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Research into the physiology of myosins - a personal odyssey

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Abstract

During my PhD, I worked on the neural regulation of mechanical properties fast and slow muscles. This led me to believe that myosins in fast and slow muscles are structurally distinct and that motor nerves regulate the expression of myosin genes. I devised a method for separating intact fast and slow myosins by gel electrophoresis and confirmed their neural regulation. The electrophoresis method was subsequently improved and used to analyse skeletal and cardiac myosin isoforms in various vertebrate species, including marsupials. This led to the discovery of neonatal myosin heavy chain (MyHC), α and β cardiac MyHCs and of the regulation of cardiac MyHCs by thyroid hormone. Antibodies were raised against 2A, 2X, 2B, masticatory and extraocular MyHCs and used to study the expression and regulation of MyHCs in jaw, laryngeal and Extraocular Muscle (EOM) fibres. Antibodies against masticatory myosin enabled the sequencing of masticatory MyHC and masticatory light chain 2 genes. Cross-bridge kinetics of fibres with different myosin isoforms were analysed. Different MyHC isoforms found in jaw-closing muscles across various species reflected evolutionary adaptations to diverse dietary intake, while MyHC expression changes in cardiac and laryngeal muscles with body mass reflected adaptations to changes in their specific metabolic rate. Transplantation experiments on masticatory and EOMs and cross-innervation experiments between laryngeal and somitic muscles revealed that their capacity to express masticatory or extraocular MyHC were myogenically determined but neural impulse patterns also influence MyHC expression. EOMs are the most complex, expressing 11 MyHC isoforms. Some EOM fibres express faster MyHCs in the endplate zone but slower MyHCs at the end segments, an arrangement helping to linearize the saccade. I suggested that during development, primary and secondary extraocular myotubes specify the synaptic inputs of the innervating neurons to generate impulse patterns which regulate the expression of their MyHCs.

Key Words: myosin gene expression, muscle contraction, neural regulation, craniofacial muscles, cardiac contractility.

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My initial interest in neuroscience research

I have enjoyed doing research into muscle physiology throughout my academic life. My entry into muscle research was not a happy personal decision, but a traumatic one beyond my control. During my student days in the medical school at the University of Sydney, I was intrigued by the physiology of the nervous system. The highlight of 3rd year physiology course in 1959 was for me a guest lecture on synaptic transmission in motoneurons given by Professor Sir John Eccles, who was the Head of the Physiology Department at the John Curtin School of Medical Research at the Australian National University. I was fascinated by the lecture, and it strongly stimulated in me a desire to pursue a research career in neurophysiology after my medical graduation. There was an opportunity after the third year in the medical course to take a year off to do a BSc (Med) course. This involved a year of supervised research and the submission of a thesis on the research work. This course could give me a foretaste of research life and I decided to take it the following year.

During the BSc (Med) year, I was very fortunate to be assigned to a neurophysiology laboratory and was supervised by Dr Bill Levick, working in the Brain Research Unit of the Head of Department, Prof. Peter Bishop, recording impulses in single ciliary nerve fibres in response to light stimulation. I enjoyed the taste of research life so much that I

firmly decided to pursue a research career after graduation instead of doing clinical medicine.

In 1962, I visited Eccles' laboratory at the John Curtin School of Medical Research and enquired about the prospect of doing postgraduate studies under his supervision. He assured me that I could get an Australian National University scholarship to do a PhD in his Department if I applied after my medical graduation. In 1963 Eccles was awarded the Nobel Prize in Physiology or Medicine, and I was very happy at the prospect of having a Nobel Laureate as a mentor. After my graduation in1964, I did apply for the scholarship and was awarded it, to commence in 1965, but I decided to defer taking it up by a year to enable me to be home with my folks in Kuala Lumpur, Malaya, as I had then been away in Australia for 9 years. During that year I held a lectureship in the Department of Physiology at the new medical school of the University of Malaya.

Opting to defer taking up the scholarship was a fateful decision! When I turned up in Canberra early in 1966, Eccles was 63 years old, and he had decided to avoid the mandatory retirement at age 65 at the Australian National University by accepting an American offer to continue his research at the Institute of Biomedical Research in Chicago. He could not supervise my PhD as he would be leaving the Department within the next few months. Eccles appointed a muscle physiologist, Dr Russel Close, as my PhD supervisor. My dream of doing brain research under his supervision was shattered. Furthermore, no one else in the Department could supervise me to do neurophysiological research. I was devastated.

Diversion into muscle research

I was assigned to work on a problem which Eccles and colleagues had pioneered a few years ago on the neural control of contractile properties of fast and slow skeletal muscles. They measured the isometric contraction times of muscles, the reciprocal of which is a crude indicator of muscle speed. They showed that all muscles of new-born kittens were uniformly slow, and that fast muscles acquired their speed progressively during the early postnatal period while the speed of developing slow muscle remained unchanged. However, after isolation of the lumbosacral spinal cord, the development of fast muscle was unaffected but the developing slow muscle became nearly as fast as the fast muscle.[1] Furthermore, cross-innervation of fast and slow muscles in the adult resulted in reciprocal changes in their speeds, suggesting that muscle speed was under neural control.[2] Dr Close had been following up this work by measuring the force-velocity properties of muscles, which best characterize muscle mechanics, during development and following cross-innervation. He showed that the maximal velocities of shortening of fast and slow muscles the rat[3] and mouse[4] were virtually identical at birth. During the postnatal period, the speed of shortening of the developing fast muscle increased by 2.5-3 times while that of slow muscle showed little change. Cross-innervation of fast and slow muscles resulted in reciprocal changes in their speeds of shortening,[5] indicating that the speeds of shortening of muscles were under neural control. My role was to extend this area of research and to explore whether other differences in properties between fast and slow muscles were also under neural regulation.

Close and I first showed that force-velocity properties of developing fast muscles of new-born kittens had already diverged from that of slow muscle to some extent.[6] We characterized the different effects of temperature on the contractile properties of rat fast and slow muscles[7] and the effects of tetanic contractions on the subsequent isometric twitches of fast and slow muscles. We showed that the isometric twitch tension of fast muscle was enhanced about twofold by a prior tetanic stimulation, a phenomenon known as Post-Tetanic Potentiation (PTP).[8] In contrast, slow muscle showed Post-Tetanic Depression (PTD), the isometric twitch was depressed by about 15% following a tetanus. We further showed that these post-tetanic effects were under neural control.[9,10] Works contributing to the elucidation of the molecular mechanisms of PTP and PTD will be referred to below.

Cross-innervation studies on fast and slow avian muscles[11,12] surprisingly did not show any change in their mechanical properties. We showed that in another lower vertebrate, the toad, innervation also did not regulate mechanical properties of muscles. In the toad, fast-twitch and slow-tonic muscle fibres are innervated by nerve fibres with low and high thresholds for electrical stimulation, respectively. Cross-innervation of fast-twitch and slow-tonic muscle fibres with the alien nerve type did not change the mechanical properties of these muscle fibres.[13] These observations show that neural regulation of muscle properties is an innovation that occurred during mammalian evolution, an advancement that allows adaptive changes in muscle properties in response to their pattern of use.

