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How to make rapid eye movements “rapid”: the role of growth factors for muscle contractile properties

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Abstract

Different muscle functions require different muscle contraction properties. Saccade-generating extraocular muscles (EOMs) are the fastest muscles in the human body, significantly faster than limb skeletal muscles. Muscle contraction speed is subjected to plasticity, i.e., contraction speed can be adjusted to serve different demands, but little is known about the molecular mechanisms that control contraction speed. Therefore, we examined whether myogenic growth factors modulate contractile properties, including twitch contraction time (onset of force to peak force) and half relaxation time (peak force to half relaxation). We examined effects of three muscle-derived growth factors: insulin-like growth factor 1 (IGF1), cardiotrophin-1 (CT1), and glial cell line-derived neurotrophic factor (GDNF). In gain-of-function experiments, CT1 or GDNF injected into the orbit shortened contraction time, and IGF1 or CT1 shortened half relaxation time. In loss-of-function experiments with binding proteins or neutralizing antibodies, elimination of endogenous IGFs prolonged both contraction time and half relaxation time, while eliminating endogenous GDNF prolonged contraction time, with no effect on half relaxation time. Elimination of endogenous IGFs or CT1, but not GDNF, significantly reduced contractile force. Thus, IGF1, CT1, and GDNF have partially overlapping but not identical effects on muscle contractile properties. Expression of these three growth factors was measured in chicken and/or rat EOMs by real-time PCR. The “fast” EOMs express significantly more message encoding these growth factors and their receptors than skeletal muscles with slower contractile properties. Taken together, these findings indicate that EOM contractile kinetics is regulated by the amount of myogenic growth factors available to the muscle.

Keywords

Extraocular muscle; Skeletal muscle; Contraction time; Insulin-like growth factor; Cardiotrophin; GDNF; Saccade

Introduction

The twitch contraction time of muscles (onset of force to peak force) varies between different skeletal muscles over a wide range (about 20–130 ms). Based on the twitch contraction time, muscles can be characterized as either “fast” or “slow” (tonic). Saccade-generating extraocular muscles (EOMs) are the fastest muscles in the human body, significantly faster than other skeletal muscles [13,26]. EOMs differ from limb skeletal

muscles in numerous properties, including myofiber types, myosin heavy chain composition, calcium release channels, innervation ratio, contraction time, and fusion frequencies [14,35,60,61].

The ranges of contraction times and fusion frequencies set apart EOMs from other skeletal muscles in all vertebrate species examined [14,25,61]. Developmental and crossover re-innervation studies have shown that muscle contraction and relaxation speed is plastic and can be regulated by innervation, presumably by inducing neuronal and/or non-neuronal signals [30,46–48,68]. Although innervating motoneurons can modify muscle contraction properties, the molecular mechanisms by which motoneurons and muscle itself control the speed of contraction have not been identified.

EOMs require particularly fast and precise force kinetics, because of the need for rapid eye movements (saccades), and for precise force regulation to coordinate eye movements that enable—in frontal-eyed animals—binocular fusion [20] within Panum's area [66]. It is not known how muscles switch from slow to fast kinetics. This likely involves changes of myosin heavy chain isoform expression, calcium handling in E–C coupling, and/or changes in levels of acetylcholine receptor and acetylcholinesterase [54]. Growth factors play an important role in muscle development and plasticity, and they may also control the transition of EOM fiber type composition, and the development of contraction speed and contractile force. Previous studies have shown that the growth factors IGF1 and CT1 can regulate within days some physiological parameters of EOM, including muscle strength [11,38,43]. Furthermore, IGF1 has been implicated in regulation of fast myosin heavy chain isoform expression as well as expression of calcium-handling proteins [32,50,57]. GDNF, another muscle-derived growth factor, affects postsynaptic insertion of acetylcholine receptors [69]. Therefore, we tested the hypothesis that these myogenic or neuron-released growth factors may also alter kinetics of muscle contraction.

Here we explored a possible link between contractile properties and altered levels of three exogenous and endogenous growth factors (IGF1, CT1, and GDNF) in a sub-acute in vivo animal model system. The motivation for this study was further derived from the fact that the speed of contraction is slowed in strabismic EOMs [34,35]. The growth factors we examined here, as well as others, may be of clinical relevance in the context of strabismus and other disorders of EOMs [45,66]. Thus, information about the molecular control of muscle contractile properties may assist in the design of growth factor-based pharmacological interventions to improve the performance of dysfunctional muscles.

Materials and methods

White Leghorn chicks of either sex were obtained from commercial suppliers. Post-hatch day 0 (P0) was the date of hatching. Seventy-seven chickens and four adult Wistar rats were used in this study. Some of the same animals previously used for force analysis [38] were further examined for kinetic analysis in this study. Animals were raised in brooders or cages in ventilated rooms with constant temperature and humidity and a 12-h light/dark cycle. Food and water were provided ad libitum. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Nevada, Reno.

Growth factor injections

Chicks received a total dose of 10 or 1 µg, in a volume of 10 µl, in two injections into the orbit spread over 4 days. On the sixth and ninth post-hatch day, chicks were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, 34 mg/kg) and chloral hydrate (150 mg/kg). On both days, a single dose of either GDNF (5 µg, *n*=4), IGF1 (0.5 µg, *n*=8, or 5 µg, *n*=10), or CT1 (0.5 µg, *n*=6, or 5 µg, *n*=6), or a single dose of IGF1 (0.5 µg) plus CT1

(0.5 μg), $n=7$, or a single dose of IGF1 (0.5 μg) plus CT1 (5 μg), $n=6$, was injected in the right dorsomedial orbit containing the superior oblique muscle. For controls, the same volume of PBS was injected ($n=7$) and compared with untreated animals ($n=6$). The volume of the superior oblique muscle is 7.34 ± 0.36 (SEM) mm^3 at this age. Based on its wide therapeutic window, only high-dose GDNF was tested, while both high- and low-dose IGF1 was examined since it can have hypoglycemic effects at supra-physiologic concentrations [42]. All three growth factors (recombinant human GDNF, IGF1, and CT1) were purchased from PeproTech Inc., Rocky Hill, NJ, USA. For control experiments, muscles were either not injected or were injected with an equivalent volume of sterile phosphate-buffered saline (PBS). Growth factors and PBS were administered in a volume of 10 μl using disposable insulin syringes (28G1/2; Becton Dickinson, NJ, USA).

