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Unchanged Neurotrophic Factors and Their Receptors Correlate With Sparing in Extraocular Muscles in Amyotrophic Lateral Sclerosis

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PURPOSE. To investigate the impact of amyotrophic lateral sclerosis (ALS) on the extraocular muscles (EOMs) by examining the distribution of neurotrophic factors (NTFs) and their receptors in EOMs and limb muscles from ALS transgenic mice.

METHODS. Muscle samples collected from transgenic mice overexpressing human superoxide dismutase type 1 mutations (SOD1G93A, the most widely used mouse model of ALS) at 50 and 150 days as well as age-matched controls were analyzed with immunohistochemistry using antibodies against brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4), glial cell line–derived neurotrophic factor (GDNF), and the neurotrophin receptors p75NTR, tyrosine kinase (Trk) receptor TrkB and TrkC, and GDNF family receptor alpha-1 (GFRα-1).

RESULTS. There was an intrinsic difference in NTF expression between EOMs and limb muscles in control mice: EOMs presented significantly lower number of neuromuscular junctions (NMJs) labeled for BDNF and NT-4 at 50 days, and for BDNF and GDNF at 150 days, compared with the control limb muscles of corresponding age. In ALS transgenic mice at 150 days, NTF expression in limb muscles was significantly changed but not in EOMs: the limb muscles presented a significant decline in the number of NMJs labeled for BDNF, NT-4, GDNF, p75NTR, TrkB, and TrkC, which was not observed in EOMs.

CONCLUSIONS. The significant differences in expression of NTFs on NMJs between EOMs and limb muscles in both control and ALS transgenic mice suggest that NTF may be involved in the pathogenesis of ALS and the resistance of EOMs to the disease.

Keywords: extraocular muscles, neuromuscular junctions, neurotrophin, amyotrophic lateral sclerosis, neurotrophic factor
bran, decreased density of acetylcholine receptors, and lack of nerve sprouting in denervated NMJs are observed only in limb muscles but not in EOMs of ALS transgenic mice.\textsuperscript{15,17}

Neurotrophic factors (NTFs) are known to be important in both nervous system development and maintenance, and NTF impairment in ALS has been brought to attention since the 1990s.\textsuperscript{18} We have in a previous study examined four different NTFs at the mRNA level: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4), and glial cell line–derived neurotrophic factor (GDNF) in EOMs and limb muscles from SOD1\textsuperscript{G93A} transgenic mice at different stages.\textsuperscript{16} Two of the NTFs, NT-3 and GDNF, are significantly upregulated in EOMs but not in limb muscles of early-stage SOD1\textsuperscript{G93A} transgenic mice. The early upregulation of the two NTFs in EOMs was proposed to be associated with the sparing of EOMs in the late stage of SOD1\textsuperscript{G93A} transgenic mice.\textsuperscript{16}

The NTFs exert their functions by binding to specific receptors; BDNF, NT-3, and NT-4 bind to the specific high-affinity tyrosine kinase (Trk) receptors TrkB (specific for BDNF and NT-4) and TrkC (specific for NT-3), whereas GDNF binds to GDNF family receptor alpha-1 (GFR\textsubscript{a}-1).\textsuperscript{19} In addition, BDNF, NT-3, and NT-4 also bind to a common neurotrophin receptor, p75\textsubscript{NTR}.\textsuperscript{20} To further evaluate the importance of NTFs in the pathophysiology of ALS, in the present study, we examined the cellular distribution of the four NTFs in EOMs and limb muscles of ALS transgenic mice at two different ages. As the NMJ is an important site in NTF retrograde transportation\textsuperscript{18,21} and a vulnerable site in ALS, especially for limb muscles, labeling of the NTFs and the respective receptors at the NMJs was quantified and compared between EOMs and limb muscles. In addition, labeling of NTFs was also evaluated on intramuscular nerve axons and muscle fibers.