I was initially bitterly disappointed at being deprived of the opportunity to pursue my passion for research in neurophysiology and for being diverted into muscle research. This turned out later to be a blessing in disguise. The role of myosin in muscle contraction was then fast emerging. Barany showed that the actin-activated ATPase activities of myosins from muscles of a wide range of speeds from diverse animal species were correlated with their maximal speeds of shortening.[14] Close collaborated with Barany and showed that following cross-innervation of fast and slow muscles, the ATPase activities of their myosins were transformed in proportion to their changes in speeds of shortening.[15] There was thus the exciting prospect of tackling the neural regulation of muscle properties at the molecular level. At the end of my PhD in 1969, I became convinced that myosins in fast and slow muscles were structurally distinct, that myosin isoforms controlled the speeds of muscle contraction, and that motor nerves controlled the expression of myosin genes in the muscle fibres they innervate. These convictions set the direction of my future research.

Development of methods for the electrophoretic analysis of intact myosin isoforms

I accepted a post-doctoral research fellowship in the Department of Pharmacology at the University of Illinois in Chicago in the laboratory of Dr Bernard Salafsky. I knew little about muscle biochemistry at that time, being a physiologist learning to do biochemistry in a Pharmacology De-

partment! During the two years of postdoctoral work, I was given a free hand in deciding the direction of the research. I initially showed that the intracellular K^+ concentration was higher in rat fast muscle fibres compared with that of slow muscle fibres, and that this difference was under neural control.[16] The main research objective was to develop specific antibodies against myosins extracted from fast and slow muscles to test the hypotheses regarding their structural difference and the neural regulation of myosin gene expression. I learned to prepare myosins from fast and slow muscles for use as antigens. To assess the purity of my myosin preparations, I tried to use polyacrylamide gel electrophoresis, a technique routinely used by another staff member in the laboratory to analyse serum proteins. I was frustrated by the fact that myosin persistently precipitated at the top of the gel. Upon reading the literature on the properties of myosin, I realized that this was due to the low ionic strength of the tris/glycine electrophoresis buffer. A high ionic strength buffer would be needed to keep myosin in solution, but the high conductance of such a buffer would not be suitable for gel electrophoresis. I struggled with this dilemma and eventually came across the work of Brahms and Brezner, who showed that myosin could be dissolved in low ionic strength solutions of strongly negative polyvalent anions, such as ADP, ATP, and pyrophosphate.[17] Simply adding 10 mmol ATP to the tris/glycine electrophoresis buffer solved the problem! It was a eureka moment!

During my tenure of the postdoctoral fellowship, I managed to demonstrate that fast and slow muscle myosins could be resolved from each other using the ATP/tris/glycine electrophoresis method, fast muscle myosin had a higher electrophoretic mobility than slow muscle myosin. I devised a method for measuring the Ca^{2+}-ATPase activity of the myosins in the electrophoresis gel and showed that fast muscle myosin had a higher Ca^{2+}-ATPase activity than slow muscle myosin. Cross-innervation of fast and slow muscles reversed these properties of their myosins.

In 1971, I was appointed lecturer in the Department of Physiology at the University of Sydney. On my way to take up my appointment, I attended a conference in the UK where I presented the preliminary electrophoresis results. The successful electrophoresis of intact myosin stirred considerable interest among the biochemists present. They quizzed me intensely regarding the electrophoretic method, but I was reluctant to divulge it for the following reasons. I would be going to an empty laboratory in a Physiology Department which I knew would not have the infrastructure for biochemical research. I would therefore not have a level playing field in continuing the work in the competitive field of muscle biochemistry in which I was a novice. Furthermore, the work [18] was then still incomplete and had yet to be published. To this day I still feel uncomfortable about withholding scientific information from colleagues on that occasion.

Research was difficult in the early years at the University of Sydney, as the Physiology Department lacked even very basic facilities for biochemical work. The spectrophotometer, ultracentrifuge and other necessary expensive equipment had to be acquired from research grants over several years. Fortunately, the electrophoresis method for intact myosin could cope with crude extracts of myosin. I was also fortunate to have a succession of talented BSc Honours and PhD students and research assistants to work in the laboratory. In the first few years, we improved on the myosin gel electrophoresis method simply by substituting ATP/tris/glycine buffer with 10 mmol pyrophosphate buffer. Initially this was a means to conserve research funds. Myosin isoform resolution was considerably improved with the pyrophosphate gel system. It opened the doorway for us to study myosin isoforms in various skeletal and cardiac muscles and their regulation.

Myosin is a hexamer with a pair of Myosin Heavy Chains (MyHCs) and 2 pairs of Myosin Light Chains (MLCs). The two MyHCs interact to form two distinct domains: a head domain consisting of a pair of globular heads each bearing an essential MLC (MLC1) and a regulatory MLC (MLC2), and an a-helical coiled coil rod domain that is involved in the assembly of bipolar thick filaments. I often wondered how pyrophosphate anions can keep myosin in solution at low ionic strength. A conserved 29-residue region named assembly competent domain was identified near the C-terminal of the MyHC as critical for filament assembly.[19] Pyrophosphate anions may interfere with the charge interactions between this region and a neighbouring myosin molecule involved in filament assembly. More recently, a highly conserved cluster of four positive amino acid residues near the assembly competent domain has also been shown to be critical for thick filament assembly.[20] It seems more likely that the four negative charges of the pyrophosphate anion very effectively interact with this cluster of four positive residues at low ionic strength to prevent myosin polymerization.

Pyrophosphate gel electrophoresis resolved avian fast myosin into three components which differed in MLC composition. It resolved avian slow muscle into 2 components with identical MLC composition, suggesting that these components differed in MyHC composition.[21,22] Fast muscle myosins of rabbit,[23] rat,[24] human[25] and mouse[26] muscles also showed 3 components with different MLC compositions. Myosin extracted from rabbit neonatal muscles showed 3 isoforms which migrated faster than those of limb fast myosin, with MLC composition similar to those of fast myosin components, suggesting that it was a new myosin isoform that differed in MyHC composition. We confirmed this by showing that the cyanogen bromide peptide map of the rabbit neonatal muscle MyHC was distinct from maps of MyHCs of fast and slow myosins, establishing it as a novel foetal or neonatal MyHC isoform.[23] Whalen and colleagues subsequently discovered another novel MyHC isoform in developing muscle, the embryonic MyHC, which was expressed during muscle development prior to the expression of foetal/neonatal myosin.[27]

Electrophoretic analyses of myosin isoforms in developing human skeletal muscles and in diseased muscles

Using pyrophosphate gel electrophoresis, we studied the expression of embryonic and foetal myosins in human muscles during development and in diseased human muscles. Human foetuses (16-20 weeks' gestation) showed the pres-

ence of embryonic and foetal myosin isoforms, all of which had higher electrophoretic mobilities than the three fast myosin isoforms. The embryonic and foetal isoforms were present in muscles from patients with Duchenne muscular dystrophy, consistent with the known presence of muscle regeneration in this disease. Foetal myosin isoforms were found in muscles from patients with spinal muscle atrophy in which there is a failure of muscle maturation.[28] Analysis of muscles from the dystrophic mouse (129 REJ) revealed only a shift of myosins towards slower migrating isoforms, but embryonic and foetal isoforms were absent, consistent with the absence of muscle regeneration in these muscles.[26]

