Alteration of Neurotrophic Factors and Innervation in Extraocular Muscles of Individuals With Concomitant Esotropia

Jie Hao,[1,2] Meixu Wang,[1,2] Jiawen Liu,[3] Mayinuer Yusufu,[4,5] Kai Cao,[1,2] and Jing Fu[1,2]

[1]Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing, China
[2]Beijing Key Laboratory of Ophthalmology & Visual Sciences, Beijing, China
[3]Industrial Engineering and Operations Research, University of California, Berkeley, California, United States
[4]Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, East Melbourne, Australia
[5]Department of Surgery (Ophthalmology), The University of Melbourne, Melbourne, Australia

Correspondence: Jing Fu, Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, No. 1 Dong Jiao Min Xiang Street, Dongcheng District, Beijing 100730, China;
fu_jing@126.com.

Received: June 9, 2023
Accepted: February 15, 2024
Published: March 5, 2024

Citation: Hao J, Wang M, Liu J, Yusufu M, Cao K, Fu J. Alteration of neurotrophic factors and innervation in extraocular muscles of individuals with concomitant esotropia. *Invest Ophthalmol Vis Sci.* 2024;65(3):1. https://doi.org/10.1167/iovs.65.3.1

Purpose. To determine whether neurotrophic factors and innervation in extraocular muscles (EOMs) were altered in different types of concomitant esotropia, and to explore the possible association between neurotrophic factors and innervation of EOMs in humans.

Methods. Patients with concomitant esotropia who required strabismus surgery were recruited from January to December 2022. Lateral rectus EOMs were obtained from patients, and controls were obtained from deceased organ donors. Immunofluorescence (IF) was performed to detect innervation of EOMs (neurofilament and synaptophysin), and immunohistochemistry (IHC) was used to detect the neurotrophic factors insulin-like growth factor-1 (IGF-1), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and neurotrophin-3 (NT-3). The positive IHC results were further verified using western blotting (WB). One-way ANOVA followed by a Dunnett's multiple comparison post hoc test was used for continuous variables and the χ^2 test for categorical variables. Spearman correlation analysis was used for the correlation analysis.

Results. We collected lateral rectus EOM samples from acute and chronic types of concomitant esotropia and controls. Consistent with IHC, WB showed that IGF-1 was significantly increased in patients with acute acquired comitant esotropia or essential infantile esotropia compared with controls. In IF, synaptophysins were significantly increased only in acute acquired comitant esotropia compared with controls. Furthermore, Spearman correlation analysis showed that the correlation between IGF-1 and synaptophysin was borderline ($P = 0.057$) for patients with acute acquired comitant esotropia.

Conclusions. Our study highlights the role of IGF-1 and altered innervation of EOMs in acute acquired comitant esotropia, suggesting that an effect of increased IGF-1 on nerve innervation may temporarily cause a compensatory increase in the strength of lateral rectus muscles.

Keywords: neurotrophic factors, innervation, extraocular muscle, concomitant esotropia

Concomitant esotropia is a manifest convergent misalignment of the visual axes.[1] The estimated global prevalence of esotropia is 0.77% (0.59%–0.95%).[2] Its major types include essential infantile esotropia, accommodative esotropia, and nonaccommodative esotropia, all of which involve an imbalanced strength of lateral and medial rectus extraocular muscles (EOMs). EOMs undergo continuous remodeling and plasticity throughout life[3] and are rich in innervation, with features of producing faster contraction rates and resistance to muscle fatigue.[3,4] They are innervated by ocular motor neurons that branch off numerous nerve fibers that form neuromuscular junctions.[5] The innervation of EOMs may have inherent plasticity at both the muscle and the motor neuron levels to maintain eye alignment. Unlike incomitant esotropia, in which the underlying etiology can be attributed to paralysis of the ocular motor system, the underlying molecular and neurophysiological basis of concomitant esotropia is extremely complex and heterogeneous, influenced by genetic and environmental factors.[6,7]

The innervation of EOMs can be regulated by changes in signaling molecules, which may be determined by EOM-specific gene expression.[8] Gene expression was found to be altered in strabismic human EOMs and tendons,[7] including the expression of neurotrophic factors that regulate the frequency and strength of muscle contractions by modulating the number of nerve fiber branches and

the density of neuromuscular junctions.[9] Neurotrophic factors[6,10] are a family of proteins that are identified and produced in the neurons and muscles, including glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), neurotrophin-3 (NT-3), and nerve growth factor (NGF). They play a critical role in regulating the survival and development of both neurons and muscle fibers and can be transported in either anterograde or retrograde directions between neurons and EOMs.[6] EOMs express GDNF and IGF-1, and the expression levels of BDNF, NT-3, and NGF are higher compared with facial, buccinator, tongue, and limb muscles.[7,10–12] The effects of neurotrophic factors on EOM contraction profiles and patterns of innervation are diverse. Abnormal signaling by neurotrophic factors may be one possible cause of strabismus.[13] In individuals with strabismus, GDNF was downregulated, IGF-1 expression was inconsistent, and BDNF expression was not changed.[7] In previous non-human primates studies,[14,15] the number of nerve fibers and the density of neuromuscular junctions can be regulated by exogenously applied GDNF, leading to eye alignment changes; however, the sustained delivery of 2 µg/day BDNF for 3 months to EOMs did not induce strabismus in infant monkeys.[16]

Previous research on neurotrophic factors in strabismus has mostly focused on the detection of gene expression in human EOMs, as well as on functional studies in animal models. However, to our knowledge, little research has assessed various neurotrophic factors at the protein level in different types of concomitant esotropia and their effects on the innervation of EOMs. Our study proposed a hypothesis that there may be abnormal protein expression of neurotrophic factors and innervation of EOMs in patients with concomitant esotropia and that a potential relationship may exist between them. Thus, our study aimed to determine whether neurotrophic factors and innervation in EOMs are altered in different types of concomitant esotropia and to explore possible associations between neurotrophic factors and innervations of EOMs in humans.

Methods

Sample Collection

A prospective study was conducted from January 2022 to December 2022, with ethics approval from the Institutional Review Board of Beijing Tongren Hospital, Capital Medical University (TREC2022-KY042). Informed consent was obtained from each adult or parental/guardian. The trial registration ID was ChiCTR2100053717. This study complied with the tenets of the Declaration of Helsinki.