## Materials and Methods

### Animals

The original SOD1\textsuperscript{G93A} transgenic mice, expressing high levels of human SOD1 containing a substitution of glycine to alanine at position 93, were obtained from Jackson Laboratory (Bar Harbor, Maine, USA) and were backcrossed with C57/BL6 BomTac mice for at least 20 generations. The SOD1\textsuperscript{G93A} transgenic mice begin to show signs of weakness of the hind limbs at 3 to 4 months of age and usually die at approximately 5 months of age.\textsuperscript{3}

Six SOD1\textsuperscript{G93A} transgenic mice, approximately 50 days old (at early presymptomatic stage), and six SOD1\textsuperscript{G93A} transgenic mice approximately 150 days old (at late terminal stage), were used in the present study. At early presymptomatic stage, the mice did not present any observable health problem or abnormal behavior, whereas at terminal stage, the mice were no longer able to stand up or change body position by themselves within 5 seconds after being put on their side. Age-matched normal healthy C57/BL6 mice served as wild-type controls: five mice approximately 50 days old and six mice approximately 150 days old.

This animal study was conducted in accordance with the European Communities’ Council Directive (86/609/EEC), complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and had the approval of The Umeå Animal Ethics Board.

### Muscle Samples

Muscle samples were collected directly after the animals were killed with an intraperitoneal injection of pentobarbital. Extraocular muscles and hind limb muscles were rapidly excised, mounted on cardboard, and immediately frozen in propane chilled with liquid nitrogen, and then stored at \textminus80°C until analysis. Series cross-sections (5-\textmu m thick) were cut in a cryostat (Reichert-Jung; Leica, Heidelberg, Germany).

### Antibodies and Immunofluorescence

All antibodies used in the present study (Table) have been previously characterized. In particular, the rabbit polyclonal antibodies against NTFs (BDNF NT-3, NT-4, and GDNF) and their receptors have been used widely\textsuperscript{22-29} and thoroughly tested.\textsuperscript{22-27} The specificity of the antibodies against BDNF, GDNF, and TrkB have been determined by immunoblots with homogenates from rodent muscle tissues, which showed that these antibodies recognized two forms of the ligands, resulting in a lower band corresponding to the mature protein and a higher band corresponding to the precursor protein.\textsuperscript{23,24} The specificity of the antibodies against NT-3\textsuperscript{24} and TrkC\textsuperscript{25,26} also have been established by immunoblots, but have not yet been verified by showing negative results in tissues from knockout mice. Preabsorption controls with excess of blocking peptides also have been performed and confirmed the specificity of these antibodies against NTFs and their receptors.\textsuperscript{22,24-27}

In addition, omission of the primary antibody (replaced with PBS or PBS/BSA) has been used to rule out any cross-reactions with the secondary antibody. In the present study, NMJs were identified by rhodamine-conjugated α-bungarotoxin (α-BTx; Molecular Probes, Inc., Eugene, OR, USA) and nerve axons were identified by monoclonal antibody M0726 against neurofilament (NF)
protein. Antibodies, AB5032 and AB5380 against anti-neural cell adhesion molecule (N-CAM) and neural nitric oxide synthase (nNOS) were used to detect denervated/degenerated muscle fibers, respectively.19

The muscle samples were processed for immunohistochemistry as previously described.12 In brief, the air-dried sections were rehydrated in 0.01M PBS, and then immersed in 5% normal goat 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 0.01M PBS containing 0.1% BSA and used at their optimal dilutions. After washing, sections were further incubated for 1 hour at 37°C with a mixture of goat anti-rabbit or goat anti-mouse secondary Alexa Fluor conjugated antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and rhodamine-conjugated α-bungarotoxin. Double labeling with anti-NTFs and anti-neurofilament (NF) was also used to simultaneously visualize NTFs and NF in nerve axons. Control sections were treated as above, except that the primary antibody was omitted. No staining was observed in control sections.

Staining Evaluation and Data Collection

Evaluation of the tissue sections with respect to general morphology and distribution of the labeling for NTFs on NMJs, nerve axons, and muscle fibers was performed throughout all sections. For quantification of labeled NMJs, double-stained sections combining α-BTx and one of the antibodies examined (α-BTx+BDNF, α-BTx+NT-3, α-BTx+NT-4, α-BTx+GDNF, α-BTx+BDNF, α-BTx+TrkB, α-BTx+TrkC, and α-BTx+GFRα-z1) were evaluated. The total area of each muscle section was examined and each NMJ identified with α-BTx was counted and evaluated as either “positive” or “negative” for the antibody used. Completely unstained NMJs and NMJs displaying a staining level identical to that of connective tissue were considered negative, as this was an unspecific level of staining present both under green and red filters. The same criteria were used to determine positive and negative muscle fibers and nerve axons. The total number of α-BTx-positive NMJs was recorded as well as the total number of NMJs that were labeled with each specific antibody.

