Adaptability of the Immature Ocular Motor Control System: Unilateral IGF-1 Medial Rectus Treatment

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PURPOSE. Unilateral treatment with sustained release IGF-1 to one medial rectus muscle in infant monkeys was performed to test the hypothesis that strabismus would develop as a result of changes in extraocular muscles during the critical period of development of binocularity.

METHODS. Sustained release IGF-1 pellets were implanted unilaterally on one medial rectus muscle in normal infant monkeys during the first 2 weeks of life. Eye position was monitored using standard photographic methods. After 3 months of treatment, myofiber and neuromuscular size, myosin composition, and innervation density were quantified in all rectus muscles and compared to those in age-matched controls.

RESULTS. Sustained unilateral IGF-1 treatments resulted in strabismus for all treated subjects; 3 of the 4 subjects had a clinically significant strabismus of more than 10°. Both the treated medial rectus and the untreated ipsilateral antagonist lateral rectus muscles had significantly larger myofibers. No adaptation in myofiber size occurred in the contralateral functionally yoked lateral rectus or in myosin composition, neuromuscular junction size, or nerve density.

CONCLUSIONS. Sustained unilateral IGF-1 treatment to extraocular muscles during the sensitive period of development of orthotropic eye alignment and binocularity was sufficient to disturb ocular motor development, resulting in strabismus in infant monkeys. This could be due to altering fusion of gaze during the early sensitive period. Serial measurements of eye alignment suggested the IGF-1-treated infants received insufficient coordinated binocular experience, preventing the establishment of normal eye alignment. Our results uniquely suggest that abnormal signaling by the extraocular muscles may be a cause of strabismus.

Keywords: extraocular muscles, insulin growth factor-1, myogenic growth factors, strabismus

Correct binocular eye alignment and conjugate gaze are critical components for normal development of the primate visual system. In children with strabismus, misalignment of the eyes during visual maturation disrupts the establishment of binocularity. If eye alignment cannot be accomplished by medical or surgical intervention, amblyopia and permanent vision loss can occur. It is estimated that between 3% and 5% of children are affected by strabismus and 1% to 2% have amblyopia, numbers that have remained unchanged for the last 70 years.^{1–5} Current treatment options are still not effective solutions for many individuals, particularly for those with large angles of misalignment.^{6,7}

One problem in the development of new treatments is the difficulty in creating animal models that mimic idiopathic childhood-onset strabismus. Early studies in cats produced strabismic amblyopia by using monocular deprivation or surgical esotropia or optically induced strabismus by using prism goggles.⁸ The nonhuman primate has become an important animal model for strabismus, but the most common methods for inducing strabismus in infant monkeys use forms of sensory deprivation, such as alternating monocular occlusion⁹ or prism goggle rearing.¹⁰ These types of sensory

manipulations are effective at producing strabismus. However, there are many children who develop strabismus in the absence of known sensory afferent defects, and producing strabismus by other mechanisms may allow us to model other potential causes for eye misalignment in children.

It is possible to induce strabismus by modifying the extraocular muscles (EOM) themselves. Using a variety of methods, a number of studies have induced strabismus by surgical manipulation of the EOM.^{11–13} Simple resection-recession surgery often proved insufficient to produce a lasting strabismus, so these manipulations included significant muscle resection, extirpation, or recession of the antagonist muscle on the same globe, or advancement of one muscle to the limbus with excision of the contralateral antagonist muscle.^{11,14,15} These models result in strabismus but with the disadvantage of injuring and disrupting the EOM.

In a series of studies in rabbits, we showed that treating a single EOM with exogenously added neurotrophic factors such as insulin growth factor-1 (IGF-1) or IGF-2, whether injected or treated continuously for months, resulted in increased muscle size and force generation capacity.^{16–18} Thus, it seemed reasonable that in a binocular animal such as the non-human

primate, sustained release of IGF-1 would produce a strabismus by strengthening one muscle, thereby mimicking the overacting/underacting agonist/antagonist relationship noted in the EOM of strabismus patients. In a first experiment, the medial rectus muscles of infant non-human primates were treated bilaterally with sustained release IGF-1 for 3 months.¹⁹ Although the treated medial rectus muscles were markedly enlarged in cross-sectional area and in innervational density, no change in eye alignment was detected. When the lateral rectus muscles were examined in these bilaterally treated monkeys, there were similar, apparently compensatory, changes in myofiber cross-sectional areas and innervational density in these untreated muscles as well.¹⁹ In this experimental design, each untreated lateral rectus was either an ipsilateral antagonist or a contralateral yoked muscle for each treated medial rectus muscle. We hypothesized that due to the continuous exposure to IGF-1 over 3 months, there was sufficient time for active concomitant changes to occur in the untreated lateral rectus muscles.