Estimation of effective doses of growth factors

To estimate the effective dose of the injected growth factors in the EOMs, each growth factor was radiolabeled [64]. Incorporation was typically between 70% and 95%, with specific activities of 40–70 cpm/pg. A spiked dose of 40–400 ng was injected in the orbits on the sixth post-hatch day as described above. Fifteen hours later, the animals were anesthetized and intracardially perfused with either 4% paraformaldehyde or PBS. The fixed superior oblique muscle was dissected, weighed on a digital analytical lab balance (Sartorius BP 110S, Göttingen, Germany), dehydrated to remove any free iodine [9], and gamma-counted. To determine if the growth factors in the EOMs remained largely intact after injection, a separate group of EOMs was tested for incorporation of the iodine isotope. The buffer-perfused muscles were lysed (lysis buffer—50 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100 protease inhibitors aprotinin, 1 $\mu\text{g}/\text{ml}$, and leupeptin, 1 $\mu\text{g}/\text{ml}$, pH 6.5), trituated, centrifuged at $10,000 \times g$, and the supernatant was precipitated with trichloroacetic acid to determine the ratio of incorporated radioactivity and free iodine. The percentage of internalized and retained growth factor was then calculated and the effective doses determined as a percentage of the total amount that was injected into the orbit. Seven days is deemed sufficient to induce changes in EOMs after growth factor treatment, apparently due to structural changes [11,38,43].

Neutralizing agents

To test the functions of endogenous growth factors, we used specific neutralizing proteins or antibodies. IGFs were blocked with recombinant human IGF binding protein 4 (IGFBP4), CT1 with polyclonal goat-anti-human CT1 antibody, and GDNF with polyclonal goat-anti-human GDNF antibody. These function-blocking agents were purchased from R&D Systems, Inc., Minneapolis, MN, USA. All three inhibitors neutralize the activity of their respective growth factor at doses that are similar to those applied here [3]. According to the manufacturer, the CT1 antibody neutralizes human CT1, and the GDNF antibody neutralizes human and rat GDNF, although we and others have documented effects of this antibody in chicken [12]. Chicks were anesthetized as described above, and a total dose of 2 μg IGFBP4 ($n=4$), 2 μg anti-GDNF antibody ($n=5$), or 2 μg anti-CT1 antibody ($n=4$) was injected into the right dorsomedial orbit, with one dose of 1 μg each on the sixth and ninth post-hatch day. The volume of each injection was 10 μl and the vector solution was filtered 0.1% bovine serum albumin in PBS. For control experiments, muscles were injected with 2 μg normal IgG (Mouse IgG₁ Isotype Control; R&D Systems), $n=4$. All injections were placed using disposable insulin syringes (28G1/2; Becton Dickinson). The neutralizing IGFBP4 was chosen for its specificity and reliability in inhibiting IGF function [23]. This inhibitor is known to have effects on the oculomotor system at the doses used here [51]. Similarly, a dose of 20 $\mu\text{g}/\text{ml}$ anti-CT1 inhibits the trophic activity of 10 ng/ml CT1 [3], and a single dose (2.5 μg) of the GDNF antibody is sufficient to increase pyknosis in oculomotor nuclei after orbital injection in chick embryos [12].

Measurement of twitch contraction time and half relaxation time

Anesthesia was induced as described above and maintained by i.m. injection of 10–25 mg/kg Nembutal every 30 min. Since twitch contraction time of fast muscles is sensitive to anoxia as well as fluctuations in temperature [24,31], the contractile properties of 76 superior oblique muscles were evaluated in situ with nerve and blood supply intact at 35°C as described in our previous work [15]. On post-hatch day 14 (5 days after the last injection into the orbit), the animals were placed on their left side and the head was immobilized with a stereotaxic frame. The insertion of the right superior oblique muscle was exposed and tied with a 6-0 silk suture. The trochlear nerve was carefully isolated from connective tissue near its entrance to the superior oblique muscle. The suture previously attached to the global portion of the superior oblique muscle was then tied to an isometric force transducer (Fort 25; World Precision Instruments, Sarasota, FL, USA). Parallel platinum electrodes (0.3 mm tip diameter, 1 mm between poles) were placed to contact the trochlear nerve ~5 mm proximal to its entry into the mid-belly of the superior oblique muscle. In situ muscle force of the superior oblique muscle was measured in response to trochlear nerve stimulation (15 V, 0.2 ms duration, Grass S48 stimulator, Quincy, MA, USA). Contractile properties were measured when the muscle was maintained at a length that achieves maximum active tension. Each stimulus train was separated by a 5-s interval. Twitch contraction time (onset to peak force) and half relaxation time (peak force to half relaxation) was measured with a data acquisition system (Digidata 1322A; Axon Instruments, Union City, CA, USA) and analyzed with Clampfit 10.1 software (Molecular Devices Inc., Sunnyvale, CA, USA). The sampling rate was 5–10 kHz, providing data points in traces every 0.1–0.2 ms. The total duration of physiological stimulation experiments per animal was 10–12 min, and the interval from induction of anesthesia to euthanasia lasted about 50 min. After recording contractile kinetics, the muscles were weighed on a Sartorius analytical balance.

