Neurotrophic Factor Delivery to Extraocular Muscle: Uncovering Mechanisms of Strabismus

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Chapter 1: Introduction

The oculomotor system is tasked with maintaining a stable visual image of the visual world on the retina during object or self-movement. This retinal image information is delivered to the brain for further processing. In foveate animals, objects of interest in the environment are centered on the area of the retina with highest visual acuity.

The oculomotor system develops through a combination of genetics and experience (reviewed in Boothe, Dobson and Teller, 1985). At birth, the basic paths of neuronal connections for this system are established. This includes the structures of the retina, which collects the sensory input, the striate cortex, where visual information from each eye converges onto binocular neurons to create disparity-based depth perception, the widespread cortical areas which further process visual information and translate this information into commands for eye movements, the cerebellum which fine tunes eye movements, and the motor output nuclei in the brainstem. For example, at birth visual input from each eye is already segregated into separate lamina in the dorsal lateral geniculate nucleus (Hitchcock and Hickey 1980), and into eye specific columns in the striate cortex (Horton and Hocking 1996b; LeVay, Wiesel, and Hubel 1980). Cells in the striate cortex also show binocular response (Chino et al. 1997).

However, after birth, these systems continue to mature as visual experience is acquired (Hubel, Wiesel, and LeVay 1977; Rakic 1976). A limited period of heightened synaptic plasticity occurs in the oculomotor system, where visual experience guides development of sensory and motor systems that control eye movements (Hubel and Wiesel 1970; Harwerth et al. 1990; Hubel and Wiesel 1963). This allows neuronal connections to tailor their development to a variable environment as they are refined. In humans, this ocular motor sensitive period establishes both sensory perception, such as visual acuity, motion perception, and stereopsis; and motor behavior, including eye alignment, gaze stability, and conjugate eye movements. As the system reaches an adult-like state, structural barriers form to preserve the new connections (Pizzorusso et al. 2002; Knudsen 2004; Bavelier et al. 2010; Dityatev, Schachner, and Sonderegger 2010).

The sensitive period for development of binocularity and stereopsis peaks at 3.5 months of age, but the period persists until four to seven years of age (Fox et al. 1980; Fawcett, Wang, and Birch 2005; Levi and Li 2009). A similar pattern of progression and sensitivity occurs in nonhuman primates, although with an abbreviated time course (Boothe, Dobson, and Teller 1985). If normal visual perception is disrupted during this sensitive period, abnormal development occurs across the entire oculomotor system, from changes in the input of retinal connections into the lateral geniculate nucleus to alterations at the end organ, the extraocular muscles (EOM) (Cynader and Hoffmann 1981; Crawford and von Noorden 1979; Cheng 2003).

STRABISMUS

Strabismus is an ocular motor disorder that affects about 3% of children (Robaei et al. 2006; Govindan et al. 2005; Stidwill 1997; Greenberg et al. 2007) and is a condition where the eyes don't properly align, which prevents fusion of gaze and precise conjugate eye movements. There are three main types of strabismus: accommodative, which is due to inability of the eyes to focus; comitant, where the strabismus angle is always constant; and incomitant, where the strabismus angle varies based on the direction the eyes are turned. Strabismus may be caused by a combination of a genetic predisposition and environmental factors (Matsuo et al. 2012.; Parikh et al. 2003; Paul and Hardage 1994; Wilmer and Backus 2009). Working together these factors may alter the ability of the visual and oculomotor systems to work synergistically during an early sensitive period to produce normal visual and oculomotor function.

Environmental factors that may contribute to strabismus include low birth weight, malnutrition, and exposure to environmental toxins (Glass 1984; Robaei et al. 2006). Some forms of strabismus may be due to paralysis or paresis of muscles, and may be linked to congenital cranial dysinnervation disorders (CCDD), including the most common, Duane syndrome. A number of the CCDD have an identified genetic basis in mutations of genes involved in development of the cranial ocular motor nuclei (such as HOXA1, PHOX2a, SALL4) and development of the cranial nerves (ROBO3, K1F21a, TUBB3)(Engle 2006; Graeber, Hunter, and Engle 2013; Tischfield et al. 2011). While CCDD is uncommon (about 7% of cases of strabismus)(Mohney 2007), these studies support the hypothesis that there is likely to be a genetic basis for the more common forms of strabismus.

Strabismus normally presents by six months of age (Birch et al. 1998; Archer, Sondhi, and Helveston 1989). Small angles of misalignment may resolve spontaneously, but larger angles persist into adulthood (Birch et al. 1998). For all patients with early eye misalignment, if the strabismus is not corrected during the sensitive period for development of binocularity, permanent misalignment results. In adults, individuals with strabismus often have diplopia, or double vision, as the mismatched input from each fovea competes for visual attention. It is common for individuals with childhood onset strabismus to suppress regions of the visual field to compensate for competition from the mismatched visual image on the foveae (Economides, Adams, and Horton 2012; von Noorden and Campos 2002). Often patients favor vision from one eye over the other, and develop loss of vision, or amblyopia, in one eye as a result of visual suppression during the sensitive period for the development of normal binocularity. The sensitive period for stereopsis overlaps with other visual sensitive periods, including global motion perception, contrast detection, and other aspects of visual acuity (Harwerth et al. 1990; Fawcett, Wang, and Birch 2005). As a result, patients with strabismus can also have other visual disorders, including nystagmus, a disorder of the gaze holding system (Tusa et al. 2002). A primary goal in treating strabismus is to align the eyes while still in the sensitive period so that visual experience can aid in the development of stereopsis and prevent amblyopia.

TREATMENT OUTCOMES

For patients with amblyopia, patching the non-dominant eye is frequently utilized to increase visual acuity and alignment of the strabismic eye. Binocular training may be combined with patching, and can be effective in promoting and maintaining fusion of gaze (Mansouri et al. 2014; S. L. Li et al. 2014).

However, this approach is most effective for patients with smaller angles of misalignment (Stewart et al. 2013). Surgical correction is a common treatment for strabismus, particularly if patching strategies fail to result in proper eye alignment. Surgery works under a paradigm where extraocular muscle (EOM) pairs are assumed to be comprised of a muscle that is overacting, and/or an antagonist that is too weak. An overacting muscle may be weakened by recession, where the muscle is disinserted and reattached on the globe further back from its initial position of insertion. In addition or alternatively, a muscle may be “strengthened” through resection, where a portion of the

muscle is removed, and then reattached to its initial insertion site. While strabismus surgery is often effective in many children, there are a number of drawbacks. Procedures typically require general anesthesia for children. Post-surgery, the EOMs often appear to adapt and the eyes often drift back to their original angle of misalignment (Yam et al. 2012); requiring patients to undergo multiple surgeries to achieve best eye alignment. For individuals with strabismus angles over ten degrees, success rates range from 27-60%, depending on the type of strabismus, if stereoacuity is used as a determinate of success, and on the age of the patients at the time of treatment (Yam et al. 2012; Choi et al. 2012). Another downside of surgery is that the operation itself disrupts the normal relation of the EOM with the orbit and surrounding tissue. The operation can cause scarring, disrupt the resting tension of the agonist-antagonist muscle pair (Rosenbaum et al. 1994), and disturb muscle twitch tension (Christiansen, Soulsby, and Seifen 1995).

Non-surgical methods offer viable alternatives to traditional surgery. For example, Botulinum toxin type A (Botox) injections have been used to weaken an EOM and aid in eye realignment (Scott 1980). Botulinum toxin works by binding irreversibly to the neuromuscular junction receptor (Kao, Drachman, and Price 1976), preventing the acetylcholine release necessary for activation of muscular contraction. Success post-injection is around 50% using stereoacuity, and 88% using realignment as the post-surgical measures (Tejedor and Rodríguez 2001). However, the effects of Botox are temporary. If patients re-establish binocular fusion, their alignment is likely to stay; however, multiple injections are commonly required. With higher doses of Botox, temporary local side effects such as ptosis and vertical strabismus often occur (Elston et al. 1985; Kowal, Wong, and Yahalom 2007). The EOM adapt to Botulinum toxin treatment. The antagonist muscle adapts by altering its length tension curve and sarcomere density (Kushner and Vrabec 1987). In the injected EOM, myofiber remodeling (Ugalde, Christiansen, and McLoon 2005) and nerve sprouting occur, which creates new neuromuscular junctions to replace the paralyzed ones (Hopkins 1984; Brown, Hopkins, and Keynes 1982; Harrison et al. 2011). Eventually the synaptic vesicle proteins that bound the Botox are replaced (Baldwin and Barbieri 2009; Ma et al. 2005), and muscle force recovers after three to four months of treatment (Dimitrova, Shall, and Goldberg 2002). Both Botox and surgery are often combined with prism goggles to allow

binocular fusion, or patching to promote post-surgery or injection success (Tejedor and Rodríguez 2001; Kutschke and Keech 2001).

Although current treatment options can be successful at restoring eye alignment, they are frequently ineffective, impermanent, and at times disruptive to the ocular motor system. There is agreement in the field that alternative pharmacological treatments for strabismus are currently needed in order to expand potential treatments and improve patient outcomes.

ANIMAL MODELS OF STRABISMUS

Natural selection has strongly favored frontal eye alignment and conjugate eye movements in mammals, which have vision as a dominant sense. Nonhuman primates or humans with early binocular disruption of visual input can still develop normal eye alignment, despite impairments in vision and stereoacuity (Hoyt 1980; Glass 1984; Harwerth et al. 1991). Despite the strong drive for development of a binocular system, there is a sensitive period over which stereopsis is refined, and when perturbations can disrupt fusion of gaze, and ultimately eye alignment.

Early studies in cats provided the first strong evidence for the ocular motor sensitive period (Hubel and Wiesel 1965; Hubel and Wiesel 1970). Disruption of normal binocular vision by monocular occlusion with the use of an opaque contact lens or lid suture within the first three months of age in cats was sufficient to impair vision and eye alignment. These experiments in older kittens or adult cats had little effect, highlighting the restricted bounds of the sensitive period.

When studying the development of the ocular motor system and characteristics of strabismus, infant monkeys provide an ideal model of human visual development, as their development course closely parallels that of humans, albeit with an abbreviated time course (Boothe, Dobson, and Teller 1985). In monkeys, visual acuity and contrast sensitivity develop to adult levels within the first year of life, and the sensitive period for spatial vision continues into the second year (Harwerth et al. 1990; O'Dell and Boothe 1997; Boothe, Dobson, and Teller 1985). Coarse stereopsis develops within the first two weeks to month of life, and fine stereopsis develops within the first 1 to 3 months. The period in which the system is most vulnerable to strabismus overlaps with the development of coarse stereopsis, within the first three to five weeks of life. The

sensitive period for binocularity is also complementary to the peak of synaptogenesis in monkey neurodevelopment at 4 months of age (Rakic et al. 1986).

Multiple experimental paradigms have defined the bounds of the sensitive period for stereopsis and provided insight into potential causes of sensory-induced strabismus. In animal models, a variety of experimental manipulations that provide dissociated visual input to each eye all disrupt eye alignment and create sensory-induced strabismus as early as 5 weeks of age, if disruptions occur around birth. However, most are effective when maintained through the first three months (Quick and Boothe 1989; Tusa et al. 1991; von Noorden and Dowling 1970). During this sensitive period, the visual system highly weights binocular experience. A daily period of normal binocular vision for as short as 2 hours is sufficient to preserve binocularity and ensure development of normal eye alignment (Wensveen et al. 2011).

If restricted visual information is given to both eyes, misalignment can result. For example, monkeys with binocular occlusion using lid sutures have only limited form vision through the thin eyelids. This disruption of visual input results in degraded sensory fusion, and also impairs motion perception and form vision. Monkeys with this rearing paradigm for the first 25-40 days of life develop strabismus, and nystagmus (an ocular motor disorder of normal gaze stabilization) (Tusa et al. 1991, 2001; For a review of neural mechanisms involved in nystagmus see Tychsen et al. 2010). Occlusion of only one eye, often experimentally produced by unilateral eyelid suture, reliably creates eye misalignment (Hubel and Wiesel 1970; Sparks et al. 1986; Quick and Boothe 1989). However, this is not an ideal model for studying strabismus, because the occluded eye lacks sensory information during the critical period of visual development, leading to severe loss of vision or amblyopia when viewing with the formerly occluded eye.

To allow development of vision and prevent amblyopia, sensory-induced strabismus has also been created with reverse eyelid suture (Tusa et al. 1991; Hubel and Wiesel 1970). In monkeys, one eye is kept closed from the first 25 days of life by lid suture. The eye is then opened, and the fellow eye closed for the next 25 days (Tusa et al. 1991). This reliably causes strabismus; however, the occluded eyes often develop nystagmus. This could be because of early visual deprivation during the sensitive period for the development of motion processing.

More recent methods for creating strabismus have the advantage that they allow both eyes to see throughout the period of visual development. One such method is

alternating monocular occlusion, where the occluded eye is alternated each day, such as with an opaque contact lens (Quick and Boothe 1989). Prisms can also be affixed using a helmet or goggles to the eyes, again preventing each eye from seeing a complementary region of the visual surround (Wensveen et al. 2011; Crawford and von Noorden 1980; Crawford 1996; Wong et al. 2003). These methods often preserve normal visual acuity in both eyes, while creating misalignment that persists into adulthood. The resulting eye misalignment from sensory-induced strabismus resembles that seen in strabismic humans (Kiorpes et al. 1996).

A disadvantage with these methods for use experimentally is that it is labor intensive to ensure that the prism goggles or contact lenses stay in place and aren't removed or disturbed by the experimental subject. Very brief periods of binocular experience during the sensitive period can be sufficient to train the developing binocular system and allow normal eye alignment to develop (Wensveen et al. 2011). An experimentally simpler method to create strabismus is to detach one eye muscle from its insertion or perform surgical resection/recession surgery of an eye/muscle pair to force misalignment (Crawford and von Noorden 1979; Kiorpes and Boothe 1980; Kiorpes et al. 1996; Economides et al. 2007). Modeling strabismus by simply cutting and repositioning the eye muscles as in strabismus surgery is difficult, as the eye muscles often adapt back to their original alignment (von Noorden and Dowling 1970). Another disadvantage of this method is that it damages the EOM, and could disrupt the normal relationship of the EOM in the orbit.

NEURAL CORRELATES OF STRABISMUS

Animal models of strabismus have provided insight into the neural correlates of strabismus, particularly for sensory-induced strabismus. Eye misalignment disrupts neuronal connections and signaling in various brain regions essential for depth perception and ocular motor commands. One major region impacted is the primary visual cortex. Separate but overlapping visual information is taken in by each retina, processed and relayed in the lateral geniculate nucleus for projection to the striate cortex, or V1. Here, visual information is segregated into monocular or eye specific regions called ocular dominance columns (ODC). Information from each eye is also merged in the striate cortex creating binocular neurons, which are important for initial visual processing of depth. Early eye misalignment has marked effects on the

composition of the striate cortex. As a result of such strabismus, a larger percentage of neurons in the striate cortex become monocular, or respond more strongly to monocular stimuli, (Hubel and Wiesel 1965; Van Sluyters and Levitt 1980; Engelmann, Crook, and Löwel 2015), most prominently in simple cells, which receive direct monocular input from the lateral geniculate nucleus (Scholl, Tan, and Priebe 2013). Neurons in the striate cortex also become less sensitive to depth (Chino et al. 1994; Smith et al. 1997), become binocularly suppressed, and have a lower membrane potential and lower firing rate to stimuli presented binocularly than monocularly (Sengpiel et al. 1994; Sengpiel and Blakemore 1994; Scholl, Tan, and Priebe 2013). This decreased activity is reflected in the pattern of cytochrome oxidase staining in the striate cortex. Cytochrom oxidase staining is reduced in V1 regions innervated by an amblyopic eye in strabismic animals compared to normal animals, which complements electrophysiological data suggesting excess monocular activity (Adams et al. 2013). Less cytochrome oxidase staining occurs in border stripes between ocular dominance columns, consistent with decreased activity of binocular cells.

Early studies suggest that strabismus may alter the structure of ocular dominance columns (Shatz, Lindström, and Wiesel 1977; Livingstone 1996) but more recent reanalysis with more subjects and improved techniques has demonstrated that nonhuman primates with sensory-induced strabismus and human patients with strabismus have normal ocular dominance columns (D. L. Adams and Horton 2006; Horton and Stryker 1993; Horton and Hocking 1996a). In strabismus, the visual system adapts to suppress areas of the visual field representation from the misaligned eye in order to prevent diplopia. This is believed to be the cause of amblyopia for individuals with strabismus. Interestingly, cytochrome oxidase staining correlates with reduced neuronal activity in suppressed visual fields. For individuals that favor use of one eye over the other, the dominant eye also has greater cytochrome oxidase staining in the monocular core zone of the dominant eye's ocular dominance column, again complementary to increased neuronal activity (Adams et al. 2013).

The disruption of binocular connections in the striate cortex affects the patterning of downstream sensory and motor areas, leading to disordered eye movement commands. Behaviorally, patients and animals with early onset strabismus have deficits in eye movements, including smooth pursuit (Tychsen and Lisberger 1986; Grasse and Lisberger 1992) and saccades (Fu et al. 2007; Walton, Ono, and Mustari 2013; Kapoula

et al. 1997). Studies suggest that deficits in smooth pursuit are correlated with disruptions of visual-motion sensitive areas, which translate into commands for smooth pursuit to the downstream brainstem. Neurons in medial temporal visual area (MT), which are normally binocularly driven, become less sensitive to binocular cues in animal models of strabismus, but retain their normal sensitivity to motion (Kiorpes et al. 1996). Downstream from MT, neurons in the medial superior temporal cortex (MST) have altered ocular and directional sensitivity (Mustari, Ono, and Vitorello 2008).

These cortical regions project to brainstem areas critical for generation of eye movements, which may in turn also have a loss of binocular neurons and unbalanced commands from each eye (Mustari and Ono 2011; Walton, Ono, and Mustari 2013). In the brainstem, the supraocular motor area (SOA) is involved in vergence. However, in animals with strabismus, the SOA also encodes strabismus angle (Das 2011), suggesting that strabismus misalignment may include altered vergence muscle tone. Recordings of the ocular motor nuclei, which directly command eye movements, show that the motor nuclei themselves encode the misaligned eye position and eye movements (Joshi and Das 2011; Fu et al. 2007; Walton et al. 2015). The oculomotor neurons themselves may be abnormal in strabismus, as is true for abducens motor neurons in strabismic monkeys, which have a lower firing rate than normal (Walton et al. 2015). These studies strongly support the hypothesis that the cause of eye misalignment for sensory-induced strabismus may be largely attributable to abnormal signaling by the ocular motor system, which is created by disrupted sensory input during the critical period for development of stereopsis.

Disruption of normal binocular viewing has vast influences on the brain, but also extends to altering properties of the extraocular muscles (EOM). Eye misalignment during early visual development in cats increases muscle fatigue and results in slower contractions (Lennerstrand 1979; Lennerstrand and Hanson 1979). Analysis of EOM specimens from human subjects with congenital strabismus and from dark reared animals show that strabismus alters the expression of a number of EOM transcripts, including decreasing genes related to muscle contractility, and increase in genes related to the extraocular matrix, such as collagen (Cheng 2003; Altick et al. 2012). Analysis of human EOM from patients with strabismus also suggests that there are a number of changes in these muscles compared to age matched controls including substantial

changes in the intramuscular connective tissue matrix (Stager, McLoon, and Felius 2013).

NEUROTROPHIC FACTORS

Neurotrophic factors are a family of signaling molecules that play a strong role in the development of neuronal connections and in neuronal survival. The role of neurotrophic factors continues in the adult system, as neurotrophic factors maintain neuronal connections (Causing et al. 1997; Novikova, Holmberg, and Kellerth 2000), influence plasticity (Schinder and Poo 2000; Snider and Lichtman 1996), modulate firing properties (Gonzalez and Collins 1997; Desai, Rutherford, and Turrigiano 1999), and play a role in response to injury (Lindsay 1988). In the neuromuscular system, neurotrophic factor signaling from muscles participates in neuromuscular junction establishment, and survival and maintenance of motor neurons.

When a developing axon reaches its muscle target, a large variety of muscle-derived neurotrophic factors promote survival of successfully connected motor neurons during the normal development period (Rind and von Bartheld 2002). These target-derived neurotrophic factors are retrogradely transported from the presynaptic site through the axon in vesicles along microtubules to the neuronal soma, where they activate signaling cascades (Dumas, Schwab, and Thoenen 1979). Here, the vesicles are sorted, and neurotrophic factors may be degraded or presented to afferent neurons through transcytosis (Dumas, Schwab, and Thoenen 1979; Rind, Butowt, and von Bartheld 2005). During establishment of neuronal muscular connections, multiple axons compete for postsynaptic sites on muscle fibers (Turney and Lichtman 2012). Muscle-derived neurotrophic factors can function as punishment and reward signals in the activity-dependent process of eliminating polyneuronal innervation during maturation of the system (Wyatt and Balice-Gordon 2003; Je et al. 2013). Target derived neurotrophic factors not only signal to promote connection of a motor neuron with its target, but also signal to influence properties of both muscle and motor neurons (T. Li, Feng, and von Bartheld 2011; Davis-López de Carrizosa et al. 2010; Chen and von Bartheld 2004).

The strong role of neurotrophic factors in promoting neuromuscular connections and influencing motor neuron and muscular properties during development makes them intriguing candidates for pharmacological treatment of various neuromuscular disorders. In the absence of nerve injury or congenital absence of innervation, the cause of

strabismus is unclear, but it is associated with abnormal muscular and neuronal properties, including abnormal weighting of neuronal inputs between each eye (Fu et al. 2007; Joshi and Das 2011; Walton et al. 2015), with resultant over- or under-acting muscles. Target-derived neurotrophic treatment of muscle and motor neurons have been suggested to present a potential pharmacological tool for treating strabismus by altering both neuronal connections and muscle properties (Anderson et al. 2006; McLoon, Anderson, and Christiansen 2006; Chen and von Bartheld 2004). Two strong candidate neurotrophic factors for increasing muscle force in the ocular motor system are insulin-like growth factor 1 (IGF-1) and brain-derived neurotrophic factor (BDNF).

INSULIN-LIKE GROWTH FACTOR-1

IGF-1 is a polypeptide synthesized by diverse tissues including the liver, bone, cartilage, and skeletal muscle. IGF-1 circulates in the blood stream, but IGF-1 is also produced and regulated locally. IGF-1 signaling is important for muscle maintenance and regeneration (Edwall et al. 1989; Coleman et al. 1995; Dobrowolny et al. 2005; Stewart and Rotwein 1996; Florini, Ewton, and Coolican 1996). In skeletal muscle, a population of quiescent mononucleated myogenic precursor cells called satellite cells is maintained for muscle repair in response to injury and disease. When activated, satellite cells proliferate and differentiate into myoblasts. Myoblasts proliferate and differentiate, and either fuse into existing myofibers to increase myofibers size, or myoblasts fuse together to generate new fibers (Hill, Wernig, and Goldspink 2003). IGF-1 promotes proliferation and differentiation of satellite cells (Barton-Davis, Shoturma, and Sweeney 1999; Engert, Berglund, and Rosenthal 1996; Adi et al. 2002) and myoblasts (Jacquemin et al. 2007; James, Stewart, and Rotwein 1996), and also stimulates muscle protein synthesis (Bell and McDermott 2000; Park et al. 2014).

IGF-1 signals through its receptor (IGF-1R), which belongs to the class of tyrosine kinase receptors. IGF-IR activation signals prominently through the RAS/MAP kinase pathway, which stimulates cell division (Coleman 2009), and the PI-3K/AKT pathway, which increases protein synthesis and muscle cell differentiation (Luo et al. 2006; Allen and Boxhorn 1989; Engert, Berglund, and Rosenthal 1996; Davis et al. 2002). Muscle tension and stretch induce IGF-1 expression, which promotes IGF-1's role in muscle maintenance (McKoy et al. 1999; Hameed et al. 2003) . A reservoir of IGF-1 is maintained extracellularly through binding to IGF-binding proteins (IGFBP1-6), which are

released during muscle injury and repair (Jennische, Skottner, and Hansson 1987; Jones and Clemmons 1995). IGF-1 treatment of skeletal muscle has been strongly documented to increase muscle mass through promotion of protein synthesis and inhibition of protein degradation resulting in increased myofiber size, and increased muscle force (Musarò et al. 2001; McLoon, Anderson, and Christiansen 2006; Anderson et al. 2006; T. Li, Wiggins, and von Bartheld 2010). This has made it a strong candidate for a treatment for conditions of muscle atrophy, such as aging and amyotrophic lateral sclerosis (Payne et al. 2006; T. M. Miller and Cleveland 2003).

IGF-1 and its receptor are expressed in EOM and the Schwann cells within nerves (Christiansen and McLoon 2006; Feng and von Bartheld 2011; Feng and von Bartheld 2010). Within EOM, IGF-1, IGF1 binding proteins, and IGF-1R are expressed along a longitudinal gradient, with highest expression distally (Feng and von Bartheld 2011). Treatment of EOM with IGF-1 creates effects similar to those seen in other skeletal muscles. In both developing chickens and rabbits, IGF-1 treatment increases muscle mass, myofiber cross-sectional area, muscle force, and increases myogenic precursor cell proliferation (as determined by increased BRDU+ nuclei)(Chen and von Bartheld 2004; Anderson et al. 2006; McLoon, Anderson, and Christiansen 2006; T. Li, Wiggins, and von Bartheld 2010). Complementary to the gradient expression of IGF-1, treatment of EOM with IGF-1 also has differential effects along the muscle from proximal to distal. In neonatal and juvenile chickens, IGF-1 treatment has the strongest influence on the proximal end of the muscle, where cross-sectional area of myofibers, number of myofibers, and number of proliferating nuclei increase with treatment (T. Li, Wiggins, and von Bartheld 2010). IGF-1's effects on EOM may extend not just to increasing muscle force but also can change other aspects of muscle kinetics. Acute IGF-1 treatment shortens half relaxation time in the muscle of birds, while blocking IGF-1 signaling with IGFBP3 prolongs twitch contraction time and half relaxation time (T. Li, Feng, and von Bartheld 2011). Long term treatment for three months in rabbits increases relaxation and contraction time, potentially reflecting the larger force created from larger fibers (Anderson et al. 2006). IGF-1 also decreases neonatal and increases developmental myosin in adult rabbit EOM, which potentially is responsible for alterations in twitch characteristics (Anderson et al. 2006). In addition to regulating muscles, IGF-1 treatment also maintains neuromuscular junctions. Treatment with IGF-1 protects against the

effects of aging, injury, or disease on neuromuscular size and morphology (Apel et al. 2010; Dobrowolny et al. 2005).

In addition to these important effects on the EOM, in the ocular motor system IGF-1 is also a retrogradely transported survival factor for ocular motor neurons. Treatment of EOM with IGF-1 rescues motor neurons from naturally occurring cell death in development (Rind and von Bartheld 2002). IGF-1 also promotes axon elongation. IGF-1 causes neurite outgrowth when applied to avian oculomotor explants (Rind and von Bartheld, 2002). In other skeletal muscles, IGF-1 treatment of skeletal muscle causes axonal sprouting (Caroni and Grandes 1990; Caroni et al. 1994; Salie and Steeves 2005). This has made IGF-1 treatment a promising candidate for promoting regeneration of injured peripheral motor nerves. In rat models of nerve transection, IGF-1 increases the number and density of axons that regenerate into injured muscle and also promotes increased myelination (Apel et al. 2010).

As IGF-1 promotes neuronal survival and axon regeneration, IGF-1 may also promote neuronal excitability and plasticity. IGF-1 delivery to sensory or motor neurons can increase spontaneous firing and postsynaptic potentials (Carro et al. 2000; Nuñez, Carro, and Torres-Aleman 2003). Similarly, the effects of exercise on neuronal plasticity and hippocampal neurogenesis is strongly tied to IGF-1 signaling (Carro et al. 2000; Trejo, Carro, and Torres-Aleman 2001). In the visual cortex, administration of IGF-1 dramatically increases neuronal plasticity in the adult, potentially by increasing neuronal excitability, and or by remodeling inhibitory extracellular matrix (Maya-Vetencourt et al. 2012).

In summary, IGF-1 treatment of EOM increases both the force of muscle contraction and increases the size of muscle fibers (McLoon, Anderson, and Christiansen 2006; Anderson et al. 2006; T. Li, Wiggins, and von Bartheld 2010; T. Li, Feng, and von Bartheld 2011). This makes IGF-1 a promising candidate to strengthen a presumed or identified underacting muscle for strabismus treatment. In addition to IGF-1's significant effects on skeletal muscle function and structure, IGF-1 also signals from muscle to motor neurons. IGF-1 has been implicated in promoting axon growth and influencing the excitability of neurons. The dual action of IGF-1 on both muscle and motor neurons that direct muscle movement makes it a promising candidate for treating ocular motor diseases like strabismus, which have a foundation in disordered muscle and neuronal signaling.

BRAIN-DERIVED NEUROTROPHIC FACTOR

Brain-derived neurotrophic factor (BDNF) is widely utilized throughout both the developing and mature central and peripheral nervous system. It is often released in a neuronal activity dependent manner, and plays a wide variety of roles, including acting as a neuronal survival factor, promoting formation of neuromuscular junctions, and promoting synaptogenesis and synaptic plasticity (Black 1999; Galuske et al. 1996). BDNF is in the neurotrophin family, and binds with highest affinity to the receptor tropomyosin-receptor-kinase (TrkB), which it shares with neurotrophin 4/5 (NT-4/5) (Klein et al. 1991). BDNF can also bind, though with lower affinity, to the other receptors in the neurotrophin family: TrkA and TrkC. The immature form of BDNF, pro-BDNF, signals through the receptor $p75^{NTR}$ (F. Yang et al. 2009).

BDNF is a target-derived retrogradely transported neurotrophic factor in the ocular motor system. BDNF transcript is present in EOM in both development and adulthood (Steljes et al. 1999; Morcuende et al. 2013; Harandi et al. 2014), and can be retrogradely transported from EOM (Steljes et al. 1999). EOM is likely the major source of BDNF for ocular motor neurons (Benítez-Temiño et al. 2004). Ocular motor neurons have receptors for TrkB and p75, although interestingly, a small percentage of motor neurons lack TrkB receptors (Benítez-Temiño et al. 2004; Steljes et al. 1999).

It is unclear if BDNF is neuroprotective for ocular motor neurons. In the developing chick system, BDNF treatment does not rescue motor neurons from normal developmental cell death (Steljes et al. 1999). However, BDNF treatment does rescue motor neurons from axotomy-induced cell death, and preserves the cholinergic phenotype of motor neurons (Morcuende et al. 2013). Complementarily, TrkB expression is upregulated after axonal lesion, implicating BDNF as important for regenerative responses (Morcuende et al. 2011). Other neurotrophic factors are more potent survival factors for ocular motor neurons (Morcuende et al. 2013; Steljes et al. 1999; Rind and von Bartheld 2002), suggesting that BDNF more likely plays a role in the regulation of synapses and firing properties.

BDNF promotes neurite outgrowth of oculomotor explants (Steljes et al. 1999). This is consistent with BDNF signaling in spinal motor neurons, where BDNF causes axon regeneration after injury (Lindsay 1988; Bregman et al. 1997; McTigue et al. 1998; Lu et al. 2012). Most dramatically, BDNF influences the firing characteristics of ocular

motor neurons. When motor neurons lose trophic support from their target, such as after axonal lesion, the connections from afferent neurons are also lost, and as a result have drastically reduced firing patterns (Sumner 1975; de la Cruz, Pastor, and Delgado-García 1996). BDNF restores synaptic contacts and normal firing patterns, but its function is specific to a subgroup of inputs. During horizontal saccades, ocular motor neurons have a three component firing pattern, with a burst related to the eye velocity during the saccade, a step component related to eye position, and a slide related to muscle tension. BDNF treatment restores neuronal encoding of eye position, but not eye velocity. BDNF also promotes recovery of contralateral excitatory synaptic potentials from the vestibular system, but not the ipsilateral inhibitory inputs (Davis-López de Carrizosa et al. 2009). This strongly suggests a role for BDNF in strongly influencing the response characteristics of motor neurons, and promoting only a subpopulation of afferent connections.

In addition to BDNF's retrograde influence on motor neuron properties, BDNF may exert influence at the level of the EOM as well, although studies are sparse. Work in skeletal muscles suggests BDNF may influence neuromuscular junctions and muscle myofibers. In other skeletal muscles, BDNF transcript and protein are expressed throughout postnatal development in both slow and fast muscles (Ip, Cheung, and Ip 2001; Nagano and Suzuki 2003; Mousavi and Jasmin 2006; Garcia et al. 2010; Henderson et al. 1993). In the diaphragm, BDNF expression is limited to satellite cells, where it signals with p75 to maintain the satellite cell population and inhibit differentiation (Mousavi and Jasmin 2006).

In contrast to the diaphragm, BDNF and TrkB have been localized to neuromuscular junctions in other skeletal muscles. In the rat levator auris longus (LAL) muscle, BDNF is present at neuromuscular junctions: postsynaptically, at the nerve terminal, and in Schwann cells (Garcia et al. 2010). Similarly, TrkB has also been localized to axons and the pre- and postsynaptic parts of neuromuscular junctions in the sternomastoid muscle (Je et al. 2013; Gonzalez et al. 1999; Garcia et al. 2010). In the developing LAL, pro-BDNF signaling through p75(NTR)-sortillin acts as a punishment signal for elimination of polyinnervation at neuromuscular junctions. Neuronal activity releases metalloproteases, which convert pro-BDNF into mature BDNF. The mature BDNF then serves as a survival factor for active neuromuscular junctions, and supports stabilization of singly innervated neuromuscular junctions (Kwon and Gurney 1996; Je et

al. 2013). BDNF also enhances neuromuscular transmission by increasing evoked endplate potentials (Pousinha et al. 2006; Mantilla, Zhan, and Sieck 2004). BDNF signaling may continue to maintain neuromuscular junctions after development. TrkB has been shown to be involved in maturation (T. Wang, Xie, and Lu 1995) and maintenance of postsynaptic acetylcholine receptors (Gonzales et al., 1999, Kulakowski et al., 2011). In the soleus muscle, knock-out of TrkB results in decreases in muscle strength and myofiber size, potentially because of resulting disrupted signaling and disassembly of neuromuscular junctions (Kulakowski, Parker, and Personius 2011).

From the literature, it is clear that BDNF is a target-derived neurotropic factor for ocular motor neurons, and plays a large role in supporting motor neuron synapses and firing characteristics. It is currently unclear what role BDNF may have at the target level, but the strong influence of BDNF on axonal outgrowth, promotion of synapses, and its implied role in muscle differentiation and neuromuscular junction maintenance makes it an attractive candidate as a potential treatment for strabismus that could be applied to EOM, which are readily accessible, and induce neuronal plasticity after retrograde transport.

EXTRAOCULAR MUSCLE

The extraocular muscles (EOM) control position, stability, and movement of the eyes. The fovea is an area in the retina with densely packed cones and an associated high concentration of retinal ganglion cells, which forms the basis for high acuity central vision. In foveate animals, EOM participate in five distinct eye movements, which aid in positioning the image of areas of interest in the environment onto the fovea. Execution of these diverse eye movements places a variety of physiological demands on extraocular muscle. The eyes muscles are fatigue-resistant, which is compatible with the fact that eyes are constantly moving to change the direction of gaze and then hold the eyes in a variety of precise locations. 1. Saccades are fast, precise movements that shift the eyes from one item of focus to another in the visual field in 20 to 200 ms, depending on angular distance. In fact, eye muscles have the fastest contractile properties of any mammalian skeletal muscle (Close and Luff 1974). Other eye movements require slower graded movements. 2. Smooth pursuit is used to track a moving object, and maintains its image on the foveae. 3. Vergence is a dysconjugate eye movement

required for aligning the optic axis of the eyes on objects in depth either close (convergent) or farther away (divergence) from a non-optical infinity reference point from to the viewer. Finally, the vestibular ocular reflex and optokinetic reflex allow stability of an image on the retinae despite movement of the head or the visual surround.

