Wnt and Extraocular Muscle Sparing in Amyotrophic Lateral Sclerosis

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PURPOSE. The extraocular muscles (EOM) and their motor neurons are spared in amyotrophic lateral sclerosis (ALS). In limb muscle, axon retraction from the neuromuscular junctions occurs early in the disease. Wnts, a conserved family of secreted signaling molecules, play a critical role in neuromuscular junction formation. This is the first study to examine Wnt signaling for its potential involvement in maintenance of normal morphology in EOM in ALS.

METHODS. Extraocular muscle and limb muscle axons, neuromuscular junctions, and myofibers from control, aging, and ALS subjects and the SOD1^{G93A} mouse model of ALS were quantified for their expression of Wnt1, Wnt3a, Wnt5a, Wnt7a, and β -catenin.

RESULTS. All four Wnt isoforms were expressed in most axon profiles in all human EOM. Significantly fewer were positive for Wnt1, Wnt3a, and Wnt7a in the human limb muscles. Similar differential patterns in Wnt myofiber expression were also seen except in the case of Wnt7a, where expression was elevated. In the SOD1^{G93A} mouse, all four Wnt isoforms were significantly decreased in the neuromuscular junctions at the terminal stage compared to values in age-matched controls. β -Catenin was activated in a subset of myofibers in EOM and limb muscle in all subjects.

CONCLUSIONS. The differences in expression of Wnts in EOM and limb muscle, particularly at the neuromuscular junction level, suggest that they play a role in the pathophysiology of ALS. Collectively, the data support a role for signaling of Wnts in the preservation of the EOM in ALS and their dysregulation and the subsequent development of pathology in the ALS limb muscles.

Keywords: extraocular muscles, Wnt, neuromuscular junctions, amyotrophic lateral sclerosis, beta-catenin, skeletal muscle, SOD1^{G93A} mice

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disease characterized primarily by loss of both upper and lower motor neurons, as well as sequential axon retraction from neuromuscular junctions.^{1–3} Amyotrophic lateral sclerosis is clinically and pathologically heterogeneous. The biological basis of the variation in age of onset, rate of progression, and site of involvement is poorly understood. However, a hallmark of all variants of ALS is the relative sparing of the EOM.^{4–6} In routine histochemistry, the majority of ALS patients have histopathologically normal cranial nerve nuclei III, IV, and VI,⁷ as well as normal eye movements when tested in the clinic. While some abnormalities in eye movements have been described, they are considered to be caused by supranuclear deficits.⁸ Approximately 10% of ALS patients survive more than 10 years, and even in these individuals, the extraocular muscles (EOM) frequently remain clinically unaffected. This contrasts sharply to the extreme wasting of the limb muscles as well as the muscles innervated by the trigeminal, facial, glossopharyngeal, vagus, accessory, and hypoglossal motor nuclei. Elucidating the cause of the relative sparing of the EOM is a key issue in our understanding of the

pathogenesis of the ALS syndrome and has the potential of opening up new avenues for therapeutic intervention.

The first structural change seen in the limb skeletal muscles of ALS patients is denervated motor endplates within affected muscles, occurring significantly before loss of alpha-motor neurons in both human ALS muscle and in the SOD1 transgenic mouse models of ALS.⁹ We recently showed that the EOM from patients with ALS had some morphological alterations compared to normal EOM, but were remarkably well preserved compared to the limb muscles from the same ALS subjects.^{10,11} The neuromuscular junctions in the human EOM maintained their nerve contacts and had a normal composition with respect to laminins, synaptophysin, and the p75 neurotrophin receptor, whereas the neuromuscular junctions in limb muscles of the same patients were severely affected.¹² Additional abnormalities associated with neuromuscular junctions in the limb muscles of the SOD1^{G93A} transgenic mouse model of ALS included fragmentation of the postsynaptic membrane, decreased density of acetylcholine receptors, and lack of nerve sprouting in denervated junctions, while the EOM neuromuscular junctions in this mouse model of ALS were spared.^{13,14} These studies

TABLE. Characteristics of ALS Subjects

Subject	Sex	Age at Death, y	Symptom Duration, mo	Diagnosis	SOD1 Genotype	Site of First Symptom
1	Male	80	31	SALS	wt/wt	Right hand
2	Male	75	321	FALS	D90A/D90A	Left leg
3	Female	64	134	FALS	D90A/D90A	Left leg
4	Male	66	13	SPBP	wt/wt	Bulbar onset
5	Female	58	35	SPBP	wt/wt	Bulbar onset
6	Male	71	17	SALS	wt/wt	Right hand

SALS, sporadic amyotrophic lateral sclerosis; FALS, familial amyotrophic lateral sclerosis; SPBP, sporadic progressive bulbar palsy; wt/wt, no known genetic mutation.

confirm the increased resistance of the EOM to the pathophysiological changes associated with limb muscle in ALS.