The sections were photographed with a Spot camera (RT KE Slider; Diagnostic Instruments, Inc., Sterling Heights, MI, USA) connected to a Nikon microscope (Eclipse E800; Nikon, Tokyo, Japan). Further processing of the images was performed using the Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA, USA).

Statistical Analysis

The percentage of NMJs labeled for each NTF and receptor in relation to the total number of NMJs was calculated for limb muscles and EOMs of each individual mouse in both the control and the transgenic groups. Means and SDs were calculated for each group and 2-way ANOVA with Tukey post hoc multiple comparisons was performed using Prism 7 software (GraphPad, San Diego, CA, USA). Statistically significant difference was considered at P ≤ 0.05.

RESULTS

Immunostaining for the four NTFs was detected in NMJs, intramuscular nerve axons and muscle fibers in EOMs, and limb muscles from both control and ALS transgenic mice at 50 and 150 days.

Neurotrophic Factors in NMJs

Brain-Derived Neurotrophic Factor. In EOMs of control mice at 50 days, 50.6% ± 19.6% of NMJs were labeled with BDNF (Fig. 1A; Supplementary Fig. S1A), whereas in the EOMs of age-matched SOD1G93A transgenic mice, the number was increased to 70.3% ± 19.5%, but the difference was not statistically significant between the two groups (Fig. 1A; Supplementary Fig. S1B). In EOMs of both control and SOD1G93A transgenic mice at 150 days, the percentages of NMJs positive to BDNF were similar, 40.2% ± 26.0% and 49.5% ± 18.0%, respectively (Figs. 1A, 2A, 2B).

In limb muscles of both control and SOD1G93A transgenic mice at 50 days, the vast majority of NMJs were labeled with BDNF 91.6% ± 8.0% and 98.9% ± 2.0%, respectively (Fig. 1A; Supplementary Figs. S1I, S1J). Similarly, in limb muscles of control mice at 150 days, most NMJs (82% ± 10%) were labeled with BDNF (Figs. 1A, 3A). However, in limb muscles of SOD1G93A transgenic mice at 150 days, only 44.9% ± 15.0% of the total number of NMJs were labeled with BDNF which was significantly lower than in limb muscles of age-matched control mice (44.9% vs. 82.0%, P = 0.01; Figs. 1A, 3B). A statistically significant difference was also observed between the percentages of NMJs labeled with BDNF in limb muscles of control mice at 50 days (P = 0.02) and 150 days (P = 0.003; Fig. 1A).

In summary, in limb muscles, the percentages of NMJs labeled with BDNF were constant in control mice from 50 days to 150 days but significantly reduced in SOD1G93A transgenic mice at 150 days. In contrast, in EOMs of both control mice and SOD1G93A transgenic mice, the values were constant from 50 days to 150 days. Moreover, significantly fewer NMJs labeled with BDNF were found in EOMs than in limb muscles in control mice at both 50 and 150 days.

Neurotrophin-4/5. In EOMs of control mice and SOD1G93A transgenic mice at 50 days, the percentages of NMJs labeled with NT-4 were very similar (57.2% ± 19.3% vs. 60.0% ± 26.6% respectively; Fig. 1B), and became slightly higher in EOMs of control mice (64.0% ± 20.8%) and SOD1G93A transgenic mice (68.8% ± 10.7%) at 150 days (Figs. 1B, 2C, 2D).

In limb muscles of both control mice and SOD1G93A transgenic mice at 50 days, labeling with NT-4 was observed in the vast majority of NMJs (94.2% ± 7.1% and 92.9% ± 6.8%, respectively; Fig. 1B, Supplementary Figs. S1K, S1L). However, although the corresponding value in limb muscles of control mice at 150 days remained relatively constant (79.6% ± 15.8%), it was only 38.1% ± 11.8% of NMJs in limb muscles of SOD1G93A transgenic mice at 150 days, a difference that was statistically significantly (P = 0.004; Figs. 1B, 3D). In addition, comparison of the percentages of NMJs labeled with NT-4 in limb muscles of SOD1G93A transgenic mice between 50 days and 150 days revealed a significant reduction in values from 50 days to 150 days (92.9% vs. 38.1%, respectively, P = 0.0002). Further comparison of the proportion of NMJs labeled with NT-4 between EOMs and limb muscles in control and SOD1G93A transgenic mice revealed significantly lower values in EOMs than in limb muscles in both control (P = 0.02) and ALS transgenic mice (P = 0.04) at 50 days (Fig. 1B).