Patients with concomitant esotropia who required strabismus surgery were recruited, including acute type (acute acquired comitant esotropia [AACE]) and chronic types (essential infantile esotropia [EIE], partially accommodative esotropia [PAE], and nonaccommodative esotropia [NAE]). Information on age, gender, and history of previous ophthalmic and systemic diseases was recorded. Ophthalmic examinations were conducted, including best-corrected visual acuity, refractive status, slit-lamp biomicroscopy, fundus photography, and prism cover and uncover tests. Patients were excluded if they met any of the following criteria: previous history of intracranial and systemic diseases, history of eye surgery, amblyopia, nystagmus, paralytic or non-paralytic EOM disorder with abnormal eye movement, or esotropia accompanied by vertical strabismus. We used lateral rectus EOMs, as resection can only be performed on the weakened horizontal muscles in patients with concomitant esotropia. Lateral rectus EOMs of the controls were obtained from deceased organ donors who had no record of meeting any of the above exclusion criteria. Briefly, lateral rectus muscles were resected behind the muscle insertion in patients with concomitant esotropia (8.64 ± 1.53 mm in AACE, 8.88 ± 1.28 mm in EIE, 8.02 ± 1.91 mm in NAE, and 7.75 ± 1.94 mm in PAE), and in the controls with the range of 8 to 9 mm. EOM samples were separated from tendons tissues, divided into fractions, and stored at −80°C or embedded in paraffin.

Immunohistochemistry and Immunofluorescence

Prior to immunostaining, hematoxylin and eosin staining was performed to test and verify that EOMs were obtained, and then deparaffinization and rehydration were performed. Antigen retrieval was performed for all antibodies using EDTA antigen retrieval buffer or citrate buffer heated to 100°C. Sections were stained with the following antibodies and dilutions for immunohistochemistry (IHC): Anti-IGF-1 (ab106836, 1:300; Abcam), which is goat polyclonal to IGF-1 and recognizes human; Recombinant Anti-BDNF antibody [EPR1292] (ab108319, 1:500; Abcam), which recognizes mouse, rat, and human; Anti-GDNF antibody (ab119473, 1 µg/mL; Abcam),which recognizes rat and human; NT-3 Polyclonal antibody (18084-1-AP, 1:100; Proteintech), which recognizes human, mouse, and rat; NF-H/NF200 Polyclonal antibody (18934-1-AP, 1:200; Proteintech); and Synaptophysin Monoclonal antibody (60191-1-Ig, 1:200; Proteintech).

Sections were then incubated with primary antibodies at 4°C in the indicated blocking buffer dilutions overnight. For IHC, sections were stained with horseradish peroxidase (HRP)-labeled Goat Anti-Rabbit IgG H&L (ab6721, 1:1000; Abcam; BDNF, GDNF, and NT-3) or HRP-labeled Donkey Anti-Goat IgG (H+L) (A0181, 1:100; Beyotime, Jiangsu, China; IGF-1) for 60 minutes at 37°C. After the sections were washed with PBS, 3,3′-diaminobenzidine (DAB) substrate solution (ZLI-9017; ZSGB Biotech, Beijing, China) was added to the slides, which were subjected to Mayer's hematoxylin staining (G1080; Solarbio Life Science, Beijing, China).

For IF, the secondary antibodies of the corresponding species of the primary antibody were successively added and incubated in the dark for 1 hour: 1:500 Goat Anti-Rabbit IgG H&L (Alexa Fluor 488) (ab150077; Abcam) and 1:500 Goat Anti-Mouse IgG H&L (Alexa Fluor 594) (ab150116; Abcam). The sections were mounted with an anti-fluorescence quenching mounting medium. Furthermore, in control tissue, except for normal control representing samples from the deceased organ donors, a blank control was designed for the omission of the primary antibodies to rule out any cross-reactions with the secondary antibody.

Based on a previous study,[16] where α-bungarotoxin was used to visualize neuromuscular junctions, we stained with double-labeling synaptophysin and α-bungarotoxin to ascertain that all α-bungarotoxin–labeled neuromuscular junctions in the EOM specimens colocalized with presynaptic components. Therefore, in addition to synaptophysin, α-bungarotoxin (00005-100UG, 1 µg/mL; Biotium, Fremont, CA, USA) was added to assist in demonstrating neuromuscular junctions. Furthermore, Anti-beta III Tubulin antibody -

Neuronal Marker (ab18207, 1 µg/mL; Abcam) was also used for co-staining to verify that the signal expression colocalized with neurofilament.

Sections were observed under an ECLIPSE Ti confocal microscope (Nikon, Tokyo, Japan), and images were collected. The nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI) were blue under the excitation of ultraviolet, the positive expression of neurofilament was fluorescein-labeled green, and synaptophysin was fluorescein-labeled red. Image J software (National Institutes of Health, Bethesda, MD, USA) converted the values into IntDen or integrated optical density and semiquantitatively analyzed the relative expression. Six visual fields were randomly taken across the sections and the grayscale value was averaged for IHC. In the morphometric analyses of neurofilament, density was calculated as the number of labeled bright-green–positive nerve fibers per square micrometer of cross-sections. In the entire cross-sections from three slides, positive staining was counted and averaged to determine statistical significance. Similarly, synaptophysin density was also determined and reported as number of labeled bright-red–positive neuromuscular junctions per cross-sectional area (μm^2).

Western Blot

The positive results obtained from IHC were further verified using western blotting (WB). Radioimmunoprecipitation assay (RIPA) buffer (R0010; Solarbio Life Science) containing protease inhibitors was added for homogenization. The supernatant was obtained after centrifugation at 14,000*g* at 4°C. Protein concentration was detected using a BCA Protein Assay Kit (PC0020; Solarbio Life Science). The sample (20 µg/µL) was added to SDS-PAGE gel for electrophoresis separation of proteins; after protein separation, it was transferred to a cellulose membrane (ISEQ00010; EMD Millipore, Burlington, MA, USA) for imprinting. After the transfer was completed, the protein-adsorbed membrane was blocked with 5% skim milk at room temperature for 1 hour, and then IGF-1 primary antibody (ab9572, 1:300; Abcam) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ab8245, 1:5000; Abcam) were added for overnight incubation at 4°C. After the primary antibody incubation was completed, a 1:1000 diluted secondary antibody (A0208; Beyotime) was added for 1 hour at room temperature, and the membrane was washed three times, after which it was transferred to a Gel Doc XR imaging system (Bio-Rad, Hercules, CA, USA). An enhanced chemiluminescence (ECL) solution (P0018FM; Beyotime) was then added, and ImageJ was used to compare the densities of the bands in the WB.