Measurement of contractile force and fusion frequency

In addition to contraction time, contractile force of the right superior oblique muscle was measured after treatment of growth factors or neutralizing agents and compared with vehicle-injected muscles and normal controls. We used the same method as described above and previously reported in detail [15]. The twitch tension and tetanic tension of the muscle was recorded by the Digidata data acquisition system with sampling rates of 5–10 kHz. The tension data were normalized by average cross-sectional area (CSA). The fiber length was determined by measuring the start (interseptal bone) to the insertion (the point tied to the force transducer). The average CSA was calculated with the formula: muscle mass (mg)/[muscle density (mg/mm³)×fiber length (mm)]. We used 1.06 mg/mm³ for muscle density [63]. In addition to the contractile force, we recorded the fusion frequency, defined as the lowest frequency of tetanic tension at which individual twitches could not be resolved. Tetanic tension was increased in increments of 50 Hz (50–450 Hz).

Tissue collection, RNA isolation, and RT-PCR

Chickens were sacrificed with an overdose of Nembutal (50 mg/kg i.m.). Rats were euthanized in an isoflurane chamber. EOMs from chicken and rat, extensor digitorum longus (EDL) (rat), and pectoralis (chicken) were dissected within 30–60 min after death and immediately frozen in liquid nitrogen. Rat EDL was selected as a representative fast-twitch skeletal muscle that is frequently compared with EOMs [2], and the pectoralis as an avian fast-twitch muscle. For gene expression studies, several EOMs were pooled because of their small size, while individual EDL/pectoralis samples were used for skeletal muscles. The tissues were homogenized in 1 ml Trizol (Invitrogen, Carlsbad, CA, USA). RNA was extracted with 200 µl chloroform and then processed with Purelink micro-to-midi total RNA isolation kit (Invitrogen), following the manufacturer's protocol. To remove genomic DNA, isolated RNA was further treated with Turbo-free DNase kit (ABI, Foster City, CA, USA).

RNA concentrations and possible protein contaminations were determined with NanoDrop 1000 (NanoDrop Products, Wilmington, DE, USA). A260/280 and A260/230 absorbance ratios were greater than 2.0. The RNA integrity was evaluated by denaturing gel electrophoresis. RNA was reverse transcribed into cDNA with Superscript III kit and Oligo(dT) primers (Invitrogen). GoTaq green master mix (Promega, Madison, WI, USA) was used for qualitative PCR. “RT minus” control was used to detect possible genomic DNA contamination, as described previously [21].

Real-time PCR (qPCR)

Primers were synthesized by Operon Inc., Huntsville, AL, USA. Several sets of LIF receptor primers were designed with Primer Express (ABI). The best primer pairs (lowest Ct, highest amplification efficiency, and stable performance) were selected for real-time PCR. The following sequences were obtained from published reports: chicken primer sequences for IGF1 and GAPDH [21], rat primer sequences for IGF-1A, GAPDH [10], CT1, IGF1 receptor [58], and gp130. LIF receptor forward primer was 5'-TCATCAGTGTGGTGGCAAGAA-3' and reverse primer 5'-GCAGGGCTCAGACCATGACG-3'. The amplification efficiency of all primer sets was within 95–105%. The coefficient of variation R^2 was above 0.99. Real-time PCR was performed on an ABI 7300 cycler with SYBR Green PCR Master Mix (ABI) according to the manufacturer's default protocol. Dissociation curves were run for each well to check for non-specific amplifications. PCR reaction products were randomly selected for sequencing to further evaluate the amplicon specificity. We used GAPDH as an internal control and verified similar expression levels between EOMs and limb muscles by normalizing against cDNA amounts measured by Nano drop. To confirm that GAPDH is a reliable RNA standard for our muscle samples, we designed primers for four other housekeeping genes: elongation factor 1A (EEF1A₁ and EEF1A₂) and ribosomal proteins (RPS13 and RPS29). Expression stability of GAPDH (geNorm software, M value=0.563) was comparable with ribosomal proteins (M values=0.459 and 0.497, respectively), and more stable than elongation factors 1A₁ and 1A₂ (M values of 0.668 and 0.919, respectively). We used GAPDH as our housekeeping gene since GAPDH is most commonly used in EOM gene expression studies [10,21,22].

Statistical analysis

All physiological data were analyzed using IBM SPSS statistics 18.0 (SPSS Inc., Chicago, IL, USA). The data are shown as mean±SEM. Statistical significance was evaluated at a confidence level of $p < 0.05$. The physiological data were tested for normal distribution with homogeneity of variance, and were analyzed with one-way ANOVA, and then LSD test for multiple comparisons. For the parameters that failed the test of variance homogeneity, Kruskal–Wallis test was used for the analysis of the mean ranking (distributions), and then Dunnett t test (two-sided) was applied for post hoc multiple comparisons. Quantitative PCR data were analyzed for statistical significance by using paired two-tailed t test.

Results

Normal twitch contraction time and half relaxation time

We measured twitch contractile kinetics of the superior oblique muscle in normal (non-injected) and control-injected chicks at the age of 2 weeks after hatching, using a sub-acute in situ protocol that maintains blood supply to the muscle during measurements. Our sampling rates allowed us to determine the onset of force, peak force, and half relaxation time course with sufficient accuracy to identify differences between groups (Table 1). The twitch contraction time of the untreated muscles ranged from 5.7 ms to 5.9 ms. This is within the range (5.5–6.0 ms) previously reported for extraocular muscles (EOMs) in birds

of this size (chicken—[15] and pigeon—[61]). The fusion frequency of the untreated muscles was consistent with previous avian EOM studies [16]. For the PBS-injected control group, the mean contraction time was not significantly different from the untreated group (Table 1). Normal IgG was used as a further control experiment for the loss-of-function study with neutralizing antibodies. Since neither of these groups differed significantly from each other, we conclude that the injection procedure itself and application of vehicle or other control solutions does not have a significant effect on twitch contraction time or relaxation time.