Wnt proteins are a family of conserved, secreted signaling molecules that play a role in neuromuscular development and regeneration.¹⁵ Several Wnt proteins are highly expressed in skeletal muscle, at the neuromuscular junction, and in motor neurons.^{16,17} These include Wnt1,^{16,18} Wnt3a,¹⁹ Wnt5a,^{16,20} and Wnt7a.^{21,22} Additionally, alterations in Wnt signaling have been implicated in neuromuscular and neurological diseases, including muscular dystrophy,²³ limb-girdle muscular dystrophy 2A,²⁴ Alzheimer's disease,²⁵ and ALS.^{26,27} These studies suggest that alterations in Wnt expression in motor nerves, neuromuscular junctions, and/or muscle fibers may play a role in the pathophysiological processes of ALS.

To gain further insight into changes in expression of members of the Wnt signaling pathway in ALS, the motor nerves, neuromuscular junctions, and myofibers were examined for the expression of Wnt1, Wnt3a, Wnt5a, and Wnt7a in muscle specimens from subjects with different genetic subtypes of ALS and in the SOD1^{G93A} transgenic mouse model of ALS. As Wnts are secreted factors that can activate signaling cascades in both the pre- and postsynaptic compartments, it is important to understand the potential source of the Wnt molecules that are altered in ALS.¹⁵ In addition, the expression of β -catenin was examined to determine whether these Wnts act through the canonical Wnt signaling pathway.²⁸

MATERIALS AND METHODS

All human muscle samples were collected at autopsy with the approval of the Research Ethical Committee of Umeå University and the Regional Ethical Review Board in Umeå, section for Medical Research, adhering to the principles of the Declaration of Helsinki. The animal study was conducted according to national and international guidelines, and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experiments and animal handling were approved by the Ethical Committee of the Medical Faculty, Umeå University, and were carried out in accordance with the European Communities' Council Directive (86/609/EEC).

Human Subjects

Extraocular muscles and samples of biceps brachii, vastus lateralis, and tibialis anterior muscles were collected from six subjects who had been diagnosed with ALS in accordance with the European Federation of Neurological Societies consensus criteria for ALS.²⁹ Detailed information about ALS subjects is given in the Table. Age-matched control muscles were obtained at autopsy from subjects with no known neuromuscular disease. Normal EOM samples from four control subjects with mean age of 41 years (ranging from 34 to 47 years) are referred to as "adult," and those from four control subjects with mean

age of 75 years (ranging from 71 to 81 years) are referred to as "elderly." Normal limb, trunk, neck, and lumbrical muscles were collected from five adults (mean age 33 years, ranging from 17 to 55 years) and from four elderly adults (mean age 76 years, ranging from 69 to 82 years).

All tissues were mounted, rapidly frozen in propane chilled with liquid nitrogen, and stored at -80°C until processed. Serial cross sections, 5 μm thick, were prepared in a cryostat (Reichert Jung; Leica, Nussloch, Germany).

Mouse Samples

The EOM and hind limb muscles from SOD1^{G93A} mice at presymptomatic (~ 50 days, $n = 4$) and terminal stages (~ 150 days, $n = 4$) were collected directly after the animals were euthanized with an intraperitoneal injection of pentobarbital and processed as above. Age-matched C57BL/6 mice served as controls ($n = 3$ for the presymptomatic group; $n = 4$ for the terminal group).

Antibodies and Immunofluorescence

Sections were processed for immunohistochemical localization of one of the following polyclonal antibodies: Wnt1, Wnt3a, Wnt5a, or Wnt7a (1:500; Abcam, Cambridge, UK). In order to localize Wnt expression within nerves, sections were colabeled with antibodies against 70 kD neurofilament (1:500, clone NR4; DAKO, Glostrup, Denmark) and laminin (1:30,000, PC128; The Binding Site Ltd., Birmingham, UK). In order to localize Wnt expression within neuromuscular junctions, sections were colabeled with rhodamine-conjugated α -bungarotoxin (α -BTx) (Molecular Probes, Inc., Eugene, OR, USA). In addition, immunostaining for Wnts as above, β -catenin (1:300; Abcam), and dystrophin (GTx15277; GeneTex, Inc., Irvine, CA, USA) was performed in consecutive sections.

Immunohistochemistry was performed on air-dried serial consecutive tissue sections rehydrated in 0.01 M PBS, and then immersed in 5% normal donkey serum (Dakopatts, Glostrup, Denmark) for 15 minutes. Sections were then incubated with the appropriate primary antibody at 4°C overnight. All antibodies were diluted in 0.01 M PBS containing 0.1% bovine serum albumin. After washing, sections were incubated for 1 hour at 37°C with donkey anti-rabbit secondary antibody (FITC) for green fluorescence, donkey anti-mouse secondary antibody (rhodamine red-X) for red fluorescence, and donkey anti-sheep secondary antibody (Cy5) for far red fluorescence at 640 nm, respectively (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Control sections were treated as above except that the primary antibody was omitted.