Analysis of cardiac myosins and their regulation by thyroid hormone

Analysis of cardiac myosins was most enjoyable and rewarding. We showed by pyrophosphate gel electrophoresis that ventricular myosin in adult rats comprised 3 electrophoretic components, V_1, V_2 and V_3 in the order of decreasing electrophoretic mobility and Ca^{2+}-activated ATPase activity.[29] These ventricular myosin isoforms had the same pair of ventricular MLCs. Analysis of the MyHCs of V_1 and V_3 showed that they have significant differences in amino acid composition and in their cyanogen bromide peptide maps. Since the peptide map of a mixture of V_1 and V_3 MyHCs did not differ from that of MyHCs from myosin containing all three components, the results show that the MyHCs of V_1 and V_3 are structurally different, designated as α MyHC and β MyHC respectively, and that the MyHC compositions of V_1, V_2 and V_3 are respectively αα, αβ and ββ.[30]

We showed that cardiac myosin isoforms were regulated by thyroid hormone. Juvenile rats expressed purely V_1, while V_2 and V_3 emerged as the animal matures. Thyroid hormone administration in the adult rat shifted isoform expression towards V_1, while hypothyroidism induced by hypophysectomy shifted ventricular myosin towards V_3.[29] In vitro analysis of radioactive amino acid uptake into myosins by ventricular strips from hypophysectomized rats indicated that administration of thyroid hormone induced the synthesis of α MyHC but suppressed β MyHC synthesis. This occurred at the level of gene transcription since these actions were suppressed by actinomycin D.[31]

In pursuing the functional significance of cardiac myosin isoforms, we showed that V_1 and V_3 isoforms differed kinetically by a factor of two, V_1 having the higher actin-activated ATPase activity.[32] Cross-bridge cycling kinetics can be measured using dynamic stiffness analysis, which yields a stiffness minimum frequency, F_{min}, which reflects cross-bridge cycling kinetics. This parameter is sensitive to rate constants for the power stroke and the rate of cross-bridge detachment.[33] In collaboration with Dr Gunther Rossmanith at Macquarie University, we showed that F_{min} for V_1 myocardium was twice that of V_3 myocardium.[34] V_1 myocardium also had a higher maximal rate of tetanic stress development and heat production,[35] and consumed twice as much ATP during isometric contractions as V_3 myocardium.[36] Thus, V_1 muscle is more powerful, while V_3 muscle is more economic. The functional significance of thyroid stimulation of V_1 and suppression of V_3 is clearly to enhance cardiac contractility and thus cardiac output to meet the metabolic demands due to the thyroid induced increase in metabolic rate. This feature is a mammalian evolutionary innovation since there is no evidence that thyroid hormone regulates avian cardiac myosin.[37]

The expression of mammalian ventricular myosin isoforms is also influenced by hypertension and the associated cardiac hypertrophy. Experimentally induced hypertension and cardiac hypertrophy increase the expression of V_2 and V_3, which regresses after normalization of blood pressure and ventricular mass.[38] This ventricular myosin response during cardiac hypertrophy would serve to reduce the metabolic load on the hypertrophic heart.

Myosin from the atrial myocardium in both eutherian [29] and marsupial [39] hearts migrate faster than V_1 in pyrophosphate gel and has a higher Ca^{2+}-ATPase activity than V_1. Atrial myosin has the same αα MyHC composition as V_1 but is associated with atrial-specific MLCs. The N-terminal of MLC1 interacts with actin during cross-bridge cycling. Atrial MLC1 ($MLC1_A$) has a weaker interaction with actin compared with that of ventricular or slow MLC1 ($MLC1_S$) of limb slow fibres.[40] This difference in MLC1 isoform expression between atrial myocardium and V_1 myocardium is associated with a 68% higher maximal velocity of shortening [41] and a F_{min} 2.5-fold higher for atrial myocardium (L Turnbull, G Rossmanith and J Hoh, unpublished observations). The speed of atrial contraction will thus always be faster than ventricular contraction. The functional significance of faster atrial contraction is that it leaves more time during the cardiac cycle for the energetically more demanding ventricular contraction.

Mechanisms of action of inotropic agents

While thyroid hormone tonically controls cardiac contractility by regulating myosin gene expression, inotropic agents influence cardiac contractility phasically through cell signalling mechanisms. We showed that adrenaline enhances F_{min} by about 50% in V_1 myocardium and 25% in V_3 myocardium, these effects were abolished by propranolol, a β-adrenergic blocker. This suggests that adrenaline, which works by enhancing the intracellular level of cyclic AMP (cAMP), enhances the rate of cycling of cross-bridges in the ventricular myocardium.[42] This enables the ventricle to enhance contractility and cope with the reduced duration of the cardiac cycle due to the chronotropic effect of adrenaline. This effect on cross-bridge cycling has been confirmed by the work of others using other methods.[43,44] We showed that dibutyryl cAMP, an agonist of cAMP, also enhanced F_{min}. Likewise, agents that block cAMP phosphodiesterase, the enzyme that catalyzes the hydrolysis of cAMP, namely, isobutylmethylxanthine (IBMX) and caffeine, also increased F_{min}. However, an inotropic agent that does not affect the intracellular level of cAMP, namely, ouabain, had no effect on F_{min}. These findings support the hypothesis that a β-adrenergic receptor-mediated increase in crossbridge cycling rate is due to an increase in intracellular cAMP level.[45]

It is well established that elevation of intracellular cAMP

following β-adrenergic stimulation of the myocardium leads to the activation of protein kinase A, which phosphorylates cardiac isoform of Myosin Binding Protein-C (MyBP-C), c-MyBP-C, and Cardiac Troponin I (c-TnI) but has no effect on ventricular Myosin Light Chain 2 ($MLC2_V$) phosphorylation. Also, the chemical phosphatase 2,3-Butanedione Monoxime (BDM) is known to dephosphorylate the β-adrenergically mediated c-MyBP-C phosphorylation but leave c-TnI phosphorylation relatively intact.[46] We showed that IBMX approximately doubled the F_{min} value of rat papillary muscle, and this action was unaffected by the addition of BDM. In the presence of IBMX and BDM, the level of phosphorylation of c-MyBP-C was not enhanced, that of $MyLC2_v$ was reduced to 60% of control, but phosphorylation of c-TnI was markedly increased. This increase in phosphorylation of c-TnI but not of c-MyBP-C associated with the increase in F_{min} indicates that c-TnI phosphorylation is the molecular basis for the enhanced cross-bridge cycling seen during β-adrenergic stimulation of the heart.[47] This agrees with the work of others using transgenic mice that express a non-phosphorylatable slow skeletal isoform of troponin I in the myocardium.[48]

There is evidence that β-adrenergic stimulation also tonically regulates cardiac contractility by modulating myosin isoform expression, since chronic administration of propranolol, a β-adrenergic blocker, leads to enhanced expression of V_2 and V_3 myosin isoforms.[49]

Endothelin is a powerful inotropic agent secreted by vascular endothelial cells. It has been shown to increase the amplitude of cardiac twitch contraction without significantly increasing the cytosolic calcium transient. We showed that endothelin had no effect on F_{min} but caused an increase in the level of incorporation of $^{32}P_i$ into ventricular $MLC2_V$ without a concurrent change in the level of incorporation of ^{32}Pi into c-TnI seen following β-adrenergic stimulation. We concluded that endothelin exerts positive inotropic effects through an increase in divalent cation sensitivity by increasing $MLC2_V$ phosphorylation.[50] This action of endothelin is analogous to the mechanism of PTP of fast skeletal muscle in which tetanic stimulation induces the phosphorylation of fast MLC2 ($MLC2_F$) which shifts OFF cross-bridges away from thick filaments to become ON cross-bridges (see later).