The EOM are extremely adaptable when their yoked and/or antagonist muscles are manipulated. In a set of experiments, rabbit superior rectus muscles were surgically resected to model strabismus surgery.^{20,21} Significant changes in myosin heavy chain isoform expression, myonuclear addition, and satellite cell activation occurred in the operated superior rectus muscle, which mirrored changes seen in surgical specimens from human EOM with previous strabismus surgery.²² Interestingly, in the rabbit specimens, reciprocal changes occurred in the unoperated but passively stretched antagonist inferior rectus muscles of the same globe. There were also changes in the contralateral yoked superior rectus muscles, despite not having been directly altered by the surgical manipulation. This strongly suggested that the central nervous system is affected when perturbations in the periphery alter muscle size and force generation capacity continuously over a period of months. Although the mechanisms that control these adaptations in EOM are not understood, our current studies suggest that it might be possible to use this capacity of EOM to remodel as a potential treatment for eye movement disorders such as strabismus.

Based on these studies, we hypothesized that unilateral treatment with sustained release IGF-1 might be more likely to cause strabismus. A unilateral treatment paradigm would allow us to parse out how the EOMs adapt to disruption in the balance of muscle pairs more clearly. One medial rectus muscle from each of four infant monkeys was treated with sustained release IGF-1 for a duration of 3 months (Fig. 1A). These unilaterally treated monkeys were assessed for changes in eye alignment during the treatment period. At the end of 3 months, all rectus muscles were assessed morphometrically for myofiber cross-sectional area, and the lateral and medial rectus muscles were analyzed for nerve and neuromuscular junction density. These data were compared to those from three age-matched sets of control rectus muscles in order to assess what, if any, compensatory changes would develop in the untreated yoked and antagonist muscles.

METHODS

Animals and Surgery for Sustained IGF-1 Treatment

Monkeys were obtained from the breeding colony at the Washington National Primate Center at the University of Washington. All experiments were approved by the University of Washington Animal Care and Use Committee. All procedures were performed in compliance with guidelines issued by

National Institutes of Health and Association for Research in Vision and Ophthalmology for use of animals in research.

Three *Macaca nemestrina* and one *Macaca mulatta* infant monkeys 1 to 2 weeks of age were photographed to document alignment prior to treatment to verify starting eye alignment. With the subjects under general inhalation anesthesia, the conjunctiva was opened near the limbus in one quadrant, and the medial rectus muscles were visualized. The eye was held stable by placing a small muscle hook under the muscles near their insertion, and a pellet (Innovative Research of America, Sarasota, FL, USA) was placed on the surface of one medial rectus muscle. The pellets become quite sticky when placed within the connective tissue around the muscles and adhere to the muscle surface. Pellet position was verified by magnetic resonance imaging (MRI) at 2 months and by visual inspection at the time of tissue collection. The pellets were 3 mm × 1.5 mm, and were calibrated for the sustained release of 2 µg of IGF-1 per day for a total of 90 days. This dose was selected based upon previous studies.^{18,19} After pellet implantation, the conjunctiva was closed with an 8-0 ophthalmic vicryl suture. Two of the treated monkeys were photographed for the determination of eye alignment at intervals during the treatment period. The two others were photographed for the determination of eye alignment changes only at 3 months after treatment.

Eye alignment was assessed by corneal light reflex. Displacement (in millimeters) between the flash reflection and the pupil center between both eyes was measured on each photograph. The degree of misalignment was then calculated using the Hirschberg ratio for macaques of 148/mm.²³ Measurements from three or more photographs were averaged for each subject and time point. Based on these photographs, the angles of eye misalignment were tracked over the first 3 months. At the end of the 3 month period, the monkeys were euthanized, and all EOMs were dissected from origin to insertion, embedded in tragacanth gum, and frozen on 2-methylbutane chilled to a slurry on liquid nitrogen. Tissue was also collected from two additional age-matched control monkeys with normal alignment. The tissue was stored at −80°C until processed. Muscle specimens from a third age-matched control monkey used in previous studies were also used in this analysis.¹⁹

Immunohistochemistry and Histological Processing

Frozen EOM were sectioned at 30 µm using a cryostat (Leica, Wetzlar, Germany), and the sections were stored at −80°C until processed. Every 20th section of all rectus muscles was stained with hematoxylin and eosin for performing myofiber cross-sectional area analysis. Every 40th section was washed in 0.01 M phosphate-buffered saline, pH 7.4, containing 0.1% Triton X-100 (PBS/TX), blocked in 10% goat serum for 1 hour and then incubated overnight at 4°C with an antibody to the slow myosin chain isoform (MyHC; Vector Laboratories, Burlingame, CA, USA). Sections were washed in PBS and incubated in secondary antibody, goat anti-mouse immunoglobulin G (IgG) labeled with Cy3 (1:1000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hour at room temperature. To visualize neuromuscular junctions, sections were double-labeled by subsequent treatment with α -bungarotoxin conjugated to Alexa Fluor 488 (1:3000 dilution; Molecular Probes; Eugene, OR, USA) overnight. A subset of sections were triple-labeled for nerve by incubation in an antibody to neurofilament (1:1000 dilution; product smi-31; BioLegend; Dedham, MA, USA) overnight and visualized with goat anti-mouse DyLight 405 (1:1000 dilution; Jackson ImmunoResearch Laboratories). All sections were washed