Morphometry

All nerves present in the cross sections of the entirety of EOM or limb muscle samples were evaluated. Coexpression of

FIGURE 1. Immunostaining for Wnt1 (A–D), Wnt3a (E–H), Wnt5a (I–L), and Wnt7 (M–P) in nerve bundles from adult control EOM (A, E, I, M), aging EOM (B, F, J, N), adult limb muscles (C, G, K, O), and aging limb muscles (D, H, L, P). The range of variation in numbers of axons positive for Wnt1 (I, J) and Wnt3a (K, L).

specific Wnt isoforms with neurofilament-positive axons was assessed morphometrically as percent of Wnt-positive axon profiles out of the population of all neurofilament-positive axon profiles. Quantification of percent of myofibers positive for each of the Wnt isoforms was assessed as percent positive out of the population of all myofibers in each cross section examined. Coexpression of specific Wnts with α -BTx-positive neuromuscular junctions was assessed morphometrically as percent of Wnt-positive neuromuscular junctions out of the population of all neuromuscular junctions. Statistical significance was determined by ANOVA and graphed using Prism 6 software (GraphPad, San Diego, CA, USA). Data were considered significant at $P < 0.05$.

RESULTS

Pattern of Wnt Expression in Motor Nerves: Human

Wnt1. In adult human EOM, $70.9 \pm 5.4\%$ of the axon profiles identified as positive for neurofilament protein also coexpressed Wnt1 (Figs. 1–3). In the EOM from subjects with ALS, there was a significant reduction to 40% of control values in the density of Wnt1-coexpressing axons, to $42.5 \pm 6.2\%$. Approximately half of the axon profiles in the orbital layer retained Wnt1 expression, but only a few axon profiles in the global layer were found to coexpress neurofilament and Wnt1. To verify that this decrease in Wnt1 expression in axons was not due to aging alone, EOM from elderly subjects were examined, where $11.6 \pm 3.0\%$ of the neurofilament-positive axons also expressed Wnt1 (Figs. 1–3). This was a 72.6% lower expression level than in the EOM from ALS subjects and 83.6% lower than in adult EOM. This demonstrates that Wnt1 is preferentially retained in the nerves in the EOM from ALS subjects.

In contrast, within the limb muscle specimens from adults and ALS subjects, the density of Wnt1-positive axons was significantly lower than in the EOM (Figs. 1, 3, 4). Despite apparent differences in the number of Wnt1-positive axon profiles between the adult, elderly, and ALS limb muscles, at $31.0 \pm 11.8\%$, $15.8 \pm 4.9\%$, and $22.9 \pm 6.4\%$, respectively, these differences were not statistically significant. This was due to the extremely wide variance between specimens, with several subjects in each of the three cohorts having no Wnt1-positive axon profiles in any of the sections analyzed. It is interesting to note that aging alone does not explain the differences in the numbers of Wnt1-coexpressing axons, as overall there were 50% fewer in the aging limb muscle but only 26% fewer in the ALS limb muscles.

In summary, Wnt1 expression in the nerves in the ALS limb muscles was approximately one-quarter that in adult limb muscles, and in EOM was approximately half the density found in the adult nerves; this was even more significantly reduced in the aging muscles. In addition, the limb muscles had approximately 50% fewer Wnt1-positive nerves than were found in EOM in both the normal and ALS specimens.

Wnt3a. Most of the adult EOM nerves expressed Wnt3a, with $81.1 \pm 4.3\%$ of the axons expressing this isoform (Figs. 1–3). In the EOM of ALS patients, the density of Wnt3a-positive axon profiles was similar to the levels in the normal EOM, at $75.8 \pm 6.0\%$. In the aging EOM, however, the density of Wnt3a-expressing nerves was significantly lower; Wnt3a coexpression was observed in $50.4 \pm 10.4\%$ of the nerves, with 33% and 37% fewer than in the axons from the adult and ALS EOM, respectively.

In the adult limb muscles, only $20.4 \pm 8.8\%$ of the axons coexpressed Wnt3a, approximately 75% less than the percentage seen in adult EOM nerves (Figs. 1, 3, 4). The coexpression pattern was only $11.5 \pm 3.5\%$ in the nerves in the aging limb muscles but essentially unchanged from control levels in the nerves from the ALS limb muscle, at $16.5 \pm 7.9\%$.

FIGURE 2. EOM from ALS subjects immunostained for Wnt (green), neurofilament (red), and laminin (white). EOM were immunostained for Wnt1 (A, B, I, J), Wnt3a (C, D, K, L), Wnt5a (E, F), and Wnt7a (G, H). Arrows indicate examples of Wnt-negative axons. There were fewer Wnt1-positive axons in the global layer (I, J) compared to the orbital layer (A, B), and the same was true for Wnt3a, where the global layer (K, L) had fewer positive axons than the orbital layer (C, D).

In summary, a large proportion of the nerves in adult EOM expressed Wnt3a, and the levels did not change significantly in the nerves in the EOM from ALS subjects. Aging resulted in approximately a 30% loss of axon profiles containing Wnt3a in the EOM nerves. In contrast, overall, the density of Wnt3a-expressing nerves in the limb muscles was significantly lower than that of EOM, with over 75% fewer Wnt3a-positive axon profiles in each of the limb muscle groups compared to their EOM counterpart.

Wnt5a. Essentially all the axons within adult EOM contained high levels of Wnt5a, and this coexpression pattern was retained in the nerves of the EOM from ALS subjects (Figs. 1–3). There was an approximately 10% difference in the number of nerves within the EOM from elderly subjects that coexpressed Wnt5a, which was $88.7 \pm 11.3\%$, compared to close to 100% expression of this Wnt isoform in normal and ALS EOM axons.