Immunohistochemical analysis of limb muscle development

We used immunohistochemical methods to study the developmental changes in myosin gene expression in fibres of fast and slow muscles in kittens.[51] During limb muscle development, muscle fibres are formed in two rounds of myotube formation from myoblasts migrating from the somites. Primary myotubes are first formed, increase in diameter and get innervated before a second wave of myoblasts forming secondary myotubes around the primary myotubes. We found that primary and secondary myotubes were characterized by different patterns of myosin gene expression. Primary myotubes in future fast and slow muscles of new-born kittens stained strongly for β-slow myosin but weakly for foetal/embryonic myosins. These myotubes were termed slow primaries. During subsequent development, foetal/embryonic myosins were withdrawn from slow primaries, and they continued to express β-slow myosin in developing slow muscle under the influence of the slow motor nerve. Only a subpopulation of slow primaries in the developing fast muscle became slow fibres, the rest became fast fibres under the influence of the fast motor nerve. In the peripheral region of developing fast muscles, a subpopulation of primary myotubes, referred to as fast primary myotubes, expressed fast myosin in addition to β-slow and embryonic/foetal myosin. These fast primary myotubes end up expressing fast myosin at maturity under the influence of thyroid hormone.[52]

The secondary myotubes stained strongly for foetal/embryonic myosins but not β-slow myosin and could be divided into two subpopulations: i) fast secondaries, in which foetal/embryonic myosins were replaced by fast myosin at maturity, and ii) slow secondaries, in which the foetal/embryonic myosins were replaced by β-slow myosin. Fast secondaries were found principally in the developing fast muscle but formed a minor component of developing slow muscle. Slow secondaries were found principally in the developing slow muscle but formed only a minor component in the developing fast muscle.[51]

It later emerged that the cellular basis of these differences in myosin gene expression in primary and secondary myotubes lies in the fact that two distinct myoblast lineages with different patterns of gene expression during myogenesis emerge sequentially from the somites: embryonic myoblasts, which form primary myotubes, and foetal myoblasts, which form secondary myotubes. Embryonic myoblasts constitutively express genes characteristic of slow fibres during myogenesis in tissue culture, while foetal myoblasts express genes characteristic of fast fibres.[53] The difference in the fate of slow primaries in the developing slow and fast muscles is now known to be due to the influences of the emerging different neural impulse patterns along the nerves to slow and fast muscles and the postnatal surge of thyroid hormone. Slow primaries in developing slow muscle receive Chronic Low Frequency Stimulation (CLFS) from the soleus nerve, which maintains β-slow myosin expression, while slow primaries in developing fast muscle respond to the postnatal surge of thyroid hormone and express fast myosins in the absence of CLFS from the fast nerve.[54]

Fibres of a given phenotype may respond differently to neural and thyroidal influences depending on their developmental origin. Surrounding the fast primaries in the periphery of fast muscles are fast secondaries which also express fast myosin in the adult, so that the peripheral fibres in a fast muscle are devoid of slow fibres. In response to hypothyroidism, only fast fibres of fast primary origin express β-slow myosin.[52]

The above observations show that classification of fibres into phenotypes does not uniquely specify their response to modulating influences. Fibres should also be classified according to their development origins, which has been referred to as their ontotype. The different properties of fibres of diverse ontotypes underlie the incomplete transformation of muscles following nerve cross-union and neural stimulation, previously attributed to the vague notion of different

adaptive ranges of fast and slow muscles.[55] The complex interplay of ontotype, neural and hormonal influences on myosin gene expression and their known molecular mechanisms during the development of limb muscle fibres have recently been reviewed.[54]

Development of antibodies to specific myosin isoforms

As masticatory and extraocular muscles express myosin isoforms not found in limb muscles, to study the expression of myosin isoforms in these muscles at the cellular level, we needed to employ immunohistochemical methods using antibodies specific to masticatory and EOM myosin isoforms. We first raised polyclonal antibodies against cat masticatory MLCs and used it to isolate a masticatory MLC2 ($MLC2_M$) clone from a cDNA library and to sequence the $MLC2_M$ gene.[56] We also raised a polyclonal antibody against masticatory MyHC and used it to isolate a masticatory MyHC clone from the cDNA library in the course of sequencing the masticatory MyHC gene.[57] This anti-masticatory MyHC antibody was also used to study the regeneration [58] and development [59,60] of cat jaw closer muscles.

We next turned our attention to the production of Monoclonal Antibodies (mabs). We initially produced mabs 4A6 and 10A10 against Extraocular (EO) MyHC and used them to study EO MyHC expression in laryngeal muscles and EOMs.[61,62] We also prepared mabs 3F10 and 1H2 to masticatory-specific isoforms of MyBP-C (m-MyBP-C) and tropomyosin (m-Tm) respectively and used them to study the expression of these proteins in cell cultures[63] and in transplanted masticatory muscles.[64]

The pyrophosphate gel electrophoresis method can only differentiate between native fast and slow myosin isoforms and their light chain-based isoforms but does not reveal the known MyHC heterogeneity of limb fast myosins. To study the expression of different isoforms of fast MyHCs, we raised mabs 2F7, 6H1 and 10F5 against MyHCs 2A, 2X and 2B respectively.[65] The specificities of these mabs were verified in representatives of four orders of eutherians (rodent, lagomorph, carnivore, and primate) as well as two orders of marsupials (diprotodonts and dasyuromorphs). These mabs, together with mab 4A6 against EO MyHC, are powerful tools for studying mammalian muscle fibre types. We have deposited their clones at the Developmental Studies Hybridoma Bank at the University of Iowa to enable other investigators to access them. We used these mabs in our studies on MyHC gene expression in limb, laryngeal and EOMs in eutherian mammals and in limb muscles of marsupial mammals, as described below.

Myosin gene expression in marsupial limb and cardiac muscles

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of limb MyHCs from seven marsupial species, spanning two marsupial orders, revealed four MyHC components, each of which specifically cross-reacted in Western blots with a mab against a corresponding eutherian MyHC. For all seven marsupial species, the relative mobility of the band identified by each mab matched that in the rat, showing that the four MyHCs are homologous to eutherian β-slow, 2B, 2X and 2A MyHCs, respectively, in the order of decreasing electrophoretic mobility.[66] Whereas small eutherian mammals like rats and mice express all 4 limb MyHCs, larger eutherians like the cat, dog and horse do not express 2B MyHC in their limb muscles.[54] Unlike eutherian mammals, large and small marsupial mammals express the full complement of limb MyHCs. [52,66,67] Remarkably, there is little or no 2B MyHC expression in the gastrocnemius and flexor digitorum profundus muscles in hopping marsupials, despite the predominance of fibres expressing 2B MyHC in most of their other hindlimb muscles studied.[67] These two muscles are involved in elastic energy saving during hopping [68] and express high levels of 2X MyHC. In sharp contrast, non-hopping marsupials strongly express 2B MyHC in their gastrocnemius muscles.[67] We suggest that the suppression of 2B MyHC expression in these muscles of hopping marsupials and the general suppression of 2B MyHC in large eutherian mammals are due to the possibility that 2X fibres are more efficient than 2B fibres in elastic energy saving. The general suppression of 2B MyHC in large eutherian mammals may be explained by the fact that in eutherians all four limbs and their muscles are involved in locomotion and elastic energy saving.

