat domain of NFs has also been implicated in progression of symptoms of ALS [23]. Interestingly, the microtubule-associated protein, tau, has also been found to be hyperphosphorylated in ALS tissue [27]. Since there appears to be a reciprocal relationship between *O*-GlcNAc and phosphate on some proteins including tau [7,20,28] we were intrigued by the possible reciprocal relationship between *O*-GlcNAc levels and phosphorylation of NFs. If phosphorylation and *O*-GlcNAc are generally reciprocal then we might observe that *O*-GlcNAc levels in a mouse model of ALS would be significantly decreased. Furthermore, if this holds true for NFs in addition to other proteins, pharmacological elevation of *O*-GlcNAc levels might be achieved by blocking the action of OGA, the enzyme responsible for removing this modification, which could reduce the hyperphosphorylation of NFs in this model.

To evaluate this possibility, we investigated the distribution of *O*-GlcNAc modified proteins in CNS and whether *O*-GlcNAc levels were decreased in G93A mSOD mice. Using an antibody that recognizes many *O*-GlcNAc modified proteins, we found that *O*-GlcNAc is particularly abundant in neurons, but is also found in oligodendrocytes and astrocytes. We did not detect *O*-GlcNAc immunoreactivity in microglia. In both oligodendrocytes and astrocytes, *O*-GlcNAc immunoreactivity was most evident in the nuclei, whereas in neurons *O*-GlcNAc immunoreactivity was found at similar levels in both the nucleus and cytoplasm, but most notably in the perinuclear region, as described previously in human astrocytes,

mouse cerebellar neurons, and mouse brain tissue [2,17,18,20]. Using immunocytochemistry with densitometry, we found that *O*-GlcNAc levels are significantly decreased in spinal cord tissue from mSOD mice as compared to controls. Furthermore, in the mSOD mice we observed lower *O*-GlcNAc levels in the remaining neurons, as well as motor neuron loss, which likely lead to decreased overall *O*-GlcNAc levels at disease end stage.

Having established that *O*-GlcNAc levels were decreased in symptomatic G93A mSOD mice and because motor neurons had significantly lower *O*-GlcNAc protein modification, we investigated whether we could modulate *O*-GlcNAc levels using a small molecule inhibitor of OGA. While PUGNAc has been widely used as an inhibitor of OGA to modulate *O*-GlcNAc in cells and tissues it is known that this compound also inhibits lysosomal β -hexosaminidases, the enzymes that cleave both GlcNAc and GalNAc from many glycosylated proteins [17]. Furthermore, PUGNAc does not increase *O*-GlcNAc levels in the CNS of treated animals [29]. Therefore, we used NButGT, a potent ($K_i = 230$ nM) and highly selective (1200-fold selectivity) small molecule inhibitor of OGA, which has been shown to increase global levels of *O*-GlcNAc modified proteins in various tissues [16,17]. We found that a three-day oral treatment with NButGT significantly increased *O*-GlcNAc levels, as measured both by western blot and immunohistochemistry. Interestingly, the treatment increased *O*-GlcNAc levels in spinal cord to similar levels, both in mSOD and control mice, when compared to untreated mSOD and control animals, respectively. This data suggests that in addition to neuronal loss, some of the reduction in *O*-GlcNAc levels in mSOD spinal cord is likely due to decreased *O*-GlcNAc modification within surviving neurons and other cells, an observation consistent with the known activation of protein kinases and increased phosphorylation of neuronal proteins in this animal model [15]. These observations were supported by immunohistochemical analysis of *O*-GlcNAc levels that showed significantly enhanced *O*-GlcNAc levels in neurons, astrocytes, and oligodendroglia in both mSOD and wt spinal cord tissue following NButGT treatment. NButGT is therefore able to modulate *O*-GlcNAc effectively in this mouse model of ALS.

To test whether inhibitor treatment was modulating *O*-GlcNAc levels on NFs, we used an antibody (NL6) that has been raised against rat *O*-GlcNAc modified NF-M [15]. As outlined above, there is considerable evidence for hyperphosphorylated NFs playing a role in the progression of ALS in humans [15]. Recent studies have shown reductions in *O*-GlcNAc modified NF-M in a rat model of ALS over-expressing mSOD [15]. Here, we observed significant reductions of NL6 immunoreactivity in mSOD versus wt mice both in NButGT treated and non-treated groups by using western blot. Our western blot results corroborate the findings reported by Ludemann et al. [15]. Interestingly, use of NButGT significantly increased *O*-GlcNAc modified NF-M in wt but not mSOD mice as determined using the NL6 antibody. Given these observations, we examined the effects of short term (three days) NButGT treatment on phosphorylation of the KSP repeats of NF-M using the SMI31 antibody, which recognizes the phosphorylated KSP epitope. Given that it has been proposed that the KSP repeat region of NF-M contains several *O*-GlcNAc modification sites, we anticipated that treatment might decrease phosphorylation of this region. However, western blot analysis did not revealed a significant decrease in the mean SMI31 immunoreactivity. No difference in mean SMI31 immunoreactivity was observed between treated and untreated mSOD mice. We speculate that this lack of difference in SMI31 immunoreactivity in mSOD mice may arise from the aggregation of hyperphosphorylated NFs in these severely affected animals [6], which may prevent dephosphorylation and subsequent *O*-GlcNAc modification. Overall, however, these results indicate that *O*-GlcNAc levels can be modulated *in vivo* to increase levels of *O*-GlcNAc on NFs. To address whether elevated *O*-GlcNAc levels induced by NButGT or other OGA

inhibitors might alter hyperphosphorylation of NFs, further long term treatment studies monitoring progression of symptoms in disease models are required to clarify this possibility.

Acknowledgments

The authors would like to thank the staff at the Animal Care Facility at Simon Fraser University for their assistance. We also thank the Canadian Institutes for Health Research (CIHR) and the Scottish Rite Charitable Foundation (SRCF) for providing funding for this work. X.S. is a recipient of a doctoral research scholarship from the CIHR and the ALS Society of Canada, and also holds a scholarship from the Michael Smith Foundation for Health Research (MSFHR). D.J.V. is a scholar of the MSFHR, a Canada Research Chair Tier II in Chemical Glycobiology and an E.W.R. Stacie Memorial Fellow.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neulet.2012.04.018>.

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