To achieve this variety of movements, there are six different EOM that move each globe, and a seventh muscle that raises the eyelid (*levator palpebrae superioris*). Of these six EOM, the four recti generally move the eye horizontally and vertically, and two oblique participate in rotational movements. The six EOM in each orbit are controlled by three different cranial nerves: the oculomotor, abducens, and trochlear nerves.

The four recti muscles are the medial, lateral, superior, and inferior rectus. They originate from the apex of the orbit at the same common tendinous ring. Each muscle inserts on the sclera, on either the medial, lateral, superior, or inferior sides of the globe, anterior to the equator. The medial and lateral rectus muscles move the eyes horizontally as an agonist/antagonist pair on each globe. The lateral rectus abducts the eye, and the medial rectus adducts the eye. Because most eye movements are conjugate, these muscles are also functionally yoked. For example, if one were to look to the left, the left lateral rectus and right medial rectus would contract together to generate a conjugate shift in gaze. Two different nerves control these functionally paired muscles. The lateral rectus muscle is controlled by the abducens nerve, and the medial rectus is controlled by oculomotor nerve, which also controls the inferior rectus, superior rectus, and inferior oblique. To generate conjugate horizontal eye movements, afferent eye movement signals in the abducens nucleus are relayed to the contralateral oculomotor nucleus via internuclear neurons, allowing simultaneous contraction of the lateral rectus and contralateral medial rectus.

Because the orbit has a pyramidal shape (axis set approximately 23 degrees away from the mid sagittal plane), the superior and inferior rectus muscles rotate the eyes toward the nose as well as move them vertically. Thus, the superior rectus muscle elevates and intorts (rotation about the optic axis) the eye, and the lateral rectus muscle depresses and extorts the eye. The vertical rectus muscles work in parallel with the two oblique muscles, the superior and inferior oblique, which play a major role in torsional movements of the eye and secondary actions in the vertical plane. In contrast to the recti, the oblique muscles originate from the bony orbit. The superior oblique also runs through a cartilaginous pulley (the trochlea), before inserting onto the sclera posterior to

the equator deep to the superior rectus muscle, and is uniquely innerved by the trochlear nerve. The inferior oblique is the only muscle whose origin is anterior in the bony orbit. It runs in a parallel path to the post-trochlear portion of the superior oblique, and inserts onto the sclera posterior to the equator.

Complementary to the wide variety of movements the EOM execute, the EOM have unique properties from other skeletal muscle, from the type of myosins and neuromuscular junctions they express, to their ability to continually remodel (McLoon, Willoughby, and Andrade 2012). The EOM arise from non-segmented cranial mesenchyme, instead of somites like limb skeletal muscles. The genes that control early development of the EOM also don't overlap with other craniofacial muscle, or the genes that control limb and body muscular formation (reviewed in Harel and Tzahor 2012). Formation of the EOM is dependent on the transcription factor Pitx2 (Diehl et al. 2006), as well as formation of the eye, optic vesicle, and periocular neural crest cells (Bohnsack et al. 2011). Thus, among skeletal muscles, the EOM are considered their own allotype, and have a distinct gene profile from other skeletal muscles (Porter et al. 2001).

The extraocular muscles are composed of two distinct layers: an orbital layer and a global layer. An intermediate layer exists between the two layers, and has a mixture of properties. In contrast to the global layer, the orbital layer has smaller fibers (Mayr 1971; Wasicky et al. 2000; Kjellgren et al. 2003), more activated satellite cells (McLoon et al. 2004), higher expression of developmental myosins (Y. Zhou, Liu, and Kaminski 2010) and more complex innervation (Pachter, Davidowitz, and Breinin 1975; Pachter 1984). Muscle fiber properties vary both between the two EOM layers, and longitudinally from the proximal to distal end of the muscle (Mayr 1971; Wasicky et al. 2000; McLoon, Rios, and Wirtschafter 1999; McLoon et al. 2011). Due to the wide variety of myofiber properties throughout the EOM, in contrast to limb skeletal muscles, EOM myofibers are difficult to classify, despite some attempts to segregate them into six fiber types (Spencer and Porter 1988; Wasicky et al. 2000). To add to the diverse and varying properties of the EOM, the EOM express nine different myosin heavy chain isoforms (MyHC) (Wieczorek et al. 1985; Asmussen, Traub, and Pette 1993), which allow for a variety of contractile properties. These include fast and slow MyHCs, alpha-cardiac MyHC, a unique EOM-specific myosin, (Pedrosa-Domellöf et al. 1992), and developmental myosins which the EOM maintain into maturity. These MyHC isoforms are co-expressed within single fibers, and their expression can vary throughout the

length of an individual fiber (Y. Zhou, Liu, and Kaminski 2010; McLoon et al. 2011). The EOM myofibers likely represent a continuum of slow-tonic to fast-twitch properties, in part due to the co-expression of multiple myosin heavy chain isoforms within single fibers (Wieczorek et al., 1985; McLoon et al., 2011; Park et al., 2010).

NEUROMUSCULAR JUNCTIONS

Another unique feature of EOMs are their neuromuscular junctions, which have two distinct types of endplates: *en plaque* and *en grappe*. *En plaque* endings are similar to those found in limb skeletal muscle, and are most prominent as a broad band in the middle two-thirds of muscle (Kupfer 1960). The smaller *en grappe* endplates are found throughout the length of the extraocular muscles and are predominately found where slow MyHCs are expressed. *En grappe* neuromuscular junctions maintain expression of the immature gamma subunit, instead of the epsilon subunit normally found in mature neuromuscular junctions and in the *en plaque* neuromuscular junctions of EOM (Horton and Stryker 1993; Kaminski, Kusner, and Block 1996; Fraterman, Khurana, and Rubinstein 2006).

The EOM myofibers are commonly grouped into two types based on their innervation (Siebeck and Krüger 1955). Singly innervated fibers (SIF) express predominantly fast MyHC isoforms, and have a centrally located *en plaque* ending. Multiply-innervated fibers (MIF) predominately express slow MyHC isoforms, have multiple *en grappe* endings throughout their length, and are most commonly associated with unmyelinated nerve fibers; these account for 10-20% of nerve fibers in the EOM (Eberhorn et al. 2005). Functional studies have strongly suggested that SIF and MIF fibers may play distinct roles in eye movements. SIFs produce large twitch contractions, and produce action potentials that propagate down the muscle fiber (Chiarandini and Stefani 1979). In contrast, MIFs are fatigue resistant, and contract during slow tonic input (Bondi and Chiarandini 1983; Hess and Pilar 1963; Chiarandini and Stefani 1979). Calcium imaging of whole EOM in the chick suggests that MIF fibers have spontaneous calcium waves associated with local contractions of the muscle fibers that are independent of activation of the nicotinic acetylcholine receptor. These calcium waves haven't been observed in SIF fibers, although both SIF and MIF fibers may exhibit spontaneous and evoked fast calcium transients that aren't correlated with fiber contraction (Feng et al. 2012). These studies provid strong evidence for differing roles of

SIF and MIF fibers in eye movements. MIFs, as predominantly non-twitch, non-fatigable fibers, may be recruited early to control eye position, and fine-tuned eye movements. In contrast, SIFs may be recruited later for larger eye movements including saccades (Feng et al. 2012; Spencer and Porter 2006).

The dichotomy of EOM myofibers as SIF or MIF oversimplifies the complexity of EOM function. There is ample evidence that individual myofibers in the orbital layer can have an *en plaque* ending in the middle of the myofiber and *en grappe* endings along the rest of the myofiber, resulting in differential electrical activity along the length of individual myofibers (Jacoby, Chiarandini, and Stefani 1989; Pachter 1984; Mayr et al. 1975). These orbital fibers were originally thought to run the full length of the muscle, but recent evidence demonstrates otherwise (McLoon et al. 2011). In the global layer, careful dissection of cat EOM fibers demonstrates that the SIF fibers are short and highly branched, and connect to MIF fibers through myomyous junctions (Mayr et al. 1975).

The distal end of EOM has specialized nerve structures called palisade endings, which refers to an unusual axon trajectory in which the axons from the midbelly course distally toward the tendon, then do a 180 degree turn, and innervate the distal end of slow fibers. It was originally thought that these endings might be a sensory input to EOM, but a flood of recent studies have shown that these endings are in fact motor, and arise from the motor nuclei (Lienbacher et al. 2011; Zimmermann et al. 2011; Zimmermann et al. 2013). The role that such high density of neuromuscular junctions may play at the ends of muscle fibers is unknown. Growing evidence suggests that for the medical rectus, these distal *en grappe* endings contribute to vergence (Wasicky, Horn, and Büttner-Ennever 2004; Erichsen, Wright, and May 2014). Distal injection of an EOM with retrograde tracer labels a cluster of neurons in the oculomotor nucleus that lie along the dorsal midline periphery of motor nuclei (although midbelly injections can also label this region) (Büttner-Ennever et al. 2001; Wasicky, Horn, and Büttner-Ennever 2004). Afferent tracer injections have supported that these motor neurons receive similar projections from pre-motor nuclei that control eye movements, but in addition uniquely receive input from the pretectum (Wasicky, Horn, and Büttner-Ennever 2004). Further, the dendrites of these neurons extend up into the Edinger Westfal and superior oculomotor area, rather than ventrally into the oculomotor nucleus (Erichsen, Wright, and May 2014). The precise role of these motor neurons has yet to be shown, but these

anatomical studies suggest that the motor neurons may receive input from circuits commanding object fixation, response to visual background, vergence, and the near response (Wasicky, Horn, and Büttner-Ennever 2004; Erichsen, Wright, and May 2014).

Distal tracer injections into the distally-located *en grappe* endings in the other EOM also preferentially label neurons in the medial dorsal area. These motor neurons are often smaller in size, and negative for parvalbumin, perineuronal nets, and SMI-32, a general marker for motor neurons (Eberhorn, Büttner-Ennever, and Horn 2006; Eberhorn et al. 2005). Expression of parvalbumin and perineuronal nets is highly correlated with high motor neuron activity (reviewed in Wang and Fawcett 2012). Absence of these markers in presumed MIF motor neurons is compatible with a proposed role for MIFs in tonic firing (Eberhorn et al. 2005). If there is a unique population of motor neurons for MIFs, one would hypothesize that there would be motor neurons with inputs that arise solely from pre-motor nuclei that determine eye position, and that the motor neurons would fire tonically, instead of with the characteristic pulse-slide-step. Neuroanatomical labeling with tracers suggests that MIF motor neurons labeled by distal injections may not receive input from excitatory nuclei commanding saccades and the VOR (Ugolini et al. 2006). However, thus far, no motor neurons have been isolated in behaving non-human primates that fire tonically, although two units within the abducens nucleus that innervated non-twitch muscle and responded only to tetanic stimulation were found in one preparation in the cat (Goldberg, Clamann, and McClung 1981). Sensitivity of motor neuron firing to eye position and eye velocity varies along a continuum suggesting that there is heterogeneity in the function of individual motor neurons that would affect different aspects of extraocular muscle contraction characteristics (Sylvestre and Cullen 1999b; Davis-López de Carrizosa et al. 2011). Structurally, motor neurons of the ocular motor system have a wide diversity of soma size and synaptic density on the soma and dendrites (Spencer and Sterling 1977). It may be that units more sensitive to the control of eye velocity are recruited later for more eccentric eye positions, and may preferentially innervate global SIF fibers. However, based on the heterogeneity of the myofibers themselves, to delineate motor neurons as solely SIF or MIF may oversimplify the ocular motor system.

CAN NEUROTROPHIC FACTORS REWIRE THE OCULOMOTOR SYSTEM?

Strabismus is a unique disorder of the ocular motor system, where normal eye alignment does not develop, resulting in disconjugate eye movements and lack of binocular vision. Neurotrophic factors such as IGF-1 and BDNF have emerged as potential candidates for treatment of ocular motor disorders. Neurotrophic factors have the ability to alter properties of EOM myofibers, neuromuscular junctions, and the motor neurons themselves through retrograde transport from the target muscle. A multi-action pharmacological agent that could be applied directly to the readily accessible EOM could present a clinically simple treatment for strabismus. In chapter 2, I build on previous work, which demonstrated that IGF-1 makes myofibers stronger, to explore if the efficacy of IGF-1 could be potentiated with pre-treatment of hepatocyte growth factor. This would enhance the potential of IGF-1 to strengthen a presumed weak muscle in strabismus, and strengthen the potential therapeutic use of IGF-1 to correct strabismus. Despite the strong evidence that IGF-1 treatment strengthens EOM, the efficacy of IGF-1 to alter eye alignment in a binocular animal model is currently unknown. In chapters 3 and 4, I test the hypothesis that persistent bilateral (Chapter 3) or unilateral (Chapter 4) IGF-1 treatment to the medial rectus of a nonhuman primate can alter eye positioning. For these studies, we treated visually normal infant nonhuman primates during the postnatal development of the ocular motor system, which allowed us not only to assess if persistent IGF-1 treatment could disrupt the eye alignment, but also to investigate if aberrant neurotrophic factor signaling during development of the ocular motor system in the EOM is a possible cause of strabismus. Finally, in Chapter 5, I extend my studies of neurotrophic factor treatment to infant nonhuman primates to test if another promising neurotropic factor, BDNF, could alter eye alignment during development of the ocular motor system.

Chapter 2: Effects of Sequential Injections of Hepatocyte Growth Factor and Insulin-like Growth Factor-I on Adult Rabbit Extraocular Muscle

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SUMMARY

To determine whether hepatocyte growth factor (HGF) and insulin-like growth factor-I (IGF-I) have synergistic effects in promoting extraocular muscle fiber growth and force generation. A superior rectus muscle of adult rabbits was treated with either a single injection of HGF or sequential injections of HGF followed one week later by IGF-I. One week after HGF alone and one week after the IGF-I injection, the SR muscles from treated and control orbits were examined for alterations in force generation as well as changes in myofiber size. Injection of HGF alone did not result in changes to muscle force, specific tension, or myofiber cross-sectional area; however, it did result in a significant increase in numbers of satellite cells. Sequential injection of HGF and IGF-I resulted in significantly increased force, specific tension, and myofiber cross-sectional areas, as well as increased numbers of satellite cells. Pre-injection with HGF augments the treatment effect of IGF-I. This is likely a result of HGF-induced activation of satellite cells. This synergistic effect should allow a reduction in IGF-I dosing required to produce a given increase in EOM force generation.

INTRODUCTION

Pharmacologic treatment of strabismus is evolving. Botulinum toxin A (BTA) was developed over three decades ago (Scott 1980) as a means of weakening skeletal muscle, while avoiding permanent alterations in the biomechanics of the ocular rotary mechanism. BTA proved effective in some forms of strabismus, but widespread adoption of BTA has been limited, due, in part, to its relatively brief duration of action and the

frequent need for reinjection. Whereas BTA only weakens extraocular muscle, growth factors can be used to strengthen them (Anderson et al. 2006; T. Li, Wiggins, and von Bartheld 2010; McLoon, Anderson, and Christiansen 2006; McLoon 2003); however, no growth factors have been approved for clinical use. Recent studies using the local anesthetic bupivacaine have shown changes in eye position, but the mechanism is unclear (Scott, Alexander, and Miller 2007; Zhang et al. 2010). This potential to increase and decrease EOM strength pharmacologically, in a way that is easily and safely administered, bears further development and study.

Recently we showed that single, direct intramuscular injections of insulin-like growth factor (IGF) -I or II into adult rabbit EOM result in significantly increased muscle force (grams) and specific tension (mN/cm^2), with most effective doses at 10 µg and 25 µg (Anderson et al. 2006; McLoon 2003). This increased muscle force was maintained by sustained delivery of IGF-I to the extraocular muscle (McLoon, Anderson, and Christiansen 2006), as IGF-I and its receptor are both expressed in adult extraocular muscle (Anderson et al. 2006; Feng and von Bartheld 2011). There is some concern over the potential effect of high doses of IGF-I in extraocular muscle because recent data has shown a possible link between the IGF-I receptor in orbital fibroblasts in active thyroid eye disease and fat expansion (Naik et al. 2015). Thus, we tested a strategy for lowering the dosage of IGF-I while maintaining equivalent force augmentation. Prior to clinical use, several important issues need to be addressed, including the dosage required for both maximal and lasting effects, as well as demonstration that no local or systemic toxicity result from sustained release of these growth factors.

Hepatocyte growth factor (HGF) stimulates mitogenesis and plays an important role in tissue regeneration (Jiang and Hiscox 1997). In skeletal muscle, HGF activates quiescent satellite cells, which are the regenerative population (Tatsumi et al. 1998; Allen et al. 1995). It also inhibits differentiation of the activated satellite cells (Gal-Levi et al. 1998). This should result in increased numbers of myogenic precursor cells in treated muscles; however, HGF alone does not result in increased myofiber growth (K. J. Miller et al. 2000), unlike IGF-I (Barton-Davis, Shoturma, and Sweeney 1999; Coleman et al. 1995; G. R. Adams and McCue 1998). Studies show that the expression of these two growth factors increases in skeletal muscle during repair, growth, and remodeling (Mueller et al. 2011; Honda et al. 2010). The present study examined whether pre-injection of HGF prior to IGF-I treatment would augment the effect of IGF-I alone by

providing greater numbers of satellite cells and therefore promoting increased myofiber size and force generation. If this injection strategy is effective, less IGF-I could be injected in order to obtain the same overall muscle-strengthening effect. The potential mechanisms of action are also investigated.

METHODS

Adult New Zealand rabbits were obtained from Bakkom Rabbitry (Viroqua, WI) and housed with Research Animal Resources in their AAALAC-approved facility at the University of Minnesota. All studies were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and followed the guidelines for animal research set forth by the National Institutes of Health. Rabbits were anesthetized with an intramuscular injection of ketamine and xylazine (1:1, 10mg/kg: 2mg/kg, respectively). Every experimental injection was accompanied by an injection of an equal volume of saline on the contralateral side. One group of 8 rabbits received a single injection of 2.5μg HGF in 100μl sterile isotonic saline into one superior rectus muscle (rmHGF, R & D, Minneapolis, MN), with a similar volume of saline injected into the contralateral superior rectus muscle. The second group of 8 rabbits received a single injection of HGF as described, followed one week later with an injection of 5μg IGF-I in 100μl sterile isotonic saline (rmIGF-I, R &D) into the same superior rectus muscle treated with HGF. This timing is the point of peak satellite cell proliferation based on *in vitro* studies (Hebert and McLoon, unpublished studies 2012). The contralateral superior rectus muscle received a second saline injection of a similar volume. As an additional control for the potential effect of injection alone causing the subsequent increased force measurements seen with the HGF/IGF-1 treatments, another group of six rabbits received bilateral injections of 100μl sterile isotonic saline followed one week later with an injection of 5mg IGF-I in 100μl sterile isotonic saline into one of the saline treated superior rectus muscle. An additional four rabbits were injected with HGF only and 4 rabbits were injected with HGF/IGF-I as described but prepared for histological analysis only.

After one week for the HGF-only treated rabbits, or two weeks for the HGF/IGF-I treated rabbits or saline/IGF-I treated rabbits, the animals were deeply anesthetized with ketamine and xylazine, followed by a thoracotomy. The SR were removed from the sclera to the apex of the orbit and analyzed for force and specific tension generation as

previously described (Anderson et al. 2006; McLoon, Anderson, and Christiansen 2006; Anderson, Christiansen, and McLoon 2008; Day et al. 2002). Maintained in oxygenated Krebs solution at 30^{o}C for the duration of the experiment, the muscles were attached to force transducers in *in vitro* incubation chambers (Aurora Scientific, Aurora, Ontario, Canada) and stimulated by flanking platinum electrodes. After length/tension curves were generated, all further tests were performed with supra-maximal stimulus intensities at optimal preload. After muscles achieved stabilization (Lynch et al. 2001), they were stimulated at frequencies of 10, 20, 40, 100, 150, and 200 Hz at a train duration of 500 ms with a two minute inter-stimulation rest and force in grams was determined. Percent differences were calculated for each stimulation frequency for each rabbit, and the mean percent difference was determined based on these values. For any given treatment, the mean values of all treated rabbits were averaged to determine the final percent difference.

The HGF only (N=8), saline/IGF-I (N=6), HGF/IGF-I (N=8), and control superior rectus muscles (N=14) were prepared for histologic examination. All superior rectus muscles were embedded in tragacanth gum, frozen in 2-methylbutane chilled to a slurry on liquid nitrogen, sectioned at 12μm in a cryostat, and stored at -80^{o}C until processed for immunohistochemistry. The following primary antibodies were used for immunostaining using our standard methods: fast, neonatal, and developmental myosin heavy chain (MyHC) isoforms (1:40; Vector Labs, Burlingame, CA); Pax7 (1:100; Hybridoma Bank, Iowa City, IA) and neurofilament (1:1000; Covance, Princeton, NJ). After blocking in normal serum, selected sections were incubated in primary antibody, rinsed in phosphate buffered saline, incubated with reagents in the Vectastain Elite ABC kit (Vector Lab), followed by incubation in diaminobenzidine using heavy metal intensification (McLoon 2003). Cross-sectional areas were measured for each of the three MyHC isoforms in both the orbital and global layers within sections taken from the muscle midbelly and within 200 μm of the end of myofibers in the tendon (referred hereafter as “in the tendon region”). Mean area and percent positive for expression of each isoform (see Figure 2.3) were calculated, with a minimum of 200 myofibers counted per layer and region. The effect on satellite cell number was assessed by analysis of Pax7-positive nuclei. Pax7 is a widely accepted marker for quiescent satellite cells (Seale et al. 2000). Pax7-positive nuclei were counted per myofiber number, with a minimum of 200 fibers counted per field for each layer and region. Neurofilament-stained

HGF, HGF/IGF-I and saline-treated superior rectus muscles were assessed by determining the area positive for axon bundles as a percent of total muscle area per cross-section, and orbital and global layers were compared in both the midbelly and tendon region.

Data were expressed as mean plus or minus the standard error of the mean. All data were analyzed for statistical significance using either an unpaired two-tailed t-test (if 2 groups were being compared) or an analysis of variance (ANOVA) and Dunn's multiple comparison tests aided by the Prism and Statmate software (Graphpad, San Diego, CA) for multiple group comparisons. An F-test was used to verify that the variances were not significantly different. Additional statistical analyses included ANOVA with Bonferroni's Multiple Comparison test. A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

Single injections of HGF alone did not result in significantly increased force generation one week after treatment, whether examined in grams (Figure 2.1A) or specific tension (not shown). In contrast, sequential injections of HGF followed one week later by IGF-I did result in significantly increased force (Figure 2.1B) and specific tension (not shown) at all stimulation frequencies. When compared to the mean percentage change in force generation observed with escalating doses of IGF-I alone (Anderson et al. 2006), the combined sequential injection of HGF and IGF-I resulted in significantly increased force generation over that seen after single injections of IGF-I regardless of dose (Figure 2.2). Injections of only HGF resulted in forces that were significantly below all doses of IGF-I alone and even more significantly below the combined HGF/IGF injections. Pre-injection with saline prior to injection with IGF-I resulted in levels similar to IGF-I alone (Anderson et al. 2006).

Mean myofiber cross-sectional areas were unchanged after HGF alone but were significantly greater after the sequential treatment with HGF and IGF-I compared to control levels, specifically in the orbital middle, and global middle and tendon regions (Figure 2.3). It should be noted that there was a great deal more heterogeneity of myofiber cross-sectional area in the HGF treated global region myofibers (Figure 2.3). This may have been due to concentrations differences along the muscle after injection. Future studies will examine how local HGF concentration might alter these effects. The

HGF-only treated superior rectus muscles had significantly more Pax7-positive nuclei per myofiber number than the untreated control muscles. This increased density of satellite cells was maintained in the HGF/IGF-I treated superior rectus muscles (Figure 2.4). No change in nerve density was seen in the HGF/IGF-I treated muscles at this short post-treatment interval (not shown).

The sequential treatment of superior rectus muscles with HGF and IGF-I resulted in few changes in fast MyHC isoform expression, with significant decrease only in the orbital tendon region (Figure 2.5A). The global layer showed significant changes in developmental and neonatal MyHC isoform composition, with significant decreases in the percentage of global layer myofibers expressing developmental MyHC isoform (Figure 2.5B) and a significant increase in neonatal MyHC isoform expression (Figure 2.5C). There was no measureable change in orbital layer fibers expressing developmental MyHC; however, the percentage of myofibers expressing neonatal MyHC did decrease significantly.

DISCUSSION

A single injection of HGF was insufficient to cause a significant change in muscle force generation one week after treatment. However, when HGF was injected intramuscularly one week prior to an injection of IGF-I, both mean myofiber cross-sectional area and force generation significantly increased. A prior injection of HGF before an IGF-I injection also resulted in significant elevation of muscle force generation above forces normally observed with a single IGF-I injection (Anderson et al. 2006). Thus combined sequential treatment by HGF and IGF-I resulted in muscles significantly stronger than after any dose of IGF-I alone (Anderson et al. 2006). This would allow for administration of a smaller amount of IGF-I to produce the same or even enhanced muscle force.

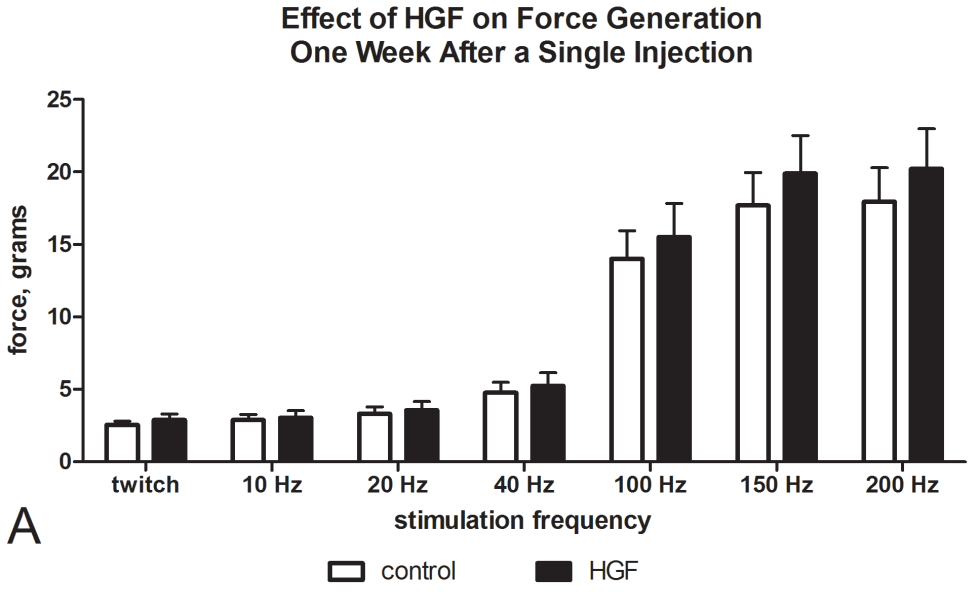
IGF-I is well known for its ability to build muscle mass and strength in skeletal muscle in a variety of conditions (G. R. Adams and McCue 1998), and has been shown to increase muscle mass and force generation capacity in, for example, mouse models of muscular dystrophy (Roth et al. 2001; Barton et al. 2002), aging (Barton-Davis et al. 1998), and muscle cachexia from cancer (Ng et al. 1992), and other chronic debilitating diseases (Yoshida et al. 2010). Several possible mechanisms may explain why injection

of HGF followed by IGF-I increased force generation greater than with IGF-I alone. HGF activates quiescent satellite cells, resulting in entry into the cell cycle and increased replication (Allen et al. 1995; Tatsumi et al. 1998). As predicted from these studies, HGF injection into rabbit extraocular muscle resulted in significant increases in satellite cell number, (Figure 2.4) which has been specifically linked to myofiber hypertrophy (Petrella et al. 2008; McCarthy et al. 2011). However, HGF treatment also inhibits skeletal muscle differentiation (Gal-Levi et al. 1998; K. J. Miller et al. 2000). IGF-I can induce satellite cell proliferation, resulting in increased number of these muscle precursor cells (Rosenthal and Cheng 1995). However, IGF-I also significantly contributes to the development of myofiber hypertrophy, increasing protein synthesis rates (Bark et al. 1998) and decreasing protein degradation pathways (Sacheck et al. 2004). This can occur even in the absence of increased satellite cell number (Barton-Davis, Shoturma, and Sweeney 1999). Sequential up-regulation of HGF followed by IGF-1 is seen in regenerating and diseased skeletal muscle (Honda et al. 2010; Hayashi et al. 2004) and has even been shown to foster regeneration of infarcted pig heart (Ellison et al. 2011). The rationale for sequential treatment is based on these collective studies.

Changes in MyHC isoform composition were most pronounced in the global layer, which produces the majority of force needed for eye movements. It is unclear how an increased percentage of global layer myofibers positive for neonatal MyHC and a decreased percentage of fibers positive for developmental MyHC would alter muscle function in light of the complex co-expression patterns of MyHC isoforms in single fibers in the extraocular muscle (McLoon et al. 2011). Nevertheless, similar MyHC isoform changes were seen after single injections of IGF-I in our previous study (Anderson et al. 2006) as well as in other studies that examined MyHC isoform composition after either exogenously supplied IGF-I treatment or other experimental paradigms that result in localized increases in IGF-I (H. Yang et al. 1997; Schertzer, Ryall, and Lynch 2006). Our previous work showed that, despite increased neonatal MyHC expression, the addition of new myonuclei into existing EOM myofibers did not correlate with neonatal MyHC fiber expression (Christiansen and McLoon 2006). Although not specifically measured in the present study, alteration of MyHC isoform expression patterns would be expected to change shortening velocity (Lowey, Waller, and Trybus 1993); thus functional sequelae would be predicted from these changes.

Both IGF-I and IGF-I receptor are expressed in extraocular muscles throughout life,[2,8] and are thought to play a role in maintaining the continuous myofiber remodeling that occurs in adult mammalian extraocular muscle (McLoon et al. 2004). Thus, the cellular machinery for rapid response to exogenously increased IGF-I is present without requiring receptor up-regulation. Recently an interrelationship was suggested between orbital tissue in Graves' ophthalmopathy and antibodies to IGF-I receptor (T. J. Smith et al. 2008). Expression was found to be co-resident with the expression of antibodies to thyroid stimulating hormone receptor (T. J. Smith 2010). However, recent studies somewhat allay the concern that exogenously added IGF-I might provide a "trigger" for an autoimmune response in the orbit. When mice were immunized with IGF-I receptor, thyroid stimulating hormone receptor, or both proteins, only immunization with thyroid stimulating hormone receptor protein alone resulted in antibodies to both thyroid stimulating hormone receptor and IGF-I receptor and moderate tissue fibrosis in orbital tissues (Zhao et al. 2011). This suggests that the precipitating factor for orbital disease is development of antibodies to thyroid stimulating hormone receptor. It should be noted that in all previous studies of IGF-I treatment of extraocular muscle report no changes in connective tissue density (Anderson et al. 2006; T. Li, Wiggins, and von Bartheld 2010; McLoon, Anderson, and Christiansen 2006; McLoon 2003). Our findings demonstrate that equivalent or greater efficacy using lower doses of IGF-I is possible in extraocular muscle.

FIGURE 2.1 (**A**) Effect on muscle force (grams) one week after a single injection of HGF compared to saline treated controls. (**B**) Effect of sequential treatment of superior rectus muscles by HGF followed one week later by a single injection of IGF-I compared to saline treated controls on muscle force in grams. Asterisk (*) indicates significantly different from control (ANOVA, Bonferroni; p≤ 0.05). N=8. Data are shown as mean plus or minus the standard error of the mean.



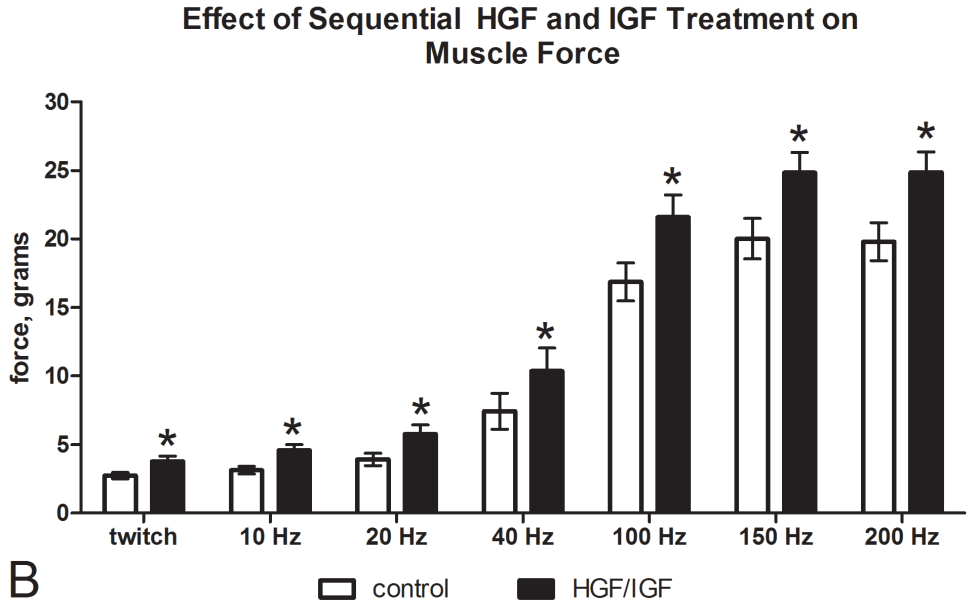


FIGURE 2.2 Comparison of percentage change of means in specific tension of either HGF alone or IGF- I alone compared to sequential treatment with HGF and IGF- I. Percentage change of injected IGF-I muscles was calculated based on Anderson et al. 2006. Asterisk (*) indicates significant difference of HGF/IGF injections compared to all doses of IGF only, HGF only and saline/IGF. σ indicates significant difference of HGF only compared to all doses of IGF only or HGF/IGF. While not shown on the graph there were significant differences between 1 and 25μgIGF-I doses, and between 10 and 25 μg IGF-I doses. N=6-8. (ANOVA, Bonferroni: p≤ 0.05).

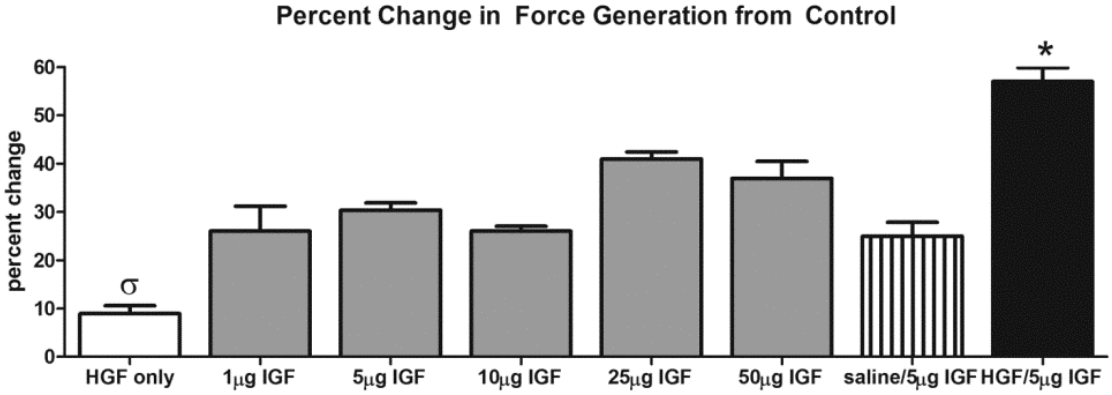


FIGURE 2.3 Mean cross-sectional areas in HGF/IGF-I co-treated superior rectus compared to saline treated controls determined on sections immunostained for fast MyHC. (**A**) Control global layer muscle fibers immunostained for fast MyHC two weeks after sequential saline injections. (**B**) Global layer myofibers examined one week after a sequential treatment of HGF followed one week later by IGF-I. Bar is 20 microns. (**C**) Mean cross-sectional areas of the middle region of the superior rectus muscles treated with saline only (control), HGF only, or sequential HGF/IGF-I. (**D**) Mean cross-sectional areas of the tendon region of the superior rectus muscles treated with saline only (control), HGF only, or sequential HGF/IGF-I. (ANOVA, Bonferroni, p≤ 0.05). N=4. White indicates control, light gray indicates HGF treatment, and black indicates HGF/IGF treatment. Data are shown as mean plus or minus the standard error of the mean. An asterisk* indicates significantly different from saline-only control muscles.