We also analysed cardiac myosins and their subunit compositions in marsupial mammals.[39] With pyrophosphate gel electrophoresis, ventricular myosin in macropodoids shows the same three isoforms, V_1, V_2 and V_3, as in eutherian ventricles.[29,30] SDS-PAGE of marsupial ventricular myosins reveals two MyHCs, the faster isoform reacts with specific anti-α-MyHC antibody while the slower isoform reacts with anti-β-MyHC antibody. This shows that the MyHC compositions of marsupial V_1, V_2 and V_3 to be αα, αβ and ββ, respectively, as for eutherian cardiac myosins.[30] Interestingly, in some members of the small Antechinus flavipes, we found 2 isoforms of α-MyHC with different Ca^{2+}-ATPase activities.[69] Marsupial atrial myosin shows αα MyHC composition but diverged from V_1 in having atrial-specific MLCs,[39] as in eutherians.[29]

We showed that marsupial ventricular myosins were also subject to thyroid regulation. Hypothyroidism in a small marsupial, which expressed virtually pure V_1, led to a shift towards V_3.[69] Analysis of ventricular myosin of the tammar wallaby during early pouch life revealed that it initially expressed pure V_3 but started to shift towards V_1 expression around the time of pouch exit,[70] coincident with the developmental surge of thyroid hormone secretion. The hormone promotes skeletal muscle maturation and enhances general metabolism. The increase in V_1 expression empowers the heart to meet the consequent enhanced cardiovascular demand, enabling the joey to cope with the heightened activity of post pouch-exit life.

Fibre types in jaw-closing muscles of carnivores

Rowlerson and co-workers first reported the discovery of a new isoform of myosin found in jaw-closing muscles of the

cat.[71] This myosin has a unique MyHC and a unique pair of specific MLCs, and was dubbed "superfast myosin" because cat jaw-closing muscle has a contraction time shorter than that of limb fast muscle and also had a very high AT-Pase activity which normally connotes high contraction speed.[14] Fibres expressing superfast myosin are associated with the development of very high stress and have a high tension cost.[72] It is appropriately expressed predominantly in carnivores to enables them to deliver a very powerful bite during prey capture.[73] However, we showed by dynamic stiffness analysis that the F_{min} of cat masticatory muscle fibres was not higher than that of limb fast fibres and thus were not "superfast".[74] Superfast myosin is thus currently referred to as masticatory myosin.

A possible molecular basis for the high stress feature of masticatory fibres is its expression of m-MyBP-C.[63,75] To appreciate this possibility and the molecular mechanisms for PTP and PTD later, a brief introduction to thick filament structure and the biology of MyBP-Cs is necessary.

In the resting sarcomere, most myosin heads are bound to the core of the thick filament, forming an ordered helical array, the two heads of each myosin molecule are folded back against the thick filament, forming a stable interacting head motif. In resting muscle fibres, these cross-bridges are in a super-relaxed state with very low Mg-ATPase activity[76] and are in the OFF state, being not competent to interact with activated thin filaments. However, a small proportion of myosin heads in resting muscle fibres are shifted away from the thick filament in an ON state with higher Mg-ATPase activity and are competent to bind activated actin filaments.

Recent research has shown that MyBP-C has a strong modulating influence on contractile function of skeletal and cardiac muscles. MyBP-C is a component of the thick filament of striated muscles localized in the middle one-third of each half-thick filament. There are 4 fibre-type specific MyBP-C isoforms: fast (f-MyBP-C), slow (s-MyBP-C), cardiac (c-MyBP-C) [77] and masticatory (m-MyBP-C).[63,75] Slow muscle fibres express only s-MyBP-C, but fast fibres express both f-MyBP-C and s-MyBP-C, while m-MyBP-C is only expressed in masticatory fibres of carnivores. While the C-terminals of MyBP-Cs bind strongly to the thick filament, the N-terminals of MyBP-C can bind either thick or thin filament depending on their phosphorylation status.

The role of c-MyBP-C in regulating muscle contractility has been more extensively investigated in cardiac muscle than in skeletal muscles. In cardiac muscle, unphosphorylated c-MyBP-C binds to both thick and thin filaments.[78] Unphosphorylated c-MyBP-C binding to the myosin cross-bridge on the thick filament near the MLC2,[79] stabilizing the OFF state of the cross-bridge. Upon β-adrenergic stimulation of the myocardium, cAMP-activated protein kinase phosphorylates the N-terminal of c-MyBP-C (in addition to c-TnI referred to above), leading to the turning of OFF cross-bridges into the ON state, enhancing cardiac contractility.[80]

In collaboration with Dr Maki Yamaguchi and colleagues at the Jikei University in Tokyo, we studied thick filament structure of masticatory fibres using X-ray diffraction analysis at the Spring8 Synchrotron Facility. We showed that in dog masticatory fibres, the proportion of cross-bridges in the ON state was significantly higher than that in fast muscle fibres.[81] This may be due to the presence of m-MyBP-C constitutively destabilizing the OFF state of masticatory myosin cross-bridges and turning them into the ON cross-bridges, thereby enhancing Ca^{2+} sensitivity and accelerating the cross-bridge attachment rate during activation which can contribute to the high stress feature of masticatory fibres.

The high stress feature of masticatory fibres may also be due to a structural feature of $MLC2_M$ which may resemble phosphorylated $MLC2_F$ in destabilizing the OF cross-bridges on the thick filament into the ON state,[82] as described below in connection with PTP. This could also lead to enhanced abundance of ON cross-bridges and enhanced Ca^{2+} sensitivity and rate of crossbridge attachment during contraction, leading to the high stress feature of masticatory fibres.

Cat jaw-closing muscles also have some slow fibres which express a jaw-specific β-slow myosin which is immunohistochemically distinct from limb slow myosin.[83] The MyHC of this myosin is identical to that of limb β-slow myosin but its MLCs are identical to those of masticatory myosin.[74] This leads to jaw-slow fibres having very different kinetic properties compared with limb slow fibres. Masticatory MLC1 ($MLC1_M$) has been shown to be identical to embryonic $MLC1_E$ as well as atrial $MLC1_A$.[84] As referred to above in connection with atrial myosin, this $MLC1_{A/E/M}$ isoform has a weaker interaction with actin during the cross-bridge cycle compared with the slow $MLC1_S$ of limb slow fibres.[40] This increases the F_{min} of jaw-slow fibres by ten-fold compared with that of limb-slow fibres.[74] The temperature sensitivity of F_{min} of jaw-slow fibres is reduced relative to that of limb-slow fibres, indicating a reduced activation energy for the cross-bridge cycling, which reflects a reduced stability of the attached cross-bridge [74] resulting in an accelerated cross-bridge detachment rate, leading to a more rapid rate of relaxation. The main function of jaw-slow fibres in jaw-closing muscles of carnivores is to produce a sustained force for holding the jaw against gravity. The rapid relaxation of jaw-slow fibres allows the carnivore to rapidly open the jaw wide prior to seizing a prey with a powerful bite.