FIGURE 1. (A) Rectus extraocular muscles and the experimental design. One medial rectus muscle (*pink*) was treated with a pellet which slowly released 2 $\mu\text{g/day}$ IGF-1 for 90 days. For horizontal eye movements, the treated medial rectus muscle adducts the eye, opposed by the antagonist lateral rectus muscle (*orange*), which abducts the eye. For conjugate eye movements, the medial rectus is also functionally yoked with the contralateral lateral rectus (*green*). (B–E) Corneal light reflex photographs of the monkeys after 3 months of sustained IGF-1 treatment. Monkeys had average eye misalignments of 12° (B), 11° (C), 15° (D), and 6° (E).

and mounted on glass slides and coverslipped with Vectashield (Vector Laboratories).

For further analysis of myosin expression, every 40th section was stained with antibody to embryonic (1:20 dilution) and neonatal (1:20 dilution) MyHC (Vector Laboratories). Sections were blocked for nonspecific binding with goat serum and avidin-biotin blocking reagents (Vector Laboratories), incubated in the primary antibody overnight at 4°C, rinsed, and then incubated with the ABC reagents (Vectastain Elite; Vector Laboratories). Sections were processed with the heavy metal intensified diaminobenzidine procedure.

Image Processing and Analysis

For visualization of nerve and neuromuscular junctions, slides were imaged with scanner laser confocal microscopy (model FV1000; Olympus, Tokyo, Japan) to sequentially capture images in the same focal plane with different filters. Neuromuscular junctions were imaged at 20 $\times$ oil immersion magnification and nerves at 10 $\times$ magnification. Z-stacks through the thickness of the sections were collected. Every 20th section was imaged for analysis. With a random starting point, every third field throughout the entire muscle specimen was taken.

Z-stacks were collapsed for each field, and the following measurements were taken using ImageJ software (National Institutes of Health, Bethesda, MD, USA): muscle area, mean diameter for myofibers positive or negative for slow MyHC, total number of neuromuscular junctions, and area of each neuromuscular junction endplate as determined by hand circling. For every fiber with a neuromuscular junction, it was also noted whether the fiber was positive or negative for slow myosin. For each field, the muscle layer (orbital, global) and region (proximal, endplate zone, distal) were noted. Neuromuscular junctions were categorized based on slow myosin expression, endplate area, and morphology to distinguish between *en grappe* and *en plaque* endplates. For *en plaque* neuromuscular junctions in the endplate zone, an additional measurement of neuromuscular junction length was performed. For assessment of neuromuscular junction size, only counts of complete neuromuscular junctions were included. For a subset of sections, every fifth field was imaged at 60 $\times$ oil immersion magnification to visualize neuromuscular junction morphology fully. Additional staining among control and experimental muscles with double-labeling of SNAP-25 (1:1000 dilution; Covance; Dedham, MA, USA) or synaptophysin (1:300 dilution; Abcam; Cambridge, MA, USA), nerve (smi-31), and α -bungarotoxin revealed that all α -bungarotoxin-

FIGURE 2. Representative MRI examination of one of the treated monkeys shows the location of the pellet on the medial rectus muscle (arrow).

labeled neuromuscular junctions in the EOM specimens colocalized with presynaptic components. Based on these results, we are confident that most α -bungarotoxin-positive neuromuscular junctions in our samples were active endplates.

For calculation of average myofiber cross-sectional area, slides were visualized with a light microscope, and fields were randomly chosen throughout the length of the muscle in both the global and the orbital layers. A minimum of 200 myofibers per section were analyzed, and a minimum of three slides per muscle specimen was measured.

For calculation of embryonic MyHC and neonatal MyHC density and mean myofiber diameter, every 40th section along the treated and control medial rectus slides was visualized using light microscopy. For analysis, every third field throughout the slide was counted with a random starting point. The percentage of fibers in each field staining positive for each myosin was determined. The average fiber diameter for fibers positive and negative for each myosin was also determined for each field. Mean myofiber size and percent positive for both embryonic and neonatal MyHC isoform expression were assessed with imaging analysis software (BioQuant Nova Prime morphometry program; BioQuant, Nashville, TN, USA).

All data were analyzed statistically (Prism software; GraphPad, San Diego CA, USA). For all measurements, comparisons were made between the treated medial rectus and control medial rectus muscle, or between untreated lateral rectus muscle from experimental subjects and control lateral rectus muscle. All data were analyzed for statistical significance using the Student's *t*-test with correction for multiple comparisons using the Holm-Sidak method, except for analysis of cross-sectional area, which was analyzed with one-way ANOVA, followed by the Tukey test to correct for multiple comparisons. Data were considered statistically significant if $P < 0.05$. For all graphs, error bars signify standard deviation.