A similar pattern of Wnt5a expression was seen in the nerves from the limb muscles, where most if not all of the nerves in adult, elderly, and ALS limb muscles coexpressed Wnt5a (Figs. 1, 3, 4).

In summary, almost all the axons within the EOM and limb muscles coexpressed Wnt5a in adult, elderly, and ALS specimens.

Wnt7a. Approximately $67.0 \pm 8.4\%$ of the nerve fibers in the EOM from adult subjects coexpressed Wnt7a (Figs. 1–3). These levels were significantly decreased in the aging EOM nerve fibers, to $7.9 \pm 1.7\%$. In the EOM of ALS subjects, $54.2 \pm 8.9\%$ of the axon profiles coexpressed Wnt7a, which was not statistically different from the coexpression levels in normal EOM despite being reduced by approximately 19.0%.

Interestingly, the density of Wnt7a-positive axons in adult limb muscle was only slightly lower than in the adult EOM, with $56.3 \pm 11.7\%$ positive for Wnt7a (Figs. 1, 3, 4). Paradoxically, the number of Wnt7a-positive axon profiles was significantly increased in the limb muscles from older individuals, where $82.8 \pm 6.2\%$ were positive for this isoform. The axon profiles in the limb muscles from ALS subjects expressed a percentage of Wnt7a-positive axons similar to that for the ALS EOM, with $47.7 \pm 11.4\%$ positive; however, there was great heterogeneity between patient specimens (Figs. 1, 3, 4). Thus, there was a 46% increase in axons coexpressing Wnt7a in the aging limb muscles and a 15% decrease in the ALS limb muscles compared to the number of Wnt7a-positive axons in the adult control limb muscle specimens.

In summary, a large percentage of the axons in adult and ALS EOM contained Wnt7a, but this was significantly reduced in the nerves from aging EOM. A similar density of Wnt7a-positive axons was present in adult and ALS limb muscle specimens, with a significant increase in axons expressing Wnt7a in the aging limb skeletal muscles, where the vast majority contained Wnt7a.

A similar pattern of immunostaining was seen in the nerves in the EOM and limb muscle tissue sections from the SOD1^{G93A} mouse model of ALS. Relative to limb muscle, for example, there was a moderate number of Wnt1-positive nerves; there were few Wnt3a-positive nerves at both 50 and 150 days in the transgenic mice; and similar to observations in the human muscles, both Wnt5a and Wnt7a were highly expressed in the nerves from both control and transgenic mouse muscles (data not shown).

Heterogeneity Between Subjects. During the analysis of Wnt coexpression patterns, it appeared that there was a wide variation in density of axons positive for Wnt1, Wnt3a, and Wnt7a between the muscle specimens. When the coexpression levels were reexamined based on high and low levels of expression, using 50% of the highest level as the dividing point, there was a large variability in the numbers of axon profiles positive for the expression of Wnt1, Wnt3a, and Wnt7a when the EOM from different subjects were compared (Fig. 3E). For example, an EOM specimen from one patient had basically no axons that were positive for any of the three Wnt isoforms, while an EOM specimen from a different patient had significant expression of Wnt1 and Wnt3a, but not Wnt7a. Furthermore, Wnt7a immunoreactivity was highly expressed in the perineurium in one EOM and one limb specimen, despite their origin from different subjects. There was no obvious correlation between the various Wnt expression levels in the axon profiles of individual muscle specimens and the form of ALS (sporadic or D90A SOD1 mutation), bulbar or spinal onset of disease, duration of disease, age of patient at the time of death, or any other known feature of the subjects' disease process.

Patterns of Wnt Expression in Muscle Fibers: Human

Wnt Expression in EOM Myofibers. In all the EOM examined, subpopulations of myofibers expressed Wnt within

FIGURE 3. Quantification of the percent of neurofilament-positive axons that expressed Wnt1 (A), Wnt3a (B), Wnt5a (C), and Wnt7a (D) in normal EOM, aged EOM, ALS EOM, normal limb, aged limb, and ALS limb muscle specimens. *Significant difference from normal EOM. ϕ Significant difference from corresponding EOM. (E) Variability in neurofilament and Wnt coexpression for the four Wnt isoforms when coexpression levels were reexamined based on high and low levels of expression, using 50% of the highest level as the dividing point. For Wnt1, Wnt3a, and Wnt7a there were subjects whose EOM were devoid of a particular Wnt, while other subjects had significantly different densities of coexpression. *Significant difference between low and high levels of expression. #Significant difference between high and mid levels of coexpression.