Development of masticatory muscles

The finding of a jaw-specific isoform of myosin stimulated our interest in studying its expression during development, its regulation in adult life and the structure of its MyHC and MLC genes. Jaw-closers develop from cells in the dorsal portion of the first branchial arch which derive from the cranial paraxial mesoderm. We analysed cat masseter muscle during perinatal development using immunohistochemical methods. This muscle, which has both masticatory and jaw-slow fibres, has four developmentally distinct myotube types, each characterized by a specific sequence of myosin gene expression during perinatal development: i) slow primaries, which initially express embryonic and β-slow MyHCs but not neonatal MyHC and end up expressing β-slow MyHC at maturity, ii) masticatory primaries, which

initially express embryonic, neonatal and β-slow MyHCs and replace these with masticatory MyHC at maturity, iii) slow secondaries, which initially also express embryonic and neonatal MyHCs and replace these with β-slow MyHC with or without masticatory MyHC, and end up expressing only β-slow MyHC at maturity, and iv) masticatory secondaries, which initially express embryonic and neonatal MyHCs which are progressively replaced by masticatory MyHC.[60] These four myotubes types appear to be homologous to the four ontotypes of developing limb fast muscle. In the developing cat posterior temporalis, which is composed homogeneously of masticatory fibres in the adult, we found only masticatory primary and masticatory secondary myotubes, both of which ended up expressing masticatory myosin at maturity.[59] The posterior temporalis is homologous to the white region of limb fast muscle which is composed homogeneously of fast fibres which develop from fast primary and fast secondary myotubes.[51] I postulate that the postnatal expression of masticatory myosin is dependent on the postnatal surge of thyroid hormone, just as the expression of fast myosin in developing limb fast muscle is thyroid hormone dependent.[54] If so, masticatory fibres of masticatory primary myotube origin would be expected to revert to β-slow myosin expression under hypothyroidism, just as adult limb fast fibres of fast primary ontotype do.[52]

Myogenic and neurogenic regulation of cat jaw-closing muscle

We investigated whether the expression of masticatory myosin was neurogenically or myogenically regulated, using electrophoretic and immunohistochemical methods. Masticatory fibres of the cat temporalis muscle were transplanted into limb fast and slow muscle beds,[58] and limb fast muscles were transplanted into the temporalis muscle bed.[85] The satellite cells in transplanted muscles underwent regenerative myogenesis and became reinnervated by the foreign nerves. Jaw muscle regenerates innervated by a fast limb muscle nerve expressed masticatory myosin but not limb fast muscle myosin, whereas the jaw muscle regenerates innervated by a slow limb muscle nerve expressed not masticatory myosin, nor limb-slow myosin, but jaw-slow myosin.[58,64] Conversely, limb fast muscle regenerate innervated by the jaw muscle nerve expressed limb fast myosin but not masticatory myosin.[85] The transplantation experiments also revealed that m-MyBP-C and m-Tm were co-expressed with masticatory myosin in regenerated muscles.[64] These results indicate that while the nerve may influence myosin gene expression, the phenotypic options for myosin gene expression during myogenesis is determined by the developmental origin of the myogenic cells. The term allotype has been introduced to classify muscles of diverse developmental origins with their unique phenotypic options for myofibrillar gene expression. This notion is supported by the observation that aneural regenerates of cat jaw muscle express masticatory myosin [86] and that myotubes in tissue cultures of cat jaw satellite cells express masticatory myosin as well as m-MyBP-C and m-Tm.[63]
The above experiments also reveal that motor nerves innervating fast and slow muscles could influence myosin gene expression of fibres of masticatory muscle allotype. Since it is known that the conversion of a limb fast muscle into a slow one following cross-innervation by a slow nerve is due to the CLFS delivered by the slow nerve,[87] it seems likely that the expression of jaw-slow myosin in masticatory muscle transplants innervated by the nerve to limb slow muscle is also mediated by CLFS. This was confirmed by the observation that CLFS of masticatory muscle fibres transformed them into jaw-slow fibres.[88]

Sequencing of masticatory MyHC and masticatory MLC2 genes

Using a polyclonal antibody raised against masticatory MLCs, we isolated a full-length cDNA clone encoding $MLC2_M$ gene ($mylc2_m$) from an expression cDNA library prepared from cat masseter muscle, and fully sequenced it.[56] The deduced amino acid sequence of $MLC2_M$ shares 58% overall identity with limb $mylc2_f$ gene, indicating that superfast $mylc2_m$ gene has diverged considerably from limb fast $mylc2_f$ gene during evolution. However, it has a 93% amino acid sequence identity with a known human MLC gene, MYL5,[89] suggesting that MYL5 is a human homologue of $mylc2_m$ gene, consistent with the expression of masticatory myosin in primates.[90] However, masticatory myosin is not expressed in human jaw-closers because of a frameshift mutation in the masticatory MyHC gene (MyHC-16).[91] The possibility remains that human jaw muscle may express jaw-slow myosin, which would include MYL5.
Screening the same cDNA library with an anti-masticatory myosin antibody combined with the use of RACE-PCR enabled us to obtain the full sequence of masticatory MyHC gene, MyHC-16.[57] Sequence comparisons at the DNA and amino acid levels show that cat MyHC-16 gene has less than 70% homology to known mammalian striated muscle MyHC genes, compared with 87-96% between other mammalian fast isoforms themselves, indicating early evolutionary divergence from these MyHC genes.[57] A phylogenetic tree of known mammalian MyHC gene sequences indicates that masticatory MyHC gene was the first mammalian MyHC gene to diverge from the invertebrate MyHC gene.

Phylogenetic plasticity of mammalian jaw-closing muscles

Masticatory myosin is expressed in the shark[73] and crocodile,[92] consistent with the ancient origin of the masticatory myosin heavy[57] and light chain[56] genes. Mammals evolved from carnivorous reptilian ancestors; the high-stress feature of masticatory fibres served these ancestors well in prey capture. We showed that the jaw-closers of some mammals continue to express masticatory myosin, e.g., dog, monkey, flying foxes and microbats, but the sheep expressed β-slow myosin.[90] Masticatory myosin is expressed in the jaw-closing muscles of carnivorous eutherian and marsupial mammals and some non-carnivorous mammals that consume nuts and hard vegetable matter.[73]

During mammalian evolution, new functional demands were made on jaw-closing muscles to cater for the wide range of diets and feeding habits. Mammals need to masticate food to facilitate rapid digestion and absorption of nutrients to support their high metabolic rate. This was achieved by replace masticatory myosin with cardiac and limb myosin isoforms.[73] The range of myosins expressed in jaw-closers of eutherian mammals includes masticatory, limb fast, foetal, β-slow and α-cardiac myosins.[73] The low tension-cost of β-slow myosin is highly appropriate for grazing animals like the sheep and cattle [90] that spend much time feeding and ruminating. Expression of all four limb fibre types in rodents [93] provides a wide range of kinetic properties to deal with their omnivorous diet.