Estimation of the effective doses of growth factors

To evaluate the retention of effective doses of exogenous growth factors in the superior oblique muscle, we injected a small amount of radiolabeled IGF1, CT1, or GDNF in the orbit. Fifteen hours later, approximately 0.06–0.6% of the growth factors was retained in the dehydrated muscle tissues, as revealed by gamma counting. Precipitation analysis of retained radiolabeled growth factors revealed that 90% of radioactivity was derived from protein-incorporated iodine, with only 10% free iodine (data not shown). Accordingly, the large majority of counts were non-degraded and presumably represented active growth factor. Based on the total amounts injected in the orbit (Table 1), more than 40.6 ng/ml IGF1 or 67.8 ng/ml CT1 was retained in the superior oblique muscles after low-dose injection (0.5 μ g IGF1 or CT1) and more than 406 ng/ml IGF1, 678 ng/ml CT1, or 426 ng/ml GDNF was retained after high-dose injections (5 μ g IGF1, CT1, or GDNF). The amount of growth factors measured in EOMs from the contralateral orbit was either below the threshold of detection or at least 10-fold lower than in the targeted EOMs. Concentrations of 10 ng/ml IGF1 and 20 ng/ml CT1 are sufficient to activate their receptors in skeletal myotubes and cardiomyocytes, respectively [33,39]. Therefore, based on the exponential reduction known from previous measurements of EOMs [11], our injections of 2 $\times$ 0.5–5 μ g growth factors yield effective doses [33,39] in the superior oblique muscles at least 15 h after injection, and likely for a total of at least 2 days after each of the two injections.

Effects of exogenous growth factors (gain-of-function) on contraction time and half relaxation time

The twitch contraction time was slightly but significantly shortened after growth factor administration (Fig. 1a; Table 1). For IGF1 and CT1, two different doses (2 $\times$ 5 μ g and 2 $\times$ 0.5 μ g) were tested. We also tested combinations of growth factors as listed in Table 1. A shorter twitch contraction time was seen after application of GDNF (2 $\times$ 5 μ g) or CT1 (2 $\times$ 5 μ g) alone, or IGF1 (2 $\times$ 0.5 μ g) plus CT1 (2 $\times$ 0.5 μ g) (Table 1). A high dose (2 $\times$ 5 μ g) of CT1 significantly ($p=0.004$) shortened the twitch contraction time, as shown by a 4% decrease compared with that of the PBS control (Table 1). GDNF (2 $\times$ 5 μ g) treatment also shortened the twitch contraction time by 6% ($p<0.001$). When treated with IGF1 alone (2 $\times$ 5 μ g or 2 $\times$ 0.5 μ g) or with low-dose CT1 (2 $\times$ 0.5 μ g), there was no significant difference in the contraction time between experimental and control groups (Table 1). To test possible synergistic effects, we combined two growth factors at doses that were not individually effective. As shown above, IGF1 (2 $\times$ 0.5 μ g or 2 $\times$ 5 μ g) alone or low-dose CT1 (2 $\times$ 0.5 μ g) alone had no effect on twitch contraction time, and low-dose IGF1 (2 $\times$ 0.5 μ g) plus high-dose CT1 (2 $\times$ 5 μ g) had no significant effect on contraction time (Table 1). The combination of low-dose IGF1 plus low-dose CT1 shortened the twitch contraction time with a 3.6% decrease ($p=0.006$) (Table 1). Thus, combining certain growth factors at doses that were not individually effective could significantly shorten contraction time.

The half relaxation time could also be shortened significantly after growth factor treatment (Fig. 1a; Table 1). The most pronounced shortening was seen with IGF1 (2 $\times$ 0.5 μ g) treatment. Compared with that of PBS control, the half relaxation time was shortened by

about 25% ($p=0.001$). CT1 ($2\times 5\text{ }\mu\text{g}$) treatment also significantly shortened half relaxation time ($p=0.025$), a decrease of 22%. After treatment with the combination of IGF1 ($2\times 0.5\text{ }\mu\text{g}$) plus CT1 ($2\times 5\text{ }\mu\text{g}$), the half relaxation time was significantly decreased by 21% ($p=0.034$). We conclude that IGF1, CT1, and GDNF have different effects on EOMs' contractile kinetics; moreover, contraction time and half relaxation time have different sensitivities to the growth factors. Differences between individual and combination treatments may reflect complex interactions between growth factor ligand binding, binding proteins, and signaling pathways [3,23].

Effects of inhibitors (loss-of-function) on contraction time and half relaxation time

To test whether endogenous growth factors play a role in the regulation of twitch kinetics, we blocked these growth factors with either neutralizing antibodies or binding proteins. As mentioned in "Materials and methods", doses used to neutralize endogenous growth factors were based on previous reports [3,11,12]. Treatment with IGFBP4 ($2\times 1\text{ }\mu\text{g}$) significantly prolonged both twitch contraction time ($p<0.001$) and half relaxation time ($p<0.001$) by 35% and 22%, respectively, when compared with those of the IgG control group (Fig. 1b; Table 1). Neutralizing endogenous GDNF also prolonged the contraction time. Compared with the normal IgG control experiment, the contraction time was increased by 20%, and this difference was statistically significant ($p=0.027$). However, the antibody against GDNF had no effect on the half relaxation time. The treatment with anti-CT1 ($2\times 1\text{ }\mu\text{g}$) did not significantly change either contraction time or half relaxation time. Since exogenous CT1 had significant effects on contraction time (see above), one possible explanation is that the antibody against mammalian CT1 does not recognize the endogenous chicken ligand (or a crucial site thereon) where it binds to the LIF- or gp130 receptor, but the observation that the antibody reduced force significantly (see below) indicates that it was able to block some of CT1's effects. Nevertheless, we conclude that endogenous IGFs and endogenous GDNF act to shorten the twitch contraction time of EOMs.