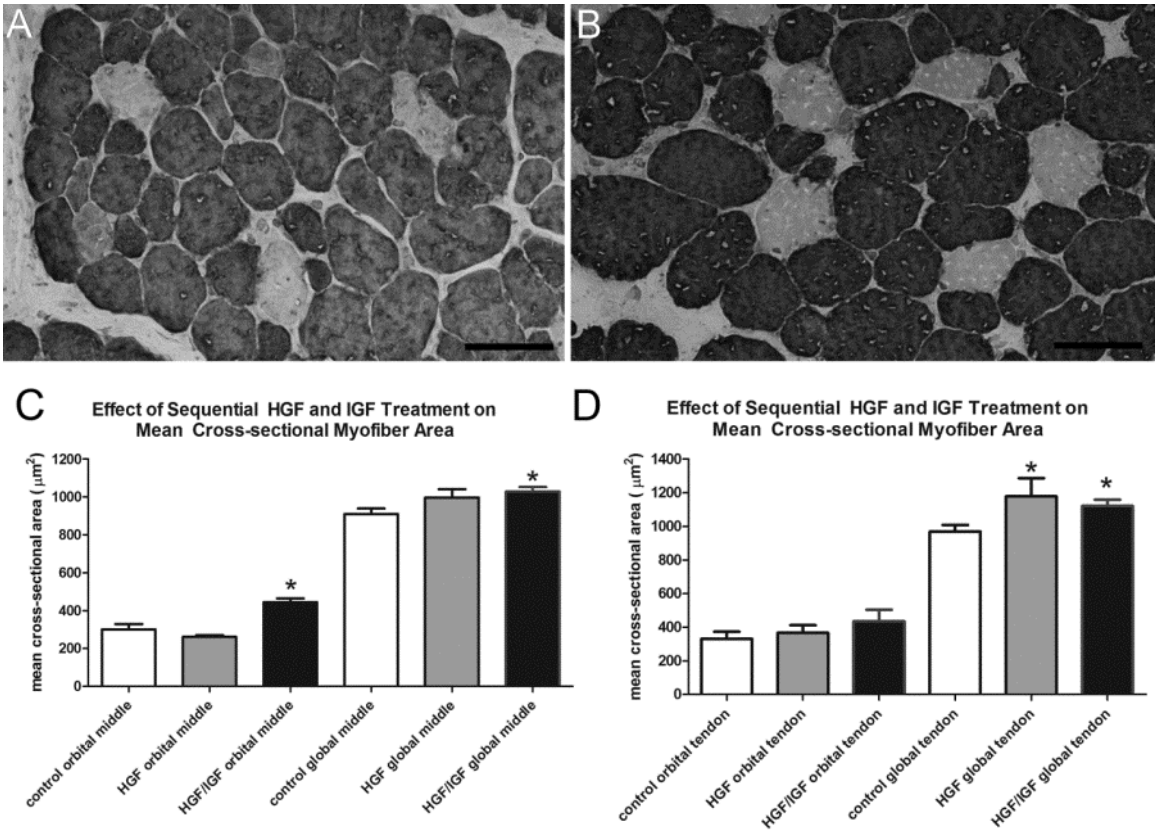


FIGURE 2.4 Density of Pax7+ satellite cells (arrows) in HGF/IGF-I co-treated superior rectus muscles compared to saline treated controls. (**A**) Saline-treated control global layer superior rectus myofibers immunostained for Pax7. (**B**) Global layer myofibers immunostained for Pax7 examined one week after a sequential HGF/IGF-I treatment. Bar is 20 microns. (**C**) Quantification of density of Pax7+ cells per number of myofibers the middle region of the muscle. (**D**) Quantification of density of Pax7+ cells per number of myofibers the tendon region of the muscle. White indicates control, light gray indicates HGF treatment, and black indicates HGF/IGF treatment. (ANOVA, Bonferroni, $p \leq 0.05$). N=8. Data are shown as mean plus or minus the standard error of the mean. An asterisk* indicates significantly different from saline-only control muscles.

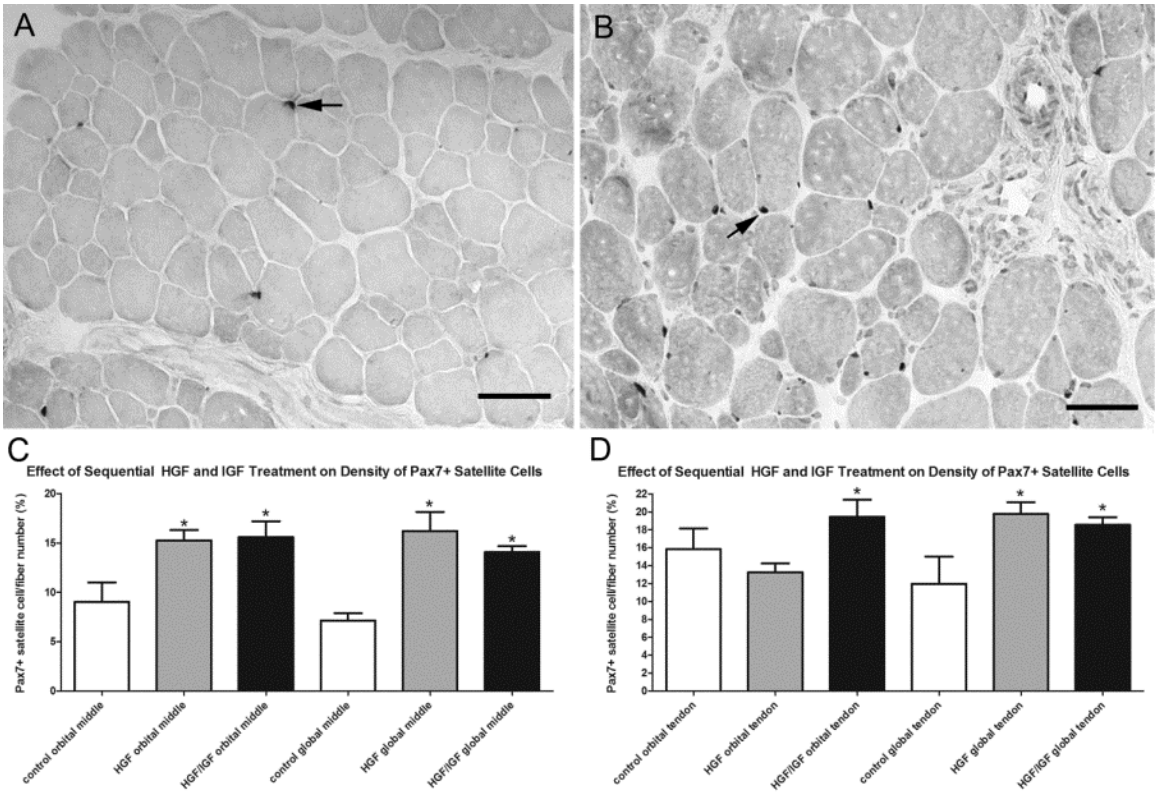
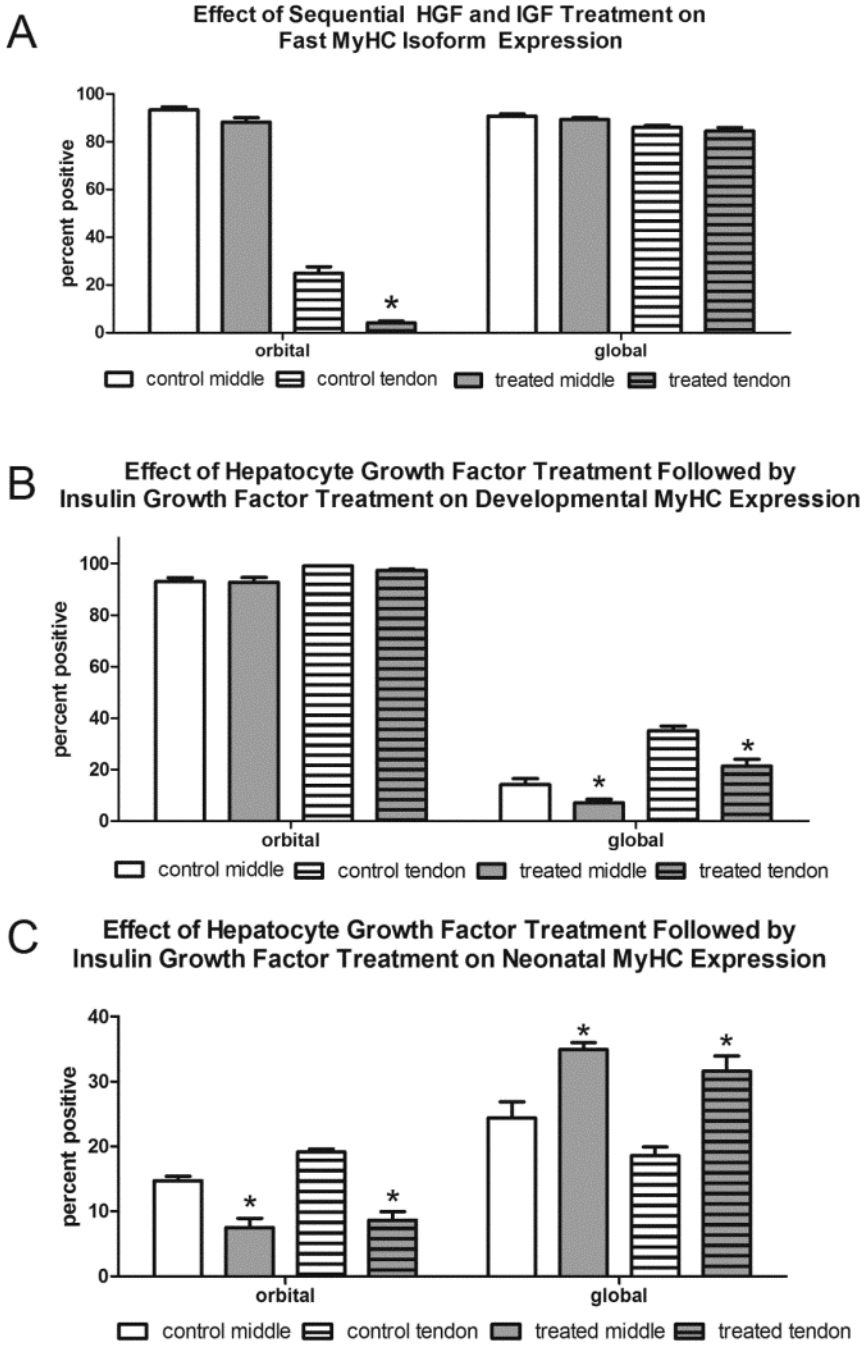


FIGURE 2.5 Quantification of percentage of myofibers expressing (**A**) fast MyHC, (**B**) developmental MyHC, and (**C**) neonatal MyHC in HGF/IGF-I co-treated superior rectus muscles compared to saline treated controls. (ANOVA, Bonferroni, p≤ 0.05). N=8. Data are shown as mean plus or minus the standard error of the mean. An asterisk* indicates significantly different from saline-only control muscles.



Chapter 3: Effects of the Sustained Release of IGF-1 on Extraocular Muscle of the Infant Non-Human Primate: Adaptations at the Effector Organ Level

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SUMMARY

The authors have demonstrated that prolonged exposure of adult rabbit extraocular muscle (EOM) to insulin-like growth factor-1 (IGF-1) results in significantly increased cross-sectional area and muscle force generation lasting over 3 months. Here the authors assess the effects on EOM of sustained IGF-1 treatment on normal binocular infant *Macaca mulatta*. Sustained-release IGF-1 pellets were implanted bilaterally in each medial rectus (MR) muscle of two normal infant non-human primates. Eye position was examined using corneal light reflex testing. After 3 months, morphometric analyses of myofiber cross-sectional area and innervation density in treated MR muscles were compared with an age-matched control and with antagonist lateral rectus (LR) muscles. After 3 months, the slow-release pellets remained at the implantation site in all four MR muscles treated. The treated MR showed pronounced increases in cross-sectional area and nerve density, mirrored in the untreated antagonist LR. Three months of bilateral sustained IGF-1 release in infant non-human primate MR resulted in increased muscle size and innervation density, mirrored in the untreated antagonist LR. It appears that bilateral MR treatment resulted in slow adaptation of both treated MR and contralateral LR muscles over time such that functional homeostasis and near-normal alignment were maintained. Further work is needed to determine what signaling mechanisms maintain proportional innervation when EOMs are forced to adapt to an externally applied perturbation.

INTRODUCTION

Strabismus is a common ocular motor disorder characterized by a misalignment of the eyes, with 3–5% of the children in the United States affected (Greenberg et al. 2007; Louwagie et al. 2009). Patching and glasses are often the first line of treatment, but surgery is required for persistent misalignment to improve the potential for recovering or preserving binocular function. Surgical treatment of strabismus entails either resection of the "underacting" extraocular muscle (EOM), recession of the "overacting" muscle, or a combination of both procedures. Surgical success rates vary, and these depend on the type of strabismus. Surgical success rates also vary if they are characterized by only their motor outcome, that is, eye position, or if both sensory and motor outcomes of surgery are measured. In children, surgical failure rates are reported to be as high as 50% (Vroman et al. 2000; Pineles et al. 2010). Botulinum toxin injections are also an effective treatment, although more often when the angle of misalignment is small (Rayner, Hollick, and Lee 1999). Botulinum toxin only weakens an "overacting" muscle. To parallel the effects of incisional surgery, a pharmacologic treatment is needed that can strengthen an "underacting" muscle. We have shown that injection or sustained release of insulin-like growth factor-1 (IGF-1) in the EOM of adult rabbits effectively increases both muscle cross-sectional area and muscle force generation (McLoon 2003; Anderson et al. 2006; McLoon, Anderson, and Christiansen 2006). Work in developing and juvenile chickens also shows that single orbital injections of IGF-1 increase the strength and mass of treated EOM (Chen and von Bartheld 2004; T. Li, Wiggins, and von Bartheld 2010).

In this study, we sought to determine whether 3 months of sustained release of IGF-1 in the EOM of a non-human primate would result in increased muscle size and alteration of eye position. The macaque monkey has binocular coordination of eye movements similar to that of humans. To move toward a phase I clinical trial of promising treatments, their effectiveness in a similarly binocular visual system is imperative. It is well known that creating strabismus in infant monkeys by surgical manipulation of the EOMs is difficult (Hubel and Wiesel 1965). Most primate models of strabismus involve perturbations of the afferent pathway in the infant monkey, including monocular deprivation (Quick et al. 1989) or rearing with prism goggles (Chino et al. 1991). Therefore, an additional goal of this study was to determine whether treatment of

both medial rectus (MR) muscles would be sufficient to induce strabismus in normal infant monkeys. The results were surprising, based on our previous studies using unilateral IGF-1 and BMP-4 (bone morphogenetic protein 4) treatment in adult rabbits (McLoon 2003; Anderson et al. 2006; McLoon, Anderson, and Christiansen 2006). Sustained IGF-1 treatment over 3 months altered muscle properties in the infant monkey, but essentially did not alter functional eye alignment. These results suggest that ongoing processes of muscle and innervational adaptation play an important role in the maintenance of binocular fusion.

METHODS

Macaca mulatta monkeys aged 1 to 2 weeks were obtained from the breeding colony at the Yerkes National Primate Research Center, Emory University in Atlanta, Georgia. All experiments were approved by the Emory University Animal Care Committee, and followed the National Institutes of Health and ARVO guidelines for use of animals in research. The infant monkeys were photographed to document orthotropic eye alignment before treatment, and all were normal. The pellets, measuring 3 × 1.5 mm, released 1 μg IGF-1/d for a total of 90 days (Innovative Research of America, Sarasota, FL). This release rate was based on a large number of previous studies examining dose effects from single injections in rabbits (McLoon 2003; Anderson et al. 2006) and the effect of pellets on rabbit EOM (McLoon, Anderson, and Christiansen 2006). Before implantation, pellets containing IGF-1 were gas sterilized. Under general anesthesia, an incision was made in the medial conjunctiva, and the MR muscles were visualized surgically. The muscles were retracted with a small muscle hook at their point of insertion, and the pellets were placed in Tenon's capsule overlying each MR muscle. The conjunctiva was closed with 8-0 ophthalmic suture. The infants were monitored visually for post-implant alignment and eye motility. Eye alignment was assessed by corneal light reflexes, which are relatively imprecise. Essentially no change in alignment was detected and confirmed by two independent observers. In addition, we obtained the rectus muscles from a 3-month-old macaque that had to be euthanized due to a trauma unrelated to the head. These muscles served as age-matched controls.

Three months after implantation, the monkeys were euthanized with a barbiturate overdose by staff veterinarians at the Yerkes Primate Center. All extraocular muscles

were dissected from origin to insertion, embedded in tragacanth gum, and frozen on 2-methylbutane chilled to a slurry on liquid nitrogen. The tissue was stored at −80°C until processed. The muscles were sectioned at 12 μm in a cryostat and processed for immunochemical visualization of fast, slow, developmental, and neonatal myosin heavy chain (MyHC) isoforms (fast and slow, 1:40; developmental and neonatal, 1:20; Vector Laboratories, Burlingame, CA). In addition, sections were selected to represent regions near the entry zone of the oculomotor nerve division into the muscles, as well as sections toward the tendon end of the muscle, and immunostained for the presence of myelinated nerve fibers using an antibody to Schwann cell myelin (1:50; Cosmo Bio Co., Tokyo, Japan) or neurofilament (1:1000; Covance, Princeton, NJ). The sections were incubated in reagents from a commercial kit (Vectastain Elite ABC kit; Vector Laboratories) and reacted using the diaminobenzidine procedure intensified by the addition of cobalt chloride and nickel ammonium sulfate solutions. Sections immunostained for the MyHC isoforms were analyzed for mean myofiber cross-sectional area and percentage of myofibers positive for each of the individual MyHC isoforms based on total fiber number. Three slides each were analyzed for each immunostain in the midregion of the rectus muscles and in the tendon region. Slides were chosen by determining distance from the tendon end of all muscle fibers, as well as close examination of fast MyHC isoform expression patterns, which are illustrative of position along the muscle length. A total of three to five fields were counted to count a minimum of 200 myofibers in both the orbital and global layers per stained slide, using the central region of each muscle section to avoid potential edge effects. In addition, total myofiber number was determined by counting every myofiber in whole muscle cross-sections, keeping the counts of orbital and global layers separate. Minimally, three slides were counted in their entirety, for the control MR and for all four IGF-1–treated MR. Schwann cell myelin and neurofilament-stained material were analyzed to measure the area occupied by nerve bundles (in μm^2) positive for myelinated axons as a ratio of total muscle area (in μm^2) per microscope field examined both for the tendon and midbelly regions. A total of three to five fields were examined per region per muscle section. Data are presented as mean ± SEM. All quantification was performed using imaging analysis software (BioQuant Nova Prime morphometry program; BioQuant, Nashville, TN).

RESULTS

After 3 months of sustained IGF-1 treatment to the MR muscles, the pellets were in position over each of the treated MR muscles (Figure 3.1). Mean muscle cross-sectional areas of global layer fibers were larger in the MR muscles after 3 months of IGF-1 treatment compared with the age-matched control muscles (Figure 3.2). In the treated MR, these fiber changes were most pronounced in the global layer in both the midregion and tendon ends of both the treated MR muscles. The antagonist lateral rectus (LR) muscles also had noticeably larger mean cross-sectional areas in the global layer compared with controls (Figure 3.2). In the global middle and tendon regions, cross-sectional areas increased approximately 2-fold in the treated MR muscles and 2.5-fold in the untreated antagonist LR muscles. It is unlikely that changes in the LR muscle were due to unintended diffusion of IGF-1 within the orbit, in part due to the extremely low release rate of IGF-1 from the pellets and potentially the confinement of the pellet within the connective tissue sheaths of the MR muscles. To test this, the superior (SR) and inferior (IR) rectus muscles were analyzed to determine whether changes in the contralateral LR could be due to diffusion of drug (Figure 3.2). The mean cross-sectional myofiber areas of both the SR and IR muscles were similar to the control MR muscles. Thus, it is unlikely that changes in the LR were due to “diffusion” of IGF-1 within the orbit. To determine whether the IGF-1 resulted in hyperplasia, all myofibers were counted in the control and IGF-1–treated EOM, and no differences were seen in total counts (Figure 3.3) or when analyzed as fiber number in the orbital or global layers (Figure 3.3).

The effect of the sustained IGF-1 treatment on innervation was also assessed. Myelinated nerve fiber densities in the treated MR muscles were increased 2-fold in both the midregion and tendon ends of the muscles (Figure 3.4). The contralateral antagonist LR muscles showed a 50-fold increase in myelinated nerve density in the tendon region of the muscles. In addition, examination of the density of neurofilament-positive axons showed a relatively similar picture (Figure 3.5). This analysis also points out some pronounced differences in nerve density between control MR and LR muscles. Although the overall nerve density is similar in the middle regions of both muscles, there is a 25% greater nerve density in the tendon region of the MR muscles. Because this is

normalized to muscle area, this difference must be based on functional differences in this region of the MR muscle compared with the LR.

IGF-1 plays a role in muscle growth, so changes in the population of myofibers expressing the two “immature” MyHC isoforms were examined. In the midregion of the treated MR muscles, there was a large decrease in the percentage of myofibers expressing embryonic MyHC (Figure 3.6). A similar trend was seen in the tendon region of the treated MR. In contrast, in the untreated antagonist LR muscles there was an increase in the proportion of embryonic MyHC positive myofibers (Figure 3.6). In the treated MR muscles, the global layer increased its expression of neonatal MyHC, whereas the orbital layer was either unchanged (midregion) or decreased (tendon region) (Figure 3.7). The levels of neonatal MyHC expression in the LR muscles were very limited in these specimens. No changes were seen in the percentage of myofibers expressing fast MyHC in any of the muscles examined (data not shown).

DISCUSSION

Bilateral treatment of the MR muscles in infant primates with sustained release of IGF-1 for 3 months resulted in changes in myofiber cross-sectional area and innervational density in the treated muscles. The nontreated antagonist LR muscles were comparably altered bilaterally. Area measurements in the IR and SR of the treated monkeys were similar to those measured in the control MR muscles; thus, it is unlikely that changes in the LR were due to “diffusion” of IGF-1 within the orbit. Compensatory changes have long been hypothesized to play a role in determining the response to strabismus treatment (Kushner and Vrabec 1987). Previous studies have demonstrated a significant increase in muscle force generation, both as measured in grams and as specific tension (force/muscle cross-sectional area) (McLoon 2003; McLoon, Anderson, and Christiansen 2006; Anderson et al. 2006). It is possible, however, that a significantly larger daily dose of IGF-1 might be able to override binocular fusion sufficiently to alter eye position. It is unknown what dose is needed to saturate all IGF-1 receptors in the infant EOM, and receptor turnover and upregulation with increased exposure to exogenously added IGF-1 may occur. It may also be that doses will vary significantly in adults compared with the infants studied here. Future experiments will address these topics.

It is striking that the size ratio difference between the LR and MR muscles is relatively unaltered compared with control in these muscles after 3 months of continuous MR treatment with IGF-1. Myelinated nerve density increased after the sustained IGF-1 treatment. Interestingly, there was a 2-fold increase in nerve density in the treated MR, whereas in the antagonist LR there was a 50-fold increase in nerve density. This is quite remarkable because the LR muscles were not exposed to exogenous IGF-1. These results suggest that pronounced adaptations occur in the antagonist LR as a consequence of sustained exogenous IGF-1 dosing to both MR muscles. These adaptations may result from increased resting tension in the LR muscles as a consequence of bigger, stronger MR muscles, or they could result from central adaptations in the version and vergence systems that maintain alignment.

It is interesting that only minimal changes were seen in MyHC isoform expression patterns except for embryonic MyHC. These changes were inversely related in the MR and LR muscles, with decreased percentages of embryonic MyHC-containing fibers in the treated MR and increases in the contralateral but untreated LR muscles. No changes were seen in fast MyHC isoform expression. In an earlier study, we showed that 1 week after a single injection of IGF-1 in rabbit SR muscle, the percentage of positive myofibers for both embryonic and neonatal MyHC isoforms decreased, with concomitant reductions is shortening velocity (Anderson et al. 2006). Future studies will include examination of muscle force and contractile characteristics in the treated non-human primate muscles.

IGF exerts an important role in muscle development and growth (Florini et al. 1986; Tollefsen et al. 1989). It is downregulated in adult noncranial skeletal muscle, but is reexpressed after muscle injury, playing a role in facilitating muscle regeneration (Jennische, Skottner, and Hansson 1987). There is a great deal of literature supporting the ability of exogenously added IGF-1 to increase muscle size and force generation in diseased and aging skeletal muscle (Barton-Davis et al. 1998; Lynch et al. 2001; Payne et al. 2006). Although we recently demonstrated that there is no correlation between myofiber diameter and force generation in single isolated normal adult EOM myofibers (McLoon et al. 2011), previous studies using both acute (McLoon 2003; Chen and von Bartheld 2004) and long-term IGF treatment (Anderson et al. 2006) demonstrated that muscle force generation is significantly increased by this treatment. In the present study, the increase in mean myofiber cross-sectional area in the treated MR muscles supports

this view. However, the coordinated changes in the antagonist muscles we observed could have the effect of maintaining normal alignment in these treated infant monkeys. It was possible that myofiber hypertrophy induced by IGF-1 treatment could also be accompanied by myofiber hyperplasia. This question was answered by examination of the mean of the total number of myofibers in control and IGF-1–treated MR that did not appear to differ, suggesting new fiber formation did not occur. This is supported by the lack of increased numbers of myofibers expressing the immature MyHC isoforms, and by our previous study showing that myofiber remodeling is not correlated with increased numbers of neonatal MyHC-expressing myofibers (Christiansen and McLoon 2006).

The growth-promoting effect of IGF-1 on nerve outgrowth is also well established. For example, exogenous addition of IGF-1 or -2 results in intramuscular nerve sprouting in normal adult skeletal muscle (Caroni and Grandes 1990). When applied to motor nerves, rapid axoplasmic transport occurs, where it presumably exerts trophic influence on the motor neurons (Hansson, Rozell, and Skottner 1987). During motor nerve regeneration supplying adult muscles, where expression is downregulated, increased expression of IGF-1 was temporally correlated with nerve growth (Hansson, Rozell, and Skottner 1987). IGF-1 promotes motor neuron survival in developing oculomotor neurons, and exogenous treatment increases neurite outgrowth in vitro (Rind and von Bartheld 2002). Thus, the increase in nerve density after 3 months of sustained IGF-1 treatment would be an expected sequela. However, the similar and coordinated increase in the nerve density in the untreated LR muscles was not expected. The LR muscle is innervated by the motor neurons in the abducens nucleus, whereas the MR is innervated by the oculomotor nucleus. These results suggest a robust ability of the ocular motor system to communicate alterations of properties in the treated neurons to the untreated motor units of the antagonist muscle. The results of our study do not point to a clear mechanism, and experiments are under way to elucidate this.

Detection of binocular visual error by the cortical system may have led to ocular motor adaptations, including changes in muscle innervation and contractility. Centrally, communication of changes between yoked muscle–neuronal pairs may have occurred. A potential candidate for this internuclear communication is the abducens interneuron population, projecting directly from the abducens motor nucleus to the contralateral oculomotor nucleus (Büttner-Ennever 2006). Retrogradely transported growth factors have been shown to alter contractile properties in treated motor units. For example, after

axotomy of the abducens nerve, exogenously added neurotrophin-3 or brain-derived neurotrophic factor each restore different aspects of firing rate characteristics in abducens motor neurons (Davis-López de Carrizosa et al. 2009). We have previously shown that treatment of rabbit EOM with IGF-1 alters the peak rate of force development (Anderson et al. 2006). This alteration in contractility is most likely due to alteration in motor neuron firing rate (Davis-López de Carrizosa et al. 2009). Recent work has confirmed these findings in juvenile chick EOM (T. Li, Feng, and von Bartheld 2011). Future work will attempt to address the potential mechanism(s) of the communication to the motor nucleus of the antagonist, yet untreated, muscle.

This bilateral sustained IGF-1 treatment, although causing both increased innervation to the muscles and increased myofiber size, did not result in the development of a strabismus. This was not an unexpected result because it is well known that the brain maintenance of binocularity is extremely robust in normal (Takagi, Trillenberg, and Zee 2001). Primate models of experimentally induced strabismus all involve significant afferent perturbations, including prolonged alternating monocular occlusion (Das and Mustari 2007; Tychsen and Burkhalter 1997) induced anisometropia (Kiorpes et al. 1998) or prism goggles (Chino et al. 1991). In an otherwise normal monkey, our results support the concept that the normal ocular motor system can adapt to changes of slow onset and retain binocular coordination by generating similar changes in antagonist muscles.

Although the factors that allow maintenance of binocular alignment in this model are likely complex and are not yet worked out, there are two most likely mechanisms. The first invokes Hering's law of equal innervation, a mechanism proposed to account for eye movement conjugacy. Hering's law postulates that to maintain conjugate gaze, the brain sends a similar command to yoked muscles. For vertical eye movements, a structural substrate does exist: premotor neurons project to yoked muscles of both eyes (Moschovakis, Scudder, and Highstein 1990). For horizontal eye movements, the picture is more complex. Internuclear neurons between the abducens nucleus and the oculomotor nuclei allow co-activation of synergistic muscles. Second, a strong body of research suggests that a large population of cranial motor neurons to the EOM selectively provide uniocular eye commands (King 2011). Although the brain sends specific signals for the left and right eyes, Hering may still be partly right, in that the ocular motor system has innate systems that control and maintain conjugacy of both

eyes. Evidence suggests that the posterior parietal cortex controls binocular coordination of saccades (Vernet et al. 2008), and that adaptations restore functional yoking of eyes to preserve conjugate gaze (Maxwell, Lemij, and Collewijn 1995). Our data demonstrate distinct properties relative to the innervation of the muscles themselves in relation to Hering's law. First, although nerve density is similar in the midregion of both the control MR and LR, within the tendon region a striking difference in innervation density is seen. This could be due to different myofiber numbers in the LR and MR muscles (Oh, Poukens, and Demer 2001), different proportions of multiply innervated myofibers in these two muscles (Mayr et al. 1975), the presence of greater numbers of short fibers within the MR (Harrison et al. 2007), or a combination of all these factors. Thus, “equal innervation” is more aptly called “proportional innervation,” and this proportionality must be maintained to retain conjugate gaze. After 3 months of sustained IGF-1 treatment, in the tendon region of the treated MR muscles innervation density increased 2-fold, although there was a 50-fold increase in the tendon region of the LR muscles in the orbit with the treated MR muscles (see Figure 3.4; 3.5). The explanation for this difference is unclear. However, it supports the view that feedback of the imposed changes in the treated MRs was communicated to the brain, resulting in LR adaptation. One hypothesis is that abducens internuclear neuronal signaling that is provided to the yoked muscles controls this process (Figure 3.8). Although further studies are needed to dissect out the potential CNS mechanisms that control these adaptations, one has to assume that the coordinated nerve outgrowth in the untreated LR muscles was due to an active process. It is interesting to point out, however, that the same treatment given to an adult strabismic monkey, in that case being administered unilaterally, resulted in large bilateral reduction in the angle of eye deviation in primary gaze (McLoon et al., unpublished data, 2010).

Another possible mechanism for maintenance of alignment in this model is an increase in divergence tone in response to the convergent error associated with treatment-related changes in the MR muscles of each eye. To maintain binocularity in the setting of bilaterally stronger, bigger MR muscles, active divergence would be required. These innervational changes may come in the form of increased neuronal firing rate, an increase in innervational density, or, more likely, both. Irrespective of whether the mechanism is principally driven by the vergence systems, the end result is maintenance of binocularity.

In summary, 3 months of sustained release of IGF-1 delivered to both MR muscles in infant monkeys resulted in pronounced bilateral increases in the mean cross-sectional areas of myofibers and in nerve density in both the treated MR muscles and the antagonist but untreated LR muscles. Central and peripheral adaptive mechanisms provide sufficient ocular motor plasticity, especially in an infant non-human primate model, to compensate for slow growth induced by sustained low levels of IGF-1 treatment of the medial rectus muscles.

FIGURES

FIGURE 3.1 Corongal slice of a magnetic resonance imaging view of IGF-1 pellets in place on the MR muscles bilaterally. Images were acquired with a 3-Tesla magnet (GE) and viewed with NeuroLens software.

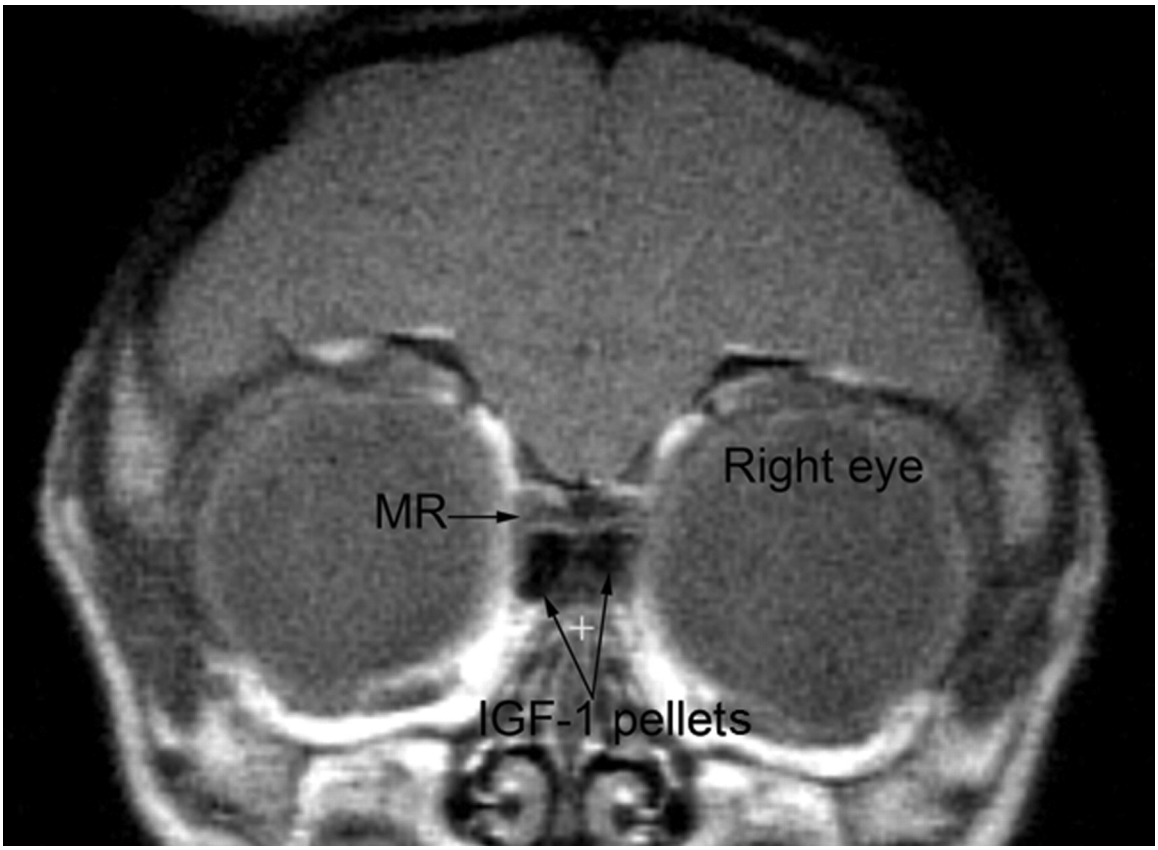


FIGURE 3.2 Three months of sustained-release of IGF-1 results in increased myofiber cross-sectional area of the treated MR muscles. (**A**) Control MR myofibers in the global layer immunostained for the presence of fast MyHC isoform. (**B**) IGF-1–treated MR myofibers in the global layer immunostained for the presence of fast MyHC isoform. Bar: 100 μm. (**C**–**F**) Quantification of mean myofiber cross-sectional areas after 3 months of sustained IGF-1 treatment in the orbital (**C**, **D**) and global (**E**, **F**) layers in the middle of the muscle (**C**, **E**) and tendon regions (**D**, **F**). IR, inferior rectus; SR, superior rectus.