In jaw-closers of marsupials, the range of myosins expressed differs from that of eutherian mammals; they express only masticatory, β-slow and α-cardiac myosins. There are only 3 types of fibres in marsupial jaw-closers: masticatory fibres, α-cardiac fibres and α/β fibres which co-express α-cardiac and β-slow myosins.[94] Masticatory fibres predominate in most marsupial species studied and are appropriate for predation or for chewing tough vegetable matter. The dunnart and the antechinus are marsupial carnivores that feed primarily on large insects and other arthropods, and all their jaw-closer fibres are masticatory.[94]

The jaw-closers of macropodids do not express masticatory myosin at all, but express α-cardiac myosin almost homogeneously.[95] Since the bettong, which is closely related to kangaroos, expressed masticatory, β-slow and α-cardiac myosins, the ancestors of kangaroos presumably also expressed these myosins, but during evolution kangaroos have suppressed the expression of β-slow and masticatory myosins, leaving pure α-cardiac myosin in their jaw-closers. Kangaroos are herbivorous foregut fermenters, in common with sheep and cattle, but they are not ruminants, and unlike sheep and cattle, they do not have a four-chambered stomach to facilitate fermentation. While sheep and cattle express the economic β-slow myosin in their jaw-closers, kangaroos express the more powerful α-cardiac myosin, enabling them to finely masticate their herbaceous food for rapid digestion.[95]

The jaw-closers of other marsupials studied express a mixture of masticatory and α/β fibres, the relative abundance of α/β fibres decreased from 60% to zero in the order: ringtail possum > brushtail possum > bettong > bandicoot > dunnart/antechinus. These variations in jaw-closer fibre type are correlated with decreasing amounts of vegetable matter in the diets of these animals.[94]

Expression and regulation of myosins in laryngeal muscles

The intrinsic laryngeal muscles of mammals have evolved to perform a diversity of functions: airway protection, regulation of respiration and phonation. These functions require muscles with different kinetic properties. The speeds of contraction of two of these muscles, the Thyroarytenoid (TA) which closes the glottis, and the Cricothyroid (CT), which tightens the vocal fold during phonation, are different, the TA has a shorter contraction time, similar in value to that of EOMs. In contrast, the CT has a longer contraction time, comparable to that of limb fast muscle.[96] Sartore and colleagues reported the existence of a new Extraocular (EO) myosin isoform found in EOM which was immunohistochemically distinct from myosins found in somitic muscles.[97] This EO myosin isoform may explain the high speed[98] and the low maximal power output of EOMs.[99] These observations led us to suspect that apparent high speed of TA might be due to the express EO myosin. We used the mab 4A6 against EO MyHC and showed that in the rabbit, EO MyHC was expressed in the TA but not in the CT, which expressed limb myosin isoforms.[61] As the TA is involved in closing the glottis, the functional significance of its expression of EO myosin is to enable the rapid closure of the glottis to enhance airway protection.

We next explored whether EO MyHC expression in laryngeal muscles was neuronally or myogenically regulated. Rat TA muscle has a vocalis division (TA-V) subjacent to the vocal ligament, and a larger external division (TA-X) involved in closing the glottis. Immunohistochemical analysis revealed that all fibres in the rat TA-X co-expressed 2B and EO MyHCs, whereas TA-V had fewer of these fibres, most fibres expressing 2X MyHC. The recurrent laryngeal nerve, which supplies both TA divisions, was transacted and reunited to allow the random reinnervation of the TA muscle fibres. Following this procedure, 16.5% of TA-X fibres expressed 2X MyHC de novo,[100] presumably due to reinnervation of TA-X fibres by nerve fibres originally innervating TA-V, suggesting that TA-X fibres were subject to neural regulation. To investigate the mechanisms regulating myosin gene expression in laryngeal muscles further, we cross-innervated the TA and the Sternohyoid (SH), a somitic muscle which expressed only limb myosin isoforms. The results showed that the SH nerve failed to suppress EO MyHC expression in 96% of TA fibres, while the recurrent laryngeal nerve failed to induce EO MyHC expression in the SH.[101] The results show that the phenotypic options for MyHC expression of laryngeal and somitic muscles are myogenically determined, and that TA and SH differ in muscle allotype.

Influence of body mass on myosin gene expression in cardiac and laryngeal muscles

It is well established that in eutherians, ventricular myosin in small animals is purely V_1, but in large animals it is purely V_3, while animals of intermediate size express all three ventricular myosin isoforms. We showed that ventricular myosins in ten species of marsupial mammals broadly reflected the body mass dependence of ventricular MyHC expression seen in eutherians.[39] These differences in cardiac myosin expression with body mass reflect the need to adjust cardiac output to match changes in metabolic rate per unit body mass, which is inversely related to body mass (see below). However, the kangaroos expressed considerably more α-cardiac MyHC for their body mass compared with eutherians of similar body mass. This is associated with the fact that kangaroos are highly athletic animals. The high α-cardiac MyHC expression en-

hances the contractility of their hearts to match their high metabolic scope and athletic lifestyle.

Body mass of animals strongly influences their basal metabolic rates. The kinetic properties of the myocardium and of the skeletal muscles controlling the respiratory rate need to be adjusted to match changes in metabolic rate with body mass. McMahon, arguing theoretically from elastic similarity, concluded that metabolic rate should scale to (body mass)$^{0.75}$ and physiological cycles should scale to (body mass)$^{-0.25}$.[102] Experimentally determined basal metabolic rates in eutherian[103] and marsupial[104] mammals do scale approximately to (body mass)$^{0.75}$, the specific metabolic rate per unit body mass would thus scales to (body mass)$^{-0.25}$. Heart rate and respiratory frequency are similarly scaled to (body mass)$^{-0.25}$,[105] in accordance with McMahon's theory, thus ensuring that the body gets adequately supplied with well oxygenated blood as animal body size changes. However, the kinetics of various myosin isoforms that constrain heart and respiratory rates scale to body mass with more modest negative exponents, being 0.175, 0.098, 0.048 and 0.041 for β-slow, 2A, 2X and 2B myosins, respectively.[106] This means that changes in kinetics of cardiac, fast and slow skeletal myosins as body mass changes are not fast enough in small animals and too fast in large animals to match the changes in metabolic rate with body mass. In the ventricular myocardium, this is compensated by the expression of the fast V_1 myosin in small animals and a shift towards the slower V_3 myosin as body mass increases.

In laryngeal muscles, the disparity between respiratory rate and kinetics of myosins expressed in laryngeal muscles as functions of body mass is compensated in small animals by recruiting faster myosins not expressed in their limb muscles: EO myosin in rats,[100] 2B myosin in cats,[107] and dogs.[108] In larger animals like horses,[109] baboons [107] and humans,[110] laryngeal muscle speed is reduced by expressing predominantly β-slow and 2A myosins. These variations in cardiac and skeletal myosin isoform expression across species illustrate the phylogenetic plasticity of both cardiac and skeletal muscles, i.e., their capacity to change the pattern of myosin gene expression in response to changing functional demands during phylogeny.

Fibre types and myosin isoforms expressed in EOMs

Muscle fibre types of EOMs and the MyHCs they express are the most complex among all skeletal muscles. EOMs are organized into two layers, a thin orbital layer facing the orbit and a more substantial global layer facing the globe. Both the orbital and global layers are made up of Singly-Innervated Fibres (SIFs) with large *en plaque* nerve endings, and Multiply-Innervated Fibres (MIFs) with small *en grappe* endings. The fibres in the orbital (o) and global (g) layers can be classified using ultrastructural and histochemical criteria into 6 fibre types: oSIF and oMIF in the orbital layer and gSIF red, gSIF intermediate, gSIF white and gMIF in the global layer.