In summary, in EOMs, the percentage of NMJs labeled with NT-4 did not show any significant difference between control mice and SOD1G93A transgenic mice at 50 days or 150 days. However, in limb muscles of ALS transgenic mice at 150 days, the percentage of NMJs labeled with NT-4 was significantly lower than in limb muscles of age-matched control mice (44.9% vs. 82.0%, P = 0.01; Figs. 1A, 3B). A statistically significant difference was also observed between the percentages of NMJs labeled with BDNF in limb muscles of control mice at 50 days (P = 0.02) and 150 days (P = 0.003; Fig. 1A).
lower compared with the value of the same muscles from aged-matched control mice. In addition, EOMs presented significantly lower percentage of NMJs labeled with NT-4 in comparison with limb muscles in both control and SOD1G93A transgenic mice at 50 days.

Neurotrophin-3. In EOMs of both control mice and SOD1G93A transgenic mice at both 50 days and 150 days, the percentages of NMJs labeled with NT-3 were comparable (control 50 days, 60.6% ± 25.7%; SOD1G93A transgenic 50 days, 62.4% ± 31.2%; control 150 days, 57.0% ± 15.3%; SOD1G93A transgenic 150 days, 64.1% ± 8.9%; Fig. 1C).

In limb muscles of control mice at 50 days and 150 days, 80.0% ± 25.0% and 82.0% ± 17.8% of NMJs were labeled with NT-3, respectively (Fig. 1C). In limb muscles of SOD1G93A transgenic mice, the values were 93.1% ± 6.3% at 50 days and 60.4% ± 15.1% at 150 days, which was 16% higher at 50 days and 26% lower at 150 days compared with the corresponding values of the control group. However, this difference was not statistically significantly different.

In summary, there were no statistically significant differences in the percentages of NMJs labeled with NT-3 for any of the comparisons made.

Glia L Line–Derived Neurotrophic Factor. In EOMs of both control and SOD1G93A transgenic mice at 50 days, 58.0% ± 13.0% and 52.2% ± 19.0% of the total population of NMJs were labeled with GDNF, respectively (Fig. 1D, Supplementary Figs. S1G, S1H). In EOMs of control mice and SOD1G93A transgenic mice at 150 days, the percentages of NMJs labeled with GDNF were 31.9% ± 24.0% and 20.9% ± 18.4%, respectively (Figs. 1D, 2G, 2H). Comparisons of the percentages of NMJs labeled with GDNF in EOMs between control mice and SOD1G93A transgenic mice did not reveal statistically significant differences at either 50 days or 150 days. However, further comparisons of the percentage of NMJs labeled with GDNF in EOMs between 50 days and 150 days revealed statistically significant differences for the SOD1G93A transgenic mice (P = 0.02; Fig. 1D), but not for the control mice.

In limb muscles of both control and SOD1G93A transgenic mice at 50 days, more than half of the NMJs were labeled with GDNF (67.3% ± 13.6% and 52.9% ± 16.3%, respectively), and there was no statistically significant difference between the two groups (Fig. 1D). In limb muscles of control mice at 150 days, the percentage of NMJs labeled with GDNF was 68.5% ± 14.8%, whereas in limb muscles of SOD1G93A transgenic mice at 150 days, the corresponding value was only 29.4% ± 4.2%, which was significantly lower (P = 0.005; Figs. 1D, 3H). Further comparison of the percentage of NMJs labeled with GDNF between EOMs and limb muscles of both control mice and SOD1G93A transgenic mice at 50 days and 150 days revealed a
significant difference only between control mice at 150 days (31.9% in EOMs versus 68.5% in limb muscles; \( P = 0.02 \)).

In summary, nearly half of the NMJs were labeled with GDNF in both EOMs and limb muscles of both control and SOD1G93A transgenic mice at 50 days as well as in limb muscles of control mice at 150 days. A significant difference in the percentage of NMJs labeled with GDNF was observed in limb muscles between control mice and SOD1 G93A transgenic mice at 150 days; in EOMs of SOD1G93A transgenic mice between 50 days and 150 days, and in control mice at 150 days between EOMs and limb muscles.