Effects of growth factors and inhibitors on contractile force

We previously reported that IGF1 and CT1 enhance the contractile force of EOMs [11,38]. To determine whether the speed-enhancing and force-enhancing effects among growth factors are linked or are independent from each other, we measured the contractile force after treatment of EOMs with the same growth factors in either gain-of-function or loss-of-function experiments.

When endogenous IGFs were neutralized with IGFBP4, the contractile force (twitch tension) of the EOM was significantly reduced (by 15%, Fig. 2a), as expected from previous gain-of-function studies [38,44]. Thus, endogenous IGFs regulate both aspects of muscle function: contractile force and twitch kinetics (contraction time and half relaxation time). When endogenous CT1 was neutralized with CT1 antibodies, the contractile force (twitch tension) was reduced (by 23.5%, Fig. 2a). However, contraction time and half relaxation time were not affected (see above), thus the CT1 antibody appears to differentially affect force and kinetics. Preliminary data indicate that combining IGFBP4 and CT1 antibody treatment together does not further reduce the twitch tension (data not shown). For the statistical analysis of these experiments, the IgG-injected group served as the control group. When the force was normalized to muscle mass, the reduction of force by IGFBP4 and CT1 antibody (data not shown) was not statistically significant. The minimal effect on both cross-sectional area and mass (Fig. 2b, c) suggests that the effect on force is largely due to changes in contractile proteins (such as myosin heavy chains [40]) rather than muscle hypertrophy.

Neither GDNF ($2\times 5\text{ }\mu\text{g}$) nor anti-GDNF ($2\times 1\text{ }\mu\text{g}$) treatment significantly altered the twitch tension (Fig. 2a) when compared with the tension of IgG or PBS controls. Thus, we

conclude that endogenous GDNF has effects on twitch kinetics but not on contractile force. In summary, some growth factors can differentially affect contractile force and kinetics of EOMs, indicating that these two effects are not linked together.

Effects of growth factors and inhibitors on cross-sectional area and muscle mass

Our previous work [38] has shown that, in gain-of-function experiments, CT1 (2×5 µg), IGF1 (2×0.5 µg) plus CT1 (2×5 µg), and IGF1 (2×0.5 µg) plus CT1 (2×0.5 µg) significantly increased muscle mass. In the present study, GDNF (2×5 µg) did not significantly increase either cross-sectional area or muscle mass (Fig. 2b, c). In our loss-of-function study, the average mass of muscles treated with IGFBP4, anti-CT1, and anti-GDNF did not change muscle mass significantly, compared with the average muscle mass of IgG controls (Fig. 2c). This means that force reductions after the treatment with the inhibitors of growth factors are not accomplished through reduced muscle mass.

Effects of growth factors and inhibitors on fusion frequencies

High doses of either of the two growth factors CT1 and GDNF increased fusion frequencies of tetanic tension as shown for CT1 (Fig. 3a), while one inhibitor, IGFBP4, reduced the fusion frequency from 250–350 Hz to 150–300 Hz, as shown in Fig. 3b. We conclude that growth factors play a role in modifying fusion frequencies in addition to contraction time.

Expression of endogenous growth factors

The above loss-of-function data show that endogenous growth factors, in particular GDNF and IGF1, are essential for fast contraction kinetics. Since nearly all skeletal muscles contract significantly slower than EOMs, one would expect EOMs to express higher levels of these growth factors and their receptors than the slower skeletal muscles. To test this hypothesis, we measured endogenous levels of mRNA encoding the three growth factors and their receptors in EOMs and in two types of striated skeletal muscles, compared with GAPDH and other housekeeping genes. Housekeeping genes were expressed at levels that were not significantly different between the two muscle types (shown for GAPDH in Fig. 4a).

We first measured IGF1 mRNA expression in an EOM, the superior oblique muscle, in 2–3-week-old chickens and compared the expression with that of the pectoralis major, which is a fast-twitch skeletal muscle that has about 1.8–5.1 times slower kinetics than EOMs, based on a previous report on avian pectoralis muscle [29]. The chicken EOM expressed 21 times more IGF1 mRNA than the pectoralis major (Fig. 4b). To determine whether this difference was consistent between species (and because a CT1 gene sequence has not been identified in birds), we compared growth factor mRNA expression between rat EOMs and rat extensor digitorum longus (EDL) muscle, a relatively fast skeletal muscle with contraction times of 11–55 ms [18].

Similar to IGF1 mRNA expression, GDNF mRNA expression was about 26-fold higher in EOM than in EDL skeletal muscle (Fig. 4c). Likewise, CT1 mRNA expression was significantly higher in EOM than in EDL (Fig. 4c). We also tested the mRNA expression of the respective growth factor receptors: IGF1 receptor, the CT1 receptors gp130 and LIF receptor, and GDNF receptors RET and GFRalpha. We found that not only the transcripts for the ligands but also for their receptors were expressed at significantly higher levels (1.5–6.5 fold) in rat EOM than in rat EDL (Fig. 4d), while transcripts for other proteins, e.g., Pfkfb1, known to be expressed at higher levels in skeletal muscle than in EOM [49], did not show this pattern (Fig. 4c). Similar results of higher levels of growth factor mRNA expression and their receptors were obtained with other housekeeping genes (RPS13, RPS29) in rat and also in other species (rabbit, data not shown).

We conclude that EOMs express transcripts for myogenic growth factors and receptors at a significantly higher level than limb skeletal muscles, and this is a common phenomenon across different species. Taken together with our electrophysiological data (gain-of-function and loss-of-function experiments), we conclude that specific growth factors are responsible for EOM's significantly faster contractions compared with those in limb skeletal muscles.