FIGURE 4. Limb muscles from ALS subjects immunostained for Wnt (green) (A, C, E, G). Merged images for Wnt (green), neurofilament (red), and laminin (white) (B, D, F, H). Note the paucity of Wnt1-, Wnt3a-, and Wnt7a-positive axons (A, C, G). The majority of the axons present coexpress Wnt5a (E, F).

their whole cross-sectional areas (Fig. 5). Wnt1 was expressed in $38.19 \pm 10.9\%$ of the myofibers in adult control EOM, but significantly increased to almost 100% in the EOM from ALS subjects (Figs. 5A, 5E). Wnt3a was expressed in only $16.3 \pm 7.9\%$ of the myofibers in adult control EOM, but, as seen with Wnt1, myofiber expression in the EOM from ALS subjects increased significantly to $95.0 \pm 1.4\%$ (Figs. 5B, 5E). Immunostaining for Wnt5a expression in individual myofibers in adult EOM was weak but was still found in $93.5 \pm 2.2\%$ of the myofibers. While expressed in only $80.4 \pm 7.8\%$ of the myofibers in the EOM of ALS subjects, this difference was not significant (Figs. 5C, 5E). In the control EOM, only $18.0 \pm 4.8\%$ of the myofibers expressed Wnt7a; this value was significantly increased in EOM myofibers from ALS subjects, with $56.5 \pm 10.8\%$ positive for Wnt7a (Figs. 5D, 5E). In summary, subpopulations of EOM myofibers in adult EOM expressed all four Wnts; and Wnt1, Wnt3a, and Wnt7a were significantly upregulated in the EOM myofibers from the ALS subjects.

Wnt Expression in Limb Myofibers. The myofibers in adult human limb skeletal muscles expressed essentially no

Wnt1 or Wnt5a (Figs. 6A, 6C, 6M). Wnt3a was expressed in $44.6 \pm 10.3\%$ of normal limb myofibers (Figs. 6B, 6M). For Wnt7a, $53.6 \pm 14.7\%$ of the myofibers were positive (Figs. 6D, 6M). In the aging limb skeletal muscle myofibers, Wnt1 expression was absent while Wnt3a immunostaining showed a mosaic patterning of fiber staining, with some myofibers very bright, some moderate, and some negative (Figs. 6E, 6F). A small group of fibers in the aging limb muscles was positive for Wnt5a, and essentially all of the myofibers were positive for Wnt7a (Figs. 6G, 6H). In the myofibers of the ALS limb muscles, there was only extremely rare immunostaining for Wnt1, a significant reduction in expression of Wnt3a to $6.0 \pm 2.3\%$ positive, and essentially no expression of Wnt5a (Figs. 6I–K, 6M). Interestingly, the number of Wnt7a-positive myofibers in the ALS limb muscles showed a large increase, to $82.0 \pm 7.7\%$; but due to the large variability between the muscles from different subjects, this was not significantly different from values in adult limb muscle (Figs. 6L, 6M).

In summary, compared to adult EOM, adult limb myofibers did not express Wnt1 or Wnt5a, but expressed higher levels of Wnt3a and Wnt7a than EOM. However in the ALS muscle specimens, there was essentially no Wnt1, Wnt3a, and Wnt5a immunostaining in individual limb myofibers. Most striking was the large proportion of myofibers expressing Wnt7a in the ALS limb muscles.

In concert with the relatively robust expression of Wnt7a within the nerves and myofibers in the adult and ALS human limb muscles, there appeared to be a concentration of Wnt7a at the sarcolemma of individual myofibers. In the adult human control limb muscle (Fig. 7A), little if any Wnt7a was seen specifically localized to the myofiber periphery. However, in the ALS specimens, the vast majority of myofibers had bright rings of Wnt7a at the sarcolemma, either partially (Fig. 7B) or entirely encircling the myofiber perimeter (Fig. 7C). Interestingly, one ALS specimen had rare myofibers that were surrounded by a ring of Wnt5a at the sarcolemma (Fig. 7D).

A similar picture was seen in the SOD1^{G93A} mouse model of ALS (Figs. 7E–H). In age-matched control mice, a small number of myofibers had bright Wnt7a immunostaining at the myofiber periphery, either partially (Fig. 7E) or entirely encircling the myofiber perimeter (Fig. 7F). By and large, myofibers with visible neuromuscular junctions were negative for Wnt7a staining (Fig. 7F, horizontal arrow). In the SOD1^{G93A} mice at 150 days, bright rings of Wnt7a were present in almost all of the myofibers (Figs. 7G, 7H), regardless of whether the myofibers were relatively normal in appearance (Fig. 7G) or pathologic (Fig. 7H). Double staining with Wnt7a and dystrophin showed that the Wnt7a was actually located subsarcolemmally (Figs. 7I–K). In summary, in the ALS limb muscle specimens, whether from human or the SOD1^{G93A} mice, bright rings of Wnt7a immunostaining were found in the subsarcolemmal position within most if not all of the myofibers.