RESULTS

Retention of the sustained release pellets was verified by MRI in the treated infant monkeys (Fig. 2). All 4 infant monkeys that received 3 months of unilateral IGF-1 treatment of the medial rectus muscle at 2 μ g/day developed exotropia, with a mean angle of $11.3 \pm 3^\circ$ (Figs. 1B–1E). Three of the four treated monkeys had clinically relevant angles greater than 10° . The fourth monkey had an incomitant strabismus, but the misalignment was evident in most of the photographs over the final month of treatment. Eye alignment was measured at intervals over the 3 months of IGF-1 treatment for two of the treated monkeys (Fig. 3). Both humans and monkeys are frequently born with eye misalignment, when visual acuity is poor and stereopsis is absent.^{24,25} For monkeys within the first

FIGURE 3. Successive measurements of eye alignment for 2 monkeys in the experimental group at intervals during the 3 months of sustained IGF-1 treatment and graphed as mean $\pm$ SD (indicated by the lighter green and blue shaded areas). Mean $\pm$ SD of the final alignment angles for the other two monkeys are also indicated. The horizontal gray box at 10° signifies the threshold for clinically relevant strabismus.

month of life, coarse stereopsis rapidly develops and the oculomotor system matures.²⁴ When experimentally creating an animal model of strabismus, it is only with significant, continual visual sensory perturbation during the development of coarse stereopsis that strabismus persists.⁹ As soon as binocular visual experience begins, there is a strong drive in the ocular motor system to use the binocular experience to produce normal eye alignment. Our longitudinal eye alignment measurements suggested that for the infant monkeys treated with unilateral IGF-1, the initial exotropia normal in newborn monkeys remained stable over time. We hypothesized that persistent IGF-1 signaling disrupted the normal drive to create coarse stereopsis, causing the binocular system to receive insufficient visual experience associated with normal eye alignment needed for the subsequent development of sensory fusion.

A secondary goal of our study was to investigate compensatory adaptations of the EOM that occur when one of the four horizontal rectus EOM that work together to maintain normal eye position and control eye movements along the horizontal plane is perturbed with continuous IGF-1 delivery. By unilaterally treating one medial rectus muscle, it allowed analysis of potential changes in the untreated ipsilateral antagonist lateral rectus and the contralateral functionally yoked lateral rectus (Fig. 1A).

Consistent with our prior study,¹⁹ unilateral IGF-1 treatment of infant monkey medial rectus muscle resulted in larger myofibers than control EOM from untreated age-matched infant monkeys (Fig. 4). This persistent trend reached significance for the treated medial rectus in the orbital layer (Fig. 4B, pink), where mean cross sectional area was $253 \pm 19.78 \mu\text{m}^2$, 75% larger than the control mean of $144.7 \pm 5.883 \mu\text{m}^2$.

Interestingly, this trend was also significant for the untreated ipsilateral lateral rectus in the global layer, where the mean myofiber area was $561.9 \pm 102.6 \mu\text{m}^2$, twice as large as control values of $276.9 \pm 121 \mu\text{m}^2$ (Fig. 4A, peach). Thus the effects of IGF-1 treatment to a single medial rectus muscle caused enlargement of the antagonist lateral rectus myofibers. There was no effect of treatment on the contralateral functionally yoked lateral rectus muscle (Fig. 4, green). It is possible that larger myofiber size of the non-IGF-1-treated antagonist muscle could be due to passive diffusion of IGF-1 into the orbit, rather than adaptation to a stronger antagonist muscle. To address this, the mean myofiber cross-sectional areas of the inferior and superior rectus muscles were also

FIGURE 4. Quantification of mean myofiber cross-sectional areas after 3 months of sustained IGF-1 treatment in the global (A) and orbital (B) layers of the muscles. Three months of sustained unilateral release of IGF-1 resulted in an increased mean myofiber cross sectional area of the treated medial rectus muscles and antagonist lateral rectus muscles (orange). No significant differences were seen in the mean cross-sectional areas of the contralateral medial and lateral rectus muscles or in any of the superior and inferior rectus muscles. Asterisks indicate significant differences between control and experimental extraocular muscles (ANOVA, Tukey text: $P < 0.05$).

determined. Because the superior and inferior rectus do not direct horizontal eye movements, they would be unlikely to adapt to perturbation of the horizontal alignment system. There was no significant increase in cross-sectional area of the vertical recti as compared to the experimental subjects' ipsilateral and contralateral orbits, nor to the control values of the horizontal rectus muscles. Based on this, it is unlikely that diffusion of IGF-1 to vertical EOM confounded our results.