Discussion

Comparison of contractile properties between extraocular and limb skeletal muscles

Twitch contraction time (onset of force to force peak) of skeletal muscle is typically about 20–130 ms, with a usual distinction between “slow” and “fast” muscle types within this range (slow—more than 100 ms; fast—less than 80 ms). To make rapid eye movements (saccades), extraocular muscles (EOMs) need to contract considerably faster than limb skeletal muscles. Previous studies on contractile parameters of skeletal muscles and EOMs have indeed revealed that EOMs contract considerably faster, nearly as fast as “superfast” song-producing muscles [52,53]. EOMs typically have twitch contraction times of about 5–15 ms (temperature dependent [6,24]) and fusion frequencies in the range of 150–500 Hz, while fast skeletal muscles contract at 20–100 ms and have fusion frequencies of 23–115 Hz, and superfast sound-producing muscles range from 3.2–7.0 ms with fusion frequencies up to 800 Hz (Fig. 5, Table 2). Such differences between EOMs and limb skeletal muscles were reported for all vertebrate species examined, from fish and frogs to birds and mammals (Fig. 5; Table 2). Our measurements of EOMs in normal 2-week-old chicks fall precisely within the expected range. Based on these data from the literature, we analyzed whether contraction time and fusion frequency are significantly correlated with each other. When compiled in a double logarithmic plot, the correlation coefficient r^2 was -0.89 ($p=0.001$) (Fig. 5). Thus, the contraction time correlates negatively with fusion frequency, with high statistical significance.

Partially overlapping but distinct growth factor effects on contractile properties

Previous work has documented effects of growth factors on EOM force [11,38,43], but potential and concurrent effects of growth factors on other aspects of muscle contraction have never been systematically examined. Our survey of growth factor effects on contraction and relaxation time revealed a remarkable variety of growth factor profiles: each of the examined growth factors has partially overlapping but not identical effects. For example, as illustrated in Fig. 6, IGF1 and CT1 both increased force in gain-of-function experiments, but GDNF did not affect force. IGF1 and CT1 both reduced relaxation time, while CT1 and GDNF reduced contraction time. In loss-of-function experiments, elimination of IGF1 or CT1 reduced force, while elimination of GDNF prolonged contraction time, and elimination of IGF1 also prolonged relaxation time. These findings indicate that the combined effects of these different growth factors can fine-tune and adjust contractile properties of muscles to make them stronger, faster, or achieve various nuances and degrees of muscle function. Thus, we propose that motor neurons and other feedback mechanisms adjust growth factor levels to regulate muscle contractile properties that ultimately optimize muscle function for diverse tasks.

Plasticity and developmental changes

It is well documented that muscles can adapt and change their contractile properties. Cross-reinnervation studies demonstrated considerable muscle plasticity and revealed neural influences on muscle plasticity. Slow muscle attained fast contractile properties after being cross-reinnervated by nerves from motoneurons that originally innervated fast muscles. Likewise, previously fast muscle acquired slow contractile properties after being cross-reinnervated by nerves that originally innervated slow muscle [46,47,68]. This influence is

presumed to be due to either nerve firing frequency and/or trophic influences (growth factor release from innervating nerves) [8,37]. Importantly, our study now identifies several plausible growth factor candidates that appear to be involved in the control of contractile kinetics.

Muscles attain their mature contraction speed gradually during post-natal or post-hatch development, with significant differences between EOMs and other skeletal muscles. For example, cat skeletal (limb) muscles attain their final (adult) contraction speed at about 10 weeks, while EOMs continue to increase contraction speed into adulthood [35]. Similarly, the contraction time of the superior oblique muscle decreases by about 40% in the first 20 days post-hatch in chickens [16]. Since such changes in contraction speed correlate with changes in the composition of myosin heavy and light chain isoforms during development [1,35], it is possible that growth factors control contractile kinetics via modification of myosin composition.

Mechanisms that control contraction speed

Several mechanisms are believed to contribute to the speed of muscle contraction, including myosin isoforms, calcium release channels, and acetylcholine receptors. The myosin heavy chain (MHC) isoforms define myofiber types and thereby influence contractile properties [17,40]. Interestingly, IGF1 promotes the expression of MHCIIb, the fastest MHC isoform, by increasing MHCIIb promoter activity [57]. Therefore, changes in myosin heavy chain expression may be produced and maintained by the relatively high level of endogenous IGF1, compared with limb skeletal muscles, and this mechanism likely contributes to the fast contractile property of EOMs.

Contraction time depends on the rate of calcium release from sarcoplasmic reticulum and influx into the cytosol. The voltage-sensing dihydropyridine receptor (DHPR) in the T-tubules as well as ryanodine receptors control calcium influx in E-C coupling. Indeed, IGF1 is known to regulate transcription of DHPRs by promoting expression of the subunit DHPR α 1s, the major subunit of DHPR in skeletal muscle [50]. Therefore, a second, not mutually exclusive way by which IGF1 may regulate contraction speed is by controlling DHPR levels in the muscle. Additional features in EOMs that are believed to contribute to fast contraction speed are the extensive T-tubules and hypertrophy of the sarcoplasmic reticulum (calcium storage) as well as high mitochondrial content [5]. Furthermore, compared with limb skeletal muscle, expression of acetylcholine receptor subunits and acetylcholinesterase are elevated, possibly due to the high innervation density of EOMs and distinct distribution of acetylcholinesterase in synapses [49,54]. Furthermore, parvalbumin, one type of calcium-binding protein, appears to be inversely regulated by IGF1 [33]. Thus, multiple molecular mechanisms may be responsible for and may cooperate in the implementation of contraction kinetics, both during muscle development and plasticity, and they seem to be at least in part controlled by growth factors such as IGF1.