Patterns of Wnt Expression in Neuromuscular Junctions and Nerve: ALS Mouse Model

The patterns of Wnt expression in the neuromuscular junctions of the EOM and limb muscles of wild-type mice and the SOD1^{G93A} mouse model of ALS were examined at 50 and 150 days of age (Figs. 8–10) and in the EOM and limb muscles from human adult, elderly, and ALS subjects. In general, the four Wnt isoforms were expressed in the human specimens, but the immunostaining was less robust than in the mouse tissue. All four Wnt isoforms colocalized with α -BTx labeling in the EOM from adult, elderly, and ALS subjects (not shown). Due to the weaker immunostaining and low number of neuromuscular junctions encountered in the human limb

FIGURE 5. EOM from ALS subjects immunostained for Wnts (green), neurofilament (red), and laminin (white). (A–D) Note that many myofibers expressed Wnts in entire fiber cross sections. Arrows indicate fibers negative for a specific Wnt isoform. (E) Quantification of the percent of myofibers from control and ALS EOM expressing a Wnt isoform. *Significantly different from control.

muscle specimens, we conducted the statistical analysis using the EOM and limb muscles from SOD1^{G93A} mice.

As identified with α -BTx staining, the vast majority of the neuromuscular junctions of the EOM and the limb skeletal muscles of all the wild-type mice at both ages coexpressed all four isoforms of Wnt (Figs. 8–10). In the SOD1^{G93A} mice, the vast majority of the neuromuscular junctions of the EOM and limb skeletal muscles coexpressed all four Wnt isoforms at the 50-day survival time. Additionally, at the 150-day survival time, the vast majority of the neuromuscular junctions of the EOM continued to coexpress all four Wnt isoforms; in contrast, in the SOD1^{G93A} mouse limb muscles, the percent of neuromuscular junctions that coexpressed Wnts dropped significantly for all four Wnt isoforms (Figs. 9, 10). The percent of coexpressing neuromuscular junctions dropped to $73.3 \pm 9.4\%$ for Wnt1, $52.0 \pm 12.5\%$ for Wnt3a, $45.2 \pm 15.7\%$ for Wnt5a, and $80.5 \pm 0.9\%$ for Wnt7a (Fig. 10). This correlated with our previous study showing that in the limb muscles of the ALS mouse model, only neuromuscular junctions at the longest survival time showed abnormal innervation; those at early stages did not.¹⁴

β -Catenin Expression

Wnt molecules act through several pathways,²⁰ and the canonical pathway is the best characterized. This involves binding of Wnt to a Frizzled receptor and the stabilization of cytoplasmic β -catenin. While a detailed analysis of the specific signaling pathways activated by Wnt was beyond the scope of the current analysis, cytoplasmic β -catenin was found in a number of myofibers in the adult control, aging, and ALS human muscles in both limb muscles and EOM (Fig. 11). In the limb muscles from the human control specimens, a mosaic pattern of staining was seen, and approximately 1/3 to 1/2 were positive for β -catenin (Fig. 11A). This pattern was largely the same for the limb muscle from both elderly and ALS subjects (Figs. 11B, 11C). A different picture emerged for the EOM specimens, where the orbital layer fibers were largely negative for β -catenin in the adult, elderly, and ALS specimens (Figs. 11D–F). In the control adult EOM, there was a scattered distribution of β -catenin-positive myofibers (Fig. 11D), and this pattern was unchanged in the ALS global layer fibers (Fig. 11F). In the muscle from the elderly ALS subjects, there was a substantial increase in the number of myofibers in the global layer positive for β -catenin. No clear correlation was seen

FIGURE 6. Limb muscle sections from adult control (A–D), elderly control (E–H), and ALS (I–L) subjects immunostained for Wnt (green), neurofilament (red), and laminin (white) for Wnt1 (A, E, I), Wnt3a (B, F, J), Wnt5a (C, G, K), and Wnt7a (D, H, L). Note that only Wnt3a and Wnt7a were expressed in the myofibers in adult limb muscles. White arrows indicate examples of negative myofibers (A). Red arrow indicates a myofiber highly positive for Wnt7a (D). Note that many more myofibers expressed Wnt3a and 7a, and a few myofibers expressed Wnt5a in elderly control muscles, and additionally that the majority of myofibers appeared to express Wnt7a (L). Note also the absence of immunostaining for Wnt 1 (I), Wnt3a (J), and Wnt5a (K) but that the majority of myofibers appeared to express Wnt7a (L) in ALS subjects. White arrow indicates a Wnt7a-negative myofiber. (M) Quantification of the percent of Wnt-positive myofibers in control and ALS limb muscles. *Significantly different from control. #Essentially no myofibers positive for this Wnt isoform. Scale bars: 40 μ m.

between β -catenin expression and any of the four Wnt isoforms examined in this study (data not shown), and this is the subject of ongoing studies.

DISCUSSION

This is the first study to examine the potential role of Wnt expression in the preferential anatomic and functional sparing of the EOM in ALS. Wnt1-positive nerves and myofibers were found at significantly greater densities in the EOM compared to limb muscles, both in normal and in ALS specimens. Wnt1 signaling helps to regulate muscle specification and neuromuscular junction formation in development,^{18,30} but recent

studies have shown that Wnt1 plays an important role in synaptic plasticity and muscle regeneration in mature animals. In both the peripheral and central nervous systems, Wnt1 appears to act both pre- and postsynaptically, controlling cytoskeletal dynamics in the innervating nerves as well as assembly and clustering of the postsynaptic apparatus.^{16,31} The presence of Wnt1 has been shown to prevent neurite elimination,³² and thus its elevated presence in ALS-resistant EOM suggests it may play a role in the selective sparing of the EOM and their innervating neurons. The potential link of Wnt1 expression to sparing of the ocular motor neurons and the EOM in ALS is particularly compelling, since early deletion of