**Neurotrophic Factors in Intramuscular Nerve Axons**

In EOMs of control mice at both 50 days (not shown) and 150 days (Supplementary Figs. S2A, S2C, S2E, S2G), labeling for all four NTFs (BDNF, NT-3, NT-4, and GDNF) was observed in intramuscular nerve axons and the labeling colocalized with labeling for NF in the nerve axons. In EOMs of SOD1G93A transgenic mice at 150 days; in EOMs of SOD1G93A transgenic mice between 50 days and 150 days, and in control mice at 150 days between EOMs and limb muscles.

Similarly, in limb muscles of ALS SOD1G93A mice at 50 days, all nerve axons were labeled with all four NTFs and most of the nerve axons coexpressed NF (not shown), whereas at 150 days, most but not all intramuscular nerve axons identified by labeling with the antibody against NF were labeled by the antibodies against the four NTFs (Supplementary Figs. S3B, S3D, S3F, S3H).

**Neurotrophic Factors in Muscle Fibers**

In EOMs of control mice at both 50 days and 150 days, all muscle fibers were labeled for the four NTFs, and the labeling was in general weak for BDNF and GDNF, and weak to moderate for NT-3 and NT-4 (not shown). Labeling for the four NTFs on muscle fibers of EOMs of SOD1G93A transgenic mice at both 50 days and 150 days was similar (not shown).

In limb muscle of control mice at 50 and 150 days and of SOD1G93A transgenic mice at 50 days, most muscle fibers were weakly labeled for NT-3 and NT-4 (not shown). In limb muscles of SOD1G93A transgenic mice at 150 days, although most muscle fibers presented similar labeling patterns as that of controls, a small number of large-sized and small-sized fibers were observed and presented different staining patterns. Although some of the large-sized fibers were unlabeled for the four NTFs, the small-sized muscle fibers were strongly labeled, especially for BDNF and/or NT-4 (Figs. 4A1,
The small-sized muscle fibers seemed to be of the fast type, as they were generally unlabeled by antibody A4.951 against slow myosin heavy chain (MyHC) (not shown). In limb muscles of control mice at 50 days and 150 days and of SOD1\textsuperscript{G93A} transgenic mice at 50 days, labeling for nNOS in muscle fibers was evenly distributed under the sarcolemma and highly concentrated at NMJs (Supplementary Figs. S4A–C), whereas labeling for N-CAM was more concentrated at NMJs but less pronounced in muscle fibers (Supplementary Figs. S4G–I), as reported previously.\textsuperscript{30} In limb muscles of SOD1\textsuperscript{G93A} transgenic mice at 150 days, however, labeling for nNOS was absent from underneath the sarcolemma in most muscle fibers (Figs. 4A3, 4B3, 4C3; Supplementary Figs. S4D, S4F). Additionally, for the small-sized muscle fibers with strong labeling for BDNF and/or NT-4, labeling for nNOS was dislocated to the cytoplasmic region in most muscle fibers (Figs. 4A3, 4B3), labeling for N-CAM became strong in small-sized muscle fibers (Figs. 4B4, 4C4), and labeling for embryonic MyHC was either strong or very weak (Fig. 4C5).

Neurotrophic Factor Receptors at NMJs

Given the finding that the percentages of NMJs labeled with BDNF, NT-4, and GDNF were significantly reduced in limb muscles of SOD1\textsuperscript{G93A} transgenic mice at 150 days, we investigated the NTF receptors in both control mice and SOD1\textsuperscript{G93A} transgenic mice at 150 days, to evaluate whether the receptors were similarly affected on NMJs of SOD1\textsuperscript{G93A} transgenic mice at 150 days.

**Figure 3.** Light microscopy images of NMJs of limb muscles (Limb) from control (WT) and SOD1\textsuperscript{G93A} transgenic mice (TG) at 150 days (150D) double-labeled with antibodies (green) against BDNF (A, B), NT-4 (C, D), NT-3 (E, F), and GDNF (G, H) and α-bungarotoxin (BTx, red). Note the weak (short arrow) or absent (long arrows) staining at NMJs in limb muscles of SOD1\textsuperscript{G93A} transgenic mice at 150 days with BDNF (B1), NT-4 (D1), NT-3 (F1), and GDNF (H1).