Previous studies have suggested that the effects of IGF-1 treatment of EOM were most prominent at the proximal end of the muscle,²⁶ which may be related to the endogenous gradient of expression of IGF-1 across the EOM.²⁷ Additionally, the size of individual myofibers varied from the proximal to distal end of the EOM and with myofiber type.²⁸ Because our gross cross-sectional counts may have masked a more nuanced effect of IGF-1 treatment on myofiber size, we performed a more detailed analysis of the medial and lateral rectus muscles by calculating fiber diameter at the proximal end, endplate zone, and distal end. The myofibers of the EOM present a continuum of properties, where multiple myosin isoforms are co-expressed throughout the EOM, and can change expression along a single fiber.^{29–32} The EOM also have diverse types of neuromuscular junctions, with *en grappe* endings that multiply innervate a myofiber where slow MyHC is expressed, and these fiber regions exhibit slow-tonic characteristics. Complementarily, *en plaque* endplates are more centrally located on myofibers where fast myosins are expressed and exhibit fast-twitch characteristics.^{33–36} Based on this common broad grouping of myofiber types, we additionally labeled myofibers with an antibody to slow MyHC to distinguish broadly between fast and slow myofiber types (Figs. 5, 6).

IGF-1 treatment resulted in significantly larger myofiber diameters for fast myofibers in the global and orbital layers

through the entire treated medial rectus muscles (Figs. 5A–5D; Figs. 6A, 6C). Slow fibers were larger than control in the proximal region in the global layer (Figs. 5B, 6B), while in the orbital layer, slow fibers were larger than control throughout the EOM except at the end the endplate zone (Fig. 6D). For the untreated antagonist lateral rectus muscle, there was a trend of larger myofibers as compared to control lateral recti, but it only reached significance for slow myofibers in the proximal and distal regions of the orbital layer (Figs. 5E–5H, 6D).

Previous studies have suggested that IGF-1 may alter expression of MyHC isoforms as well as alter muscle contractile characteristics. The EOM express nine different myosin isoforms. Many are coexpressed, and expression of individual isoforms can vary along a fiber's length.^{29,31,32} We used immunolabeling of myofibers to assess whether IGF-1 treatment altered expression of slow or developmental MyHC expression. The percentage of myofibers expressing slow MyHC was unchanged with IGF-1 treatment (Fig. 7). To further assess the effects of prolonged IGF-1 treatment on MyHC isoforms, the medial rectus muscles were also labeled for expression of the “developmental” myosins, embryonic MyHC or neonatal MyHC (Figs. 8, 9). No significant change in expression of these myosins was found along the muscle length from proximal to distal along the muscle, nor between the orbital and global layers (Figs. 8A, 10A, 10B). As nearly all orbital layer fibers express embryonic MyHC,^{28,29,31} counts are shown only for the global layer.

Although no change in MyHC expression was observed in the treated EOM, because IGF-1 treatment created such a robust increase in myofiber size, we also assessed the effects of IGF-1 on myofiber diameter on myofibers that expressed either embryonic or neonatal MyHC isoforms. No change in myofiber diameter was seen in the global layer on fibers where

FIGURE 5. Confocal images of representative myofibers immunostained with an antibody against the slow MyHC isoform (*red*) and α -bungarotoxin (*green*) to visualize neuromuscular junctions. Myofibers from control (A, C) and IGF-1-treated (B, D) medial rectus muscles in the global (A, B) and orbital (C, D) layer of the proximal region. (E–H). Myofibers from control (E, G) and experimental untreated antagonist lateral rectus muscle in the global (E, F) and orbital (G, H) layers in the proximal region. Note the apparent increases in myofiber diameter in the IGF-1-treated muscle fibers. Bar = 25 μ m.

FIGURE 6. Three months of sustained IGF-1 resulted in increased myofiber diameter of both the slow and the fast myofibers. Quantification of mean myofiber diameter of myofibers positive for slow MyHC isoform (**B**, **D**) and negative for slow MyHC isoform (**A**, **C**), from the global (**A**, **B**), and orbital layers (**C**, **D**) of muscle from the proximal to distal end of the medial and lateral rectus muscles. Asterisks indicate significant differences between control and experimental medial or lateral rectus muscle (*t*-test, Holm-Sidak; $P < 0.05$).

embryonic MyHC was expressed (Fig. 8B). In contrast, myofibers negative for embryonic MyHC had significantly larger myofiber diameter, from $24.7 \pm 1.1 \mu\text{m}$ in the proximal region to $22.8 \pm 1.8 \mu\text{m}$ in the distal region (Figs. 8C, 8E).

Expression of neonatal MyHC was correlated with a different pattern of change in fiber diameter than that seen in myofibers expressing embryonic MyHC. In the orbital layer, all fibers that expressed neonatal MyHC had significant increases in fiber diameter (Figs. 9E, 10D), whereas fibers negative for neonatal MyHC had no change in fiber diameter (Fig. 10F). In the global layer, similar to what was seen with embryonic MyHC, only fibers negative for neonatal MyHC in the proximal region showed significant increases in myofiber diameter (Figs. 9B, 10E).