Data Analysis

Continuous variables, such as age, amount of deviation, and the expression of neurotrophic factors, were expressed as mean ± SD. For the comparisons of continuous variables among patients with concomitant esotropia and the controls, one-way ANOVA followed by Dunnett's multiple comparison post hoc test was used to determine significance. The one-way ANOVA can only reveal that there is a difference in the overall comparison among the means of all groups and does not specify which particular groups have differing means. To identify these specific differences, further post hoc testing is needed. There are many methods for multiple comparisons. Of them, Dunnett's multiple comparison post hoc test is suitable for comparing a specific group with other groups. In this study, we employed Dunnett's test to compare different types of esotropia with the controls. The χ^2 test was used for categorical variables. Spearman correlation analysis was used to evaluate the correlation between neurotrophic factors and innervation of EOMs in concomitant esotropia patients and the controls. The correlations between age and the expression of neurotrophic factors were also analyzed. Statistical analysis was performed with SPSS Statistics 26.0 (IBM, Chicago, IL, USA). A two-sided *P* value of less than 0.05 was considered to be statistically significant.

Results

Basic Characteristics

All of the samples were lateral rectus EOMs resected from patients with concomitant esotropia and the controls, including 18 patients with AACE, 16 patients with EIE, 27 patients with NAE, 20 patients with partially accommodative esotropia, and 21 controls (Table 1).

Immunofluorescence Analysis

Immunofluorescence (IF) was performed to detect the expression of neurofilament and synaptophysin in lateral rectus muscles. Neurofilament represents nerve fibers, and synaptophysin demonstrates neuromuscular junction.

Table 1. Basic Characteristics of Controls and Patients With Concomitant Esotropia

Variables	AACE	EIE	NAE	PAE	Controls	*P*
Age (y)	18.17 ± 15.10	9.50 ± 15.95	12.70 ± 12.06	4.10 ± 1.21	49.0 ± 13.21	<0.001*
Sex (female/male), *n*	5/13	8/8	12/18	10/10	4/17	0.155
Deviation, 33 cm (PD)	65.29 ± 19.96	65.33 ± 19.32	62.59 ± 17.62	61.25 ± 19.19	—	0.887
Deviation, 6 m (PD)	65.29 ± 19.96	63.00 ± 17.30	61.48 ± 17.20	59.75 ± 18.74	—	0.820
SE OD (D)	–2.59 ± 3.91	0.28 ± 2.86	–0.10 ± 2.98	2.7 ± 1.57	—	<0.001*
SE OS (D)	–2.46 ± 3.87	0.65 ± 2.55	–0.23 ± 2.88	2.81 ± 1.84	—	<0.001*
History (y)	1.80 ± 1.80	9.5 ± 15.95	5.03 ± 6.16	2.07 ± 1.19	—	0.030*
Recession (mm)	7.03 ± 2.53	6.53 ± 2.99	6.33 ± 1.99	6.88 ± 2.44	—	0.780
Resection (mm)	8.64 ± 1.53	8.88 ± 1.28	8.02 ± 1.91	7.75 ± 1.94	—	0.172
n	18	16	27	20	21	—

Unless otherwise indicated, the data are shown as mean ± SD. One-way ANOVA was used to determine the significance of continuous variables. OD, right eye; OS, left eye; PD, prism diopters; SE, spherical equivalent.

* A two-sided *P* value of less than 0.05 was considered to be statistically significant.

Investigative Ophthalmology & Visual Science

Table 2. One-Way ANOVA Followed by Multiple Comparisons for Neurotrophic Factors and Innervation in EOMs

Variable	*F*	*P*	Multiple Comparisons With Control	*P*
GDNF	0.929	0.451	AACE vs. C	0.961
			EIE vs. C	1.000
			NAE vs. C	0.977
			PAE vs. C	0.485
BDNF	1.732	0.151	AACE vs. C	0.336
			EIE vs. C	0.686
			NAE vs. C	0.392
			PAE vs. C	0.963
IGF-1	3.371	0.013*	AACE vs. C	0.040*
			EIE vs. C	0.015*
			NAE vs. C	0.380
			PAE vs. C	0.995
NT-3	0.942	0.444	AACE vs. C	0.981
			EIE vs. C	1.000
			NAE vs. C	0.971
			PAE vs. C	0.414
NEFH	3.318	0.014*	AACE vs. C	1.000
			EIE vs. C	0.776
			NAE vs. C	0.208
			PAE vs. C	0.385
SYN	9.096	<0.001*	AACE vs. C	<0.001*
			EIE vs. C	0.829
			NAE vs. C	0.992
			PAE vs. C	0.540
NEFH-to-SYN ratio	5.007	0.001*	AACE vs. C	0.603
			EIE vs. C	0.717
			NAE vs. C	0.025*
			PAE vs. C	1.000

One-way ANOVA followed by a Dunnett's multiple comparison post hoc test was used to determine significance of continuous variables. C, control; F, ANOVA analysis statistic; NEFH, neurofilament, heavy polypeptide; SYN, synaptophysin.

*A two-sided *P* value of less than 0.05 was considered to be statistically significant.

Synaptophysin is a presynaptic protein located in synaptic vesicles that can be used as a marker of the neuromuscular junction.[16,17] With one-way ANOVA, synaptophysin density appeared to be significantly different in all types of esotropia ($P < 0.001$), but further Dunnett's post hoc tests showed that the difference was significant only in AACE ($P < 0.001$). Moreover, one-way ANOVA showed that, among patients with all types of concomitant esotropia and the controls, there were significant differences in the density of neurofilament structures ($P = 0.014$) and the ratio of neurofilament to synaptophysin structures ($P = 0.001$). Furthermore, according to Dunnett's multiple comparison post hoc test, compared to the controls, neurofilaments in patients with all types of concomitant esotropia were not expressed at levels that were significantly different. For patients with NAE compared to the controls, the ratio of neurofilament to synaptophysin was significantly higher ($P = 0.025$), but their neurofilaments and synaptophysin levels were not significantly different (Table 2, Fig. 1). Furthermore, when verifying the position of the neuromuscular junction and muscle fiber under a confocal microscope, where synaptophysin and α-bungarotoxin display co-staining, the signals were almost similar in controls, as well as co-staining with neurofilaments and beta-III tubulin (Fig. 2).