**p75\textsuperscript{NTR}.** In EOMs of both control and SOD1\textsuperscript{G93A} transgenic mice at 150 days, the vast majority of NMJs were labeled with p75\textsuperscript{NTR} (95.0% ± 5.5% and 95.5% ± 5.0%, respectively; Figs. 5A–D, 5K). In limb muscles of control mice at 150 days, a similar percentage of NMJs (97.5% ± 6.0%) was labeled with p75\textsuperscript{NTR} (Figs. 5E, 5F, 5K). However, in limb muscles of SOD1\textsuperscript{G93A} transgenic mice at 150 days, the percentage of labeled NMJs was only 61.0% ± 14.7%, which was significantly lower (37% lower, \(P = 0.0001\)) than that in limb muscles of age-matched control mice (Figs. 5G–K).

**Tyrosine Kinase Receptor B.** In EOMs of both control mice and SOD1\textsuperscript{G93A} transgenic mice at 150 days, most NMJs were labeled with TrkB, and there was no statistically significant difference in the percentage of positively labeled NMJs between the control and SOD1\textsuperscript{G93A} transgenic group (Fig. 6A). In limb muscles of control mice at 150 days, the vast majority of NMJs (94.1% ± 5.2%) was labeled with TrkB (Figs. 6A, 6D); however, in limb muscles of SOD1\textsuperscript{G93A} transgenic mice at 150 days, only 61.0% ± 4.8% NMJs were labeled with TrkB, which was significantly lower (35% less, \(P < 0.0001\); Fig. 6A). Most NMJs labeled with TrkB were also labeled with BDNF or with NT-4, whereas a small number of NMJs labeled with TrkB were unlabeled with NT-4 (Figs. 6E–G).

**Tyrosine Kinase Receptor C.** In EOMs of control mice and SOD1\textsuperscript{G93A} transgenic mice at 150 days, the number of NMJs positive to TrkC was 78.5% ± 6.0% and 76.2% ± 9.7%, respectively (Fig. 6B). In limb muscles of control mice at 150 days, 92.6% ± 8.8% of NMJs were positive to TrkC (Figs. 6B,
6H), whereas in limb muscles of SOD1 G93A transgenic mice at 150 days, only 49.7% of NMJs were positive to TrkC, which was significantly lower (46% less, \( P < 0.0001 \), Fig. 6B).

Presence of NT-3 labeling but absence of TrkC labeling was occasionally observed in NMJs of limb muscles from SOD1G93A transgenic mice at 150 days (Figs. 6I–K).

**Glial Cell Line–Derived Neurotrophic Factor Family Receptor-α1.** In EOMs of control mice and SOD1G93A transgenic mice at 150 days, comparable numbers of NMJs were labeled with GFR-α1 (68.0% ± 12.5% and 78.6% ± 9.3%, respectively; Fig. 6C). In limb muscles of control mice at 150 days, approximately 97.5% ± 5.6% of NMJs were labeled with GFR-α1 (Figs. 6C, 6L), whereas in limb muscles of age-matched SOD1G93A transgenic mice, the corresponding value was 87.9% ± 3.6%, which was 10% less, but not statistically significant (Fig. 6C). Neuromuscular junctions with the presence of GFR-α1 but absence of GDNF were more frequently encountered than NMJs with simultaneous expression of GFR-α1 and GDNF (Figs. 6M–O).

**DISCUSSION**

The present study is, to the best of our knowledge, the first to systematically examine the distribution of four different NTFs in EOMs and limb muscles of control mice and SOD1G93A transgenic mice at early (50 days) and terminal (150 days)
stages. The major findings include the following: in the control mice, there was an intrinsic difference in NTF expression on NMJs between EOMs and limb muscles; and in the SOD1G93A transgenic mice at terminal stage, the expression of NTFs and their receptors on NMJs was significantly decreased in limb muscles, but not in EOMs, in comparison with age-matched control mice.

Extraocular muscles differ from all other limb and trunk muscles in terms of developmental origin, innervation, structural organization, fiber type composition, and gene expression profile.31–35 The present study demonstrated the uniqueness of the EOMs with regard to NTFs in NMJs between EOMs and limb muscles; and in the SOD1G93A transgenic mice at terminal stage, the expression of NTFs and their receptors on NMJs was significantly decreased in limb muscles, but not in EOMs, in comparison with age-matched control mice.