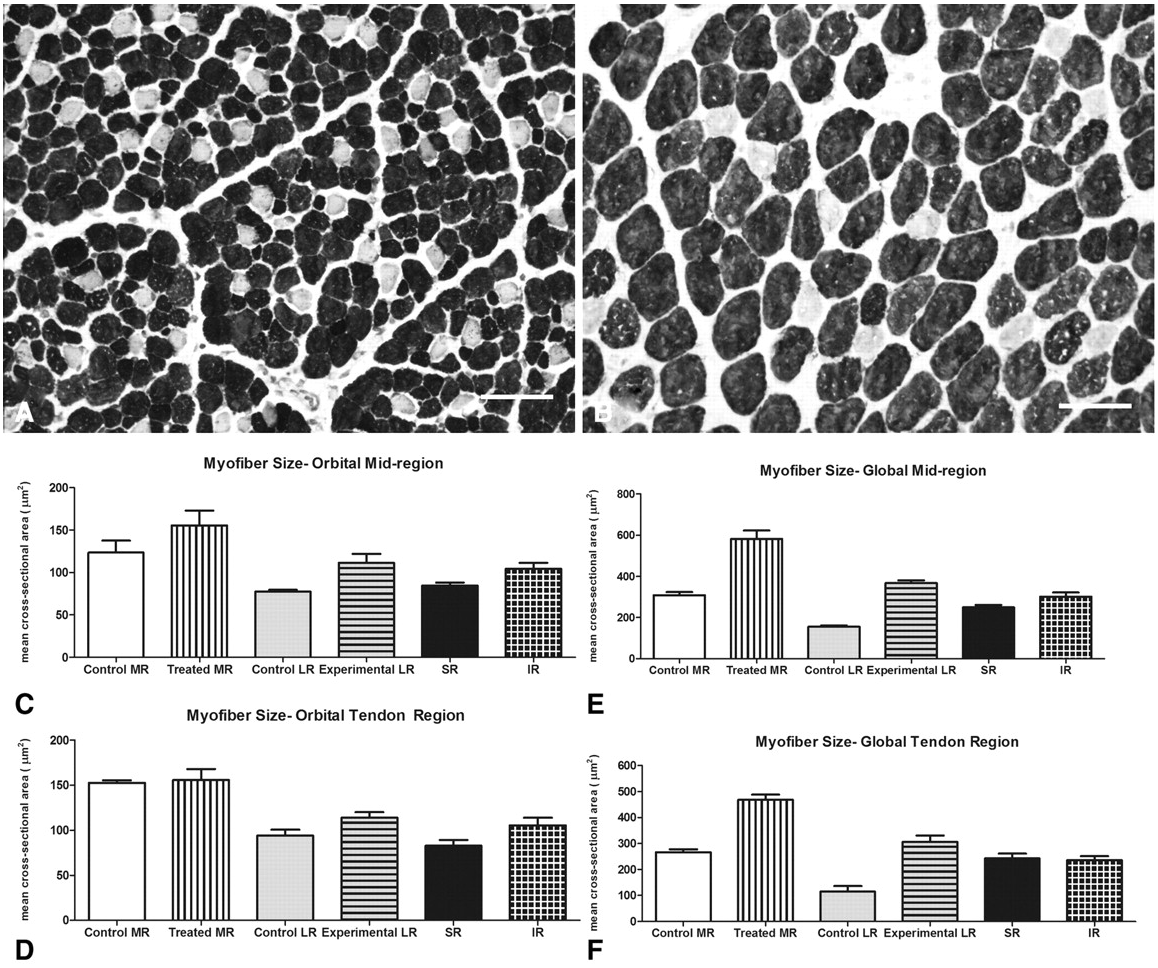


FIGURE 3.3 (**A**) Total myofiber number in control and IGF-1–treated MR muscles. (**B**) Total myofiber number in the orbital and global layers of control and IGF-1–treated muscles.

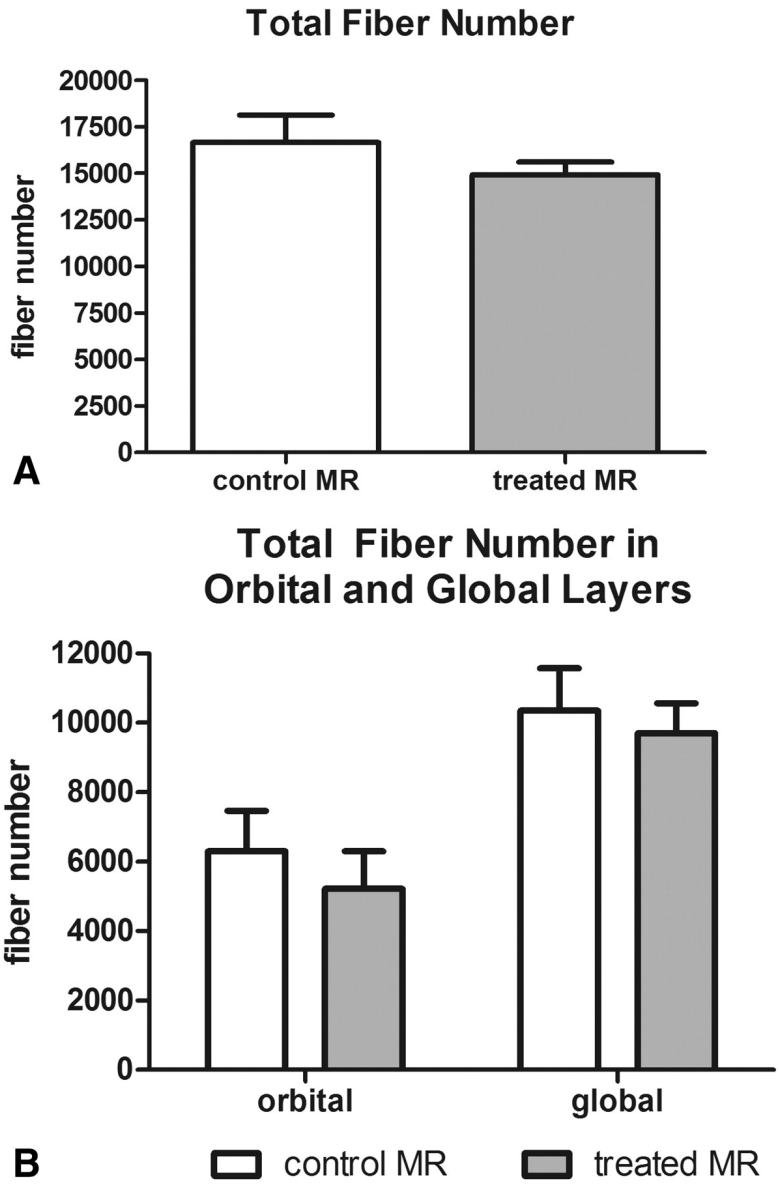


FIGURE 3.4 Three months of sustained release of IGF-1 results in increased myelinated nerve density per muscle area in the treated MR muscles. Each photomicrograph shows global layer on the left side and orbital layer on the right side. (**A**) Control MR myofibers immunostained for the presence of myelinated nerve using an antibody specific for Schwann cells. (**B**) IGF-1–treated MR muscle. Bar: 100 μm. (**C**, **D**) Quantification of myelinated nerve density/myofiber cross-sectional area after 3 months of sustained IGF-531 treatment in (**C**) the midregion of the muscle and (**D**) in the tendon region.

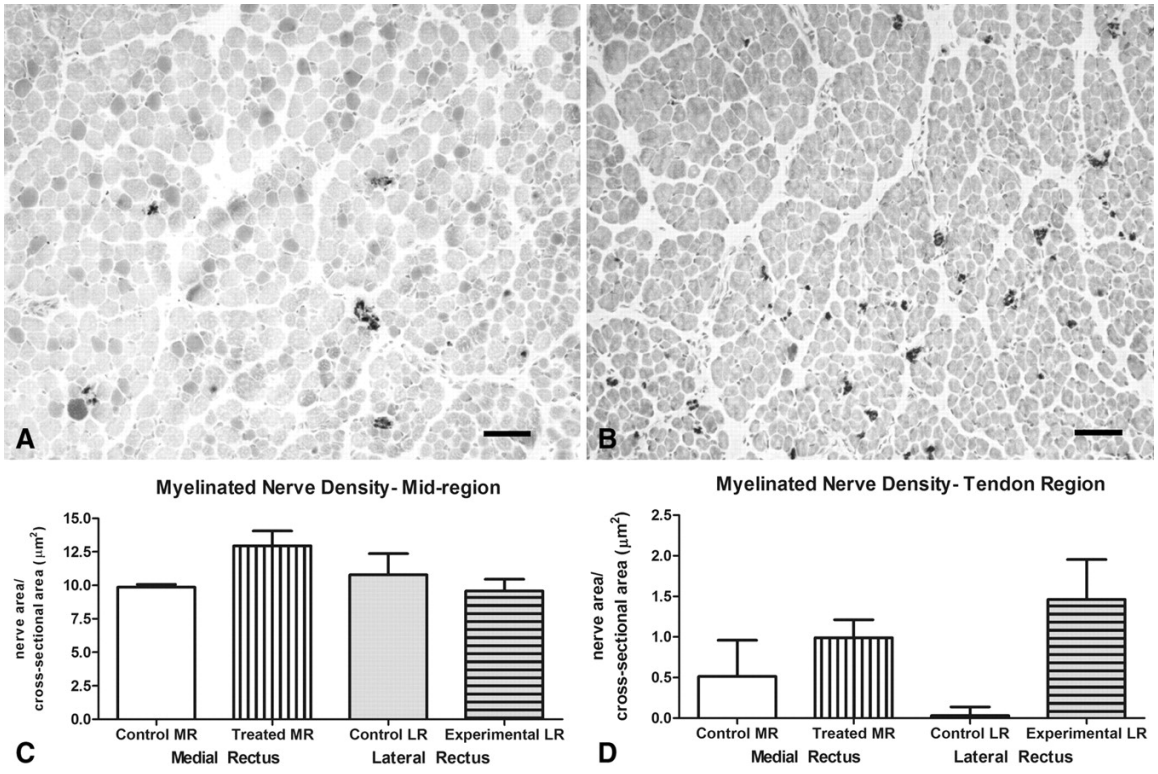


FIGURE 3.5 Quantification of total axon density/myofiber cross-sectional area after 3 months of sustained IGF-1 treatment in the midregion (**A**) and the tendon region (**B**).

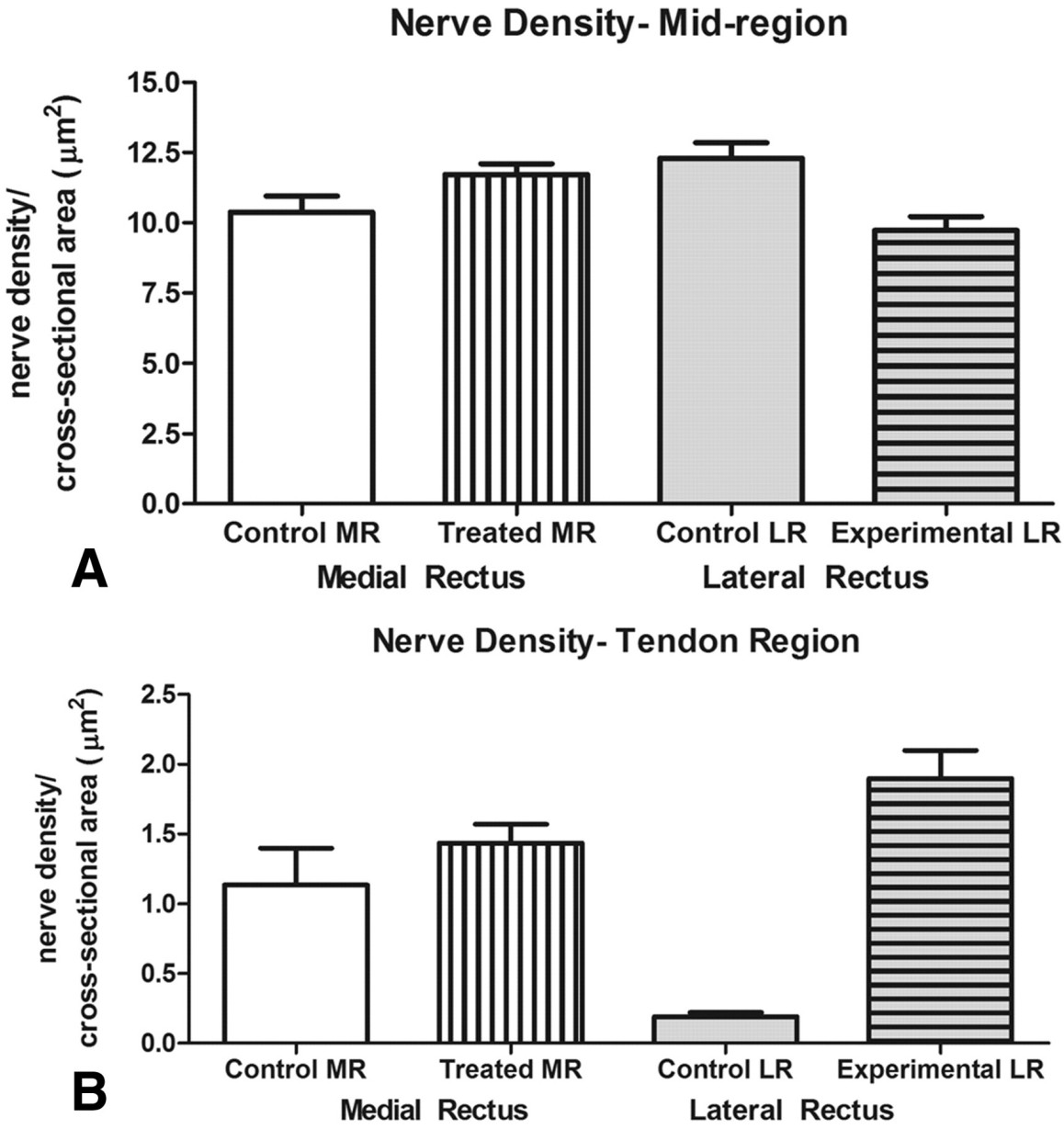


FIGURE 3.6 Quantification of embryonic MyHC isoform expression after 3 months of sustained IGF-1 treatment. # indicates data not included. Exp, experimental LR opposite to a treated MR.

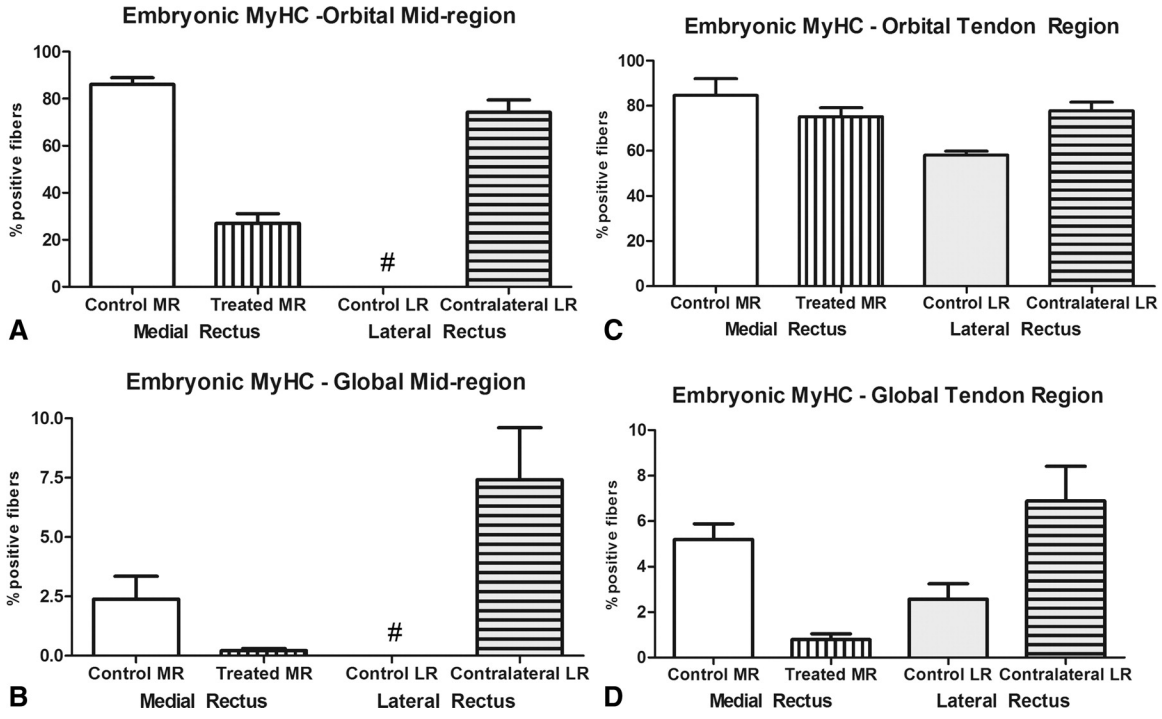


FIGURE 3.7 Quantification of neonatal MyHC isoform expression after 3 months of sustained IGF-1 treatment.

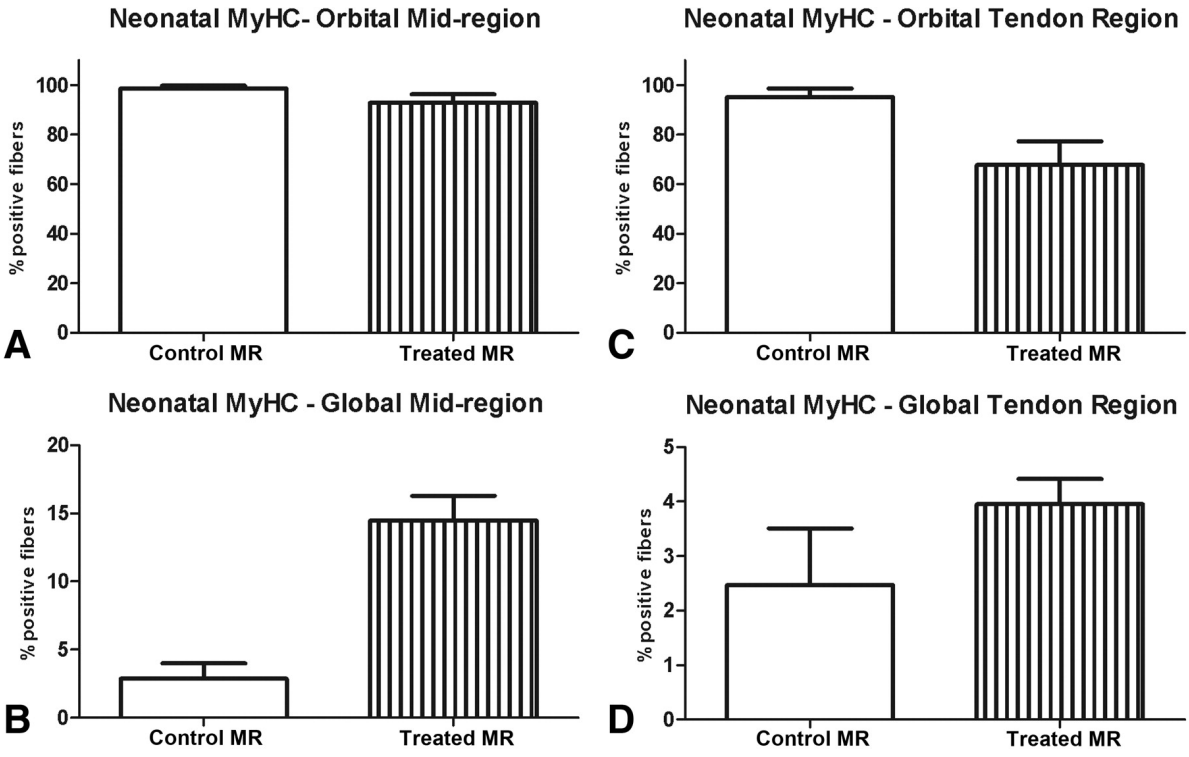
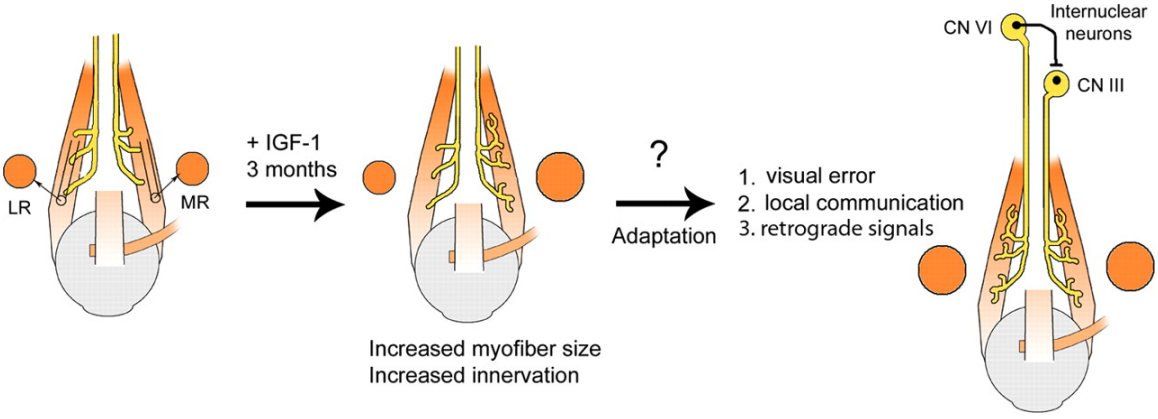


FIGURE 3.8 Schematic model depicting potential pathways for communication of changes in the treated MR muscles to the abducens motor nucleus, in turn increasing nerve outgrowth into the contralateral but untreated LR.



Chapter 4: Adaptability of the Immature Ocular Motor Control System: Unilateral IGF-1 Medial Rectus Treatment.

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SUMMARY

Unilateral treatment with sustained release IGF-1 to a single medial rectus muscle in infant monkeys was performed to test the hypothesis that strabismus would develop in correlation with changes in extraocular muscles during the critical period for development of binocularity. Sustained release IGF-1 pellets were implanted unilaterally on a medial rectus muscle in normal infant monkeys during the first 2 weeks of life. Eye position was monitored using standard photographic methods. After three months of treatment, myofiber size, myosin composition, neuromuscular junction size, and innervation density were analyzed morphometrically in all horizontal rectus muscles and compared to muscles from age matched controls. Sustained IGF-1 treatment during the first three months of life resulted in strabismus for all treated subjects; three of the four had a clinically significant strabismus of over 10 degrees. Both the treated medial rectus and 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. Sustained IGF-1 treatment to extraocular muscle during the sensitive period for development of orthotropic eye alignment and binocularity was sufficient to disturb ocular motor development, resulting in strabismus in the infant monkeys. This could be due to altering fusion of gaze during an early sensitive period. Serial measurements of eye alignment suggest 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 EOM may be a cause of strabismus.

INTRODUCTION

Correct binocular eye alignment and conjugate gaze is a critical component needed for the 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 3-5% of the childhood population are affected by strabismus, and 1-2% suffer from amblyopia, numbers that have remained unchanged for the last 70 years(Apel et al. 2010; Nordloew 1964; Graham 1974; Friedman et al. 2009; Thompson et al. 1991). Current treatment options are still not effective solutions for many individuals, particularly for those with large angles of misalignment (Abbasoglu, Sener, and Sanac 1996; Yam et al. 2012).

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 using monocular deprivation, surgical esotropia, or optically induced strabismus using prism goggles (Crawford et al. 1996). The non-human primate has become a common animal model for strabismus, but the most common methods for inducing strabismus in infant monkeys use forms of sensory deprivation, such as alternating monocular occlusion (Tusa et al. 2002) or prism goggle rearing (Crawford and von Noorden 1980). These types of sensory manipulations are very good 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 themselves. Using a variety of methods, a number of studies have induced strabismus by surgical manipulation of the extraocular muscles (von Noorden and Dowling 1970; Harwerth et al. 1983; Kiorpes and Boothe 1980). 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 (von Noorden and Dowling 1970; Kiorpes et al. 1996;

von Noorden and Crawford 1981). These models result in strabismus, but with the disadvantage of injuring and disrupting the EOM.

In a series of studies in rabbits, we have shown that treating a single EOM with exogenously added neurotrophic factors such as insulin growth factor-I (IGF-I) or IGF-II, whether injected or treated continuously for months, resulted in increased muscle size and force generation capacity (McLoon 2003; Anderson et al. 2006; McLoon, Anderson, and Christiansen 2006). Thus, it seemed reasonable that in a binocular animal such as the non-human primate, sustained release of IGF-I 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-I for 3 months (Willoughby, Ralles, et al. 2012). While 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 (Willoughby et al., 2012). In this experimental design, each untreated lateral rectus was either an ipsilateral antagonist or a contralateral yoked muscle for the treated medial rectus muscle. We hypothesized that due to the continuous exposure to IGF-I over three months, there was sufficient time for active concomitant changes to occur in the untreated lateral rectus muscles.

The extraocular muscles 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 (Christiansen and McLoon 2006; Christiansen, Antunes-Foschini, and McLoon 2010). 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 (Stager, McLoon, and Felius 2013). Interestingly, in the rabbit specimens, reciprocal changes occurred in the unoperated but passively stretched antagonist inferior rectus muscle of the same globe. There were also changes in the contralateral yoked superior rectus muscle, despite its not having been directly altered by the surgical manipulation. This strongly suggests that the central nervous system is affected when perturbations in the

periphery alter muscle size and force generation capacity. While 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-I might be more likely to cause strabismus. A unilateral treatment paradigm would allow us to parse out how the EOM 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-I for a duration of three months (Figure 4.1A). These unilaterally treated monkeys were assessed for changes in eye alignment during the treatment period. At the end of three 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 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 University Animal Care and Use Committee. All procedures were performed in compliance with guidelines issued by the National Institutes of Health and the Association for Research in Vision and Ophthalmology for use of animals in research.

Three *Macaca nemestrina* and one *Macaca mulatta* infant monkeys aged 1-2 weeks were photographed to document orthotropic alignment prior to treatment to verify normal eye alignment. Under general inhalation anesthesia, the conjunctiva was opened near the limbus in a 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) was placed on the orbital surface of one medial rectus muscle. The pellets become quite sticky when placed

within the connective tissue around the muscles, and do not move. Position was verified by MRI at two months and by visual inspection at the time of tissue collection. The pellets were 3 x 1.5mm, and calibrated for the sustained release of 2µg of IGF-1 per day for a total of 90 days. This dose was selected based upon previous studies (McLoon et al., 2006; Willoughby et al. 2012). After pellet implantation, the conjunctiva was closed with a 8-0 ophthalmic vicryl suture. Two of the treated monkeys were photographed for determination of eye alignment at intervals during the treatment period. The two others were photographed for determination of eye alignment changes only at 3 months of treatment.

Eye alignment was assessed by corneal light reflex. The displacement (mm) 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 of 14 degrees/millimeter for macaques (Quick et al., 1989). The displacement (mm) 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 14 degrees/millimeter for macaques. 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 three months. 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 prior studies was also used in this analysis (Willoughby et al., 2012).

IMMUNOHISTOCHEMISTRY AND HISTOLOGICAL PROCESSING

Frozen extraocular muscles were sectioned at 30 µm parallel to the long axis of the fibers using a Leica cryostat, and the sections were stored at -80^{o}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 0.01M phosphate buffer, pH 7.4 containing 0.1% Triton X-100 (PBS/TX), blocked in 10% goat serum for one hour, and then incubated overnight at 4°C with an antibody to slow heavy myosin chain (MyHC) (Vector Laboratories, Burlingame, CA). Sections were

washed in PBS and incubated in secondary antibody, goat anti-mouse IgG labeled with Cy3 (1:1000; Jackson ImmunoResearch Laboratories; West Grove, PA) for one hour at room temperature. To visualize neuromuscular junctions, sections were double labeled by subsequent treatment with alpha-bungarotoxin conjugated to AlexaFluor®488 (1:3000; Molecular Probes; Eugene, OR) overnight. A subset of sections were triple labeled for nerve by incubation in an antibody to neurofilament (1:1000; smi-31; BioLegend; Dedham, MA) overnight, and visualized with goat anti mouse DyLight® 405 (1:1000; Jackson ImmunoResearch Laboratories). All sections were washed 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) and neonatal (1:20) 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 (Olympus; FV1000) to sequentially capture images in the same focal plane with different filters. Neuromuscular junctions were imaged at 20x oil immersion, and nerve at 10x. 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 the analysis software Image J (NIH; http://imagej.nih.gov/ij): muscle area, mean diameter for myofibers positive or negative for slow MyHC, total number of neuromuscular junctions, and the area of each neuromuscular junction endplate as determined by hand circling. For every fiber with a neuromuscular junction, it was also noted if the fiber was positive or negative for slow myosin. For each field, the muscle layer (orbital, global) and region (proximal, endplate zone, distal- toward the orbit) were noted. Orbital layer was distinguished from the global both by smaller myofibers, and positive immunostaining for embryonic myosin on flanking stained sections. 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 5th field was imaged at 60x oil immersion to visualize neuromuscular junction morphology fully. Additional staining among control and experimental muscle with double-labeling of SNAP-25 (1:1000; Covance; Dedham, MA) or synaptophysin (1:300; abcam; Cambridge, MA), nerve (SMi-31), and α-bungarotoxin revealed that all α-bungarotoxin labeled neuromuscular junctions in the EOM specimens co-localized with presynaptic components. Based on this, we are confident that the majority of 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 orbital layers. A minimum of 200 myofibers were analyzed per section, and a minimum of three slides were measured per muscle specimen.

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 was performed with imaging analysis software (BioQuant Nova Prime morphometry program; BioQuant, Nashville, TN).

All data were analyzed statistically (Prism Software, GraphPad, San Diego CA). 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 a 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 the standard deviation.

RESULTS

The retention of the sustained release pellets was verified by MRI in the treated infant monkeys (Figure 2). All four infant monkeys that received 3 months of unilateral IGF-1 treatment of the medial rectus muscle at 2 µg/day developed an exotropia, with a mean angle of 11.3 ±3^{o} (Figure 4.1B-E). Three of the four treated monkeys had clinically relevant angles greater than 10^{o}. The fourth monkey had an incomitant strabismus (angle varied over time), but the misalignment was evident in most of the photographs over the final month of treatment. Eye alignment was measured at intervals over the three months of IGF-1 treatment for two of the treated monkeys (Figure 4.3). Both humans and monkeys are frequently born with eye misalignment, when visual acuity is poor, and stereopsis is absent (O'Dell and Boothe 1997; Archer, Sondhi, and Helveston 1989). For monkeys, within the first month of life, coarse stereopsis rapidly develops and the oculomotor system matures (O'Dell and Boothe, 1997). When experimentally creating an animal model of strabismus, it is only with aggressive continual visual sensory perturbation during the development of coarse stereopsis that strabismus persists (Tusa et al., 2002). As soon as 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 suggest that for the infant monkeys treated with unilateral IGF-1, the initial exotropia normal in newborn monkeys remained stable over time. We hypothesize 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-I 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 (Figure 4.1A).

Consistent with our prior study (Willoughby et al., 2012), unilateral IGF-1 treatment of infant monkey medial rectus muscle resulted in larger myofibers than control EOM from untreated age-matched infant monkeys (Figure 4.4). This persistent trend reached significance for the treated medial rectus in the orbital layer (Figure 4.4B, pink), where mean cross sectional area was 253 ± 19.78 μm^2, 75% larger than the control mean of 144.7 ± 5.883 μm^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 ±102.6 μm^2, twice as large as control values of 276.9 ±121 μm^2 (Figure 4.4A, 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 (Figure 4.4, green). It is possible that larger myofiber size of the non-IGFI 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 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 the rectus muscles 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 (T. Li, Wiggins, and von Bartheld 2010), which may be related to the endogenous gradient of expression of IGF-1 across the EOM (Feng and von Bartheld 2011). Additionally, the size of individual myofibers varied from the proximal to distal end of the EOM and with myofiber type (McLoon, Rios, and Wirtschafter 1999). 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 (Rubinstein and Hoh 2000; Jacoby et al. 1990; Y. Zhou, Liu, and Kaminski 2010; McLoon et al. 2011). 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. Complimentarily, *en plaque* endplates are more centrally located on myofibers where fast myosins are expressed, and exhibit fast-twitch characteristics (Chiarandini and Stefani 1979; Wasicky, Horn, and Büttner-Ennever 2004; Stirn Kranjc, Smerdu, and Erzen 2009; Feng et al. 2012). 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 (Figures 4.5, 4.6).

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 (Figure 4.5A, B; Figure 4.6A, C). Slow fibers were larger than control in the proximal region in the global layer (Figure 4.5B; 4.5B), while in the orbital layer, slow fibers were larger than control throughout the EOM except at the end the endplate zone (Figure 4.6D). 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 (Figure 4.5 E-H; Figure 4.6D).

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 co-expressed, and expression of individual isoforms can vary along a fiber's length (Rubinstein and Hoh 2000; Y. Zhou, Liu, and Kaminski 2010; McLoon et al. 2011). 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 (Figure 4.7). To further assess the effects of prolonged IGF-1 on MyHC isoforms, the medial rectus muscles were also labeled for expression of the "developmental" myosins, embryonic MyHC or neonatal MyHC (Figure 4.8, 4.9). No 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 (Figure 4.8A; 4.10A, B). As nearly all orbital layer fibers express embryonic MyHC (McLoon et al., 1999; Rubenstein et al., 2000; Zhou et al., 2010), counts are only shown 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 embryonic MyHC was expressed (Figure 4.8B). In contrast, myofibers negative for embryonic MyHC had significantly larger myofiber diameter, from 24.7 ± 1.1 μm in the proximal region to 22.8 ± 1.8 μm in the distal region (Figure 4.8C, E).

Expression of neonatal MyHC was correlated with a different pattern of change in fiber diameter than seen in myofibers expressing embryonic MyHC. In the orbital layer, all fibers that expressed neonatal MyHC had significant increases in fiber diameter (Figure 4.9E; 10D), while fibers negative for neonatal MyHC had no change in fiber diameter (Figure 4.10F). 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 (Figure 4.9B; 4.10E).

IGF-1 is known to promote neurite growth and nerve sprouting (Caroni and Grandes 1990; Apel et al. 2010; Rind and von Bartheld 2002), and maintains neuromuscular junction morphology in aging and diseased animals (Dobrowolny et al. 2005; Payne et al. 2006). Our previous study suggested that IGF-1 promoted increased innervation in the distal end of the muscle, but this study was limited due to a low number of subjects (Willoughby et al., 2012). 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 changes in 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. While the *en plaque* neuromuscular junctions on fast myofibers sometimes appeared larger at first examination, they were not statistically different from control (Figure 4.11). 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.