IHC Analysis

One-way ANOVA showed that, among patients with all types of concomitant esotropia and the controls, there was a significant difference in IGF-1 signal ($P = 0.013$) when followed by a Dunnett's multiple comparison post hoc test compared with the controls; only the IGF-1 signal was significantly increased in patients with AACE ($P = 0.04$) and EIE ($P = 0.015$). However, no significant differences in neurotrophic factors were found between other types of concomitant esotropia and the controls (Table 2, Fig. 3).

WB Analysis

Based on the positive results from IHC, further IGF-1 analysis was performed using WB for AACE ($n = 13$), EIE ($n = 16$), and controls ($n = 14$). One-way ANOVA showed that, among patients with the two types of concomitant esotropia and the controls, there was a significant difference in IGF-1 content ($P < 0.0001$). Furthermore, results of a Dunnett's multiple comparison post hoc test showed that, compared with the controls, the IGF-1 signal was significantly increased in patients with AACE ($P < 0.0001$) and EIE ($P = 0.023$). These data were consistent with the IHC results (Fig. 4).

Association Between IGF-1 and Innervation of EOMs

Because of the findings of significantly increased IGF-1 in AACE, as well as significantly increased synaptophysin, the potential relationship between IGF-1 and synaptophysin was further analyzed in AACE. Figure 2 confirms the existence of IGF-1 and synaptophysin in the EOM specimens in AACE (Figs. 2G–2I). In a non-human primate model,[18] it was shown that a continuous release pellet of IGF-1 resulted in an increased density of neuromuscular junctions. Although Spearman correlation analysis showed the correlation between IGF-1 and synaptophysin was borderline ($n = 17$; $P = 0.057$) for patients with AACE, we suggest that, within a certain range, there could be a possible connection between increased IGF-1 and increased synaptophysin in the acute type. However, there were no significant differences in synaptophysin for other chronic types of concomitant esotropia compared to controls, indicating that this hypothesis cannot be extended to other chronic types of esotropia.

Discussion

Neurotrophic factors are essential for neuronal survival during development, and they mediate synaptic and morphological plasticity.[19] Neurotrophic factors are present in axons and dendrites of growing neurons and in pre- and postsynaptic terminals of neurons.[17] In a previous strabismus animal model, multiple slender nerve fibers were connected to a neuromuscular junction, and the number increased from 4.2% to 21.3% in neuromuscular junctions with more than one axon due to the effects of neurotrophic factors.[14] The findings on neurotrophic factors also indicated its involvement in trophic signaling alterations in the EOMs,[20] which can trigger a microcascade of plasticity at the muscle and motor neuron levels.[6] It has been demonstrated that continuous treatment with a neurotrophic factor has the potential to correct eye alignment in a sustained manner, which may override compensatory mechanisms in the ocular motor system.[14,18,21]