We propose that the difference in NTFs between the EOMs and limb muscles in control mice is intrinsic, most likely due to the difference in developmental origin and innervation between the two muscles.35–36 Whether this intrinsic difference in the expression of NTFs on NMJs is correlated with the sparring of the EOMs in ALS is thus apparently worth exploring further.

In limb muscles of SOD1G93A transgenic mice at 150 days, the expression of BDNF, NT-4, and GDNF on NMJs was significantly decreased in comparison with the same muscles of control mice at both 50 days and 150 days. Fischer et al.5 reported that the first structural change in limb muscles of the SOD1 G93A transgenic mouse model is denervation of the NMJs, occurring long before loss of alpha-motor neurons. We have recently observed, in both ALS patients and ALS transgenic mice, severely affected NMJs in limb muscles but not in EOMs.13,15 Similarly, the NMJs in EOMs of both ALS patients and the ALS transgenic mouse model were spared and the nerve contacts were well maintained with normal composition in neurofilament, synaptophysin, laminins, and Wnt isoforms.12–15 Administration of BDNF, NT-3, and GDNF in neonatal rats can significantly rescue extraocular motor neurons and extraocular muscles from axotomy-induced cell death,24 suggesting a potential neuroprotective effect of NTFs on oculomotor neurons and the EOMs in ALS.

Neurotrophic factors have been proven to play crucial roles in regulating synapse development, growth, and plasticity: BDNF triggers synaptic potentiation, growth, and repair,37,38 whereas GDNF is associated with hyperinnervation of NMJs and multiple endplate formation.39,40 In addition, administration of exogenous BDNF and NT-4 lead to enhanced neuromuscular transmission in the adult rat.41 Taking these into consideration, the present results show that NTFs were
significantly reduced on NMJs in limb muscles but were unchanged in EOMs of SOD1G93A transgenic mice at 150 days, suggesting that the NTFs may be closely associated with the maintenance of nerve-muscle connections and sparing of the EOMs and the wasting of limb muscles in the SOD1G93A transgenic mice.

The present study revealed significant reduction of the receptors p75 NTR and TrkB at NMJs of limb muscles in SOD1G93A transgenic mice at 150 days, indicating simultaneous declines in BDNF and NT-4 and their specific receptors. However, such simultaneous declines were not observed between NT-3 and TrkC, or between GDNF and GFR-α1 in SOD1G93A transgenic mice at 150 days. Asynchronous changes have been reported in other studies. In muscles of ALS patients, the mRNA level of GDNF was significantly changed, but the mRNA level of its receptor GFR-α1 was unchanged. Similarly, in spinal motor neurons of ALS patients, mRNA level of NT-3 was declined but its receptor TrkC was unchanged. Whether the simultaneous declines in the NTFs and their specific receptors in limb muscles of SOD1G93A transgenic mice at 150 days are coincident or whether there is a causal relationship between the two events is currently unknown. Interestingly, a study on BDNF and TrkB in aging mice revealed that although endogenous BDNF was less available at NMJs of diaphragm at 18 months of age, reduction in TrkB occurred at 24 months of age, indicating a sequential relationship between changes in NTF and its receptor.

Neurotrophic factors were present in the muscle fibers of both EOMs and limb muscles, generally with no significant difference between control and SOD1G93A mice at either early or terminal stages. However, BDNF and/or NT-4 were significantly increased in some small-sized limb muscle fibers of 150-day SOD1G93A transgenic mice. To further characterize the small-sized fibers showing increased BDNF and/or NT-4, immunostaining with nNOS and N-CAM also was performed. The immunoreactivity with nNOS mostly disappeared at the periphery but increased in the sarcoplasmic region of these muscle fibers, along with increased N-CAM. Previous studies