DISCUSSION

Unilateral sustained release of IGF-1 to a single medial rectus muscle of infant macaques within the first postnatal week and continued for three months resulted in strabismus in our subjects. Three of four treated monkeys maintained a clinically relevant strabismus of greater than 10 degrees. 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 (McLoon, Anderson, and Christiansen 2006; Anderson et al. 2006; Willoughby, Christiansen, et al. 2012). Treatment of EOM with IGF-1 increased both twitch and tetanic muscle tension (Chen and von Bartheld 2004; McLoon, Anderson, and Christiansen 2006; Anderson et al. 2006; T. Li, Wiggins, and von Bartheld 2010), 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 separate muscles, the medial and lateral recti in each orbit, innervated by two different cranial nuclei, abducens and oculomotor, have to 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 (Christiansen and McLoon 2006; Christiansen, Antunes-Foschini, and McLoon 2010) or chemically with botulinum toxin (Ugalde, Christiansen, and McLoon 2005). The current treatments for strabismus that only alter the extraocular muscles (surgery, botulinum toxin) may in fact have low success rates because of the robust adaptation of the

oculomotor system. 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 to drive normal eye alignment. 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 (Lee and Carroll 2007; Shenker et al. 2003; Song et al. 2012; Song et al. 2014; S. Zhou 2000). This adaptation is mediated through interneurons in the spinal cord, and interestingly has even been hypothesized to be communicated through changes in neurotrophic signaling(Lee and Carroll 2007; Song et al. 2012). Based on the literature, 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 (Clendaniel and Mays 1994), resulting in compensatory adaptation. Future studies are needed to uncover the mechanisms which may mediate this adaptation.

We did not observe complimentary 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 with strabismus surgery undergo changes in neuronal drive to extraocular muscle (Agaoglu et al. 2015; Pullela et al. 2015). However, in the present study, adaptation of the EOM was restricted to the treated orbit, which may explain how all of our treated infant monkeys had developed strabismus at the end of three months. This view is consistent with our prior study, which found that treatment of both medial rectus muscles of infant monkeys with IGF-1 resulted in normal alignment (Willoughby et al. 2012). 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 a previous study, we treated two infant monkeys bilaterally with IGF-1, and observed substantial increases in nerve density in the distal region of untreated antagonist muscle (Willoughby et al., 2012). Based on this previous finding and the implicated role of IGF-1 in promoting neurite outgrowth (Caroni and Grandes 1990; Rind and von Bartheld 2002; Apel et al. 2010), we assessed the effects of IGF-1 on nerve and neuromuscular junction density, but no found measurable changes after three 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 (Caroni et al., 1990; Apel et al., 2010; Payne et al., 2010). 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 neurotropic 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-innervation (Nguyen et al. 1998; Zwick et al. 2001; Chen et al. 2003) or where hyper-innervating effects would hypothetically be useful. Alternatively, any influence of IGF-1 on EOM neuromuscular junction size or complexity might have manifested only short term, and thus would not be evident after three months of adaptation.

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 of EOM caused increases in slow, developmental, and neonatal MyHC isoform expression (Christiansen et al 2010). Elevated levels of thyroid hormone also resulted in increased expression of slow MyHC, and decreased expression of neonatal and developmental MyHC isoforms (Harrison, Lee, and McLoon 2010). Disruption of the transcription factor Pitx2 caused a decrease in slow MyHC expression as well as smaller slow myofibers (Y. Zhou, Liu, and Kaminski 2011; Hebert, Daniel, and McLoon 2013). A number of studies showed that IGF-1 treatment of limb skeletal muscle had no effect on MyHC isoform expression (McKoy et al. 1999; Musarò et al. 2001; Payne et al. 2006). 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 (Anderson et al., 2006). 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 (Pette and Staron 1997; Bottinelli and Reggiani 2000). 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 three months of sustained IGF-1 treatment (Anderson et al., 2006). 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 three months of treatment in non-human primates. It is of note 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 three 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 is improved in adult strabismic monkeys by resection/recession surgery, no immediate changes in motor neuron firing rates occur despite an improvement in eye alignment (Agaoglu et al. 2015; Pullela, Agaoglu, Joshi, Agaoglu, Coast, et al. 2015). We hypothesize that this is due to short term adaptations at the muscle level (Anderson et al., 2006). However, by 1 month, there was a significant increase in neuronal drive that was associated with worsening of the strabismic angle (Agaoglu et al. 2015; Pullela, Agaoglu, Joshi, Agaoglu, Coast, et al. 2015). 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.

An important aspect of our study is that the infant monkeys were treated during the sensitive period for the development of stereopsis (Boothe, Dobson, and Teller 1985; Harwerth et al. 1990). By treating the system during the sensitive period when it has increased responsiveness to environmental input, full and proper development of the binocular system was likely disrupted. From our longitudinal analysis of eye alignment over time for IGF-1 treated monkeys (Figure 4.3), it appears that IGF-1 treatment maintained the exotropia the infants were born with. We hypothesize that abnormal IGF-1 signaling disrupted the oculomotor system sufficiently, so that during the sensitive period for 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 et al., 2012). 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 (Chen et al., 2004; McLoon et al., 2006). 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 (Thoenen and Sendtner 2002). 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 normal eye alignment.

FIGURES

FIGURE 4.1 (**A**) Diagram of the rectus extraocular muscles and the experimental design. One medial rectus muscle (pink) was treated with a pellet which slowly released 2µg/day of IGF-1 for 90 days. For horizontal eye movements, the treated medical 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 (**B**) 6° (Monkey 1), (**C**) 12° (Monkey 2), (**D**) 19 ° (Monkey 3) and (**E**) 11° (Monkey 4).

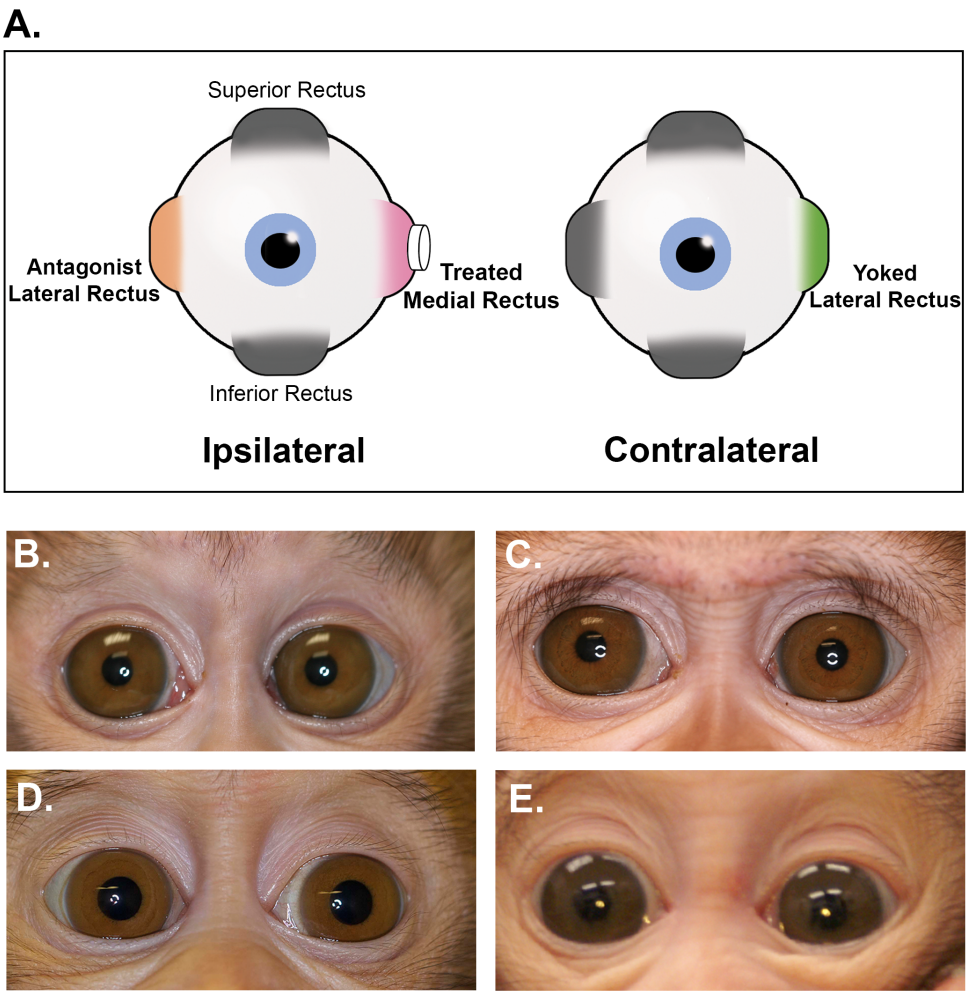


FIGURE 4.2 Representative magnetic resonance imaging examination of one of the treated monkeys shows the location of the pellet on the medial rectus muscle (arrow).

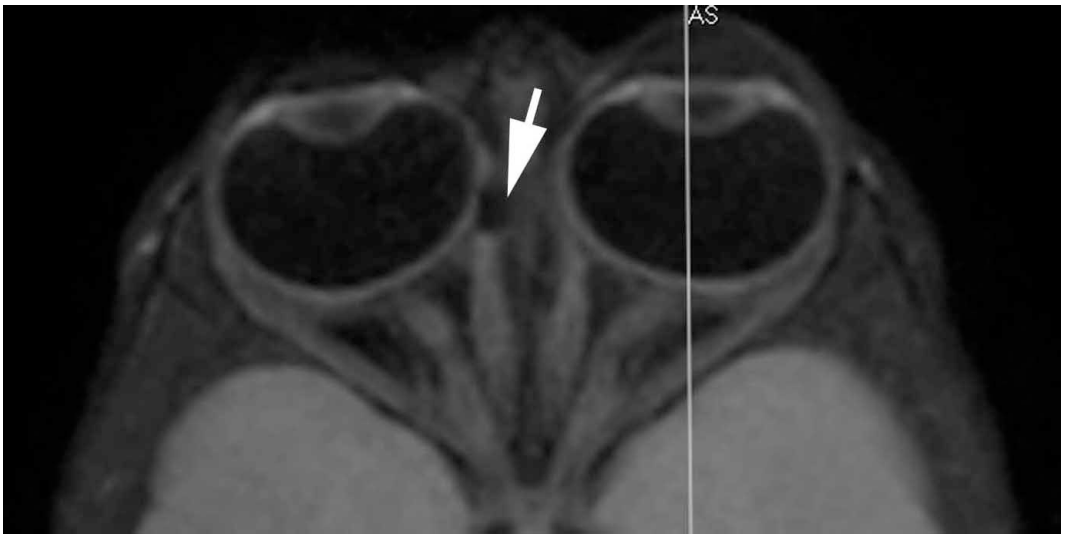


FIGURE 4.3 Successive measurement of eye alignment for two monkeys (monkey 1, monkey 2) in the experimental group at intervals over the three months of sustained IGF-1 treatment. The mean of the final alignment angles for the two other monkeys (monkey 3, monkey 4) are also indicated in the ±SD. The horizontal grey box at ten degrees signifies the threshold for a clinically relevant strabismus.

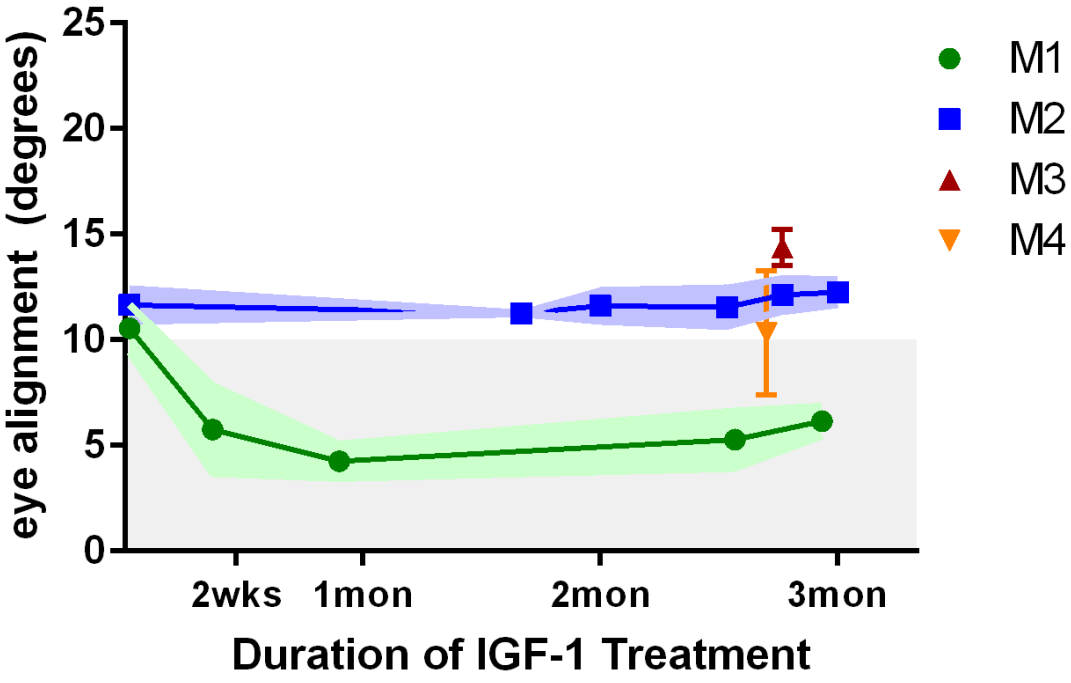


FIGURE 4.4 Quantification of mean myofiber cross-sectional areas after 3 months of sustained IGF-1 treatment in the global (**A**) and orbital (**B**) layers of the muscles. Three months of sustained-release of IGF-1 resulted in an increased mean myofiber cross sectional area of the treated medial rectus 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 differences between control and experimental EOM (ANOVA, Tukey; $p < 0.05$).

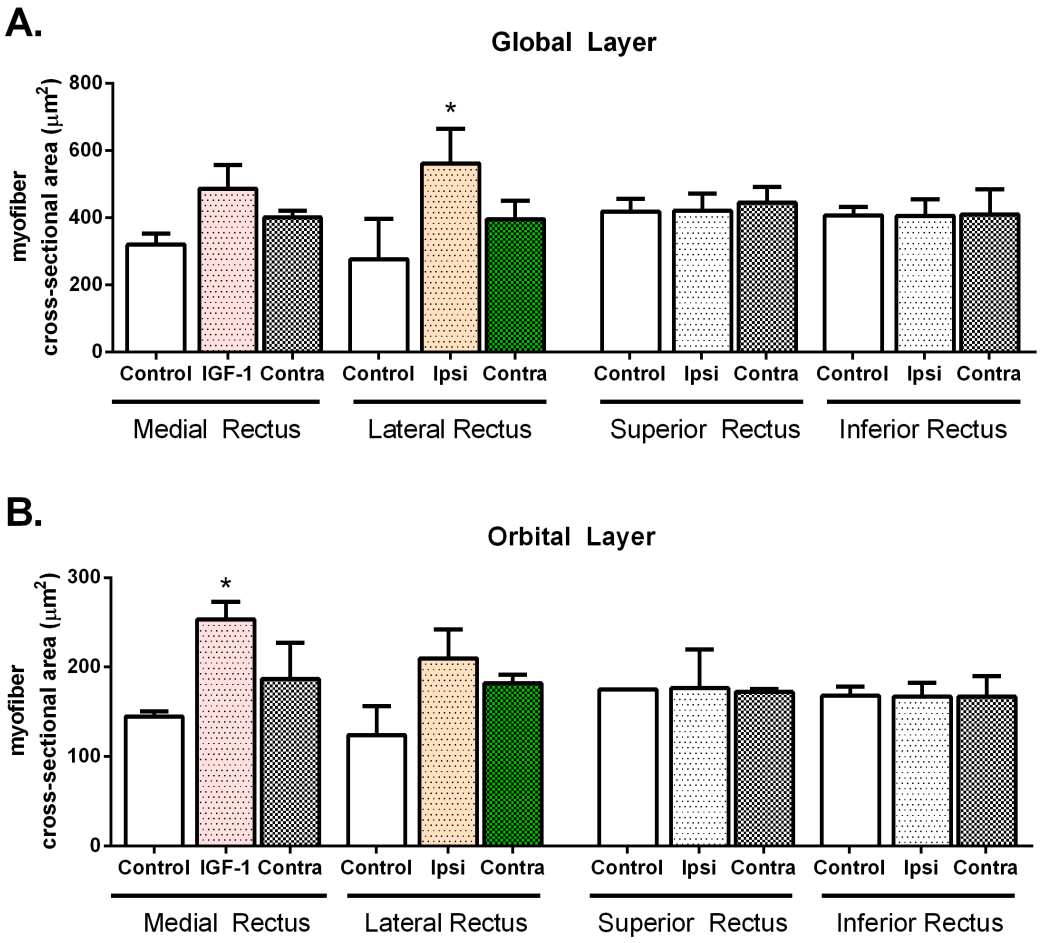


FIGURE 4.5 Confocal images of representative myofibers immunostained with an antibody against the slow MyHC isoform (red) and alpha-bungarotoxin (green) to visualize neuromuscular junctions. Myofibers from control (**A**, **C**) and IGF-1 treated (**B**, **D**) medical rectus muscles in the global (**A, B**) and orbital (**C**, **D**) layer. (**E**-**H**). Myofibers from control (**E**, **G**) and experimental untreated antagonist lateral rectus muscle in the global (**E**, **F**) and orbital (**G**, **H**) layers. Note the apparent increases in myofiber diameter in the IGF-1 treated muscle fibers. Bar: 25μm.

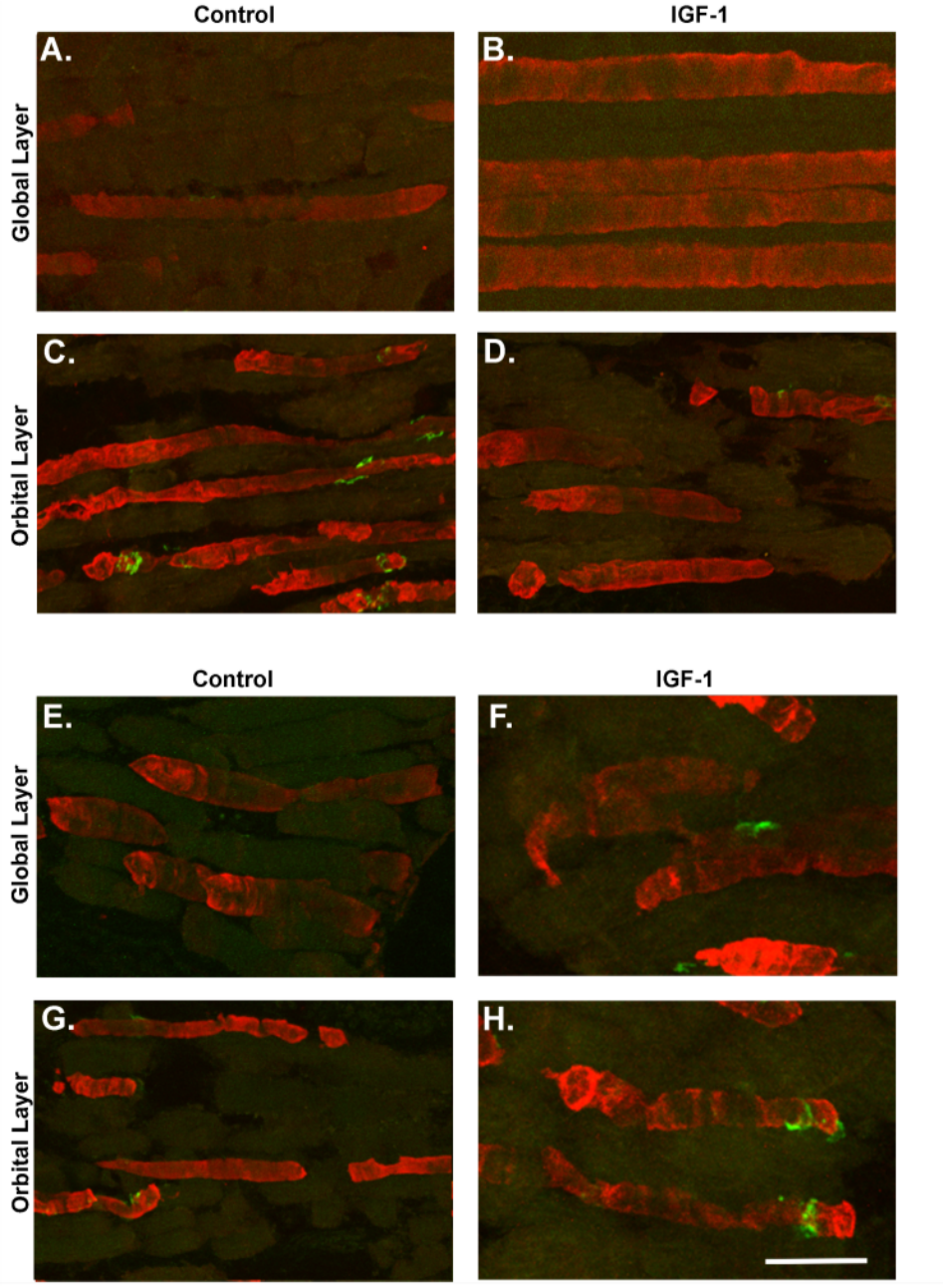


FIGURE 4.6 Three months of sustained IGF-1 results in increased myofiber diameter of both slow and fast myofibers. Quantification of mean myofiber diameter of myofibers positive for slow MyHC isoform (**B**, **D**) and negative for slow MyHC isoform (**A**, **C**), from the global (**A**, **B**), and orbital layer (**C**, **D**) of muscle from the proximal to distal end of the medial and lateral rectus muscles. Asterisks indicate differences between control and experimental medial or lateral rectus muscle (T-test, Holm-Sidak; $p < 0.05$).

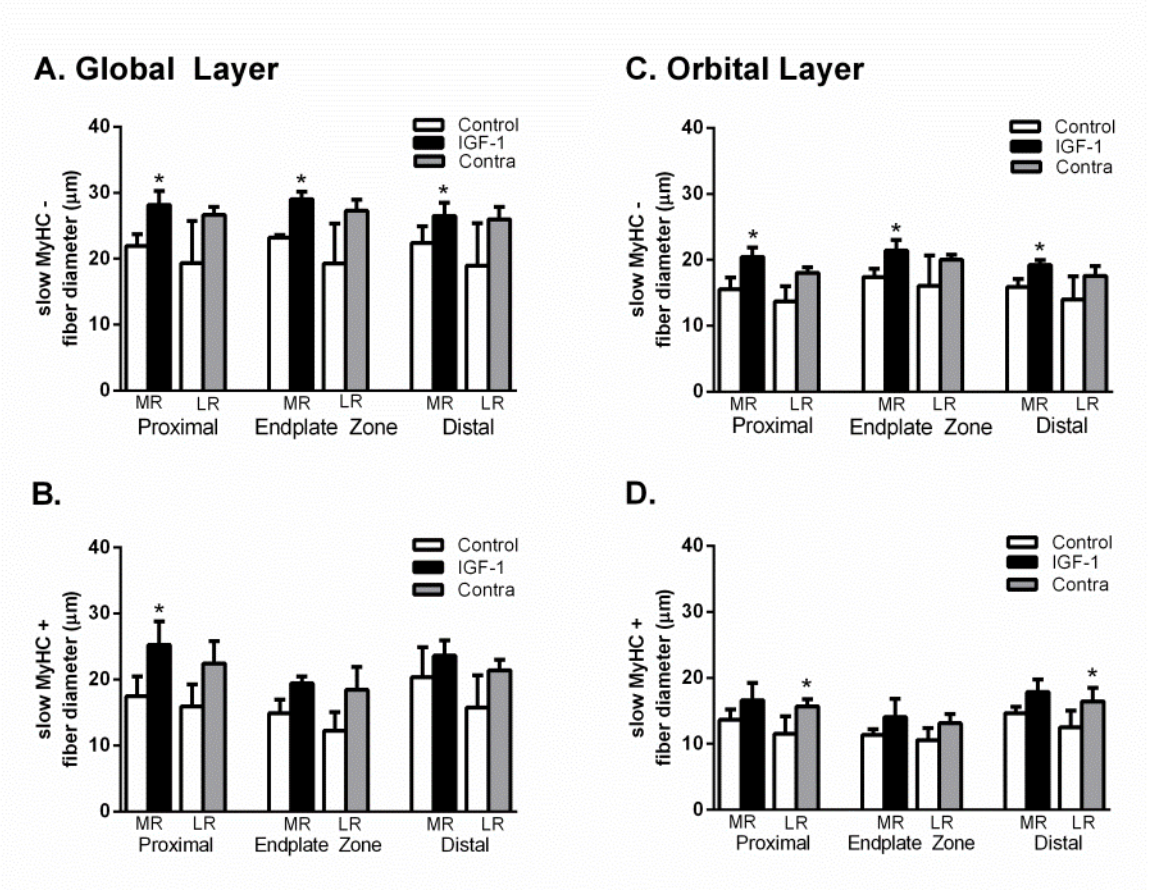


FIGURE 4.7 Quantification of the percent of myofibers expressing slow MyHC isoforms in the global (**A**) or orbital (**B**) layers. No significant difference was seen.

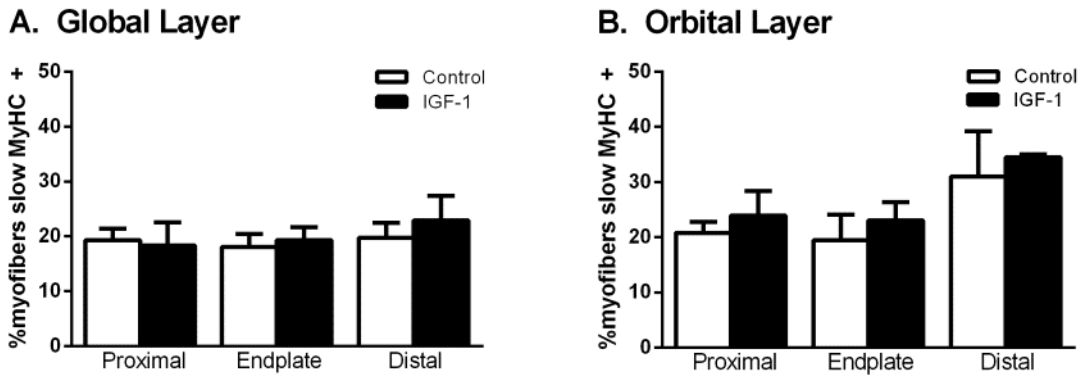


FIGURE 4.8 Sustained release of IGF-1 had no effect on percentage or size of myofibers expressing the embryonic MyHC isoform in the global layer. (**A**) Quantification of the percent of myofibers expressing the embryonic MyHC isoform. (**B**-**C**) Quantification of the mean diameter of myofibers positive (**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 (T-test, Holm Sidak; $p < 0.05$).

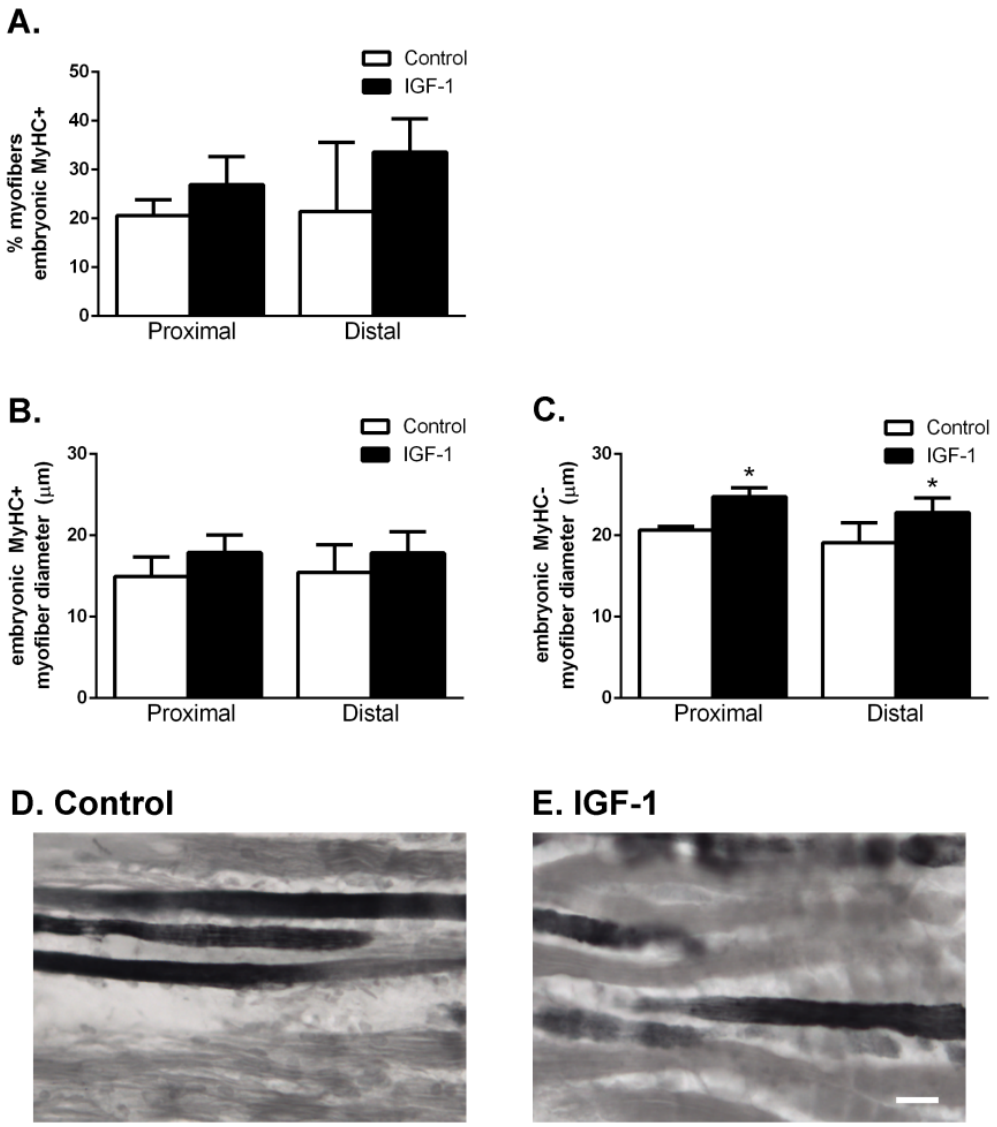


FIGURE 4.9 Myofibers expressing neonatal myosin have larger diameter, but do not change in frequency. (**A**) Scatterplot of all counted myofibers from control (black circles) and IGF-1 treated (grey circles) medial rectus. IGF-1 treatment caused a slight shift toward larger myofibers (x axis), but the percentage of fibers expressing the neonatal MyHC isoform (y axis) was fairly heterogeneous. Photomicrograph of immunostaining with an antibody to the neonatal MyHC isoform in the global (**A**, **B**) and orbital layer (**C**, **D**) of control (**A**, **C**) and IGF-1 treated (**B**, **D**) medial rectus muscles. Bar: 25μm.

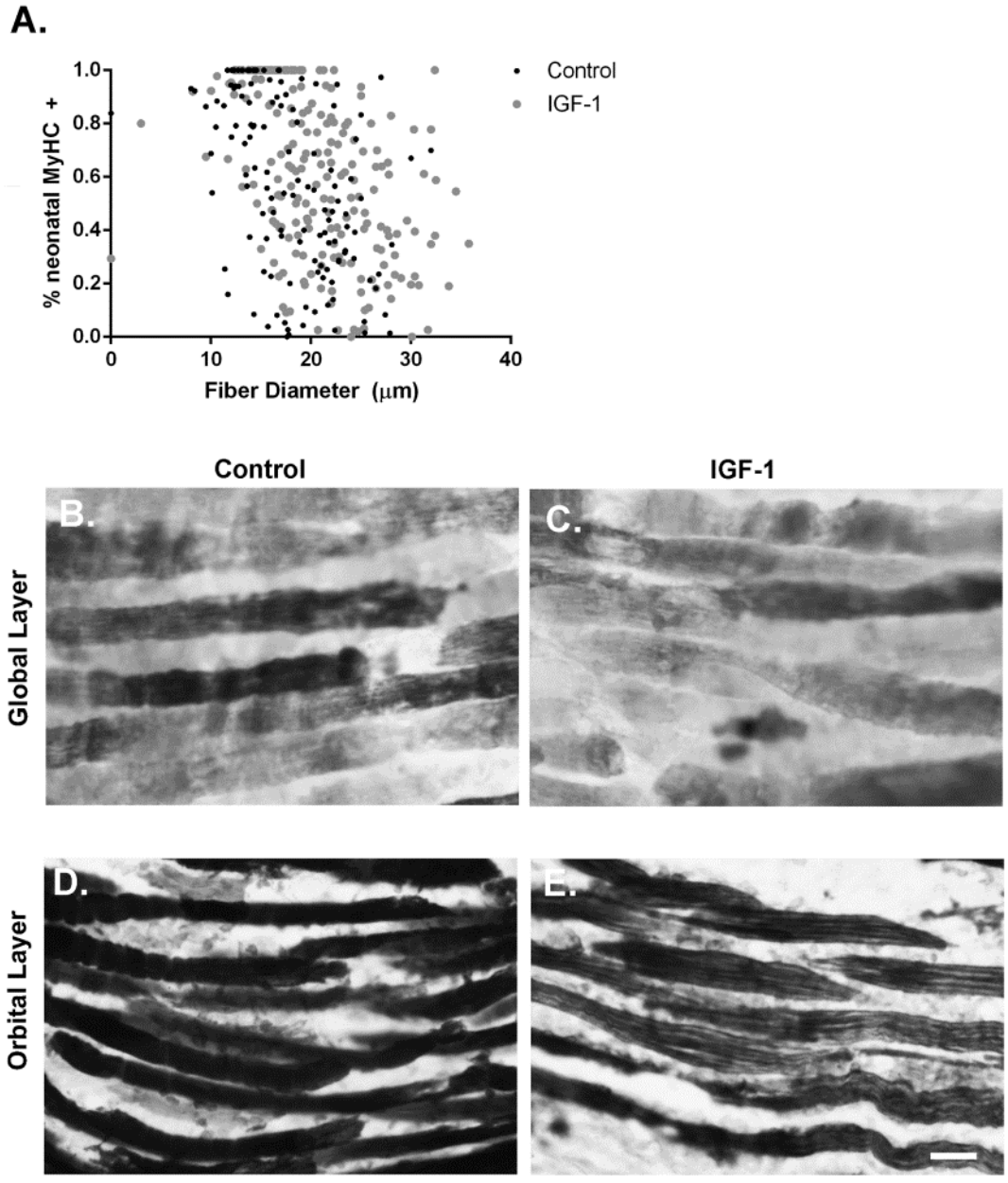


FIGURE 4.10 Quantification of the percentage of myofibers expressing the neonatal MyHC isoform. Quantification of the percentage of myofibers expressing the neonatal MyHC isoform in the global (**B**) and orbital (**C**) layer. (**C-F**) Mean myofiber diameter of myofibers in the global (**C**, **E**) or orbital (**D**, **F)** layers for myofibers positive (**C**, **D**) or negative (**E**, **F**) for the neonatal MyHC isoform. Asterisks indicate significant difference between control and experimental medial rectus (T-test, Holm Sidak; $p < 0.05$).