Investigative Ophthalmology & Visual Science

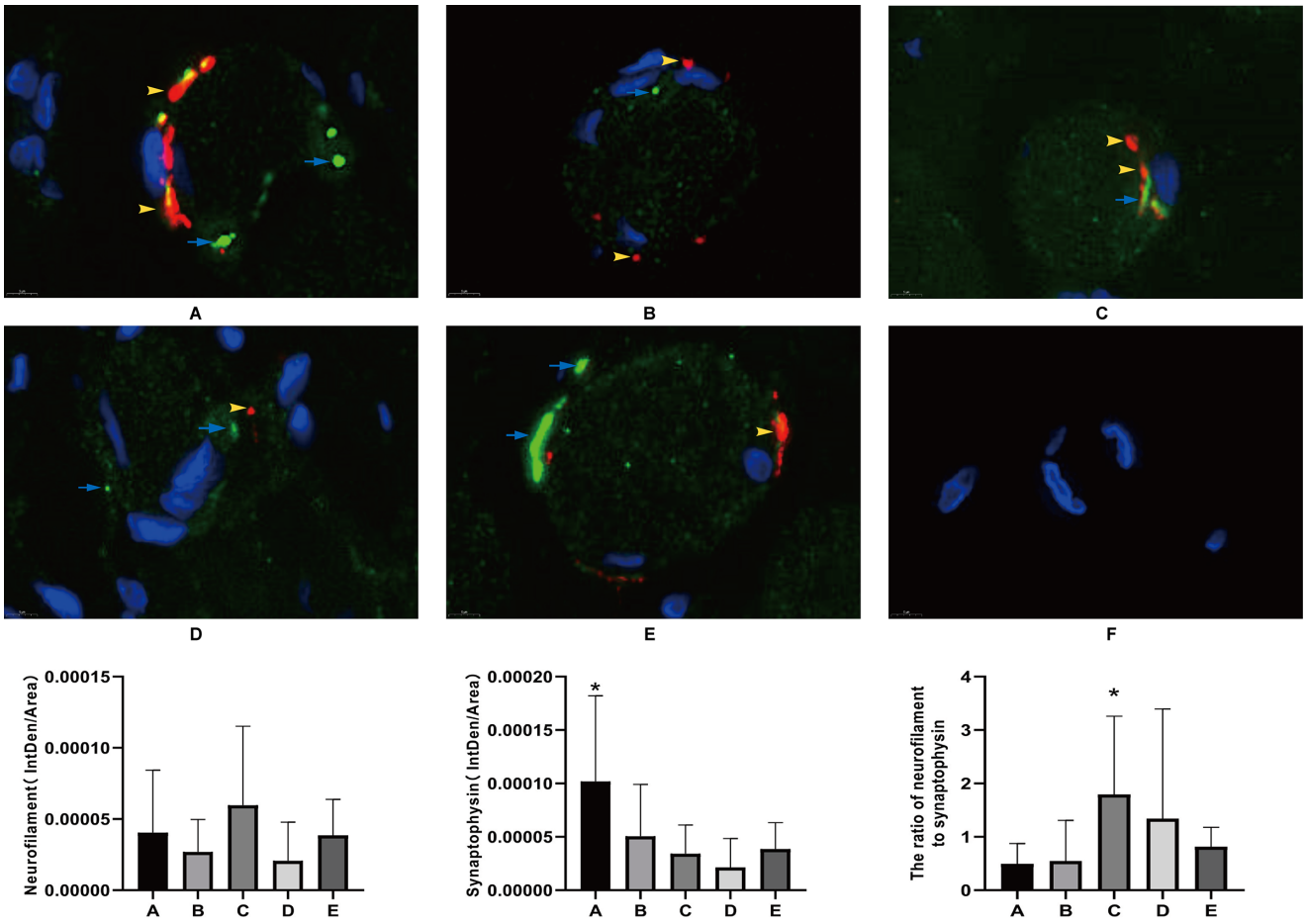

Figure 1. The expressions of neurofilament (*green*), synaptophysin (*red*), and nucleus (*blue*) in patients with concomitant esotropia compared with controls by immunofluorescence staining. The *Y*-axes of the graphs, from *left* to *right*, represent the expression of neurofilament and synaptophysin and the ratio of neurofilament to synaptophysin. From *left* to *right* on the *X*-axis of each graph, the displayed histograms correspond to the figure parts: AACE ($n = 17$) (**A**), EIE ($n = 16$) (**B**), NAE ($n = 25$) (**C**), PAE ($n = 20$) (**D**), normal control ($n = 21$) (**E**). The blank control shows omission of the primary antibodies (**F**). The *blue arrowheads* in the figures indicate neurofilament (*green*), and the *yellow arrowheads* indicate synaptophysin (*red*). IntDen, integrated density. The standard deviations are shown as *error bars* in the histograms. $^*P < 0.05$.

IGF-1 is synthesized from multiple sources, including the EOM itself, the systemic circulation, innervating motoneurons, and Schwann cells within nerves, which may be the most prominent source of IGF-1 for EOMs.[22] In a previous study, the mRNA expression level of IGF-1 in EOMs was 21 times higher than that in other skeletal muscles.[8] In a study of strabismic human EOMs, microarrays showed that IGF-1 gene expressions did not change but was significantly upregulated 5.3-fold, based on the method of quantitative PCR (qPCR),[20] but other studies have demonstrated mixed results based on qPCR or PCR arrays.[7,23] Such results obtained by different methods demonstrate the complexity of IGF-1 expression. It is still controversial whether IGF-1 enhances neurite growth. IGF-1 may play an important role in synaptic maintenance and synaptic plasticity.[24] It was thought that, in developing EOMs and regenerating nerves, IGF-1 increased neurite growth.[25,26] However, the effect of exogenous IGF-1 on innervation density was not thought to extend beyond an adjustment of innervation to match the increased myofiber size in infant monkey and adult EOMs.[13,18] Animal experiments[13,18] have shown that continuous IGF-1 treatment results in muscle fiber enlargement and altered innervation density, that neuromuscular junctions can be increased by 55%, and that the area of the neuromuscular junction is expanded. After injection of 5 µg IGF-1 into EOMs, the contraction force of EOMs can be increased by 81%, whereas reduced IGF-1 in EOMs can weaken the force by 34%,[9] highlighting the role of IGF-1 in enhancing the strength of EOMs. However, besides the species differences, it is worth noting that various potentially confounding parameters may contribute to the complexity of possible scenarios. Among them, age is an important predictor of neuromuscular recovery after peripheral nerve injury, although IGF-1 was reported to increase axon number, diameter, and density in regenerated nerves of both young and aged animals.[26]

Until now, most of the previous studies have examined increased levels of exogenous IGF-1, and less is known about the levels and effects of endogenous IGF-1. In our study, endogenous IGF-1 was significantly increased in lateral rectus muscles in patients with AACE and EIE compared with controls. Significantly increased synaptophysin was only detected in AACE; however, the increased synaptophysin was not detected in other chronic types of