Mechanisms that regulate muscle relaxation

EOMs are able to sustain a series of individual twitch contractions at high frequency without tetanic fusion. The velocity of Ca²⁺ transient change is one of the factors that determine the contractile kinetics of muscle [53]. Rapid muscle relaxation is crucial for this contractile property that depends on calcium reuptake from myofiber cytosol. EOMs handle high Ca²⁺ loads very efficiently, more efficiently than limb skeletal muscle, and rapidly bring internal Ca²⁺ back to resting levels [70]. Interestingly, growth factors such as IGF1 appear to influence this process [32]. Consistent with those data, both our gain-of-function experiments (exogenous IGF1) and our loss-of-function experiments (blocking endogenous IGF1) showed that IGF1 dramatically alters half relaxation time of EOMs.

How do different growth factors control contractile properties?

As already mentioned, IGF1 signals through multiple pathways to affect contractile properties: IGF1 alters MHC expression through the signaling of PI3K, GSK-3 β , and β -catenin [57], modifies calcium release from sarcoplasmic reticulum by regulating transcription of DHPR, via calcineurin and calcium calmodulin [71], and alters relaxation time by regulating SERCA expression via PI3K and Akt signaling [32]. Less is known about how the two other growth factors, CT1 and GDNF, affect contractile properties. CT1 exerts effects on target tissues through its gp130 and LIF receptors, by stimulating the JAK–STAT pathway. However, CT1 may also activate the PI3K/Akt signaling pathway, similar to IGF1. This may explain some of the common effects between IGF1 and CT1 on EOM contractile properties. Interestingly, GDNF promotes the insertion and stabilization of postsynaptic AChRs through MAPK, CREB, and Src kinase activities [69]. We show that EOMs express 26 times more GDNF mRNA than limb skeletal muscles do, which could at least in part explain the higher expression of AChRs in EOMs compared with skeletal muscles [49]. This could also be the reason why exogenous GDNF and neutralization of endogenous GDNF altered the contraction time of EOMs in our study. Our preliminary data (yet unpublished) indicate that another myogenic growth factor, neurotrophin-4, can also increase EOM muscle contraction speed, similar to GDNF. This suggests that multiple growth factors act in concert to alter contractile properties.

Potential additional sources of factors that modify contraction speed

Our quantification of the expression of growth factors in the EOMs should not be taken to imply that muscle itself is the only source of relevant growth factors, and that those growth factors act exclusively in an autocrine manner. Several additional sources and routes of growth factors may contribute to control contractile properties of muscle, including the nerve itself, as well as a systemic (vascular) route. Indeed, some growth factors, trophic factors, and cytokines can be transported anterogradely in nerves, including motor nerves [65,67]. Our recent study on IGF1 showed that this growth factor is prominently expressed by Schwann cells in oculomotor nerves, and IGF1 protein can be transported from the nerve to EOMs [21]. Nevertheless, our current study shows that the EOMs per se express much more IGF1, CT1, and GDNF than limb skeletal muscles. Therefore, these muscle-derived growth factors and cytokines likely affect EOMs in an autocrine manner.

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Fig. 1.

Typical traces of twitch contraction time (onset of force to peak force) and half relaxation time of a normal superior oblique extraocular muscle (EOM) in a 2-week-old chicken, compared with either growth factor treated muscle (gain-of-function, **a**) or with endogenous growth factor neutralized (loss-of-function, **b**). The tension is plotted against time, with arrows indicating the duration of the contraction time and half relaxation time. **a** Typical traces of twitch contractions of EOM with PBS or CT1 treatment. **b** Typical traces of twitch contractions of EOM with treatment of IgG control or IGFBP4

Fig. 2.

Twitch tension and muscle mass of superior oblique muscles from 2-week-old chickens treated with IGFBP4 2×1 µg, CT1 antibody 2×1 µg, GDNF antibody 2×1 µg, or GDNF 2×5 µg. **a** Twitch tension in gain-of-function (GDNF) and loss-of-function studies (IGFBP4, Anti-CT1, Anti-GDNF). IGFBP4 and CT1 antibody, but not GDNF antibody significantly decreased twitch tension (* $p < 0.05$). **b** The cross-sectional area (CSA) after growth factor neutralization or GDNF treatment. **c** The muscle mass after growth factors neutralization or GDNF treatment. There were no significant differences between control groups and experimental groups. Error bars=SEM; number (n) of independent experiments for each group=4, except for GDNF ($n=5$)

Fig. 3.

Examples of effects of exogenous trophic factors or neutralization of endogenous trophic factors on fusion frequency and contraction time of extraocular muscles (EOMs) in juvenile chickens. **a** $2 \times 5 \mu\text{g}$ CT1 not only shortened the contraction time but also elevated the fusion frequency. **b** $2 \times 1 \mu\text{g}$ IGFBP4 treatment prolonged the contraction time. Data points are mean $\pm$ SEM for contraction time (x-axis) and fusion frequency (y-axis). Number of independent experiments is $n=7$ (PBS), $n=6$ (CT1), $n=4$ (IgG), and $n=4$ (IGFBP4). CT1 cardiotrophin-1, IGFBP4 insulin-like growth factor binding protein 4, PBS phosphate-buffered saline

Fig. 4.