IGF-1 is known to promote neurite growth and nerve sprouting^{37–39} and maintains neuromuscular junction morphology in aging and diseased animals.^{40,41} Our previous study suggested that IGF-1 promoted increased innervation in the distal end of the muscle, but that study was limited due to a

low number of subjects.¹⁹ Based on these studies, we analyzed the treated muscle specimens in the current study for changes in nerve density, neuromuscular junction density, and neuromuscular junction size. No significant changes in either nerve or neuromuscular junction density were seen (data not shown). The EOM has two types of neuromuscular junctions. The *en grappe* endings primarily synapse on slow myofibers and dot the entire length of the EOM. The second are *en plaque* endings, which synapse primarily on fast myofibers and concentrate in the middle third of myofibers in the endplate zone in monkeys and humans. Both types of neuromuscular junctions were considered separately. Although the *en plaque* neuromuscular junctions on fast myofibers sometimes appeared larger at first examination, they were not statistically different from control (Fig. 11). There was no difference when neuromuscular junction size was normalized to myofiber size (data not shown), suggesting that any potential increase in neuromuscular junction size was likely compensatory to the myofiber hypertrophy.

FIGURE 7. Quantification of the percentage of myofibers expressing slow MyHC isoforms in the global (**A**) or orbital (**B**) layers. No significant differences were seen. (Student's *t*-test, Holm Sidak; $P < 0.05$).

FIGURE 8. Sustained release of IGF-1 had no effect on the percentage or size of myofibers expressing the embryonic MyHC isoform in the *global layer*. (A) Quantification of the percentage of myofibers expressing the embryonic MyHC isoform. (B, C) Quantification of the mean *diameter* of myofibers positive (B) or negative (C) for the embryonic MyHC isoform. (D, E) Representative fields in the *global region* of the medial rectus immunostained for embryonic MyHC from control (D) or IGF-1-treated (E) muscle. Asterisks indicate significant differences between control and experimental medial rectus muscles (Student's *t*-test, Holm Sidak; $P < 0.05$).

DISCUSSION

Unilaterally sustained release of IGF-1 to a single medial rectus muscle of infant macaques within the first postnatal week and continued for 3 months resulted in strabismus in our subjects. Three of four treated monkeys maintained a clinically relevant strabismus of greater than 10° . We hypothesize that IGF-1 treatment of one medial rectus muscle disrupted the development of normal eye alignment in a binocular animal. This finding uniquely suggests that some cases of strabismus may be based on disrupted molecular signaling between the EOM and their innervating motor neurons.

The most prominent effect we observed from IGF-1 treatment of the medial rectus muscles was larger myofibers, which extended the results from our previous studies in rabbits on the effects of IGF-1 treatment on EOM properties.^{17–19} Treatment of EOM with IGF-1 increased both twitch and tetanic muscle tension,^{17,18,26,42} which is consistent with our current finding that IGF-1 increased the diameter of both slow and fast myofibers.

A primary task of the oculomotor system in animals with frontal vision is to maintain the image of an object of interest on both foveae. This so-called motor fusion requires precise control of eye alignment permitting sensory fusion of images in the visual cortex to support disparity based depth perception. The oculomotor system also makes use of conjugate eye movements to maintain binocular alignment of a target moving in the frontal plane. For horizontal eye movements, four

FIGURE 9. Myofibers expressing neonatal myosin had larger diameters but did not change in frequency. (A) Scatterplot of all measured fields from control (black circles) and IGF-1-treated (gray circles) medial rectus muscles. IGF-1 treatment caused a slight shift toward larger myofibers (*x axis*), but the percentage of fibers expressing the neonatal MyHC isoform (*y axis*) was fairly heterogeneous. Photomicrograph of immunostaining with an antibody to the neonatal MyHC isoform are shown in the proximal region of the global (B, C) and orbital (D, E) layers of control (B, D) and IGF-1-treated (C, E) medial rectus muscles. Bar = 25 μm.

separate muscles, the medial and lateral recti in each orbit, innervated by two different cranial nuclei, abducens and oculomotor, must produce precise alignment and movements in a coordinated fashion.