**Figure 6.** Quantification of the percentage of NMJs labeled with TrkB (A), TrkC (B), and GFR-α1 (C) in EOMs (EOM) and limb muscles (Limb) in control (WT) and SOD1G93A transgenic (TG) mice at 150 days (150D). (D–O) Light microscopy images of NMJs of limb muscles from control (D, H, L) and SOD1G93A transgenic mice (E–G, I–K, M–O) at 150 days double-labeled with antibodies (green) against TrkB (D, E), TrkC (H, I), and GFR-α1 (L, M) and α-bungarotoxin (BTx, red). Note the presence of TrkB (arrowhead in E) but the absence of NT-4 (arrow in G) at the same motor endplate (F); the absence of TrkC (arrow in I) but the presence of NT-3 (arrowhead in K) at the same motor endplate (J); and the presence of GFR-α1 (arrowhead in M) but the absence of GDNF (arrow in O) at the same motor endplate (N) in limb muscles of SOD1G93A transgenic mice. *P < 0.05.
have shown that N-CAM is normally expressed at NMJs in healthy muscles but becomes concentrated to the sarcoplasmic region in denervated and regenerated fibers, \cite{30,45,46} whereas nNOS is normally expressed in the sarcolemma of healthy muscles but absent from denervated muscle fibers. \cite{63} We propose that the small-sized muscle fibers with muscle fiber loss of BDNF and/or NT-4 were denervated fibers in the case they were negative with the antibody against embryonic MyHC, or regenerating muscle fibers in the case they were positive with the antibody against embryonic MyHC.

We recently examined mRNA expression of NTFs in the same muscle tissues and in the same ALS mouse model and found early upregulation of NT3 and GDNF mRNA in EOMs, which we propose to be protective from ALS. The results of the alterations of NTFs between the mRNA and protein levels in the present study were not completely comparable (Supplementary Table S1). Discrepancy between mRNA and protein levels also have been observed in BDNF, NT-3, NT-4, and GDNF during postnatal development in rat hindlimb muscles. \cite{47} Glial cell line-derived neurotrophic factor mRNA is expressed at the highest level at birth and decreases dramatically in the first 3 weeks, whereas GDNF protein is very low at birth and increases during the first 2 weeks and is then maintained at 3 months of age. \cite{47} The differences in NTF alterations between mRNA and protein levels may be due to the following: (1) that the mRNA expression detected with quantitative RTPCR is a sum of all structures within the muscle tissue, whereas in the present study the NTFs were visualized in individual structures, rather than an immunoblot analysis; (2) NTF alterations in each individual structure were different (i.e., NTFs were decreased at NMJs but increased in atrophied muscle fibers); and (3) the mRNA level may not reflect the rate of protein synthesis and the steady state of protein content. \cite{68} Nevertheless, both our previous and present findings suggest that NTFs may play a crucial role for the sparingness of EOMs and the vulnerability of limb muscles in ALS.

Neurotrophic factors have earlier been considered the most promising growth factors in clinical treatment of ALS; however, clinical trials failed to demonstrate therapeutic effects. \cite{14,49} Brain-derived neurotrophic factor was effective in phase I and II studies for ALS patients, but failed to show survival improvement in a large-scale phase III trial. \cite{51} As pointed out in some studies, the failure of these clinical trials might be due to improper administration sites or target tissue. \cite{49} Conventionally, ALS was believed to be a pure motor neuron disease. Thus, in almost all clinical trials, the targets of NTF delivery were the degenerating motor neurons and the NTFs were administered through viral delivery or direct infusion into the brain or spinal cord. However, the present data suggest that the NMJs and the skeletal muscles might be critical delivery sites for the treatment of ALS with NTFs. Another possible cause of failure of previous clinical trials may relate to improper NTF selection. \cite{48,50} Brain-derived neurotrophic factor has been mostly used in clinical trials, mainly based on preclinical results from other experimental mouse models (wobbler mice and axonotomy or progressive motor neuronopathy), \cite{52} rather than SOD1 mutant models. In the present study and in our previous study, \cite{16} we revealed that BDNF does not seem to be closely associated with ALS, and instead, GDNF and NT-4 seem to be better candidates. \cite{16,53,54} Finally, previous failures in clinical trials of ALS involving NTFs might be related to improper time of treatment. \cite{55} An overview of previous clinical trials revealed that most treatments of ALS patients using NTFs were performed when the typical symptoms of ALS appeared. \cite{56} However, data show that alterations in skeletal muscles and NMJs occurred very early. \cite{58} Similarly, very recently we showed early decrease of NT-4 at the mRNA level in limb muscles but increase of GDNF and NT-4 at the mRNA level in EOMs already in early-stage SOD1\textsuperscript{G93A} transgenic mice. \cite{66} We propose that these early changes in NTFs may be critical for the fate of motor neurons and muscle fibers later in ALS. Thus, a raising possibility is that successful treatment of ALS patients with NTFs is unrealistic, as it would require administration at a very early stage, before the onset of ALS symptoms and diagnosis.

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