FIGURE 7. Wnt immunostaining at myofiber peripheries in human adult (A) and ALS (B–D) muscles and in the SOD1^{G93A} mouse model of ALS (E–K). Wnt7a expression was relatively uniform in individual myofibers in adult limb muscles (A) but appeared to be expressed at high levels around the sarcolemma (laminin in white), partly ([B] arrows) or entirely ([C] arrows), in ALS subjects. Note one myofiber positive for Wnt5a at the sarcolemmal surface ([D] arrow). A similar picture was seen in the limb myofibers from control mice (E, F) and the SOD1^{G93A} mouse model of ALS (G–K). Control limb muscle specimens in cross section showing differential levels of Wnt7a immunostaining at the sarcolemmal surface ([E, F] arrows) and ALS mouse limb muscle showing increased expression of Wnt7a around the sarcolemmal surface (G, H). Note the significantly different level of muscle pathology in the muscle specimens from different SOD1^{G93A} mice, despite being at 150 days of age at the time of euthanasia. (I–K) Wnt7a (I) and dystrophin (J) immunostaining with merged image (K) in limb muscles from the SOD1^{G93A} mouse model of ALS showed that Wnt7a was within the sarcolemma of individual myofibers in the SOD1^{G93A} mouse model of ALS.

FIGURE 8. Wnt immunostaining at the neuromuscular junctions (NMJs) in EOM from control and the SOD1^{G93A} mouse model of ALS. Specimens were examined from control and ALS mouse muscles at 50 days (*two left columns*) and 150 days (*two right columns*) immunostained for Wnt isoforms (*green*) and α -BTx (*red*) to mark NMJs. Specimens were immunostained for Wnt1 (**A, B**), Wnt 3a (**C, D**), Wnt5a (**E, F**), and Wnt7a (**G, H**). Note that all the NMJs in the EOM from the control and ALS mouse muscles coexpressed each of the four Wnt isoforms at both 50 and 150 days of age.

FIGURE 9. Wnt immunostaining at the neuromuscular junctions in limb muscles from control and the SOD1^{G93A} mouse model of ALS. Specimens were examined from control and ALS mouse muscles at 50 days (*two left columns*) and 150 days (*two right columns*) immunostained for Wnt (*green*) and α -BTx (*red*) to mark NMJs. Specimens were immunostained for Wnt1 (A, B), Wnt 3a (C, D), Wnt5a (E, F), and Wnt7a (G, H). In the control muscles, all NMJs at both 50 and 150 days of age coexpressed Wnt. In the ALS muscles from these mutant mice at 50 days of age, the majority of the neuromuscular junctions coexpressed the Wnt isoforms, although some NMJs could be found that did not coexpress any one of the four isoforms. At 150 days in the ALS mouse limb muscles, there was a reduction in the density of NMJs that coexpressed Wnt3a and Wnt5a.

FIGURE 10. Quantification of the density of NMJs in limb muscles from control and the SOD1^{G93A} mouse model of ALS immunostained for Wnt1 (A), Wnt3a (B), Wnt5a (C), and Wnt7a (D). For all four Wnt isoforms, asterisk indicates significantly different from all other limb measurements.

Wnt1 at the embryo stage resulted in the absence of cranial nerves III and IV and disruption of the aneural EOM.^{33,34}

In all the EOM specimens, the density of Wnt3a-expressing axons was significantly elevated over that seen in the limb specimens; in fact, over 7-fold more Wnt3a-expressing axons were seen in all three groups of subjects. While the number of Wnt3a-positive myofibers was relatively low in the normal EOM, almost 100% of the myofibers expressed this isoform in the ALS specimens. The normal limb muscle specimens had twice the number of Wnt3a-positive myofibers, but this dropped to 6% in the ALS specimens, in sharp contrast to the marked increase in the ALS EOM. Similar to Wnt1, Wnt3a plays a role in promoting nerve outgrowth.^{35,36} In addition, it plays an important modulatory role in the formation of neuromuscular junctions, including number and size.³⁷ These processes, if supported by elevated levels of Wnt3a, could potentially be involved in maintenance of the innervated neuromuscular junctions found in ALS EOM compared to ALS limb muscles.^{12,14}

The pattern of Wnt5a expression is the most enigmatic of the results of this study, as the density of Wnt5a-positive axon profiles was equally high in all EOM and limb muscles.

However, there was a striking difference in myofiber expression of this isoform, as the myofibers in the EOM were almost all positive for Wnt5a while the control and ALS limb muscles were essentially devoid of this isoform. Wnt5a has been shown to mediate growth factor-dependent axonal branching and extension in certain neuronal populations^{38–40} and, interestingly, plays a role in remodeling postsynaptic regions.⁴¹ Wnt5a also plays a role in specification and survival of motor neurons in development⁴² and during in vitro differentiation of stem cells.⁴³ Despite the high levels of expression of Wnt5a in the axons themselves, the significant reduction of Wnt5a in the neuromuscular junctions in the limb muscles of the SOD1^{G93A} mouse model of ALS and the very high density of Wnt5a-positive myofibers in the human control and ALS EOM specimens, coupled with the absence of Wnt5a in the limb muscle fibers, suggest that this isoform may be working specifically at the neuromuscular junctions in ALS EOM to prevent their degeneration.