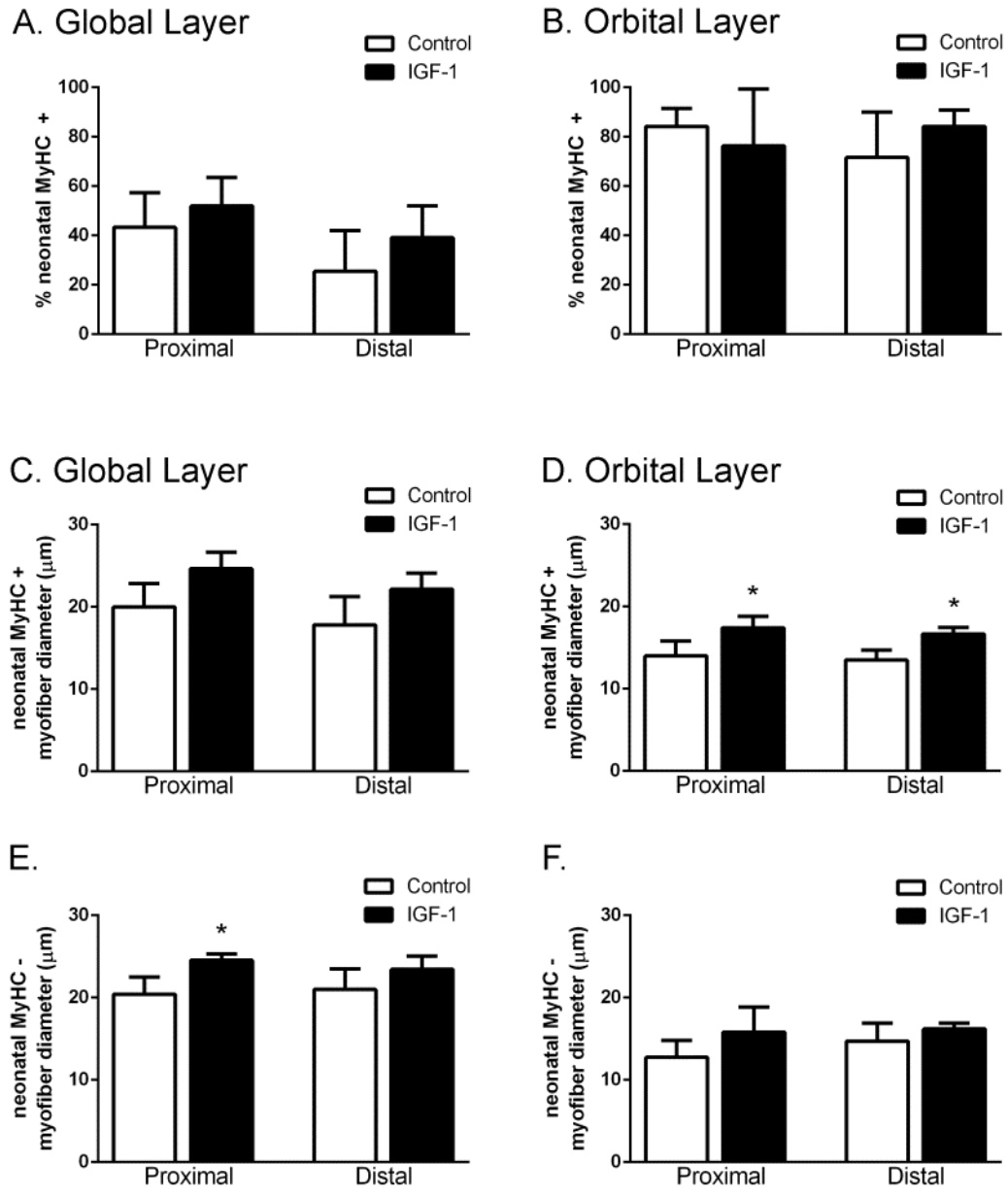
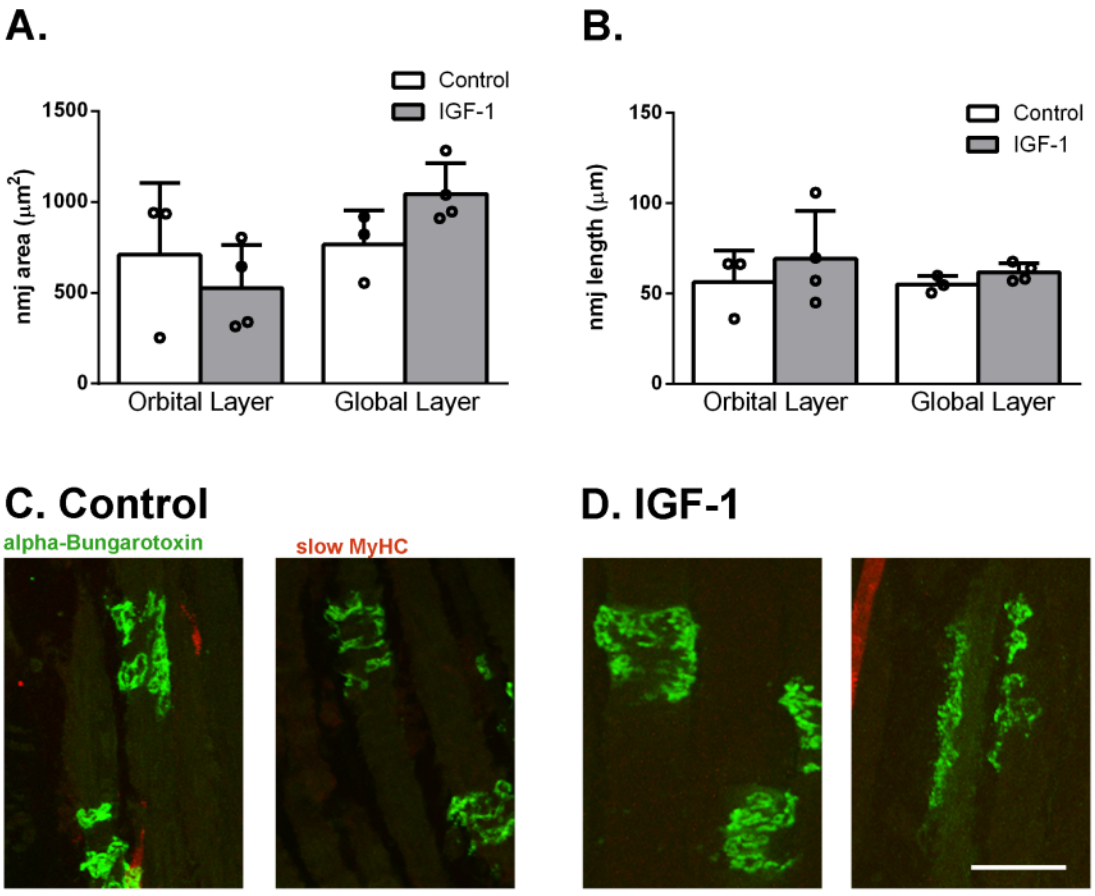


FIGURE 4.11 Quantification of neuromuscular junction area (**A**) and length (**B**) for *en plaque* neuromuscular junctions in the medial rectus muscles after sustained IGF-1 treatment. (**C**-**D**) Confocal images of representative *en plaque* neuromuscular junctions from control (C) and IGF-1 treated (D) medial rectus muscles. Neuromuscular junctions are labeled with alpha-bungarotoxin (green), and slow myofibers are immunostained with an antibody to the slow MyHC isoform (red). Bar: 50μm.



Chapter 5: Adaptation of Slow Myofibers: The Effect of Sustained BDNF Treatment of Extraocular Muscles in Infant Non-Human Primates

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SUMMARY

Neurotrophic factors have emerged as a potential treatment for oculomotor disorders because of diverse roles in signaling to muscles and motor neurons. Unilateral treatment with sustained release BDNF to a single lateral rectus muscle in infant monkeys was performed to test the hypothesis that strabismus would develop in correlation to extraocular muscles changes during the critical period for development of binocularity.

The lateral rectus muscles of one eye in two infant macaques were treated with sustained delivery of BDNF for three months. Eye alignment was assessed using standard photographic methods. Muscle specimens were analyzed to examine the effects of BDNF on the density, morphology, and size of neuromuscular junctions, as well as myofiber size. Counts were compared to age-matched controls. No change in eye alignment occurred with BDNF treatment. As compared to control muscle, neuromuscular junctions on myofibers expressing slow myosins had a larger area. Myofibers expressing slow myosin had larger diameters, and the percentage of myofibers expressing slow myosins increased in the proximal end of the muscle. BDNF expression was examined in control EOM, and observed to have strongest immunoreactivity outside the endplate zone. We hypothesize that the oculomotor system adapted to sustained BDNF treatment to preserve normal alignment. Our results suggest that BDNF treatment preferentially altered myofibers expressing slow myosins. This implicates BDNF signaling as influencing the slow twitch properties of extraocular muscle.

INTRODUCTION

Childhood strabismus is a disorder characterized by a misalignment of the eyes such that normal binocularity does not develop if left untreated. This is very common, and is present in 3-5% of children worldwide (Greenberg et al. 2007; Louwagie et al. 2009). If one considers only non-paralytic, non-anisometropic strabismus, the etiology of the majority of the cases of childhood strabismus is not understood. In addition, it is difficult to produce strabismus in a normally binocular infant non-human primate without significant visual or motor perturbations. The most effective methods for producing strabismus in non-human primates have used sensory deprivation in the form of prism goggles (Crawford and von Noorden 1980) or alternating monocular occlusion (Tusa et al. 2002) during the first three months of life – the critical period for the development of binocularity (Hubel and Wiesel 1970; Harwerth et al. 1990). Additionally resection/recession surgery also has resulted in strabismus in infant-non-human primates (Crawford and von Noorden 1979; Kiorpes and Boothe 1980; Kiorpes et al. 1996; Economides et al. 2007). However, surgery disturbs the normal muscle and connective tissue relationships within the orbit (Hertle, James, and Farber 2015), and makes this model of strabismus problematic relative to trying to understand the underlying cause as well as when attempting to develop new treatment approaches.

We have been studying the use of neurotrophic factors and the changes they induce in extraocular muscle function and structure. Neurotrophic factors are signaling molecules that can have significant influences on skeletal muscle and motor neurons, inducing changes in muscle growth, progenitor cell proliferation, and nerve outgrowth (Caroni et al. 1994; Shavlakadze et al. 2005). Our working hypothesis is that sustained treatment of an extraocular muscle (EOM) with neurotrophic factors would produce functional alterations in the EOM and their innervating motor neurons. These alterations could be manifested by adaptations in the treated muscle and also in either yoked or antagonist/agonist muscle pairs. One such neurotrophic factor is IGF-1, which has direct effects on EOM and is known to be retrogradely transported (Hansson, Rozell, and Skottner 1987). Previous work in adult rabbits showed that sustained treatment with IGF-1 of an extraocular muscle resulted in both significantly increased muscle size and force generation (McLoon et al. 2006). Similar results were shown after single injections of IGF-1 into the orbits of chicks (Chen and von Bartheld 2004). These studies support the

hypothesis that modulating neurotrophic factor levels in the periphery could have significant functional effects relative to eye position and eye movements. We recently showed that unilateral treatment with sustained release of insulin-like growth factor I (IGF-I) to one medial rectus muscle in newborn infant non-human primates produced a strabismus (Willoughby et al. 2015). Interestingly, beyond changes in myofiber cross-sectional area after the three months of sustained IGF-1 treatment, there were no significant changes in neuromuscular junction size or amount of innervating nerve (Willoughby et al. 2015) despite the development of strabismus.

IGF-1 is just one of a large number of neurotrophic factors that are expressed in motor neurons and/or skeletal muscle and have the potential to modulate ocular motor function. Another important neurotrophic factor is brain derived neurotrophic factor (BDNF), which is found in skeletal muscle, Schwann cells, and neuromuscular junctions (Steljes et al. 1999; Garcia et al. 2010). BDNF has been shown to promote neurite outgrowth specifically in developing oculomotor and other brainstem neurons (Steljes et al. 1999; Salie and Steeves 2005). BDNF also has important effects on neuromuscular junction maturation (T. Wang, Xie, and Lu 1995; Garcia et al. 2010) and function (Mantilla, Zhan, and Sieck 2004; McGurk et al. 2011). In addition, when applied to an axotomized abducens nerve in adult cats, BDNF was shown to be neuroprotective, and it also promoted reinnervation by afferents onto the abducens motor neurons. These effects were associated with restoration of the tonic firing properties of the abducens motor neurons (Davis-López de Carrizosa et al. 2009; Morcuende et al. 2013). Complementary findings provided further compelling evidence supporting a role for BDNF in signaling to the visual ocular motor system. For example, BDNF was shown to be able to reactivate ocular dominance plasticity in adult visual cortex in cases of monocular deprivation during early development (Mandolesi et al. 2005; Baroncelli et al. 2010).

The present study examined the potential efficacy of 3 months of unilateral sustained BDNF treatment of a lateral rectus muscle in infant monkeys in an attempt to produce a strabismus. We also examined the expression of BDNF in normal infant monkey EOM. Eye alignment was determined at the end of the study. Myofiber size changes as compared to EOM from normal age-matched control monkeys were determined.

Because BDNF has diverse signaling roles at the neuromuscular junction, including promotion of neurite outgrowth (Salie and Steeves 2005), maturation of neuromuscular junctions (Je et al. 2013) and potentiation of neuromuscular transmission (Mantilla, Zhan, and Sieck 2004; Pousinha et al. 2006). We analyzed the effect of sustained BDNF treatment on neuromuscular junction localization and innervational density, neuromuscular junction localization, and size. In contrast to leg skeletal muscle, EOM has a continuum of myofiber types that vary between the orbital and global layer, and from the proximal to distal ends of the muscle (Rubinstein and Hoh 2000; Jacoby et al. 1990; Y. Zhou, Liu, and Kaminski 2010; McLoon et al. 2011; McLoon, Rios, and Wirtschafter 1999). Within this broad range of properties, myofibers can coarsely be divided into myofibers expressing slow or fast MyHC isoforms, which are related to the type of neuromuscular junctions and myofiber shortening velocity (Rubinstein and Hoh 2000; Khanna et al. 2003; Kjellgren et al. 2003; Fraterman, Khurana, and Rubinstein 2006). Fast myofibers predominately have centrally located *en plaque* endings, and slow fibers have smaller *en grappe* endings along their length, although both myofibers types can be multiply innervated and have both types of neuromuscular junctions(Chiarandini and Stefani 1979; Wasicky et al. 2000; Stirn Kranjc, Smerdu, and Erzen 2009; Feng et al. 2012). To simply the complexity of EOM for analysis, we stained EOM fibers for slow myosin to help differentiate between neuromuscular junctions on these two broad myofiber types.

METHODS

ANIMALS AND SURGERY FOR SUSTAINED BDNF TREATMENT

Infant monkeys were obtained from the breeding colony at the Washington National Primate Center at the University of Washington. All experiments received approval from the Animal Care and Use Committee at the University of Washington, and were performed in compliance with guidelines for use of animals in research from both the National Institutes of Health and the Association for Research in Vision and Ophthalmology.

Two *Macaca nemestrina* infant monkeys aged 1-2 weeks received unilateral treatment of BDNF to one lateral rectus muscle with a pellet prepared to provide the sustained release of 2μg of BDNF per day for 90 days. Under general anesthesia, an

incision was made in the lateral conjunctiva in order to visualize the lateral rectus muscles. A small muscle hook was used to lift the muscles at their point of insertion to allow a sustained release pellet (Innovative Research of America, Sarasota FL) to be placed on the surface of one lateral rectus muscle, over the orbital layer. This dose was selected based upon previous studies of sustained neurotrophic factor treatment to monkey EOM (Willoughby et al., 2012), and on previous studies using anterograde delivery of BDNF to motor neurons (Novikova, Holmberg, and Kellerth 2000; Gordon 2012; Morcuende et al. 2013). After pellet implantation was performed, the conjunctiva was closed using 8-0 ophthalmic suture.

Three months after pellet implantation, the corneal light reflex was used to determine eye alignment (Quick and Boothe 1989). After deep sedation with ketamine, the monkeys were euthanized with an overdose of barbiturate anesthesia by a staff veterinarian. All twelve EOMs were dissected from origin to insertion, embedded in tragacanth gum, and frozen in 2-methylbutane chilled to a slurry on liquid nitrogen. Tissue was also collected from two additional age-matched control monkeys with normal alignment, which were also used as controls in a complementary paper (Willoughby et al., 2015). The tissue blocks were stored at -30°C until processed.

WESTERN BLOT FOR TRKB PROTEIN

Due to the difficulty of obtaining control nonhuman primate infant extraocular muscles and brainstem, and the need to process the treated tissues for histological analysis, whole tissue lysate samples of mouse EOM and brain were obtained to examine normal protein levels of TrkB, a receptor for BDNF. The collected mouse tissues were homogenized on ice in N-PER neuronal protein extraction reagent (Thermo Scientific, Waltham, MA) with Complete® Protease Inhibitor Cocktail (Roche). Homogenized samples were centrifuged at 10,000 rpm for 10 minutes. Supernatants were collected, and protein concentration was determined by bicinchoninic acid (BCA) protein assay (Thermo Scientific). 50 µg of protein were separated on 12% mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA), and transferred to nitrocellulose. Blots were subsequently blocked in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for one hour at room temperature, and then incubated overnight at 4 °C in a primary antibody to TrkB (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), and to GAPDH (1:10,000; Meridian Life Sciences, Memphis, TN.) for a loading control. The following

day, blots were washed in TBST (1x Tris-buffered Saline, 0.1% Tween 20), and then incubated for 30 minutes at room temperature in the secondary goat anti-mouse-IR700, and goat anti-rabbit-IR800 (1:1,000; Rockland, Limerick, PA). The blot was imaged with an Odyssey Infrared Imaging System (LI-COR Biosci.). Densitometry values were obtained using Odyssey instrument software.

IMMUNOHISTOCHEMISTRY AND HISTOLOGICAL PROCESSING

The extraocular muscles from the infant monkeys were sectioned frozen at a thickness of 30 μm , cut parallel to the long axis of the fibers, using a Leica cryostat, and the sections were stored at -80^{o}C until processed. Every 30th section was stained with hematoxylin and eosin (H and E) to be used for myofiber area measurements. For visualization of neuromuscular junctions, every 40th section was washed in 0.01M phosphate buffered saline (PBS), pH 7.4 containing 0.1% Triton X-100 (PBS/TX), blocked in 10% goat serum for one hour, and incubated overnight at 4°C with an antibody to the slow heavy myosin chain isoform (MyHC) (Vector Laboratories, Burlingame, CA) or to synaptophysin (1:300; abcam; Cambridge, MA). Sections were washed in PBS, followed by incubation in secondary antibody, goat anti-mouse IgG labeled with Cy3 (1:1000; Jackson ImmunoResearch Laboratories; West Grove, PA) for one hour at room temperature. To visualize neuromuscular junctions, sections were double labeled by incubation with α-bungarotoxin conjugated to AlexaFluor®488 (1:3000; Molecular Probes; Eugene, OR) overnight. A subset of sections were double or triple labeled for nerves by incubation in an antibody to neurofilament (1:1000; smi-31; BioLegend; Dedham, MA) overnight, and visualized with goat anti mouse DyLight® 405 or donkey anti mouse AlexaFluor®488 (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA).

Using the above procedures, human inferior oblique specimens were immunostained for TrkB (1:50 sc-8316; Santa Cruz), a receptor for BDNF. Human tissue and three to four sections throughout each control lateral rectus were also immunostained for BDNF (1:100; abcam; 1:100 sc-546, Santa Cruz; 1:50; Promega; Madison, WI) to assay neurotrophin and receptor distribution through the EOM. Sections were visualized with the secondary antibodies goat anti rabbit Rhodamine Red or donkey anti sheep Cy3 (Jackson). To further characterize BDNF distribution, BDNF was doubled labeled with either αbungarotoxin conjugated to AlexaFluor®488 as described

above, or with a variety of myosin isoforms using the above procedure. Antibodies against myosins from the Developmental Studies Hybridoma Bank (Iowa City, IA) were: 2x MyHC (1:20), embryonic MyHC (1:40), slow MyHC (1:1000), and neonatal MyHC (1:20). Antibodies obtained from Acbam were αcardiac MyHC (1:10) and 2a MyHC (1:100). MyHC isoforms were visualized with labeling by the secondary antibody donkey anti mouse AlexaFluor®488 (Jackson ImmunoRes.). All sections were washed and mounted on glass slides and coverslipped with Vectashield (Vector Laboratories).

IMAGE PROCESSING AND ANALYSIS

For calculation of mean myofiber cross-sectional areas, fields were randomly selected and hand circled with our morphometry system (Bioquant, Nashville, TN) using the H and E stained sections. A minimum of 200 myofibers were measured in both the orbital and global layers.

Immunostaining for BDNF, TrkB and myosin heavy chain isoform (MyHC) expression were visualized using a confocal scanning microscope at 40x oil immersion when sections were co-labeled with neuromuscular junctions or nerve, or were visualized with an epifluorensce microscope at 20x for all other cases. Representative fields were chosen for visualization.

For visualization of nerve and neuromuscular junctions, slides were imaged with scanner laser confocal microscopy (Olympus; FV1000) to sequentially capture images in the same focal plane with different filters. Neuromuscular junctions were imaged at 20x oil immersion. Every 40th section was imaged for analysis. With a random starting point, every third field throughout the entire muscle specimen was taken. Additionally, every fifth field was imaged at 60x oil immersion to capture neuromuscular junction morphology at higher power.

Z-stacks were collapsed for each field, and the following measurements were taken using the analysis software Image J (NIH; http://imagej.nih.gov/ij): muscle area, mean diameter for myofibers positive or negative for slow MyHC, total number of neuromuscular junctions, and the collapsed area of each neuromuscular junction endplate as determined by hand circling. For every fiber with a neuromuscular junction, it was also noted if 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 properties to distinguish between *en grappe* and *en plaque* endplates. Additional staining among control and experimental muscle with double-labeling of SNAP-25 (1:1000; Covance; Dedham, MA) or synaptophysin (1:300; abcam), nerve (smi-31), and α-bungarotoxin revealed that all α-bungarotoxin labeled neuromuscular junctions in the EOM specimens co-localized with presynaptic components. Based on this, we are confident that the majority of α-bungarotoxin positive neuromuscular junctions in our samples were active endplates. For determination of neuromuscular junction area, only fibers with complete neuromuscular junctions were included. Counts were conducted blinded to treatment. Data are presented as mean ± SD.

RESULTS

ANALYSIS OF EYE ALIGNMENT

Because of the evidence that BDNF is a target-derived neurotrophin that signals to oculomotor neurons (Steljes et al. 1999; Davis-López de Carrizosa et al. 2009; Morcuende et al. 2013), we hypothesized that sustained BDNF delivery to infant monkeys during the critical period for development of the binocular system would perturb oculomotor development and maturation, resulting in strabismus. However, sustained delivery of BDNF for three months to one lateral rectus muscle did not result in obvious misalignment of eye position at end of treatment. Corneal light reflex photographs of monkeys after 3 months of sustained 2μg/day BDNF treatment demonstrated that one monkey had a possible microstrabismus of 8° (Figure 5.1A), while the second treated monkey had normal alignment, <5° (Figure 5.1B).

EFFECT OF BDNF ON NEUROMUSCULAR JUNCTION

When all myofibers from the BDNF-treated lateral rectus muscles were examined, there was no change in neuromuscular junction density (data not shown). Interestingly, there was a prominent trend towards larger neuromuscular junctions on slow myofibers (Figures 5.2A, B, F, G; 5.3A) with average endplate area 35% larger, which was 65.6 ± 8.9 in control lateral rectus and 92.6 ± 1.5 μm in the BDNF-treated lateral rectus muscles. This trend was most pronounced in the endplate and distal regions of the global layer (Figure 5.3C), and proximal and distal region of the orbital layer (Figure 5.3E). When neuromuscular junction size was normalized to the diameter

of the myofibers, the trend persisted, suggesting this effect was not due to neuromuscular junction that was just in proportion to the increased myofiber size. In contrast, there was no observed difference in the area (Figure 5.3B), length, or complexity of *en plaque* neuromuscular junctions on fast myofibers (Figures 5.2C-E, H-J).

EFFECT OF BDNF ON MYOFIBER SIZE

Because of the demonstrated role of BDNF in altering slow neuromuscular junctions (Gonzalez et al. 1999; Kulakowski, Parker, and Personius 2011; Je et al. 2013) and the expression of BDNF in satellite cells in diaphragm and limb skeletal muscle (Mousavi and Jasmin 2006), we also investigated whether BDNF treatment had an effect on the size of myofibers. Complementary to the effect we observed on neuromuscular junctions, BDNF treatment resulted in larger myofibers diameter in slow myofibers (Figures 5.4; 5A) as compared to control lateral rectus muscles. This trend was most dramatic in the endplate region of the global layer (Figures 5.4B, E; 5C), where slow myofibers maintained their fiber diameter throughout the length of the muscle. In contrast, no difference between the fiber diameters of fast myofibers was observed between experimental and control lateral rectus EOM (Figure 5.5B) in either the global (Figure 5.5D) or orbital (Figure 5.5F) layer.

BDNF treatment resulted in larger diameter of myofibers expression the slow MyHC isoform compared to the untreated control lateral rectus muscles. However, we hypothesized that sustained BDNF treatment may cause compensatory adaptation in the functionally paired but untreated extraocular muscles. For this analysis, myofiber areas were determined on H and E stained sections of all the medial and lateral rectus muscles from the control and BDNF-treated animals. Mean myofiber cross-sectional area throughout the entire EOM suggests that the myofibers were larger as compared to control lateral rectus muscles. Myofibers were 60% larger in the orbital layer, going from 123.5 ± 26.2 to 233.1 ± 23.7 μm^2, and 48% larger in the global layer, from an average myofiber area of 276.3 ± 110.5 to 452.2 ± 23.9 μm^2 in the treated lateral rectus. However, changes in area were not just seen in the treated lateral rectus muscles, but also in the ipsilateral antagonist medial rectus to the treated lateral rectus (Figure 5.6). The untreated antagonist medial rectus myofibers were 31% larger in the orbital layer (143.2 ± 11.5 μm^2 compared to 197.9 ± 35.1 μm^2), and 15% larger in the global layer

(329.8 ± 42.2 μm^2 compared to 382.4 ± 56.4 μm^2) as compared to control medial rectus EOM. These results are consistent with our prior findings and suggest that the developing ocular motor system has the ability to adapt to perturbation to one of its muscles in order to preserve eye alignment.

EFFECT OF BDNF ON SLOW MYOFIBER SIZE AND DENSITY

Complementary to the pronounced effect of BDNF on fiber diameter and neuromuscular junction size on slow myofibers, the percentage of myofibers expressing slow myosin in the proximal region of the muscle was also higher in BDNF treated lateral recti in both the global and orbital layer (Figure 5.7). The percentage of myofibers positive for slow myosin was larger by 35%, from 20.6 ± 0.3% in control lateral rectus to 29.5 ± 4.5 % in BDNF-treated lateral rectus.

EXPRESSION OF BDNF IN EOM

BDNF treatment caused pronounced changes in the treated slow myofibers. We hypothesized that BDNF would be preferentially localized to slow myofibers in normal EOM. Immunostaining with three different BDNF antibodies in both human and monkey EOM confirmed localization of BDNF to EOM myofibers. BDNF appeared diffusely throughout the cytoplasm in many myofibers in the proximal and distal regions of the muscles, but was preferentially expressed in some myofibers over others, creating a mosaic pattern in both monkey (Figures 5.8A, B, D, F) and human (Figure 5.9A) muscle specimens. In some regions, BDNF was most intensely localized to the outer membrane of myofibers (Figures 5.8C, D, E; 5.9C). BDNF expression was often slightly stronger in the orbital layer as opposed to the global layer (Figure 5.9). Toward the midbelly of the muscle, BDNF immunoreactivity was drastically reduced, with less staining in the cytoplasm and outer cell membrane (Figures 5.8G-I).

The mosaic pattern of BDNF immunoreactivity in the distal and proximal ends of the EOM suggested that BDNF expression could be related to myofiber type. To test if BDNF expression was related to fiber MyHC composition, lateral rectus muscle was co-labeled by immunostaining for six different MyHC isoforms. The EOM express nine different myosin isoforms, which are often co-expressed, and vary in expression along a fiber's length (Rubinstein and Hoh 2000; Y. Zhou, Liu, and Kaminski 2010; McLoon et al. 2011). In the distal and proximal regions of the muscle, BDNF was often co-localized

with the fast MyHC type2x and type2a (Figure 5.10A, B). Less co-localization was present with the "developmental" myosins embryonic (Figure 5.10C) or neonatal MyHC (Figure 5.10D). The least overlap was observed with α-cardiac MyHC (Figure 5.10F), and surprisingly, there was little co-localization with myofibers expressing slow MyHC (Figure 5.10E).

BDNF was localized to neuromuscular junctions (Figure 5.11) and nerve (Figure 5.12) as visualized with double labeling with α-bungarotoxin and an antibody to neurofilament. However, not all endplates had prominent BDNF immunolabelling (Figure 5.8I; 5.11), and BDNF was not expressed in all nerve fibers. No clear trend was observed in BDNF intensity of immunostaining in the neuromuscular junctions and nerve in the proximal to distal extent of the muscles examined (not shown).

EXPRESSION OF BDNF RECEPTOR TRKB

Mature BDNF predominantly signals through the receptor tyrosine receptor kinase B (TrkB) although BDNF does signal with lower binding affinity to other Trk receptors and $p75^{NTR}$ (Barde, Edgar, and Thoenen 1982; Barbacid 1994; F. Yang et al. 2009). To confirm that main receptor for BDNF was present in the EOM, and therefore present for the exogenously applied BDNF signaling in the EOM, the presence of TrkB in adult mouse EOM was demonstrated with western blot (Figure 5.13A). TrkB protein expression was localized to EOM with immunollabeling in human inferior oblique specimens. Similar to BDNF, TrkB was present diffusely within individual myofibers, and also expressed in the outer cell membrane (Figure 5.13). Co-localization of TrkB with BDNF demonstrated that fibers expressing BDNF were frequently positive for TrkB (Figure 5.13). As with BDNF, TrkB was present at neuromuscular junctions (Figures 5.14A, C) and in nerve (Figures 5.15B, C). However, as observed with BDNF localization, some neuromuscular junctions (Figure 5.14A) and nerve (Figure 5.15A) had low to no immunoreactivity for TrkB.

DISCUSSION

BDNF is known to have direct effects on EOM and to promote muscle innervation in some animal models. We were interested in evaluating BDNF treatment of macaque EOM because of the utility of this model in studies of strabismus. We treated the lateral

rectus muscles of two infant monkeys with 2μg/day of BDNF for three months via a sustained-release pellet. One goal was to assess if unilateral BDNF treatment could alter eye position. We found that sustained BDNF treatment during the sensitive period for development of binocularity did not result in clinically significant strabismus, defined as >10 degrees. However, analysis of the treated EOM suggests that BDNF treatment did alter characteristics of the EOM. The most pronounced effect was on slow myofibers. On average, slow myofibers had larger diameters, larger neuromuscular junctions, and there was increased percentage of myofibers positive for slow MyHC at the proximal end of the treated muscles. These results strongly suggest that BDNF signaling controls the characteristics of slow myofibers, and may uniquely contribute to the complex phenotype of EOM. In contrast, we found no significant effects of BDNF treatment on fast myofibers.

Development of the visual system in macaques closely mirrors humans (Boothe, Dobson, and Teller 1985; Harwerth et al. 1990), making macaques a useful model to study strabismus and the effects of neurotrophic factor signaling on the developing binocular system. In a prior study, we treated infant macaque medial rectus muscles bilaterally with 1μg/day of IGF-1 for three months (Willoughby et al., 2012). The treated monkeys had normal eye alignment, despite larger myofibers after IGF-1 treatment. Our analysis suggested that binocularity developed because there were coordinated alterations of myofiber size in the treated and non-treated muscles over the 3 month duration of treatment, presumably through communication within the ocular motor system in the brain. Our results complement this study, as normal eye alignment developed in the infant macaques after sustained BDNF treatment despite increases in myofiber area and larger neuromuscular junctions in the treated lateral rectus muscle. Further, the myofiber area measurements suggest that there were coordinated changes in the non-treated muscles that were sufficient to compensate for the increased fiber size in the treated muscles.

In humans and monkeys, the two eyes move as a functional pair in order to create sensory fusion and depth perception. The oculomotor system is heavily biased to develop with normal eye alignment. Creating animal models of strabismus is difficult and requires persistent sensory deprivation during the sensitive period of the binocular system (Crawford and von Noorden 1980; Quick and Boothe 1989; Tusa et al. 2002).

The oculomotor system has a robust ability to adapt to perturbations to preserve eye alignment.

In our recent study, we found that an increased dose of 2 μg/day IGF-1 unilaterally delivered for three months to the medical rectus muscle of three infant macaques resulted in a clinically significant strabismus at the end of treatment (Willoughby et al., 2015). In all three infant monkey studies, we observed adaptation in a subset of untreated EOM, including the antagonist medial rectus muscle in the same orbit after unilateral IGF-1 treatment, and the antagonist muscle in the fellow eye in bilateral IGF-1 treatment. In the current study, myofiber area was larger in the treated lateral rectus, but also in the ipsilateral antagonist medial rectus and contralateral lateral rectus (Figure 5.6). Adaptation between paired EOM has also been observed after strabismus surgery modeled in rabbits (Christiansen and McLoon 2006; Christiansen, Antunes-Foschini, and McLoon 2010). It is possible that the dose of the BDNF treatment was not sufficient to functionally change eye movement or alignment. However, we hypothesize that after sustained BDNF treatment to one EOM, adaptations occurred in the paired untreated EOM that were sufficient to allow formation of normal eye alignment in the treated subjects. Collectively, these studies strongly support the view that the central nervous system has the ability to respond to changes in EOM force and shortening velocity produced by the exogenous addition of neurotrophic factor, and adapt over time to maintain homeostasis between antagonist/agonist paired EOM. The mechanisms by which this occurred have yet to be delineated, but likely involve signaling between internuclear neurons in the motor nuclei.

The EOM produce a variety of eye movements, which in part is reflected in the large continuum of myofiber properties, anatomical, biochemical and physiological (Asmussen et al. 2008; McLoon et al. 2011; Davis-López de Carrizosa et al. 2011). Sustained and lower velocity movements such as smooth pursuit or fixation on a visual target require slow and sustained contraction and a non-fatigable muscle (Asmussen and Gaunitz 1981; Fuchs and Binder 1983; Prsa, Dicke, and Thier 2010). Eye muscles also engage in some of the fastest contractions of skeletal muscle (Close and Luff 1974), which are recruited for quick eye movements such as saccades. The organization of the motor neurons provides for a continuum of phasic and tonic units that presumably reflect the different trophic background received from distinct types of myofibers within the EOM.

Sustained BDNF treatment to EOM uniquely altered myofibers expressing slow myosin, which are implicated in the more tonic or step phase of contraction (Bondi and Chiarandini 1983; Chiarandini and Stefani 1979; Hess and Pilar 1963; Sylvestre and Cullen 1999a). BDNF did not alter the overall density of neuromuscular junctions, even though in culture BDNF promoted neurite outgrowth of ocular motor neurons and stabilized neuromuscular junctions (Steljes et al. 1999; Je et al. 2013). However, the EOM is richly innervated compared to other skeletal muscles, with multiply innervated fibers and very small motor units. It is possible that normal neurotrophic signaling modifies EOM properties through changing myofiber type and potentiating neuromuscular junctions rather than by complete re-patterning of muscle innervation (Pette and Staron 2001; Pette and Staron 1997; Wolpaw and Carp 1994).

The sustained BDNF treatment of the infant lateral rectus muscles resulted in larger neuromuscular junctions on slow myofibers as compared to those in control lateral rectus endplates. However, the sustained BDNF treatment did not have an effect on the size or morphology of *en plaque* neuromuscular junctions localized to fast myofibers (Figure 5.2, 5.3). It is well established that neuromuscular junctions are subject to local, positive feedback loops between various synaptic regulatory factors that translate activity into structural changes at neuromuscular synapses (Loeb et al. 2002; Cano et al. 2013). These studies show the modulatory effect of BDNF on synapse structure and active zone size, for example. In other skeletal muscles, BDNF and its receptor TrkB were shown to influence both neuromuscular maturation and maintenance (Kwon and Gurney 1996; Je et al. 2013; T. Wang, Xie, and Lu 1995; Gonzalez et al. 1999; Kulakowski, Parker, and Personius 2011). In addition, BDNF treatment to skeletal muscle increases evoked endplate potentials (Pousinha et al. 2006; Mantilla, Zhan, and Sieck 2004). Functional studies are needed to extend our finding to determine the functional result of the increased endplate size seen after the sustained BDNF treatment in order to determine if BDNF enhances neuromuscular transmission. Further, it is unknown if BDNF signals to all neuromuscular junctions in the EOM, or only a subset, such as *en grappe* endplates.