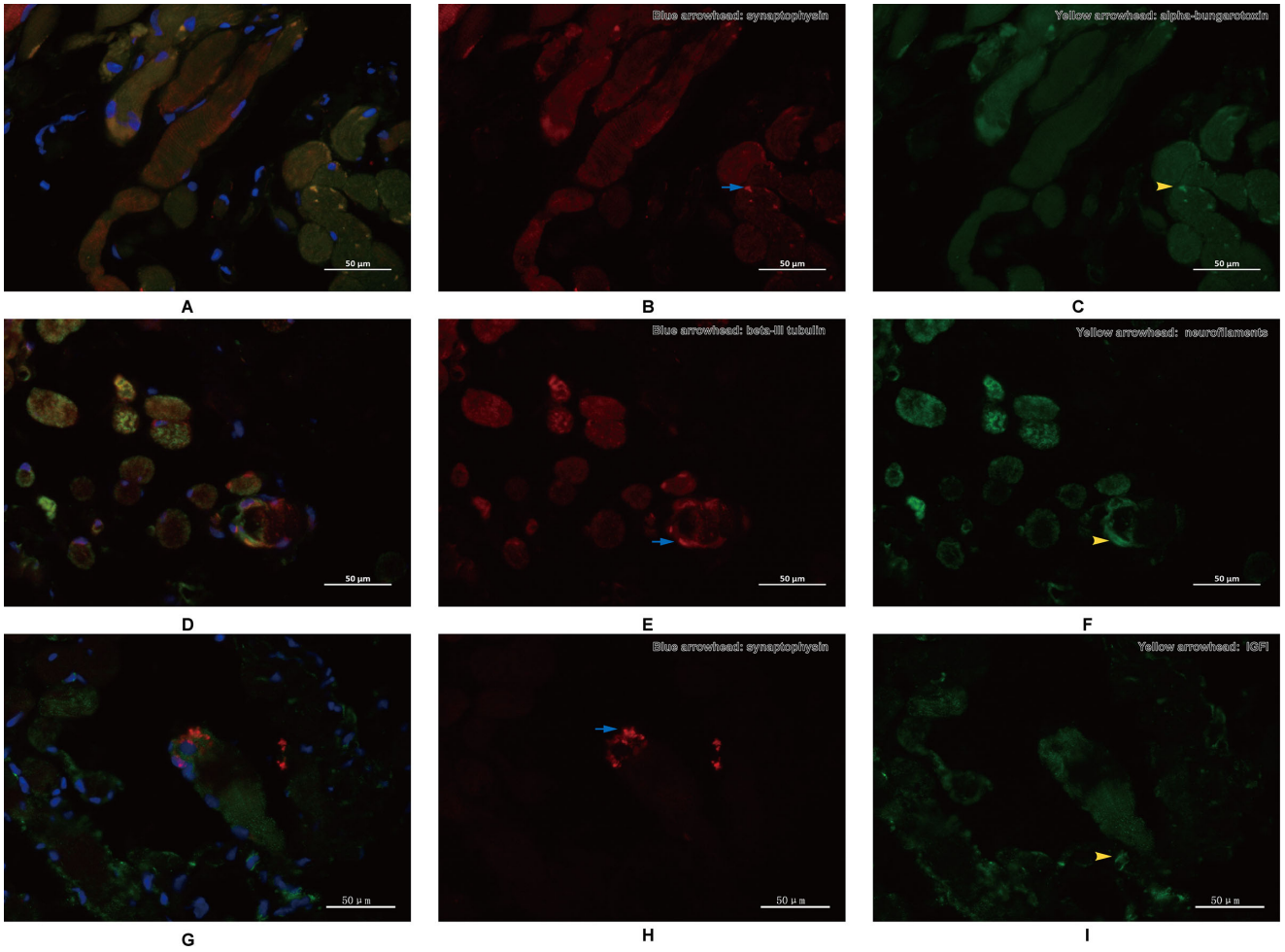

Figure 2. (**A–C**) Through immunofluorescence staining in controls, α-bungarotoxin was used for co-staining to verify the signal colocalized with synaptophysin. (**D–F**) Beta-III tubulin was double-labeled with neurofilament to test the colocalized signal expression. (**G–I**) Double-labeling of IGF-1 and synaptophysin confirmed the existence of two components in the EOM of the AACE. The *blue arrowheads* indicate the positive synaptophysin, beta-III tubulin, and synaptophysin in panels **B**, **E**, and **H**, respectively. The *yellow arrowheads* indicate the positive α-bungarotoxin, neurofilament, and IGF-1 in panels **C**, **F**, and **I**, respectively.

concomitant esotropia. This finding reflects the complexity of pathogenesis and reveals differences in the underlying molecular and neurophysiological bases of eye misalignment. AACE is a special type of esotropia that occurs suddenly, and the pathogenesis of AACE is still unclear. The medical history for AACE in our study was 1.80 ± 1.80 years, which was obviously shorter than other chronic types of concomitant esotropia (EIE, 9.5 ± 15.95 years; NAE, 5.03 ± 6.16 years; PAE, 2.07 ± 1.19 years). This suggests that the acute imbalance between horizontal rectus muscles may cause an increase in endogenous IGF-1, thereby promoting a possible temporary increase in synaptophysin and thereby a compensatory enhancement of lateral rectus muscle strength to overcome esotropia. This temporarily elevated synaptophysin may return to its original level over time, as in other types of concomitant esotropia.

The pathogenesis of concomitant esotropia is still unclear. Our study could not determine whether endogenous IGF-1 may be involved in the original development of the misalignment or is part of a secondary response that maintains the misalignment. Yet unknown factors ultimately weaken the strength of lateral rectus muscles relative to the medial rectus muscle, resulting in concomitant esotropia. One hypothesis is that, for the innervation of EOMs, the sudden imbalance between medial and lateral rectus muscles may secondarily stimulate IGF-1 to enhance neuromuscular junction density in AACE. The effect of increased IGF-1 on nerve innervation in lateral rectus muscles may temporarily cause a compensatory increase in muscle strength.

Except for IGF-1, this study did not find significant changes in protein levels of GDNF, BDNF, and NT-3 in EOMs of patients with esotropia. In a previous study, neurotrophic factors were clearly shown to be present in the EOM[27]; however, the roles of GDNF, BDNF, and NT-3 in the development of concomitant esotropia remain controversial. The effects of neurotrophic factors on ocular motor neuronal function vary in development as well as in the maintenance and plasticity of the adult ocular motor system.[6] GDNF is present in EOMs, and the mRNA expression of GDNF is 26-fold higher in EOMs than that in other skeletal muscles.[8] Overexpression of GDNF by muscle greatly increased the number of motor axons innervating neuromuscular junctions in neonatal mice.[28] In a previous study, the mRNA expression of GDNF was reduced in concomitant strabismus