Growth factors and their receptors were expressed at higher levels in extraocular muscles (EOMs) than in limb skeletal muscles. **a** Housekeeping genes (GAPDH) were expressed at levels that were not significantly different between EOMs and limb skeletal muscles. **b** EOMs expressed 21 times more IGF1 mRNA than the pectoralis major muscle in juvenile chicken. **c** There was significantly more growth factor (IGF1A, IGF1B, CT1, and GDNF) mRNA expression in the EOMs than the EDL muscles in adult rats. *Pfkfb1*, however, a gene prominent in limb skeletal muscles, was expressed much more in rat EDL muscles when compared with rat EOMs. **d** Similar to the growth factors, their receptors were also expressed at significantly higher levels in the EOMs than the EDLs. *Pect.* pectoralis major, *EDL* extensor digitorum longus, *IGF1* insulin-like growth factor 1, *CT1* cardiotrophin 1, *GDNF* glial cell line-derived neurotrophic factor, *Pfkfb1* 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1, *gp130* glycoprotein 130, *LIFRa* leukemia inhibitory factor receptor alpha, *GFRa* glial cell line-derived neurotrophic factor family receptor alpha 1, *RET* ret proto-oncogene. Error bars=SEM. Number of independent experiments: *n*=4 (**a**), *n*=3 (**b**), *n*=4 (**c**, **d**). **p*<0.05 was considered statistically significant

Fig. 5.

Contraction times and fusion frequencies of skeletal muscles (*open squares*) and extraocular muscles (*red dots*), and superfaster sound-producing muscles (*filled black squares*) from various vertebrate species plotted on a double logarithmic scale. Note that limb skeletal muscle and extraocular muscle properties minimally overlap and form a straight regression line. Data points were derived from the reports listed in Table 2

Fig. 6. Synopsis of growth factor effects on contractile properties of extraocular muscle from 2-week-old chickens. To define profiles of the three growth factors (IGF1, CT1, and GDNF), their effects are compiled for four muscle properties: force, mass, twitch contraction time (TCT), and half relaxation time (HRT). *Black bars* indicate loss of function; *gray bars* indicate gain of function. Changes in properties are indicated as % difference from normal

Table 1

Experimental treatment, contraction and relaxation times, and estimated effective doses

Treatment	Dosage (on days 6 and 9)	Sample number (n=)	Mean TCT (ms)	SEM	Statistical significance compared w/PBS	Mean 1/2 RT (ms)	SEM	Statistical significance compared w/ PBS	Estimated concentration (pg/mg=ng/ml)
Normal		6	5.84	0.04	N.S.	6.70	0.24	N.S.	
PBS control		7	5.91	0.01		7.96	0.28		
IGF1	0.5 µg	8	5.84	0.04	N.S.	5.92	0.30	p=0.001	40.6
	5 µg	10	5.80	0.02	N.S.	6.89	0.32	N.S.	406
CT1	0.5 µg	6	5.80	0.05	N.S.	7.07	0.45	N.S.	67.8
	5 µg	6	5.68	0.05	p=0.004	6.19	0.24	p=0.025	678
GDNF	5 µg	4	5.56	0.06	p<0.001	6.83	0.62	N.S.	426
IGF1 and CT1	IGF1 0.5 µg+ CT1 0.5 µg	7	5.70	0.04	p=0.006	6.46	0.30	N.S. (p=0.065)	40.6+67.8
	IGF1 0.5 µg+ CT1 5 µg	6	5.75	0.02	N.S.	6.25	0.35	p=0.034	40.6+678
					Compared w/ IgG				
IgG control	1 µg	4	5.96	0.25		7.44	0.37		
IGFBP4	1 µg	4	9.09	0.16	p<0.001	10.31	1.36	p<0.001	
Anti-CT1	1 µg	4	5.78	0.13	N.S.	7.34	0.94	N.S.	
Anti-GDNF	1 µg	5	7.40	0.66	p=0.051	6.71	0.66	N.S.	

N.S. not significant, PBS phosphate-buffered saline, 1/2 RT half relaxation time, SEM standard error of the mean, TCT twitch contraction time

Table 2
Comparison of contraction times and fusion frequencies in different types of vertebrate muscles

Species	Temp.	Muscle	Contraction time (ms)	Fusion frequency (Hz)	Reference
Extraocular muscles					
Catfish	23	LR, MR, SO, IO	12	150–170	[36]
Frog	21.5	SR	15	120	[4]
Frog	21.5	SO	18	90	[4]
Pigeon	41	LR	6.0	190–250	[61]
Chicken	38	SO	5.5	300–400	[16]
Rat	35	IR	3.8–5.1	500	[13]
Ferret	?	LR	16.7	120–220	[7]
Ferret	?	LR	16.1	157	[56]
Rabbit	35	IO	6.4	300	[5]
Rabbit	20–23	SR	8.4	70	[24]
Cat	36	MR	7.5	350	[14]
Squirrel monkey	?	LR	5.2	150–260	[28]
Monkey	37	LR	7–8.5	500	[25]
Skeletal muscles					
Frog		Ilioibularis	32	60	[4]
Lizard		Ilioibularis	21–25	55–58	[27]
Lizard		Peroneus	23	56	[27]
		Fast	50–80	60	[41]
		Slow	100–200	16	[41]
Pigeon		Gastrocnemius	31–42	23–32	[61]
Rat		Styloglossus	13.8	109	[62]
Mouse		EDL	34.2	76.7	[1]
Mouse		Diaphragm	61.9	58.8	[1]
Ferret		Soleus	84	37	[55]
Cat		Soleus	94–120	31–33	[14]
Cat		Gastrocnemius	39	100	[14]
Cat		EDL	23–38	108–115	[14]

“Superfast” sound-producing muscles

Species	Temp.	Muscle	Contraction time (ms)	Fusion frequency (Hz)	Reference
Toadfish	21	Swinbladder muscle	5	~300	[59]
Insect	25–35	Singing muscle	4.6–7.0	>200	[31]
Starling	39	Syringeal muscle	3.2–3.7	600–800	[19]

EDL extensor digitorum longus, *IO* inferior oblique, *IR* inferior rectus, *LR* lateral rectus, *MR* medial rectus, *SO* superior oblique, *SR* superior rectus, *Temp* temperature, ? no temperature reported