The sustained BDNF treatment to the infant lateral rectus muscle was also associated with larger slow myofibers (Figures 5.3, 5.4). Slow myofibers are generally smaller in diameter towards the midbelly of the muscle, where large *en plaque* endplates are concentrated. In the BDNF treated muscle in the present study, the slow myofibers

were slightly larger throughout the EOM, but the most dramatic increase in myofiber size was observed in the endplate zone.

In the diaphragm, BDNF was shown to be expressed in satellite cells, and was specifically shown to play a role in maintenance of the myogenic precursor pool (Mousavi et al., 2006). Although the pattern of immunollabeling in myofibers did not suggest enriched BDNF in satellite cells, it is possible that BDNF treatment encouraged myonuclear addition selectively of slow myofibers, particularly in the endplate zone, where endogenous levels of BDNF appeared to be less than in the proximal and distal regions of the muscles. Alternatively, if BDNF signaling potentiated neuromuscular signaling at slow neuromuscular junctions, which are present along the entire fiber length, it is possible that the higher activity level at the synapse may have promoted larger myofibers, which would be most pronounced in the endplate region, where slow myofibers are proportionally the smallest.

Based on the changes induced by sustained BDNF treatment on the properties of the slow myofibers, we hypothesized that BDNF expression in control lateral rectus muscles would be localized to this population of fibers. We confirmed protein expression of BDNF in both infant monkey and adult human EOM with immunollabeling (Figure 5.9). Surprisingly, immunollabeling of BDNF in control monkey lateral rectus muscle revealed that BDNF was seldom co-localized to the slow myofibers (Figure 5.10E). Co-expression of BDNF with five other MyHC isoforms was investigated. The EOM express nine different myosin heavy chains, which often overlap in expression (Wieczorek et al. 1985; Asmussen, Traub, and Pette 1993; Pedrosa-Domellöf et al. 1992; Y. Zhou, Liu, and Kaminski 2010; McLoon et al. 2011). BDNF was most often co-expressed with 2x and 2a MyHC, which execute fast muscle contractions. Interestingly, both 2a MyHC and BDNF expression in myofibers are drastically reduced in the endplate region of the EOM (Figure 5.9 G-I; Kjellgren et al., 2003; Zhou et al., 2010). The functional sequelae of this co-expression pattern are unknown.

Recent studies suggest that neurotrophic factors differentially promote specific afferent inputs and firing patterns in neurons (Galuske et al. 2000; Adamson, Reid, and Davis 2002; McAllister, Katz, and Lo 1999; Davis-López de Carrizosa et al. 2010). In the ocular motor system, axotomized abducens motor neurons underwent synaptic stripping. However, delivery of BDNF to the lesioned nerve preferentially restored the tonic or step phase of firing of the treated motor neurons, but not the phasic firing (Davis-López de

Carrizosa et al., 2009). Our findings that BDNF immunolabelling was strongest in areas outside the location of the *en plaque* neuromuscular junctions, and that BDNF influenced properties of slow myofibers preferentially supports a role for BDNF both in the periphery and in motor neurons that would promote slow, sustained eye movements.

In addition to influencing the properties of neurons, neurotrophic factors also influence the properties of muscles (Chen and von Bartheld 2004; McLoon, Anderson, and Christiansen 2006; T. Li, Wiggins, and von Bartheld 2010; Feng et al. 2012). Muscle-derived BDNF is expressed in the developing EOM and retrogradely transported to oculomotor neurons (Steljes et al. 1999; Morcuende et al. 2013). BDNF mRNA is present in adult mouse EOM (Harandi et al., 2014). We localized BDNF protein to individual myofibers infant monkey but also in adult human EOM with immunolabelling, confirming that BDNF expression is maintained in adult EOM (Figure 5.8, 5.9), and highly implicates BDNF as an important signaling molecule in the mature EOM. In other skeletal muscle, BDNF has been differentially localized to satellite cells (Mousavi and Jasmin 2006), the cell cytoplasm, edge of fibers, and at the synapses (Garcia et al. 2010; Sakuma et al. 2001). Similar to these studies, we observed BDNF both in the myofiber cytoplasm, around the cell membrane, and in neuromuscular junctions.

Although BDNF was not uniquely localized to slow myofibers, the highest level of expression was in the orbital layer, and in the proximal and distal regions of the muscle. This demonstration of the region-specific expression of BDNF is similar to that seen for other neurotrophic factors in the EOM. For example, IGF-1 was expressed in EOM as a gradient along the muscle length, with highest expression in the distal region of the muscle (Feng and von Bartheld 2011). The distribution pattern of BDNF along the EOM has interesting functional implications, as BDNF myofiber staining was strongest in areas of the muscle hypothesized to be more involved in the tonic or step phase of muscle contraction (Wasicky, Horn, and Büttner-Ennever 2004; Spencer and Porter 2006). Large twitch movements are hypothesized to be executed predominantly by the large *en plaque* endplates on fast myofibers of the global layer (Chiarandini and Stefani 1979; Feng et al. 2012), which was the region with the lowest expression level of BDNF.

In addition to the presence of BDNF within individual myofibers, BDNF was present at neuromuscular junctions, in nerve terminals, and in nerve fiber bundles. It is interesting that not all nerve and neuromuscular junctions were immunolabeled for BDNF expression. In addition, BDNF reactivity was seen in both *en plaque* and *en*

grappe neuromuscular junctions located proximally and distally in the muscles. About 20% of abducens motor neurons were shown to lack the TrkB receptor (Benítez-Temiño et al. 2004), which suggests that a subpopulation of motor neurons may not use BDNF as a neurotrophin. A more detailed analysis is needed to more fully delineate if BDNF expression at neuromuscular junctions is related to specific neuromuscular junction morphology or myofiber type. Further studies are needed to determine what controls BDNF expression and the role of BDNF signaling within the EOM. We also confirmed with both western blot and immunolabelling that TrkB, the highest affinity receptor for mature BDNF, is present in adult EOM. This further supports an active role of BDNF in the maintenance of mature EOM properties. Further studies are needed to determine the localization of the receptors TrkB and $p75^{NTR}$ in the EOM. This information would provide more insight into how the patterning of this neurotrophin and its receptors contributes to the varied properties of the EOM across its length.

In summary, sustained delivery of BDNF to infant monkey EOM resulted in changes in characteristics of slow myofibers. Compared to control lateral rectus muscles, neuromuscular junctions on slow myofibers had larger areas, the size of slow myofibers was larger, and the percentage of fibers expressing slow myosin was higher in the proximal region of the muscle. Despite these changes, the sustained BDNF treatment throughout the first three months of development of the binocular system did not result in strabismus. We hypothesize that the ocular motor system adapted to BDNF treatment to preserve eye alignment. Based on the morphological changes observed in EOM after BDNF treatment, further studies are needed to examine if BDNF changed muscle contractile properties. Larger slow myofibers could result in slower saccades, a longer duration or stability during fixation, or more precise eye movements and better gaze holding. These results support a role for BDNF in promoting the slow sustained contraction properties of extraocular muscle, and implicate BDNF as a potential novel treatment for ocular motor disorders where stabilization of eye movements would be desirable.

FIGURES

FIGURE 5.1 Corneal light reflex photographs of monkeys after 3 months of sustained 2µg/day BDNF treatment. One monkey (**A**) had a possible microstrabismus of 8°, (**B**) while the second treated monkey had normal alignment, <5°.

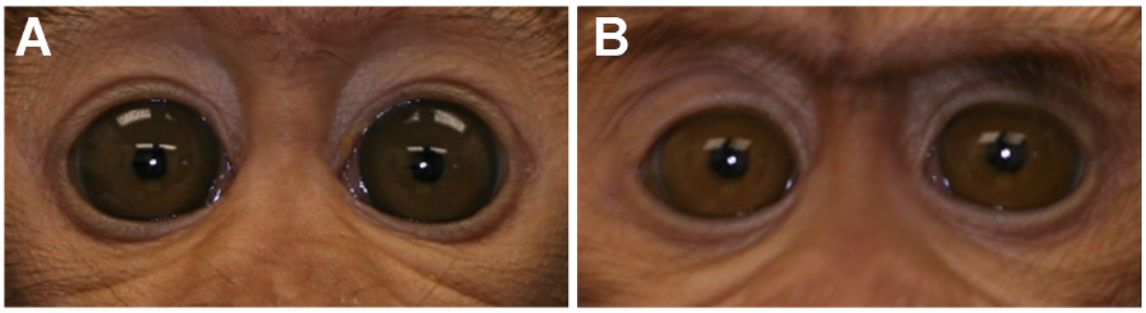


FIGURE 5.2 Confocal images of representative neuromuscular junctions immunostained with an antibody to slow MyHC isoform (red) and alpha-bungarotoxin (green) to visualize the postsynaptic component of neuromuscular junctions. Compared to control muscle (**A**, **B**), neuromuscular junctions on slow myofibers in BDNF-treated muscle had larger endplates (**F**, **G**). No change was observed in the size or morphology of *en plaque* neuromuscular junctions on fast myofibers between control (**C**-**E**) and BDNF-treated (**H**-**J**) muscles. Bar: 25μm.

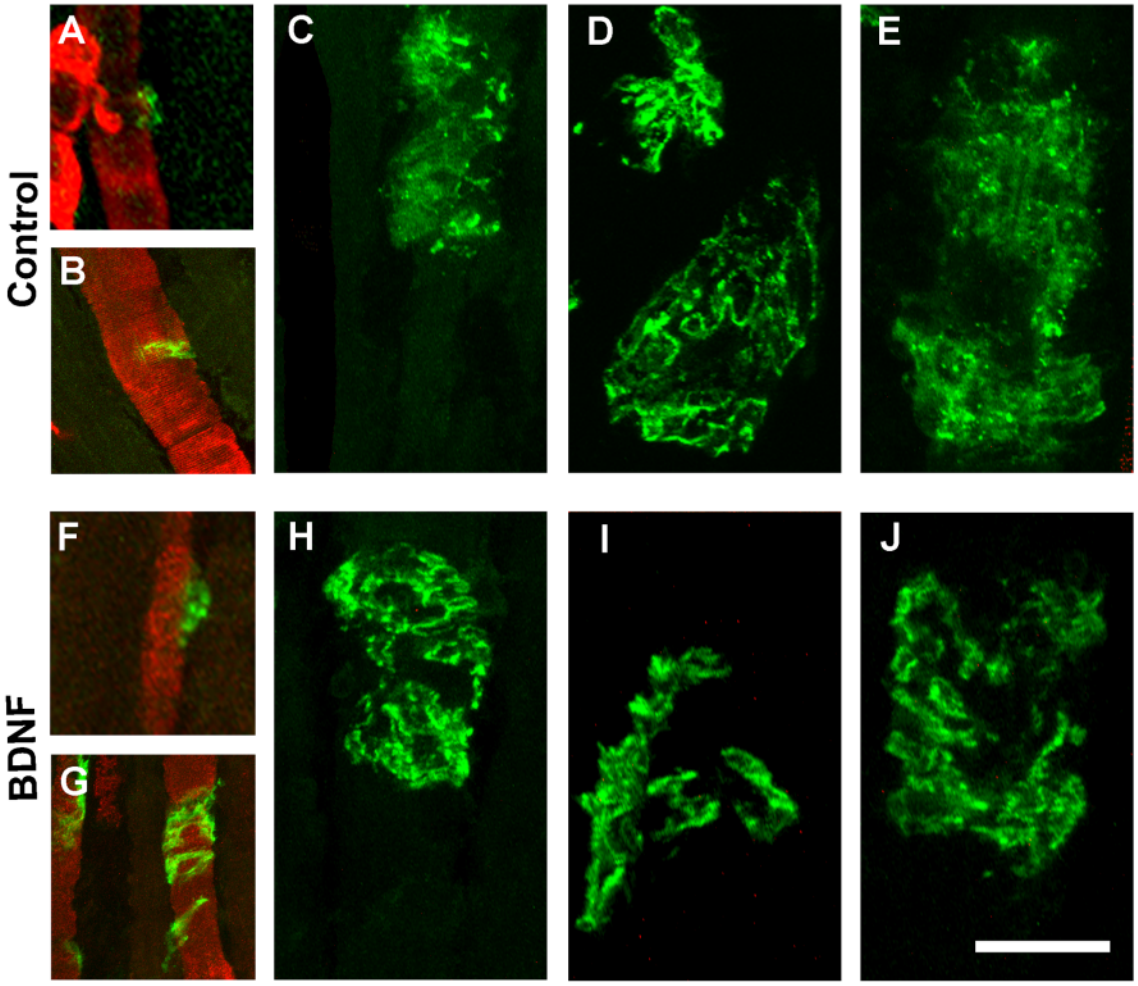


FIGURE 5.3 BDNF treatment results in large endplates on slow myofibers. (**A**). Histogram of the area of all quantified neuromuscular junctions on slow myofibers. For the two control subjects, 1861 and 2170 neuromuscular junctions were counted. For the two BDNF–treated lateral rectus muscles, 797 and 865 neuromuscular junctions were counted. (**B**) Histogram of the area for individual *en plaque* neuromuscular junctions on fast myofibers. Quantification of average neuromuscular junction area (**C**, **E**) or the average area normalized to myofiber size (**D**, **F**) in the global (**C**, **D**) and orbital layer (**E**, **F**) for neuromuscular junctions on slow myofibers. Note that even when compensating for differences in myofiber size, the trend of larger neuromuscular junctions persisted.

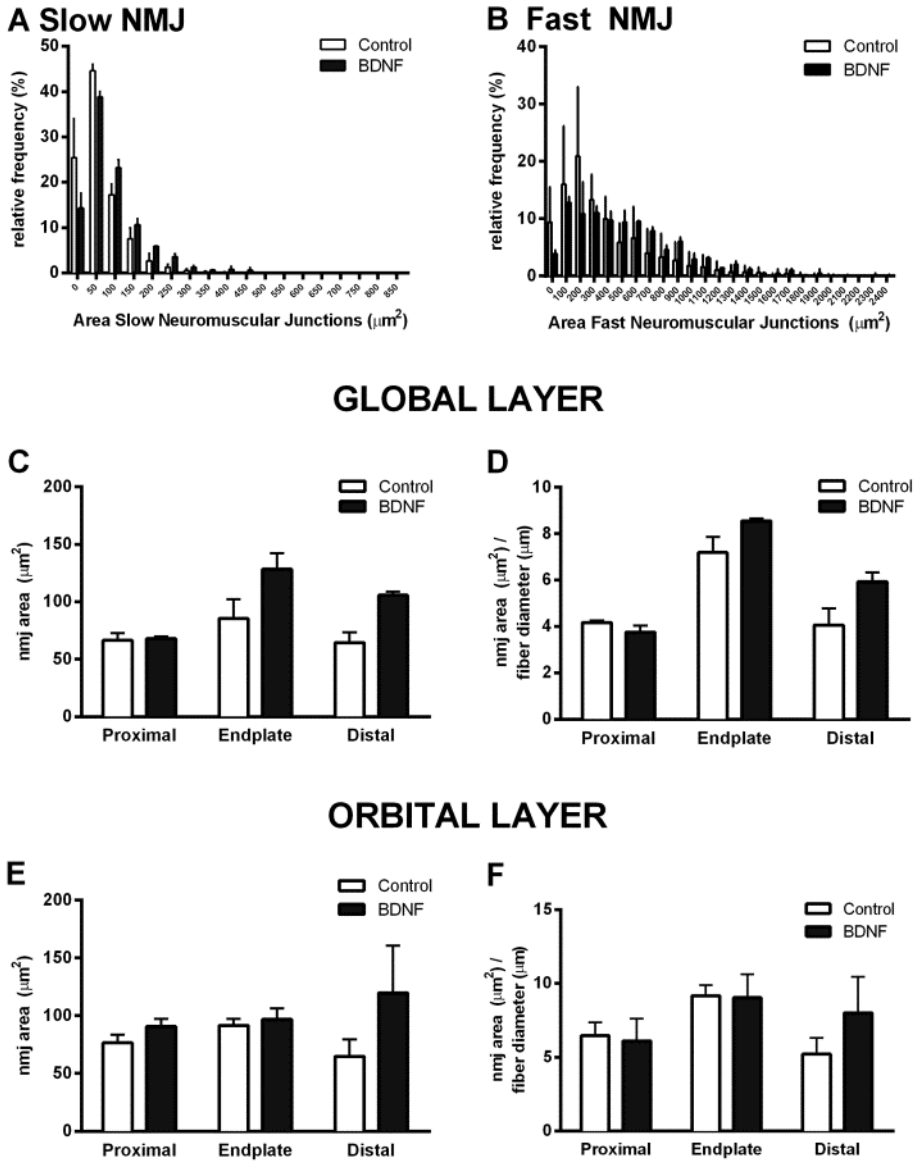


FIGURE 5.4 Confocal images of representative fields immunostained with an antibody to slow MyHC isoform (red) and alpha-bungarotoxin (green). (**A**-**F**) Myofibers from the global layer, from control (**A**-**C**) and BDNF-treated (**D**-**F**) muscle. Fields are shown from the proximal (**A**, **D**), endplate (**B**, **E**) and distal (**C**, **F**) regions of the muscle. Note the larger slow myofiber diameter particularly in the endplate region of BDNF-treated muscle. (**G**-**L**) Myofibers from the orbital layer, from control (**G**-**I**) and BDNF-treated (**J**-**L**) muscle. Fields are shown from the proximal (**G**, **J**), Endplate (**H**, **K**) and distal (**I**, **L**) regions of the muscle. Bar: 25μm.

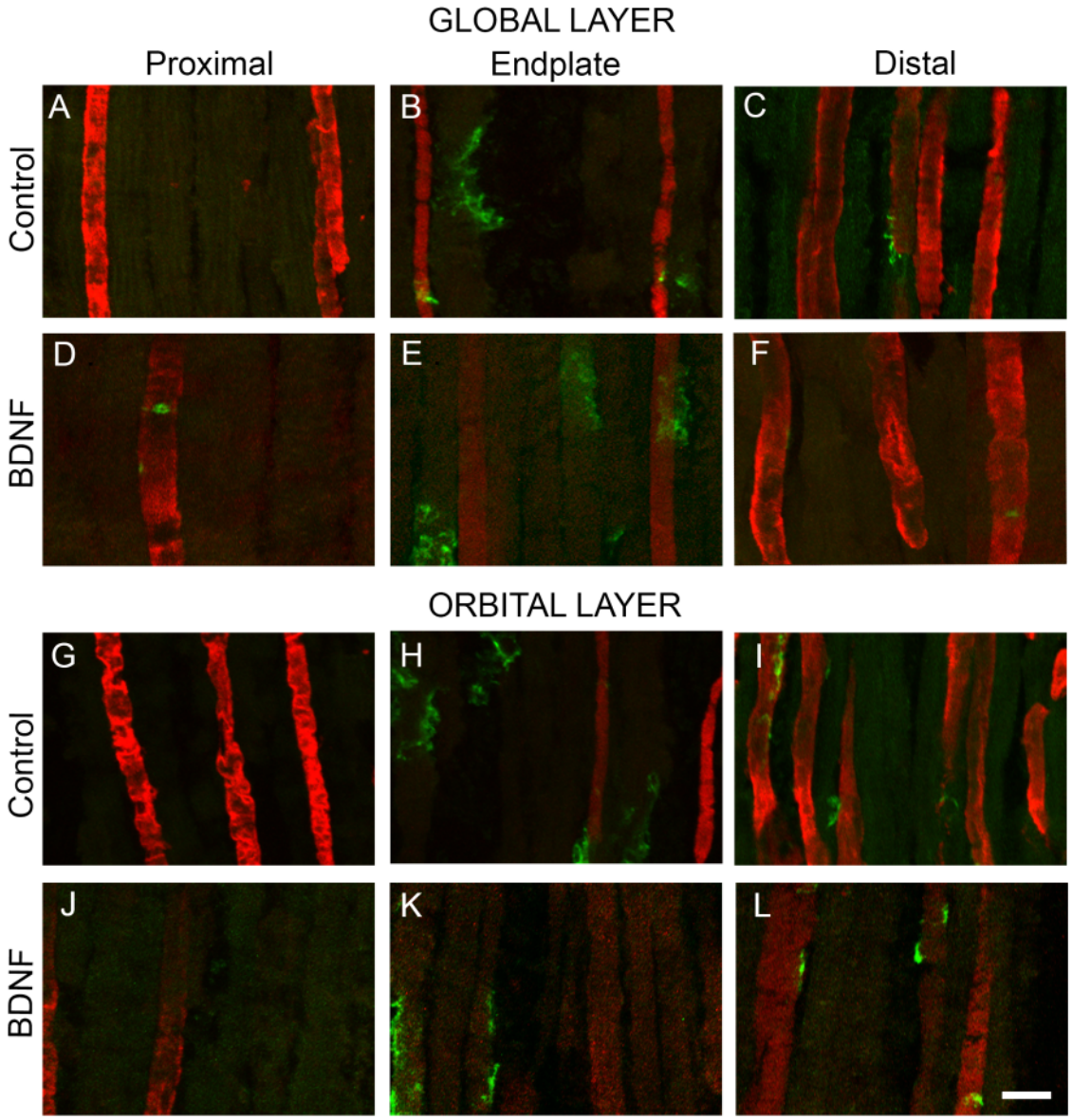


FIGURE 5.5 Mean diameter of myofibers positive for slow MyHC is larger in BDNF-treated EOM. (**A**). Histogram of the mean slow myofiber diameter from all measured fields. (**B**). Histogram of the mean fast myofiber diameter from all measured fields. (**C**, **E**) Average myofiber diameter of myofibers positive for slow myosins in the global (**C**) and orbital (**E**) layer. Myofibers were larger in diameter with BDNF treatment, most prominently in the endplate region of the global layer. (**D**, **F**). Average myofiber diameter of myofibers negative for slow myosin in the global (**D**) and orbital (**F**) layers. No change in myofiber diameter was seen.

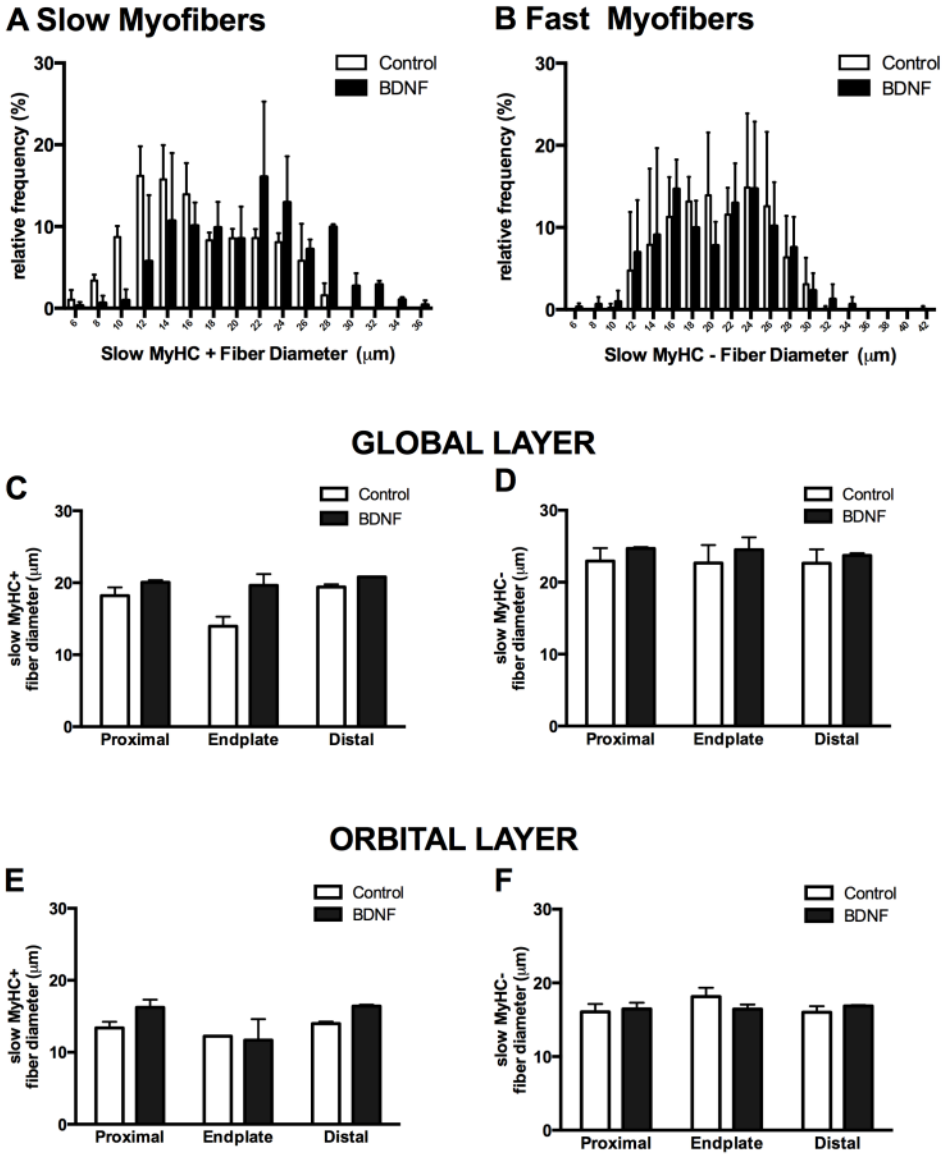


FIGURE 5.6 Mean myofiber cross-sectional area of the treated lateral rectus muscles compared to the antagonist MR, agonist MR muscle, and normal age-matched control muscles.

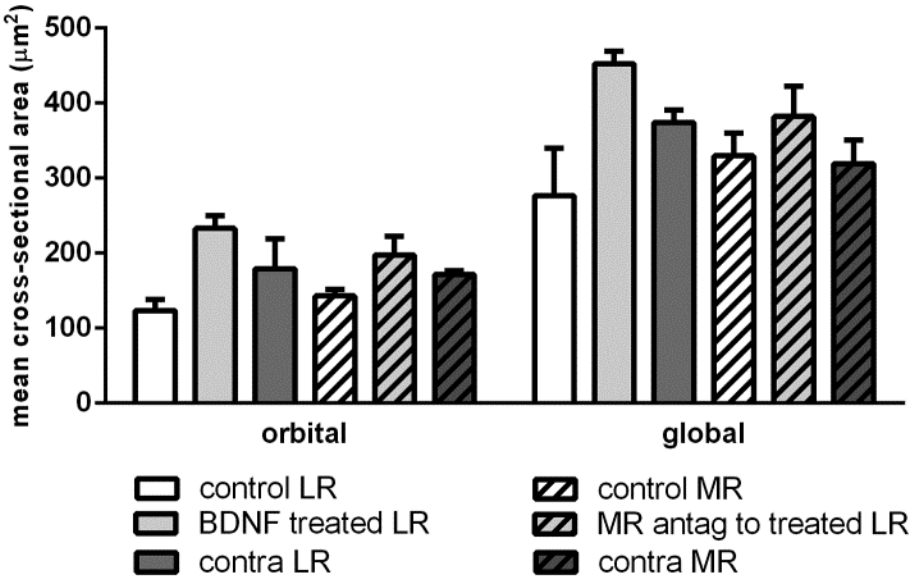


FIGURE 5.7 Quantification of the percentage of myofibers that express slow myosin in the global (**A**) and orbital (**B**) layer. Myofibers in the proximal region had a larger percentage of slow myofibers in BDNF treated muscle.

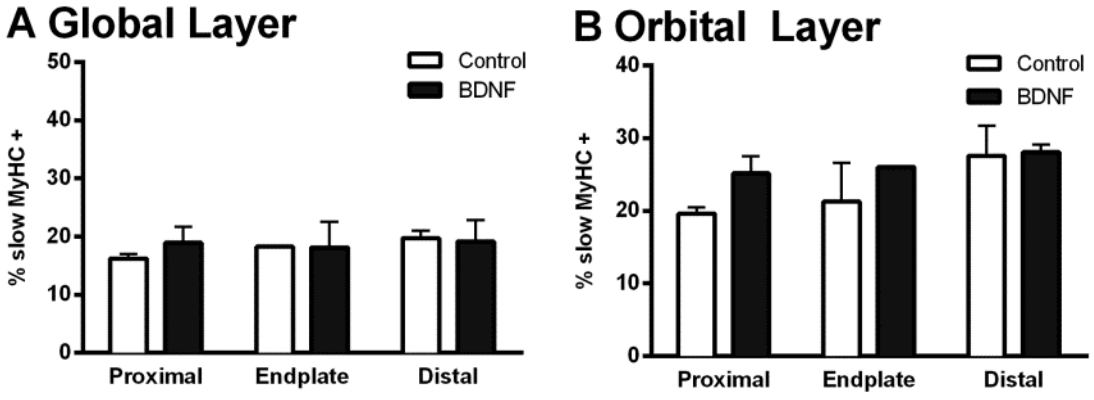


FIGURE 5.8 Confocal images of representative fields immunostained with antibody to BDNF in control monkey lateral rectus. (**A**-**C**) The proximal region of EOM had pronounced immunollabeling for BDNF. Myofibers often had diffuse immunoreactivity throughout the cytoplasm, with intensity that varied between myofibers . Note the labeling of presumed nerve fibers as well (**A**, **B)**. In some proximal regions, BDNF was highly expressed at the cell membrane (**C**). (**D**, **E**, **F**) The distal region of EOM had intense immunollabeling for BDNF. Myofibers had variable labeling for BDNF within the cytoplasm (**E**, **F**). For some distal regions, staining was very strong at the outer membrane of myofibers (**D**, **E**). (**G-I**) BDNF immunoreactivity was less positive in the midbelly and endplate zone of the EOM. (**G**) BDNF labeling was very light into the endplate zone. (**H**) Neuromuscular junctions were labeled with alpha-bungarotoxin (green) to mark the endplate zone. (**I**) Merged image to show an absence of BDNF staining. Note that for this field, there was also no BDNF labeling at the neuromuscular junction. Bar: 75μm.

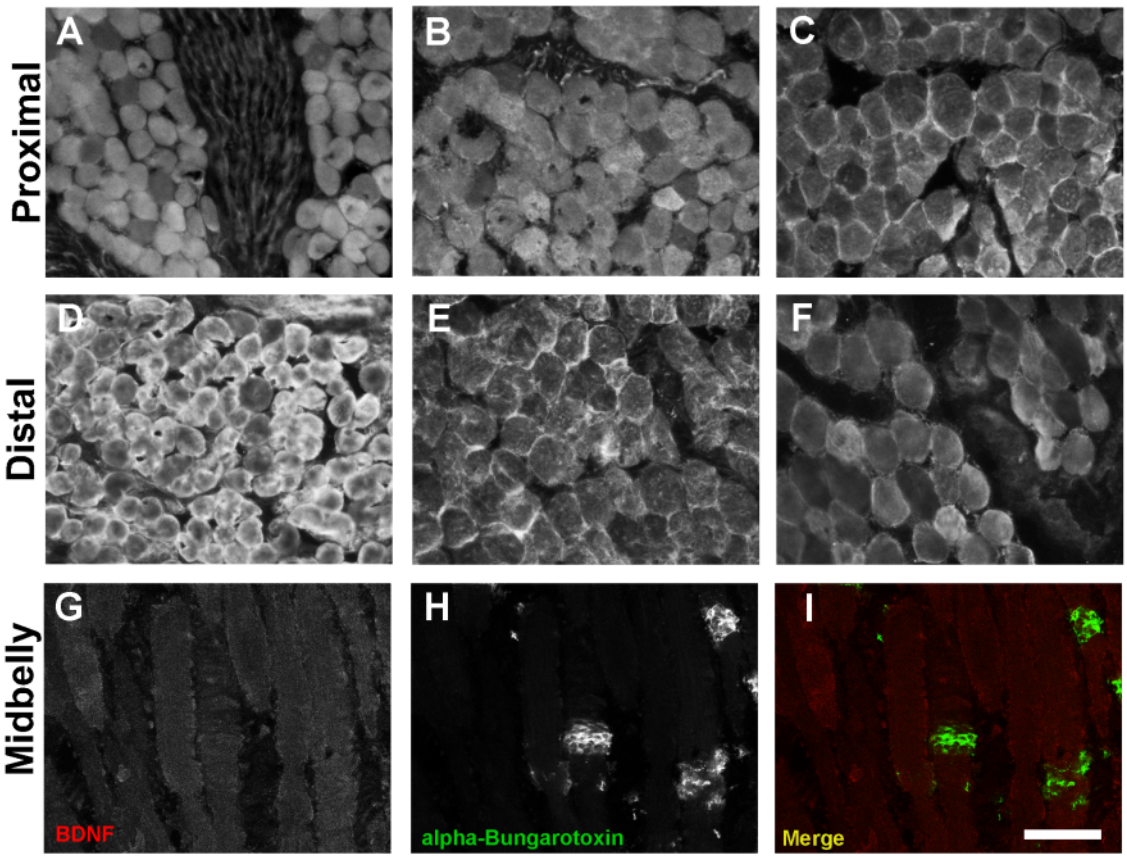


FIGURE 5.9 BDNF was highly expressed in the orbital layer in proximal and distal regions in EOM. (**A**) Representative immunolabelling of BDNF in adult human inferior oblique muscle. More pronounced labeling was visible in the orbital layer (asterisk) as compared to global layer. Varied labeling in the cytoplasm of myofibers was present, as well as labeling at the outer cell membrane of a subset of myofibers. (**B**, **C**) Representative fields from infant monkey lateral rectus muscle from the orbital layer. (**B**) Note the more prominent BDNF labeling in the orbital layer (asterisk).

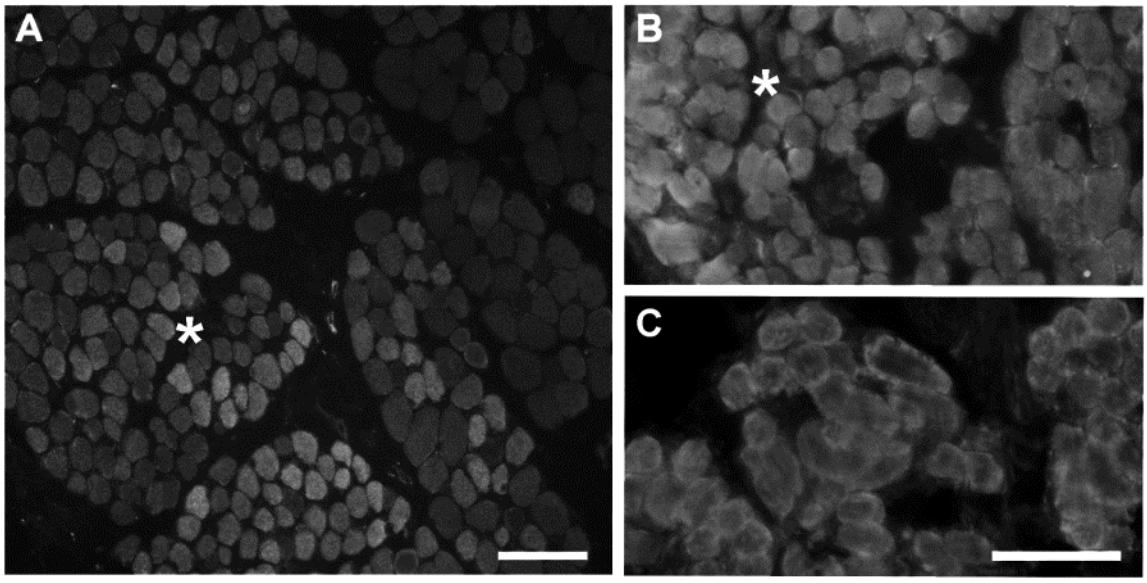


FIGURE 5.10 Representative images of co-expression of BDNF (center, red) with different myosin heavy chain isoforms (green, left) in control monkey lateral rectus. (**A**) BDNF was highly co-localized with 2x MyHC. (**B**). BDNF with highly co-localized with 2a MyHC. (**C**) Some BDNF positive myofibers were also positive for embryonic MyHC (arrow), but this co-expression pattern was inconsistent. (**D**).The pattern of BDNF expression was not related to the expression of neonatal MyHC. (**E**) Few myofibers positive for slow MyHC were positive for BDNF. (**F**) Few myofibers positive for BDNF were also positive for alpha-cardiac MyHC. Bar: 75μm.