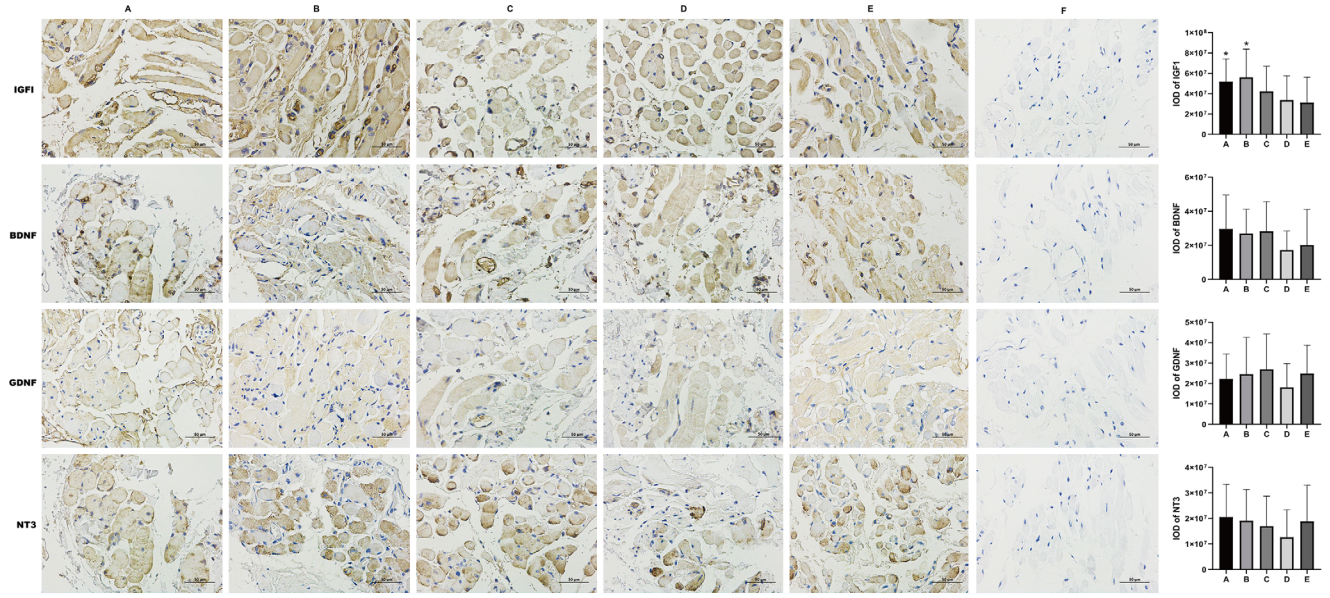

Figure 3. The expression of neurotrophic factors (BDNF, GDNF, IGF-1, and NT-3) in patients with concomitant esotropia compared with controls by immunohistochemistry staining. The *Y*-axes of the graphs displayed from *top* to *bottom* show the expression levels of IGF-1, BDNF, GDNF, and NT-3. The histograms on the *X*-axis of each graph correspond to AACE (**A**), EIE (**B**), NAE (**C**), PAE (**D**), normal control (**E**). The blank control shows omission of the primary antibodies (**F**). Positive staining is shown in *brown*. The corresponding sample numbers were $n = 18$, 15, 25, 17, and 20 for IGF-1; $n = 16$, 12, 24, 18, and 18 for BDNF; $n = 16$, 12, 24, 16, and 19 for GDNF; and $n = 16$, 14, 25, 15, and 20 for NT-3. IOD, integrated optical density. The standard deviations are shown as *error bars* in the histograms. $^*P < 0.05$.

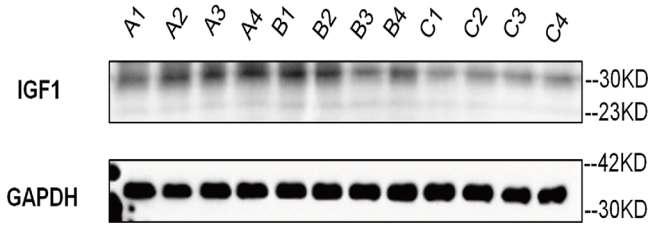

Figure 4. Increased expression of IGF-1 in AACE (**A1**–**A4**) and EIE (**B1**–**B4**) compared with the controls (**C1**–**C4**) as revealed by western blotting.

by qPCR.[20] Endogenous GDNF can alter the contraction of EOMs,[9] leading to faster contraction,[8] and reducing GDNF expression in EOMs can decrease the frequency of muscle contraction.[9] Another important subgroup of neurotrophic factors is the neurotrophin family, including BDNF and NT-3, which are implicated in different aspects of the myogenic process.[10] In a primate animal model, unilateral oversupply of exogenous BDNF to horizontal EOMs did not induce strabismus but showed a significant increase in myofiber size in the slow myosin heavy chain–expressing fibers.[16] Exogenously applied BDNF and NT-3 could restore afferent synapses on the injured motor neurons and differentially regulate firing patterns in these neurons.[19,29] NT-3 was found to be involved in muscle regeneration,[10] and NT-3 increases muscle fiber diameter in the neurogenic muscle from the Trembler-J mice.[30]

Our study had some limitations. First, the age of the controls was obviously older than that of patients with esotropia, as control samples were collected from deceased organ donors. However, in a previous study, it was reported that the effect of age was not significant and that medial and lateral rectus muscles displayed similar changes in protein and gene expression.[7] We further analyzed the relationship between neurotrophic factors and age in different types of concomitant esotropia, and no significant correlations were found (all $P > 0.05$). Second, medial rectus muscles cannot be obtained from patients with esotropia due to ethical considerations. Only lateral rectus muscles can be obtained, because this enhances the strength of the weakened muscles in patients with concomitant esotropia, which, to some extent, restricts the evaluation of neurotrophic factors and analysis of innervation in medial rectus muscles in concomitant esotropia patients. Third, the interval between death of the organ donors and fixation of EOM samples was within 4 hours. According to a previous study,[31] care must be taken to ascertain the effect of postmortem intervals on protein levels. Although in most studies such intervals may be adequate to quantitate the total levels of many proteins, care is required in the interpretation of phosphoprotein levels and the activity of enzymes regulated by phosphorylation. Our study largely dealt with morphology, and the postmortem interval has a major impact on the levels of proteins in the phosphorylated state. Fourth, the study by Zhou et al.[32] showed that the intramuscular nerve-dense regions were at a position between 31.69% ± 0.67% and 56.01% ± 0.63% of the lateral rectus muscle belly length, where the branches formed an overlapping innervation region at the center of the muscle belly. Due to the limitation of resected muscle length within a certain range during strabismus surgery, the center of the nerve-dense region could not be evaluated in this study. Last but not least, six areas of each EOM sample were randomly chosen and examined throughout the entire cross-sections, including both orbital and global layers. For our analyses, muscle cross-sections

were generally uniform but the orbital and global layers were not analyzed separately. In future studies, different layers could be analyzed separately for layer-specific information.

Conclusions

This study found that IGF-1 was significantly increased in patients with AACE and EIE, and synaptophysins were significantly increased in the EOMs of patients with AACE. We hypothesize that there may be a possible connection between increased IGF-1 and increased synaptophysin in the acute type but not in chronic types of concomitant esotropia. Further mechanistic research is warranted to explore the effects of IGF-1 on the innervation of EOMs in different types of concomitant esotropia.

Acknowledgments

The authors thank Chao Yan; Li Wang; Yi Qin; Zhibao Zhang; and Xu Zhang, from Beijing Tongren Hospital for their support with samples collection and analysis.

Supported by grants from the Young Scientists Fund of the National Natural Science Foundation of China (82101174), Beijing Hospitals Authority Youth Program (QML20230205), and Capital Health Development Research Special Project (2022-1-2053).

Disclosure: **J. Hao**, None; **M. Wang**, None; **J. Liu**, None; **M. Yusufu**, None; **K. Cao**, None; **J. Fu**, None