Previous studies suggested that the EOM were highly sensitive to perturbation and adapted when one of their functionally paired muscles was altered, either surgically^{20,21} or chemically with botulinum toxin.⁴³ Current treatments for strabismus that alter only the EOM (by surgery or botulinum toxin) may in fact have low success rates because of the robust adaptation of the oculomotor system.^{20,21} In this study, we found that unilateral IGF-1 treatment of the medial rectus muscle resulted in correlated changes in the untreated ipsilateral antagonist lateral rectus; both muscles showed increased myofiber size. We hypothesize that this change was due to adaptations by the oculomotor system that normally drives eye alignment in development. It is well documented for limb skeletal muscle that injury or exercise of one muscle results in cross-transfer effects to the contralateral muscle and the antagonist muscle in the same limb.^{44–48} This adaptation is mediated through interneurons in the spinal cord and, interestingly, has been hypothesized to be communicated through changes in neurotrophic signaling.^{44,46} Based on published reports, we hypothesize that, in our study, signaling from oculomotor neurons innervating the “stronger” IGF-1-treated muscle communicated with the functionally paired ipsilateral abducens nucleus through oculomotor internuclear neurons,⁴⁹ resulting in compensatory adaptation. Future

FIGURE 10. Quantification of the percentage of myofibers expressing the neonatal MyHC isoform in the global (A) and orbital (B) layers. (C-F) Mean myofiber diameters of myofibers in the global (C, E) or orbital (D, F) layers of myofibers positive (C, D) or negative (E, F) for the neonatal MyHC isoform. Asterisks indicate significant differences between control and experimental medial rectus muscles (Student's *t*-test, Holm Sidak; $P < 0.05$).

studies are needed to uncover the mechanisms which may mediate this adaptation.

We did not observe complementary adaptation in the contralateral functionally yoked medial and lateral rectus muscles in the opposite orbit. This was surprising because we hypothesized that we would find adaptation across all four recti muscles responsible for horizontal eye movements. However, in this study, our analysis was limited to morphological changes. It is possible that adaptations in the functionally yoked lateral rectus muscle may have occurred that were outside our analysis, including changes in neuronal drive to the extraocular muscle. Functional analysis such as measurement of neuronal firing rates or muscle contractile characteristics may reveal other manifestations of plasticity. Exciting preliminary results support this possibility, as animal models that received previous strabismus surgery were shown to undergo changes in neuronal drive to extraocular muscle (Agaoglu MN, et al. *IOVS* 2015;56:ARVO E-Abstract 5222 and Pulella M, et al. *IOVS* 2015;56:ARVO E-Abstract 5221). However, in the present study, adaptation of the EOM was restricted to the treated orbit, which may explain why all of our treated infant monkeys had developed strabismus at the end of 3 months. This view is consistent with our previous study, which found that bilateral treatment of both medial rectus muscles of infant monkeys with IGF-1 resulted in normal alignment.¹⁹ Further studies are needed to clarify both the mechanisms underlying adaptation

of extraocular muscle and the extent and mechanisms for adaptation in the oculomotor system in the brain.

In a previous study, we treated two infant monkeys bilaterally with IGF-1, and observed substantial increases in nerve density in the distal region of the untreated antagonist muscle.¹⁹ Based on this previous finding and the implicated role of IGF-1 in promoting neurite outgrowth,³⁷⁻³⁹ we assessed the effects of IGF-1 on nerve and neuromuscular junction density, but found no measurable changes after 3 months of IGF-1 treatment. IGF-1 has been found to maintain neuromuscular junction morphology and size in aging or injured limb muscle, but IGF-1 treatment to normal muscle had no effect on neuromuscular junction size or complexity.³⁷⁻³⁹ Our findings suggest that the role of muscle-derived IGF-1 in the maturing ocular motor system is primarily as a myogenic growth factor, promoting myofiber hypertrophy and increased muscle force. This makes IGF-1 an appealing treatment for strengthening a “weak” EOM. Other neurotrophic factors such as GDNF may be more likely candidates to promote nerve sprouting and formation of new neuromuscular junctions, and may be better candidates for treating ocular motor disorders of hypo-innervation⁵⁰⁻⁵² or where hyper-innervating effects would hypothetically be useful. Alternatively, any influence of IGF-1 on EOM neuromuscular junction size or complexity might have been manifested only in the short term, and thus would not be evident after 3 months of adaptation.

FIGURE 11. Quantification of neuromuscular junction area (A) and length (B) for *en plaque* neuromuscular junctions in the medial rectus muscles after sustained IGF-1 treatment. (C–D) Confocal images of representative *en plaque* neuromuscular junctions from control (C) and IGF-1-treated (D) medial rectus muscles. Neuromuscular junctions were labeled with α -bungarotoxin (green), and slow myofibers were immunostained with an antibody to the slow MyHC isoform (red). Bar = 50 μ m.

We did not find an effect of IGF-1 on patterns of MyHC expression in this study. Previous studies showed that the EOM change their MyHC expression in response to environmental perturbations. Surgical recession or tenotomy of EOM caused increases in slow, developmental, and neonatal MyHC isoform expression.²¹ Elevated levels of thyroid hormone also resulted in increased expression of slow MyHC, and decreased expression of neonatal and developmental MyHC isoforms.⁵³ Disruption of the transcription factor Pitx2 caused a decrease in slow MyHC expression as well as smaller slow myofibers.^{54,55} A number of studies showed that IGF-1 treatment of limb skeletal muscle had no effect on MyHC isoform expression.^{41,56,57} However, in a prior study, rabbit EOM injected with IGF-1 resulted in decreased neonatal MyHC expression, and alterations in percent of myofibers positive for embryonic MyHC in the global layer in a pattern that changed from the middle to proximal end of the treated muscle.¹⁷ These adaptations occurred rapidly, and were seen within the first and second weeks after treatment.