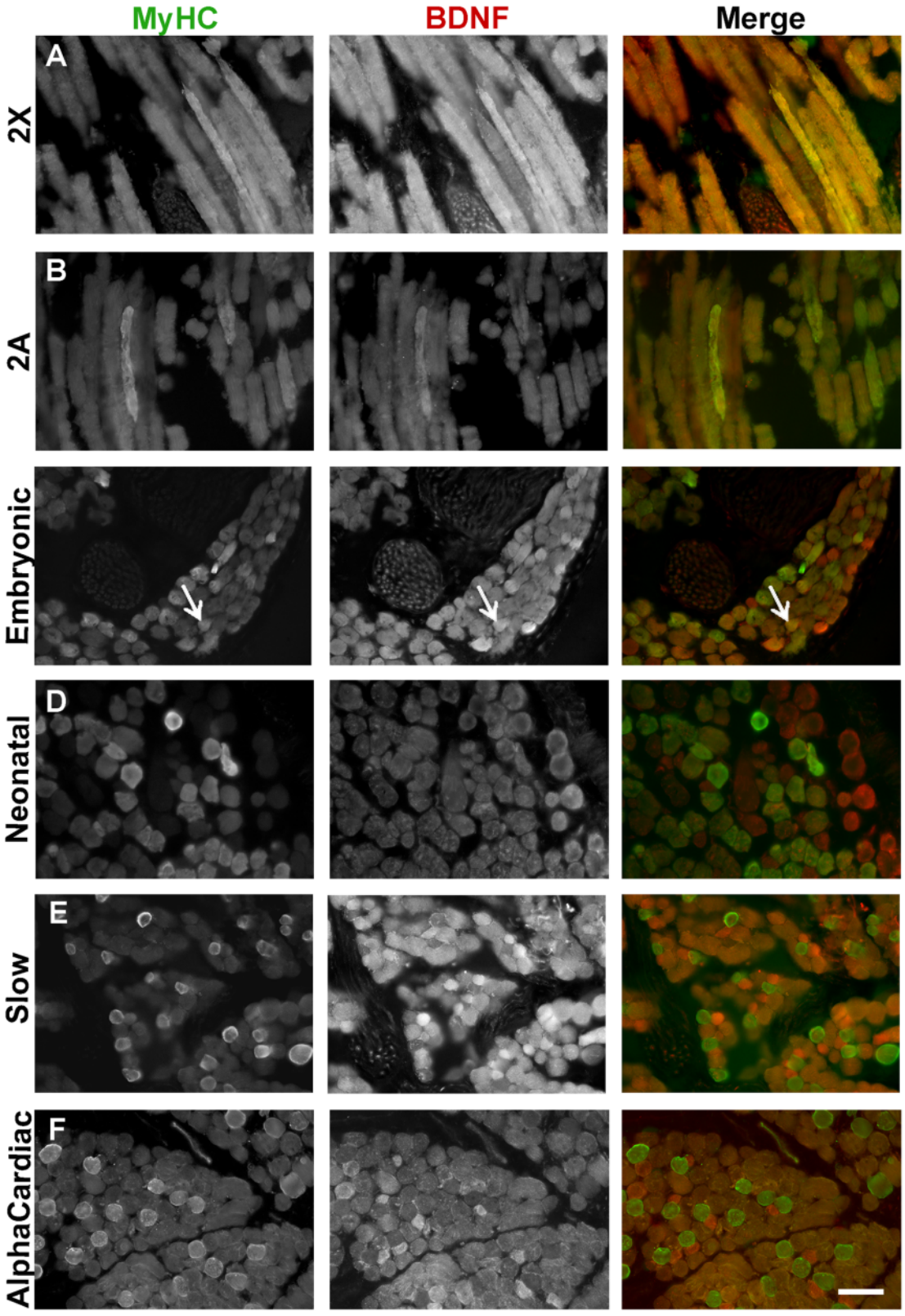

MyHC
BDNF
Merge
A
2X
B
2A
Embryonic
D
Neonatal
E
Slow
F
AlphaCardiac

FIGURE 5.11 Representative confocal images of myofibers co-labeled for BDNF (red) and neuromuscular junctions (green). (**A**) Example neuromuscular junction without BDNF staining. (**B**) Example neuromuscular junction with immunostaining at the endplate. Bar: 25μm.

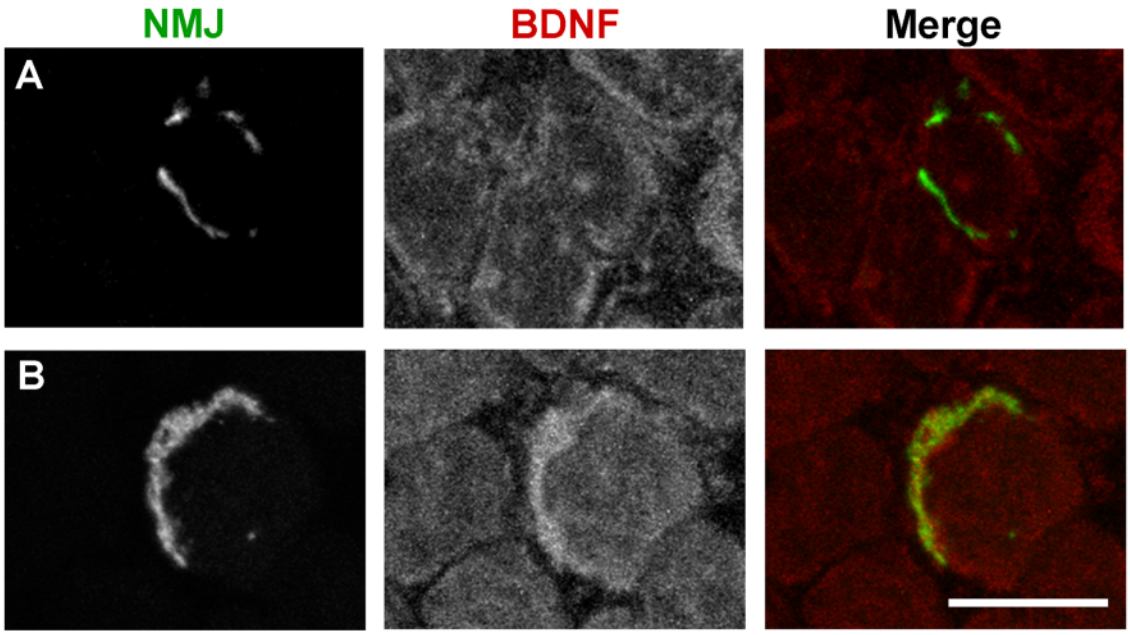


FIGURE 5.12 Representative confocal images of myofibers co-labeled for BDNF (red) and neurofilament to label nerve (green) (arrows). Bar: 25μm.

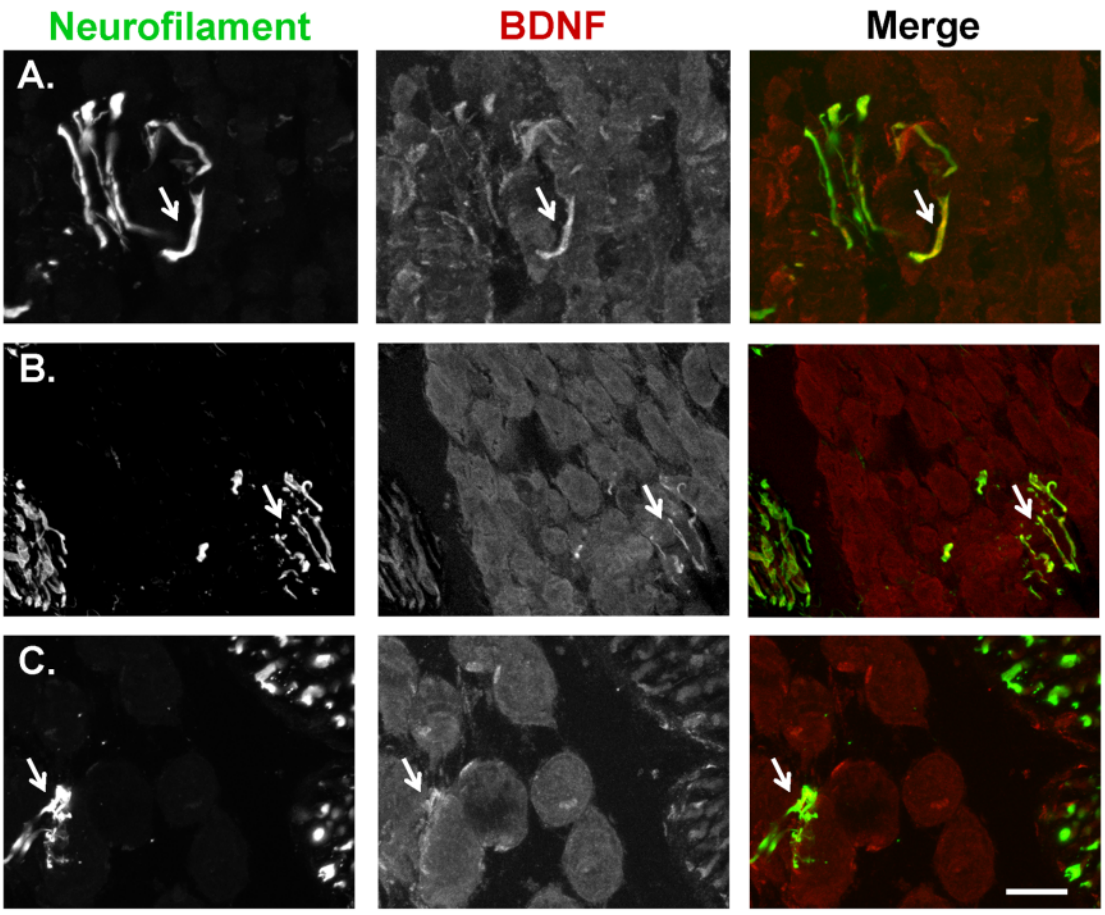


FIGURE 5.13 (**A**) Western Blot demonstrating TrkB protein expression (green) in mouse extraocular muscle. Brainstem (BS) served as a positive control, GAPDH as a loading control (red). (**B-D**) Immunostaining with antibodies to BDNF (red) and its receptor TrkB (green) in adult human inferior oblique muscle. BDNF immunollabeling was generally present in myofibers with TrkB staining, although myofibers were not always equally bright for both. (**B**) Example of nerves positive for BDNF, but negative for TrkB. (**C**) Example of the mosaic pattern of BDNF and TrkB expression in EOM. The expression pattern of BDNF and TrkB were similar but did not always overlap. (**D**) A nerve bundle and myofiber positive for both BDNF and TrkB. Bar: 25μm.

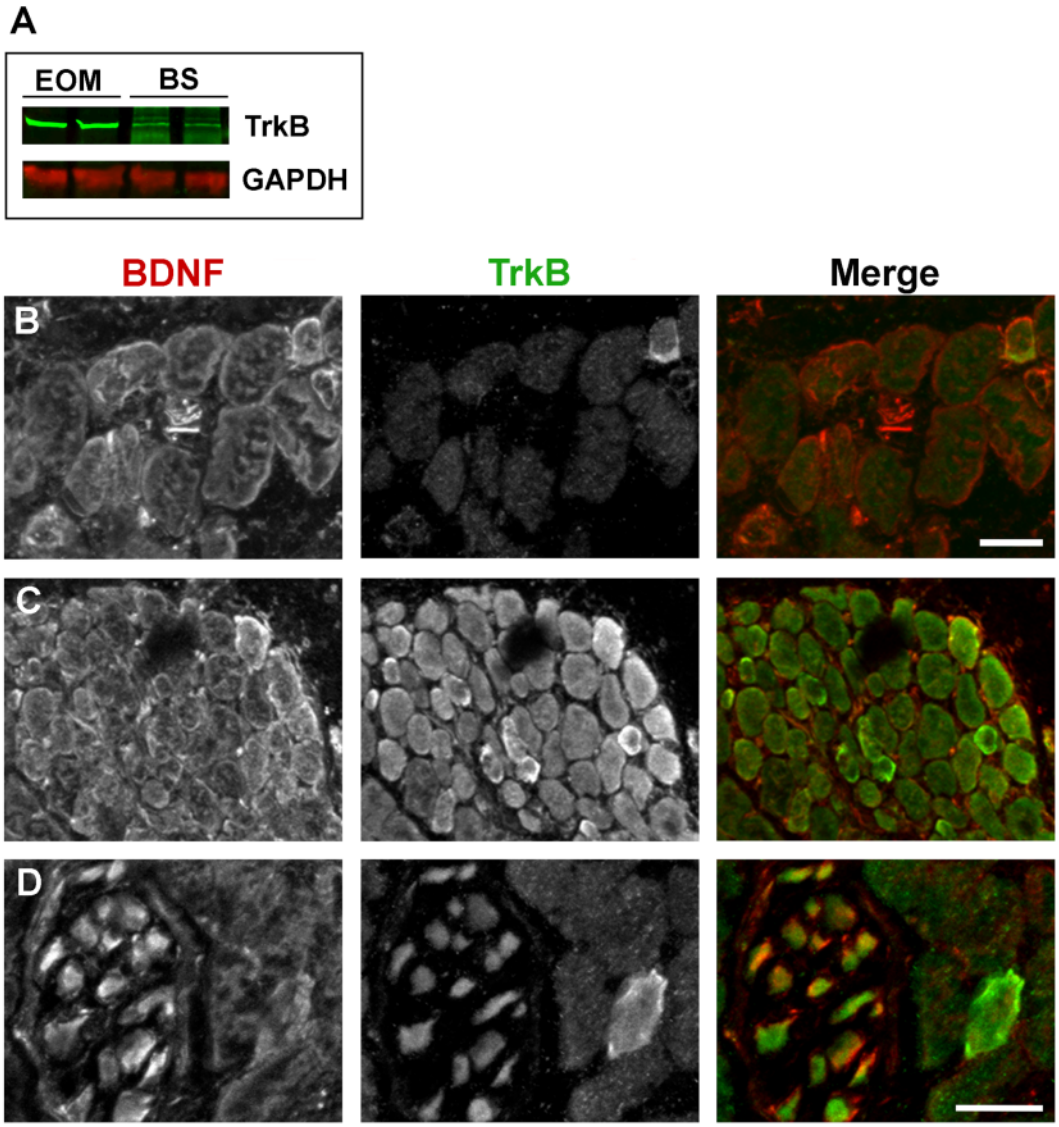


FIGURE 5.14 Representative confocal images of myofibers co-labeled for neuromuscular junctions (green) and TrkB (red) in adult human inferior oblique. (**A**) Myofiber negative for TrkB in the cytoplasm, but with faint TrkB staining at the endplate. (**B**) Example neuromuscular junctions with light to no labeling with TrkB, despite one myofiber being lightly positive for TrkB. (**C**) Higher power image of a myofiber positive for TrkB with intense labeling at the cell membrane and positive for TrkB at the endplate. Bar: 25μm.

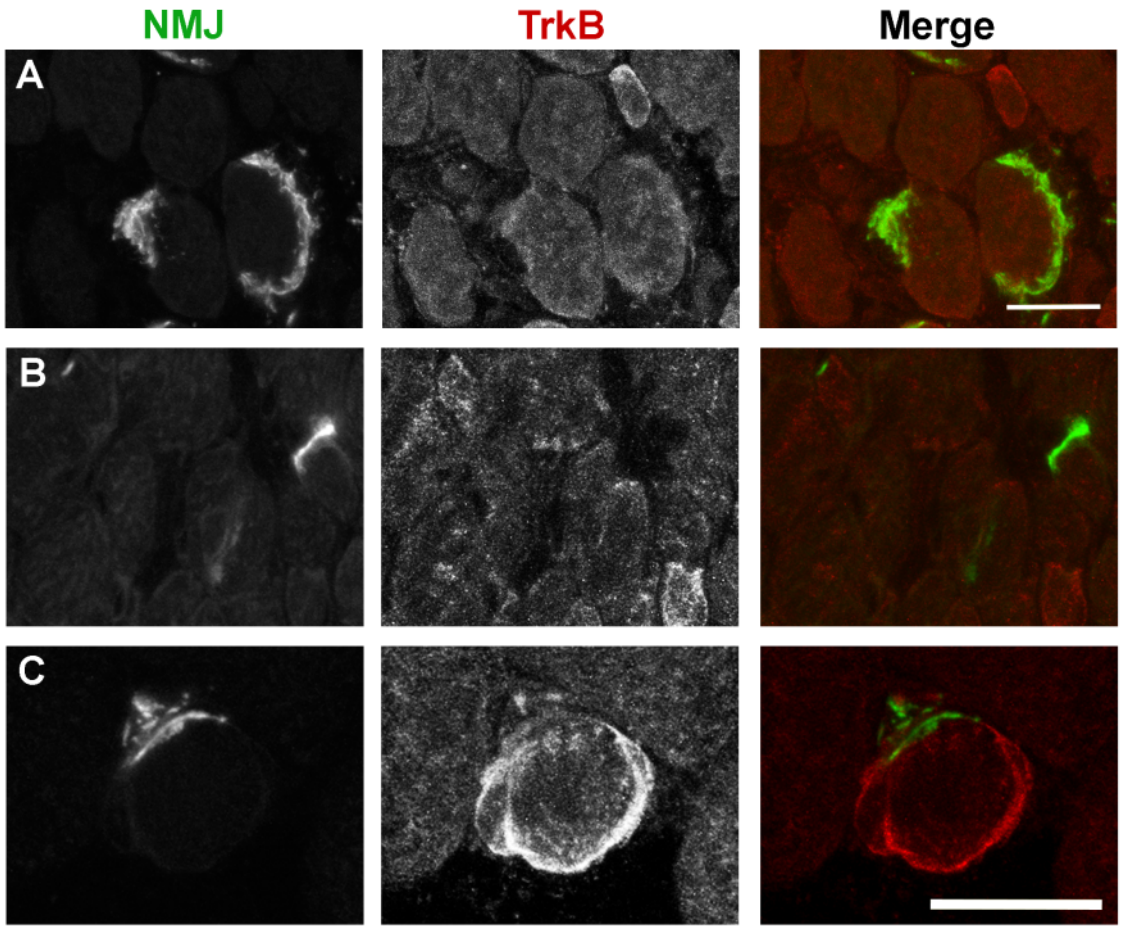
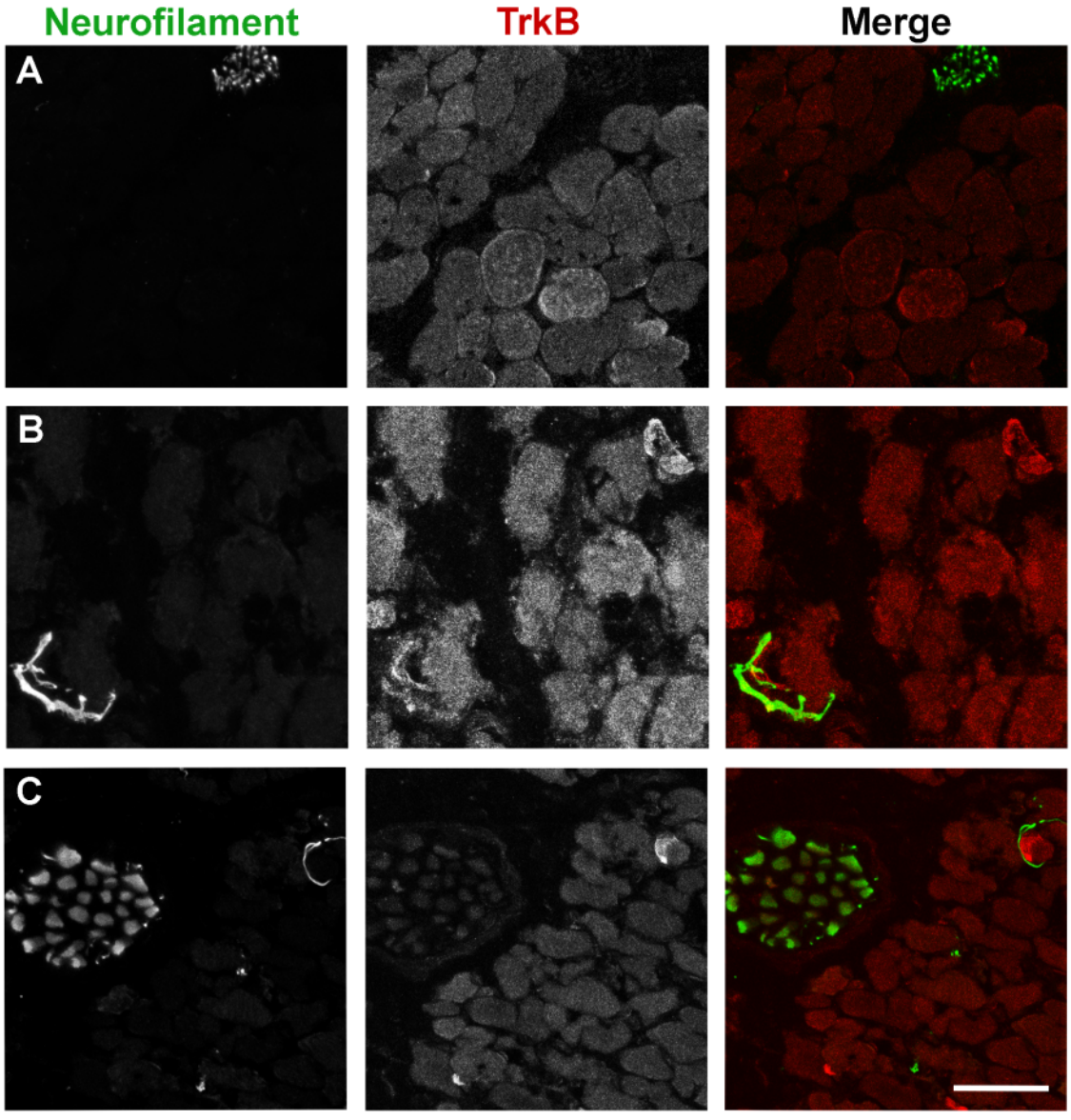


FIGURE 5.15 Representative confocal images of myofibers co-labeled for neurofilament to label nerve (green) and TrkB (red) in human inferior oblique. (**A**) Nerve bundle negative for TrkB. (**B**) Example of a nerve presumably innervating a neuromuscular junction that is positive for TrkB. (**C**) A fiber bundle positive for TrkB. Note that some nerve fibers appeared to have more intense immunolabelling for TrkB than others. Bar: 75 µm.



Chapter 6: General Discussion

SUMMARY OF CURRENT WORK

Strabismus is an ocular motor disorder characterized by eye misalignment, where clinically the EOM muscle pairs are presumed to be “overacting”, and “underacting”. There is currently a need for effective treatments that could act to strengthen an underacting muscle to aid in establishing eye alignment. Neurotrophic factors, such as IGF-1 have emerged as potential treatment options that could strengthen EOM. In Chapter 2, I show that the strengthening effects of IGF-1 on EOM can be potentiated with pretreatment with HGF, resulting in larger increases in muscle forcer with lower doses of IGF-1. In studies described in Chapters 3 and 4, I tested the hypothesis that making a muscle stronger with IGF-1 treatment could alter eye alignment in the infant non-human primate. In Chapter 3, I describe studies where infant non-human primates were treated bilaterally with sustained delivery of IGF-1 for three months during the “critical period” of the binocular system development. At the end of the three months period, neither of the treated infants developed a strabismus. However, myofiber size increased in both the treated EOM and the untreated antagonist muscles. I hypothesize that over the three months of sustained treatment, the ocular motor system adapted to maintain eye alignment. The hypothesis that the oculomotor system adapts to treatment of one EOM was further investigated in Chapters 4 and 5 this time using a unilateral treatment paradigm. Unilateral treatment to one EOM allowed examination of the pattern of adaptation across all four functionally paired EOM that direct horizontal eye movements. The work in Chapter 4 demonstrates that sustained unilateral treatment of a single extraocular muscle with IGF-1 can create strabismus in treated infant monkeys. Results suggest that the ocular motor system adapted to treatment by increasing the myofiber size of the ipsilateral antagonist muscle, but no changes were seen in the contralateral orbit. I hypothesize that unilateral treatment of IGF-1 was sufficient to overcome ocular motor adaptation and cause strabismus. In Chapter 5, a second set of infant non-human primates were treated unilaterally, but with sustained delivery of BDNF instead of IGF-1. BDNF was chosen because like IGF-1, it is a strong neurotrophic factor for the ocular motor system (Steljes et al. 1999; Davis-López de Carrizosa et al. 2009;

Morcuende et al. 2013). At the end of the three month treatment period, no strabismus developed. However, there were marked changes to a subpopulation of myofibers that contain slow myosin heavy chain isoform (MyHC). These results have interesting implications for the role of BDNF in selectively influencing tonic properties of the EOM.

RELEVANCE OF CURRENT WORK TO A TREATMENT FOR STRABISMUS

Previous studies demonstrated that IGF-1 treatment of EOM in rabbit and chick robustly increased myofiber size, muscle force, and contraction speed, resulting in a "stronger" muscle (T. Li, Wiggins, and von Bartheld 2010; McLoon, Anderson, and Christiansen 2006; Anderson et al. 2006). Based on these studies, IGF-1 was hypothesized to be a good candidate for a treatment for strabismus, by "strengthening" a presumed underacting muscle (Anderson et al. 2006; Chen and von Bartheld 2004). In Chapter 2, prior findings were extended by demonstrating that pre-treatment of EOM with hepatocyte growth factor resulted in a larger increase in muscle force with lower doses of IGF-1. This result enhances the potential for IGF-1 to be a feasible treatment option for ocular motor disorders including strabismus, because a pronounced effect can be achieved with relatively low doses given directly to the muscle. This would decrease the chance for treatment to result in unintended negative side effects that have been seen with large systemic doses of this neurotrophic factor.

In evaluating the potential of neurotrophic factors as a possible treatment for strabismus, a limitation of prior studies is that treatments were in lateral eyed animal models (chicken, rabbit), while strabismus is a disorder in animals with frontal eye alignment and foveae. In the next series of experiments (Chapters 3-5), I treated non-human primate EOM with neurotrophic factors, allowing us to look at the effects of treatment in a binocular animal model that more closely resembles that of humans.

In Chapter 4, we treated four infant non-human primates unilaterally with sustained delivery of IGF-1 for a duration of three months. At the end of the three months period, three of the monkeys had a clinically relevant strabismus of greater than 10 degrees. This exciting result has two major implications for strabismus treatment. First, the cause of strabismus is not well known, excluding cases due to paralysis or paresis of muscles, or congenital cranial dysinnervation disorders. This study demonstrated that aberrant signaling from the EOM during the development of coarse stereopsis is sufficient to perturb establishment of eye alignment and create strabismus. Analysis of gene

expression from strabismic patient muscle tissue suggesting that many genes, including mRNA for IGF-1, may be differentially expressed in extraocular muscle with strabismus. This work extends this correlational study to suggested that altered extraocular muscle signaling can cause strabismus.

Based on longitudinal measurements of eye alignment in two of the IGF-1 treated infant monkeys over time, we hypothesize that sustained IGF-1 treatment disrupted conjugate eye movements sufficiently to prevent sensory fu sion of the eyes. We hypothesize that the ocular motor system received inadequate normal binocular experience over the course of treatment. This exciting result suggests that therapies targeted toward balancing aberrant signaling of extraocular muscles could aid in improving abnormal eye alignment in strabismus. Second, this result demonstrates that the effects of neurotrophic factor treatment can extend beyond simply changing the properties of a solitary muscle, but actually alter how the whole horizontal eye alignment system functions. These results support the hypothesis that neuronal drive actually controls the strabismus (Das and Mustari 2007).This strongly suggests that neurotrophic factor treatment could be an effective way to correct eye misalignment permanently.

This current work has also provided strong support for the efficacy of sustained IGF-1 delivery via small pellets. For all eight treated non-human primates, the pellet remained correctly positioned on the EOM for the duration of the three months of treatment. Pharmacological delivery of drugs to EOM is preferable to surgical alteration of the EOM, which changes the muscle insertion, arc of rotation, and the muscle resting length and twitch tension (Clark and Demer 2002; Rosenbaum et al. 1994). The implantation of a pellet is a quick procedure. Additionally, the sustained release paradigm doesn't require the patient to return to the clinic for multiple treatments, as is required with the popular pharmacological treatment with Botulinum toxin A injections (Gardner et al. 2008). Sustained release pellets also provide local delivery, avoiding potential toxicity that could occur from systemic delivery.

An important next line of research will be to obtain an animal model with strabismus, to test if making EOM "stronger" with sustained IGF-1 can alter eye alignment sufficient to realign the eyes, and if the change in eye alignment can be maintained once drug delivery has been stopped. Studies suggest the oculomotor system heavily weights binocular experience (Mansouri et al. 2014; S. L. Li et al. 2014; Wensveen et al. 2011), supporting the view that if eye alignment can be corrected

sufficiently long enough for binocularity to develop, then it should be maintained. We hypothesize that if IGF-1 treatment corrected eye misalignment in patients, then other neuronal processes would maintain binocularity even after IGF-1 treatment ended.

IMPLICATIONS FOR THE ADAPTABILITY OF THE OCULOMOTOR SYSTEM AND EXTRAOCULAR MUSCLE

As discussed in Chapters 3 and 5, bilateral IGF-1 or unilateral BDNF treatment to horizontal rectus muscles did not create strabismus in experimental infant monkeys, despite substantial changes in the treated EOM. We hypothesize that over the course of sustained treatment, the untreated functionally paired EOM were able to adapt similarly in order to preserve eye alignment, despite the fact that the system was being challenged with abnormally high amounts of neurotrophic factor. Analysis of muscle specimens from all three studies with treated monkey EOM demonstrated that IGF-1 or BDNF treatment not only increased myofiber size in the treated muscles, but in untreated muscle pairs as well (Chapters 3, 4, 5). This finding demonstrates the plasticity of extraocular muscles, and supports that EOM function as a unit through coordination at the brainstem level.

It is well documented in the literature that in limb skeletal muscle, exercising or injuring one muscle results in cross-transfer effects to the contralateral muscle on the other side of the body (Song et al. 2012; Song et al. 2014; S. Zhou 2000; Lee and Carroll 2007; Shenker et al. 2003) and to the antagonist muscle in the same limb (Falla et al. 2007; Graven-Nielsen, Svensson, and Arendt-Nielsen 1997; Johansson and Sojka 1991; Song et al. 2014; McGurk et al. 2011; Song et al. 2013). This plasticity is likely communicated through interneurons in the spinal cord, and studies suggest that specific growth factors may be the signaling mechanism (Lee and Carroll 2007; Song et al. 2014; Song et al. 2013). Our current work adds to this hypothesis by providing insight to how this phenomenon manifests in the ocular motor system. However, eye muscles are unique in this respect from leg skeletal muscle because the twelve EOM from two eyes must work conjugately and with extreme accuracy.

One difficulty with surgery as a treatment for strabismus is that after surgically repositioning the EOM, over time the EOM often adapt back to their prior misalignment (Yam et al. 2012). Electrophysiological recordings from nonhuman primate animal models of sensory-induced strabismus have demonstrated that eye misalignment is

driven by the motor neurons (Das and Mustari, 2007; Das 2012; Walton, Ono, and Mustari 2013; Joshi and Das 2011; Das 2011). These studies strongly suggest that strabismus includes deficits not just at the extraocular muscle level, but in how the central nervous system commands conjugate eye position and movements. Thus, when strabismus surgery “fixes” muscle tension by altering the position of the muscle insertions on the sclera, it isn’t addressing the more complex relationship between the muscle and motor neuron commands. Recent studies in nonhuman primate models of strabismus, in fact, suggest that motor neurons adapt to the alignment changes induced by surgery to drive the ocular motor system back to the prior misalignment in both the treated and untreated paired motor nuclei (Agaoglu et al., 2015; Pullela et al., 2015). Our results of substantial myofiber adaptation in untreated muscle are consistent with these findings.

When the horizontal eye movement system was treated unilaterally with neurotrophic factor, strong adaptation occurred via larger myofibers in the antagonist muscle, which is passively pulled by contraction of the treated muscle. It is likely that additional adaptations occurred in the functionally paired horizontal recti in the contralateral orbit that were outside of the measurements of our study. Future studies are needed to more thoroughly investigate how the horizontal recti change as a unit when the properties of one are altered. However, it is also very likely that functional adaptations may occur such as in potentiation of neuromuscular junctions as well as alterations in neuronal firing patterns.

An advantage of using neurotrophic factors as a treatment for strabismus is that they change EOM properties through using endogenous molecular signaling, and further have the ability to signal to and influence motor neurons through retrograde transport. It is possible that neurotrophic factor treatment could be sufficient to overcome adaptations to surgical treatment. In fact, the results from Chapter 4, where strabismus is created by unilateral IGF-1 treatment, strongly suggest that persistent neurotrophic factor treatment can be sufficient to overcome oculomotor adaptations.

IMPLICATIONS FOR HOW NEUROTROPHIC FACTORS CONTROL PROPERTIES OF EXTRAOCULAR MUSCLE

These studies have contributed fundamental knowledge of how neurotropic factors maintain the complex properties of EOM. For example, earlier studies showed that

different neurotrophic factors have differing effects on muscle contraction: GDNF, IGF-1 and CT-1 all shortened muscle contraction, but only IGF-1 and CT-1 also increased muscle force. With respect to motor neurons, BDNF, NGF, and NT-3 all uniquely promoted alterations in the firing characteristics of abducens motor neurons to which they were applied exogenously (Davis-López de Carrizosa et al. 2009; Davis-López de Carrizosa et al. 2010). It is likely that, in turn, these neurotrophic factors uniquely influence myofiber properties.

Our results in Chapter 5 suggest that in addition to promoting the return of tonic afferents onto the treated motor neurons, BDNF also promoted the expression of tonic and sustained contractile muscle properties. Distribution of BDNF protein in the muscle in regions outside the endplate zone is consistent with a role for BDNF signaling in more tonic, slow eye movements. The demonstration that BDNF treatment preferentially increased slow MyHC expression and numbers of slow-expressing myofibers, and increased myofiber and endplate size only on slow myofibers further supports this hypothesis. Further functional studies are needed to determine if the sustained BDNF treatment altered tonic contraction characteristics of the EOM. We hypothesize that BDNF treatment would result in slower saccades, more sustained and stable fixation, and improved maintenance of eye position.

NT-3 signaling to abducens motor neurons acts complementary to BDNF, promoting phasic firing related to quick eye movements and saccades (Davis-López de Carrizosa et al. 2009). We hypothesize that in contrast to the effects of BDNF, treatment of EOM with NT-3 would preferentially influence fast myofibers and specifically *en plaque* neuromuscular junctions. Future studies are needed to delineate the different roles of the various neurotrophic factors expressed in the extraocular muscles that have the potential to influence myofiber types and thereby control movements of the eyes. These future studies will contribute critical knowledge of how the unique complex phenotype of the EOM is established and maintained.

Finally, although previous work had strongly demonstrated that IGF-1 signals directly to the treated EOM themselves to create muscle hypertrophy, the study included in this thesis (Chapters 3, 4) are the first work to show that the effects of IGF-1 translate to a non-human primate, strengthening the potential for IGF-1 to provide feasible treatment options for human oculomotor disorders. We also found that sustained treatment of IGF-1 had no dramatic effect on altering the myofiber types expressed. This

strongly suggests that in healthy normal EOM, IGF-1 does not play a large role in determining myofiber type, but generally signals to promote maintenance of muscle properties such as size and force generation capacity (Musarò et al. 2001; Clemmons 2009; McLoon, Anderson, and Christiansen 2006; Jacquemin et al. 2004).

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