References

1. Kaur K, Gurnani B. Esotropia. In: *StatPearls [Internet]*. Treasure Island, FL: StatPearls Publishing; 2023.
2. Hashemi H, Pakzad R, Heydarian S, et al. Global and regional prevalence of strabismus: a comprehensive systematic review and meta-analysis. *Strabismus*. 2019;27:54–65.
3. Verma M, Fitzpatrick K, McLoon LK. Extraocular muscle repair and regeneration. *Curr Ophthalmol Rep*. 2017;5:207–215.
4. Rodríguez MA, Sandgren Hochhard K, Vicente A, Liu J-X, Pedrosa Domellöf F. Gene expression profile of extraocular muscles following resection strabismus surgery. *Exp Eye Res*. 2019;182:182–193.
5. Bjorke B, Weller KG, Jones LE, et al. Oculomotor nerve guidance and terminal branching requires interactions with differentiating extraocular muscles. *Dev Biol*. 2021;476:272–281.
6. Rudell JC, Fleuriet J, Mustari MJ, McLoon LK. Childhood onset strabismus: a neurotrophic factor hypothesis. *J Binocul Vis Ocul Motil*. 2021;71:35–40.
7. Agarwal AB, Feng CY, Altick AL, et al. Altered protein composition and gene expression in strabismic human extraocular muscles and tendons. *Invest Ophthalmol Vis Sci*. 2016;57:5576–5585.
8. Li T, Feng CY, von Bartheld CS. How to make rapid eye movements "rapid": the role of growth factors for muscle contractile properties. *Pflügers Arch*. 2011;461:373–386.
9. Chen J, von Bartheld CS. Role of exogenous and endogenous trophic factors in the regulation of extraocular muscle strength during development. *Invest Ophthalmol Vis Sci*. 2004;45:3538–3545.
10. Carrero-Rojas G, Benítez-Temiño B, Pastor AM, Davis López de Carrizosa MA. Muscle progenitors derived from extraocular muscles express higher levels of neurotrophins and their receptors than other cranial and limb muscles. *Cells*. 2020;9:747.
11. Hernández RG, Silva-Hucha S, Morcuende S, de la Cruz RR, Pastor AM, Benítez-Temiño B. Extraocular motor system exhibits a higher expression of neurotrophins when compared with other brainstem motor systems. *Front Neurosci*. 2017;11:399.
12. Feng CY, von Bartheld CS. Expression of insulin-like growth factor 1 isoforms in the rabbit oculomotor system. *Growth Horm IGF Res*. 2011;21:228–232.
13. Willoughby CL, Fleuriet J, Walton MM, Mustari MJ, McLoon LK. Adaptability of the immature ocular motor control system: unilateral IGF-1 medial rectus treatment. *Invest Ophthalmol Vis Sci*. 2015;56:3484–3496.
14. Fleuriet J, Willoughby CL, Kueppers RB, Mustari MJ, McLoon LK. Eye alignment changes caused by sustained GDNF treatment of an extraocular muscle in infant non-human primates. *Sci Rep*. 2020;10:11927.
15. Willoughby CL, Christiansen SP, Mustari MJ, McLoon LK. Effects of the sustained release of IGF-1 on extraocular muscle of the infant non-human primate: adaptations at the effector organ level. *Invest Ophthalmol Vis Sci*. 2012;53:68–75.
16. Willoughby CL, Fleuriet J, Walton MM, Mustari MJ, McLoon LK. Adaptation of slow myofibers: the effect of sustained BDNF treatment of extraocular muscles in infant nonhuman primates. *Invest Ophthalmol Vis Sci*. 2015;56:3467–3483.
17. Stanga S, Boido M, Kienlen-Campard P. How to build and to protect the neuromuscular junction: the role of the glial cell line-derived neurotrophic factor. *Int J Mol Sci*. 2020;22:136.
18. McLoon LK, Christiansen SP, Ghose GM, Das VE, Mustari MJ. Improvement of eye alignment in adult strabismic monkeys by sustained IGF-1 treatment. *Invest Ophthalmol Vis Sci*. 2016;57:6070–6078.
19. Davis-López de Carrizosa MA, Morado-Díaz CJ, Tena JJ, et al. Complementary actions of BDNF and neurotrophin-3 on the firing patterns and synaptic composition of motoneurons. *J Neurosci*. 2009;29:575–587.
20. Altick AL, Feng CY, Schlauch K, Johnson LA, von Bartheld CS. Differences in gene expression between strabismic and normal human extraocular muscles. *Invest Ophthalmol Vis Sci*. 2012;53:5168–5177.
21. Fitzpatrick KR, Cucak A, McLoon LK. Changing muscle function with sustained glial derived neurotrophic factor treatment of rabbit extraocular muscle. *PLoS One*. 2018;13:e0202861.
22. Feng C, Von Bartheld CS. Schwann cells as a source of insulin-like growth factor-1 for extraocular muscles. *Muscle Nerve*. 2010;41:478–486.
23. Zhu Y, Deng D, Long C, Jin G, Zhang Q, Shen H. Abnormal expression of seven myogenesis-related genes in extraocular muscles of patients with concomitant strabismus. *Mol Med Rep*. 2013;7:217–222.
24. Benítez-Temiño B, Davis-López de Carrizosa MA, Morcuende S, Matarredona ER, de la Cruz RR, Pastor AM. Functional diversity of neurotrophin actions on the oculomotor system. *Int J Mol Sci*. 2016;17:2016.
25. Rind HB, von Bartheld CS. Target-derived cardiotrophin-1 and insulin-like growth factor-I promote neurite growth and survival of developing oculomotor neurons. *Mol Cell Neurosci*. 2002;19:58–71.
26. Apel PJ, Ma J, Callahan M, et al. Effect of locally delivered IGF-1 on nerve regeneration during aging: an experimental study in rats. *Muscle Nerve*. 2010;41:335–341.
27. Harandi VM, Gaied AR, Brännström T, Pedrosa Domellöf F, Liu JX. Unchanged neurotrophic factors and their

Investigative Ophthalmology & Visual Science

receptors correlate with sparing in extraocular muscles in amyotrophic lateral sclerosis. *Invest Ophthalmol Vis Sci*. 2016;57:6831–6842.

28. Nguyen QT, Parsadanian AS, Snider WD, Lichtman JW. Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. *Science*. 1998;279:1725–1729.
29. Davis-López de Carrizosa MA, Morado-Díaz CJ, Morcuende S, de la Cruz RR, Pastor AM. Nerve growth factor regulates the firing patterns and synaptic composition of motoneurons. *J Neurosci*. 2010;30:8308–8319.
30. Yalvac ME, Amornvit J, Chen L, Shontz KM, Lewis S, Sahenk Z. AAV1.NT-3 gene therapy increases muscle fiber diameter through activation of mTOR pathway and metabolic remodeling in a CMT mouse model. *Gene Ther*. 2018;25:129–138.
31. Li J, Gould TD, Yuan P, Manji HK, Chen G. Postmortem interval effects on the phosphorylation of signaling proteins. *Neuropsychopharmacology*. 2003;28:1017–1025.
32. Zhou G, Zhou J, Wang D, Yang S. Overall distribution pattern of the intramuscular nerves of the extraocular muscles and its clinical implications. *Int J Morphol*. 2021;39:1412–1419.

Investigative Ophthalmology & Visual Science