Wnt7a had a different profile of expression compared to the other isoforms examined in this study. The density of Wnt7a-positive axon profiles and neuromuscular junctions in control and ALS specimens from EOM remained high, but similar levels

FIGURE 11. Immunostaining for β-catenin in human limb muscles (A–C) and EOM (D–F) from control (A, D), aging (B, E), and ALS subjects (C, F). All the limb muscle specimens showed a mosaic pattern of β-catenin staining, with the greatest density of positive fibers and positive nuclei in elderly subject specimens (A–C). In the EOM specimens, the orbital layer fibers were mostly negative, although many nuclei positive for β-catenin were present in the adult control specimens. The global layer in all three specimens showed a mosaic pattern of staining, with approximately 1/3 of the myofibers positive for β-catenin in the adult control and ALS specimens, and almost all of the global layer fibers positive for β-catenin in the elderly subjects.

were seen in the number of Wnt7a-coexpressing axons in the control and ALS limb specimens. Interestingly, the density of positive myofibers in the control EOM was low but was increased 3-fold in the ALS EOM. In the limb specimens, the density of Wnt7a-positive nerves and myofibers remained high, but the density of Wnt7a-positive neuromuscular junctions of the SOD1^{G93A} mouse was significantly decreased. Concomitant with these changes, Wnt7a appeared to be localized to the periphery of the vast majority of the myofibers, in particular the smaller muscle fibers, raising the possibility either that it is preferentially upregulated in denervated and atrophic muscle fibers or that secretion of Wnt7a is inhibited in these myofibers. Wnt7a is known to regulate presynaptic assembly and remodeling of incoming axons via retrograde signaling.^{44,45} While little work has been done examining Wnt7a at the neuromuscular junction, studies in the motor regions of the brain suggest that Wnt7a plays an important role in regulating plasticity at the presynaptic terminal. Additionally, when added exogenously, Wnt7a has been shown to induce myofiber hypertrophy, reducing myofiber damage in a mouse model of muscular dystrophy.^{22,46} This raises some interesting questions for further study, as in the case of ALS where its paradoxical upregulation appears to be insufficient to prevent loss of neuromuscular junctions and myofiber atrophy in the ALS limb muscles.

β -Catenin was expressed in a subset of myofibers in all the muscle specimens examined, with a differential distribution of expression in the EOM specimens. There was no apparent correlation of the β -catenin-positive myofibers with any single Wnt isoform. However, the expression of β -catenin demonstrates that a subset of the myofibers use the canonical Wnt signaling pathway.²⁸ Further studies are needed to determine the functional sequelae of the upregulated β -catenin, as it has been associated with many processes in muscle; these include the regulation of acetylcholine receptor clustering, presynaptic function,⁴⁷ and axonal remodeling.⁴⁸ Ongoing studies in the laboratory are examining whether the β -catenin-positive fibers represent a distinct subtype, that is, fast versus slow, or if they correlate with one of the Wnt isoforms not included in this study.

The complexity of the patterns of Wnt immunostaining, as well as the complex β -catenin staining patterns, is interesting in light of our increasing understanding of the complexity of fiber types in the mammalian EOM.^{49–51} This includes the complex coexpression patterns of myosins in the slow tonic and slow-twitch myofibers. The issue of “myofiber type” is made even more complex when other aspects of myofiber diversity are considered, such as the coexpression of glycolytic and oxidative enzymes within single EOM myofibers⁵² and the uncoordinated expression within single myofibers of myosin heavy-chain isoforms and myosin-binding protein C isoforms or SERCAs.^{53,54} In addition, our preliminary studies suggest that other isoforms of the Wnt family of molecules are expressed in the EOM.^{15–17} These studies are ongoing in our laboratories.

In the last decade, dysregulation of Wnt signaling has been increasingly implicated in a number of degenerative diseases of the central nervous system.⁵⁵ For example, downregulated Wnt signaling has been associated with neuronal dysfunction in Alzheimer's disease.⁵⁶ In the SOD1^{G93A} mouse model of ALS, Wnt3a was found to be upregulated in both neurons and glial cells in the spinal cord.⁵⁷ It should also be noted that the only drug available to treat ALS, riluzole, which acts on human muscle acetylcholine receptors,⁵⁸ is an enhancer of Wnt/ β -catenin signaling.⁵⁹ Collectively these studies support the need to examine Wnt signaling pathways in nerve and muscle tissues of ALS subjects in more detail in order to better understand the potential causes of the degenerative pathology associated with ALS in limb muscles and the functional sparing

in the EOM. The differential pattern of expression of Wnt1 and Wnt3a in the EOM supports our hypothesis that they play a role in their preferential sparing in ALS subjects. Further studies are ongoing, but the current analysis supports the hypothesis that dysregulation of Wnt signaling pathways is likely to play an important role in the pathophysiology of ALS.

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