Myosin expression is related to the shortening velocity of skeletal muscle.^{58,59} We found that long-term IGF-1 caused additional changes from those seen after short-term treatment. The total contraction time and time to maximum force only became significantly shorter after long term IGF-1 treatment (1 month), and half relaxation time became significantly longer than control EOM only after 3 months of sustained IGF-1 treatment.¹⁷ Thus, it is not completely surprising that the changes observed in MyHC expression in rabbits after 1 week of IGF-1 treatment were not present after 3 months of treatment in non-human primates. It is noteworthy that the only myofiber type where a change in diameter was not seen was in regions where embryonic or neonatal MyHC was expressed in the global layer. Additional studies are needed to address if this has functional significance.

It is possible that more nuanced changes in MyHC isoform co-expression and localization, and possibly neuromuscular junction placement, may alter muscle characteristics in a way

that is outside the sensitivity of our counting methods. Alternatively, development of a stable strabismus after 3 months of sustained IGF-1 exposure could be due to subsequent changes in neuronal drive to the horizontal rectus muscles. A recent study showed that when eye alignment was improved in adult strabismic monkeys by resection/recession surgery, no immediate changes in motor neuron firing rates occurred despite an improvement in eye alignment (Agaoglu MN, et al. *IOVS* 2015;56:ARVO E-Abstract 5222 and Pallela M, et al. *IOVS* 2015;56:ARVO E-Abstract 5221). We hypothesize that this was due to short term adaptations at the muscle level.¹⁷ However, by 1 month, there was a significant increase in neuronal drive that was associated with worsening of the strabismic angle (Agaoglu MN, et al. *IOVS* 2015;56:ARVO E-Abstract 5222 and Pallela M, et al. *IOVS* 2015;56:ARVO E-Abstract 5221). These studies support the hypothesis that immediately after a short-term perturbation of the periphery there is significant muscle remodeling, but eventually the ocular motor control system overrides changes at the muscle level to return the system to its previous steady state strabismus angle. However, in our treatment model of sustained release IGF-1, the continual exposure to IGF-1 appears to have decorrelated the visual input sufficiently during the critical period that it was able to override ocular motor system adaptations, resulting in a potentially permanent change in eye alignment. In other words, the hypothesis that the IGF-1 led to early onset eye misalignment, which would disrupt binocularly correlated retinal images sufficiently to lead to a potentially permanent change in eye alignment.

An important aspect of our study is that the infant monkeys were treated during the sensitive period for the development of stereopsis.^{60,61} By treating the system during the sensitive period when it has increased responsiveness to environmental input, complete and normal development of the binocular system was likely disrupted. From our longitudinal analysis of eye alignment over time for the IGF-1 treated monkeys (Fig. 3), it appears that the unilateral IGF-1 treatment maintained the

exotropia with which the infants were born. We hypothesize that abnormal IGF-1 signaling disrupted the oculomotor system sufficiently, so that during the sensitive period for the development of binocularity, the eyes were unable to converge and achieve sensory fusion.

Treatment of a mature ocular motor system with normal eye alignment could yield very different effects. We hypothesize that a normal adult binocular system challenged with IGF-1 would adapt to preserve eye alignment. However, an adult ocular motor system with eye misalignment appears to be sensitive to IGF-1 treatment, as treatment of adult monkeys with strabismus induced by sensory deprivation during infancy resulted in improved angle of eye alignment (McLoon LK, et al. *IOVS* 2012;53:ARVO E-Abstract 6341). Collectively, these studies support the view that exogenously added IGF-1 has the potential to serve as a treatment for childhood onset strabismus.

In summary, we found that sustained treatment with IGF-1 during the sensitive period in the development of the binocular system resulted in strabismus in visually normal infant monkeys. This finding strongly suggests that treatment of EOM with IGF-1 can alter eye position by increasing muscle size, and from earlier studies, increasing muscle force development.^{17,42} Future studies are needed to apply this treatment to animal models of strabismus to pinpoint dose and duration for achieving eye alignment. A strong advantage of our treatment paradigm is local IGF-1 delivery, which avoids the serious side effects caused by systemic administration of IGF-1.⁶² In addition, this approach could provide a noninvasive treatment method that is sustained but not permanent, and so would avoid overcorrection, which could produce strabismus in the opposite direction. Finally, our study has implications for a potential cause of idiopathic childhood onset strabismus, as we demonstrate that disrupted molecular signaling via IGF-1 after birth in the EOM can disrupt the development of normal eye alignment.

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