The EOMs express a complex mixture of 11 MyHC isoforms: the four isoforms found in adult limb muscles (2A, 2X, 2B, β-slow MyHCs), foetal/neonatal-MyHC,[111] embryonic MyHC,[112] α-cardiac MyHC[113] and four EOM-specific isoforms: EO MyHC,[114] slow-tonic MyHC,[115] slow B MyHC[116] and non-muscle myosin IIB MyHC.[117]

The expression of such a wide range of MyHCs in EOMs is expected to generate fibres with a very wide range of mechanical properties. We compared the kinetic properties of single rabbit EOM fibres with those of limb fibres using dynamic stiffness analysis. Limb fast fibres have F_{min} values ranging from 10 to 26 Hz while F_{min} for limb slow fibres is 0.5 Hz. EOM fibres do have F_{min} values in the range for limb fast fibres, but in addition have F_{min} values below (4-9 Hz) and above (27-33 Hz) the range for limb fibres.[118] The wider range of kinetic characteristics in EOM fibres compared with limb fibres can be attributed to their expression of embryonic and neonatal MyHCs (low F_{min}) and EO MyHC (high F_{min}) in addition to isoforms present in adult limb muscles.

Distribution of MyHCs in EOM fibre types and functional significance

We initially examined the distribution of MyHC isoforms in the levator palpebrae superioris and retractor bulbi which share their developmental origins with EOMs. These muscles in the rabbit express EO and limb fast MyHCs. The levator palpebrae superioris of monkey and cat also express EO MyHC but this isoform is absent in cat retractor bulbi and rat levator palpebrae superioris and retractor bulbi.[114]

We next investigated the expression of MyHCs in EOM fibre types of the rat and rabbit. In the rat, gSIFs express 2A, 2X or 2B MyHC and show no longitudinal variation in MyHC distribution. However, rat oSIF and oMIF both express faster MyHCs at the endplate zone and slower ones at the end-segments: oSIFs express EO MyHC at the end-plate zone and co-express β-slow and embryonic MyHCs at the end-segments while oMIFs express β-slow MyHC at the endplate zone and embryonic MyHC at the end-segments.[119] The expression of EO MyHC at the central segments of oSIFs in the rat emerge over the first few weeks postnatally.[120]

Orbital fibres of the rabbit are also characterised by the expression of fast MyHCs in the endplate zone, flanked by segments with slower MyHCs. Rabbit oSIFs also express EO MyHC at the endplate zones, the flanking segments express embryonic and 2A MyHCs. The endplate zones of rabbit oMIFs express β-slow and embryonic MyHCs but express embryonic and slow-tonic MyHCs in the flanking end segments.[62]

We propose that the functional significance of having kinetically fast central segments flanked by slower end segments in oMIFs and oSIFs is to linearize the rapid eye movement during a saccade. As the agonist starts to contract at the beginning of the saccade, the rapid relaxation of the central segments of the orbital fibres of the antagonist reduces the load on the agonist to allow the initial acceleration of the eyeball, while the kinetically slow end segments help to break the eyeball rotation towards the end of the saccade.[62,121]

Palisade endings of gMIFs and functional significance

The gMIFs have structures called palisade ending at their myotendinous junctions. These structures resemble immature Golgi tendon organs and are innervated by the same motoneuron innervating the parent gMIF. Non-muscle myosin IIB is found at the centre of thick filaments of gMIFs. I suggest that the functional significance of the super-slow contracting non-muscle myosin IIB myosin is to permit the transmission of passive tension along gMIFs to palisade endings when the EOM is acting as an antagonist during a saccade. Activation of palisade endings elicits neural impulses that trigger axon reflexes on gMIFs, generating sustained tension that play important roles during fixation and vergence.[121] Since gMIFs are polyneuronally innervated, the gMIF/palisade ending complex acts like a motoneuron with afferent synaptic inputs from several gMIF motoneurons, integrating not their endplate potentials, but their tension contributions to stimulate palisade endings to generate neural impulses to gMIFs innervated by its motoneuron. Since each palisade ending can only influence tension output of the motor unit of which it is a part, and the control over this gMIF/palisade ending complex is vested in several gMIF motoneurons innervating the complex, the system allows the gMIF motoneuron pool to exercise exquisitely fine control over EOM tension.

Palisade endings are more abundant in the medial recti and in the inferior recti of front-eyed but not in lateral-eyed animals.[122] These EOMs are involved in convergence movements. The greater abundance of palisade endings would enable these EOMs to develop a strong axon reflex tension during convergence. This would allow the gMIF motoneurons of these EOMs to exercise exquisitely fine control over ripple-free and highly fatigue-resistant tension of the gMIF of these EOMs, and thus the precision in controlling eye position during vergence.[121]

Regulation of MyHC expression in EOMs

To investigate mechanisms regulating the expression of MyHCs in EOMs, we i) transplanted EOMs into a limb fast muscle bed to allow it to be reinnervated by limb muscle nerve and ii) allowed EOMs to regenerate *in situ* to be reinnervated by the oculomotor nerve. The results showed that the patterns of expression of MyHCs of the two types of regenerates were rather similar during the first few weeks, expressing the relatively slow embryonic, β-slow/slow-tonic and 2A MyHCs, but differed substantially in the longer term. The *in-situ* regenerates at 10 weeks additionally expressed the faster 2X, 2B and EO MyHCs. At 22 weeks, fibres in *in-situ* regenerates expressing fast 2B and EOM MyHCs became progressively more dominant and clearly distinct from fibres expressing slow β-slow/slow-tonic MyHCs. By 42 weeks, most of the fibres co-expressed EOM and 2B MyHCs, while expression of 2X and 2A MyHCs weakened. In contrast, transplanted regenerates innervated by limb fast muscle nerve only transiently expressed 2X and 2B MyHCs after 20 weeks but never expressed EO MyHC even up to 42 weeks.[121]

Contrary to earlier work,[123] the above results showed that EOM satellite cells can express EO MyHC during myogenesis, thus supporting the notion that EOMs are a distinct allotype. EOM regenerates differ from regenerating masticatory fibres which express masticatory MyHC even without innervation [86] and in tissue cultures of satellite cells of masticatory muscle.[63] This is because fibres in jaw-closers in carnivores have only 2 degrees of freedom for MyHC expression: masticatory or jaw-slow myosin, which can be adequately catered for by a default mechanism for masticatory myosin expression and a neural mechanism for controlling jaw-slow myosin expression by CLFS.[88] EOMs require a much more sophisticated myogenic and neurogenic mechanisms for controlling the expression of 11 MyHCs. The emergence of distinct fast and slow fibres in the *in-situ* regenerates suggested that there were distinct lineages of satellite cells associate with MIFs and SIFs, which were derived from primary and secondary myotubes, respectively. These results suggest that neural impulses from the limb muscle nerve can only induce limb muscle MyHC isoforms, the capacity of oculomotor nerve to induce EO MyHC can be linked to the fact that unlike limb fast nerve, it can deliver very high frequency impulses likely to be necessary to induce EO MyHC expression.[121]

Oculomotor neurons receive neurotrophins from muscle fibres they innervate for their survival. These neurotrophins specify their synaptic input, and thus the impulse patterns they can generate.[124] It is thus likely that synaptic inputs of slow-tonic and phasic motoneurons innervating MIFs and SIFs, respectively, are modulated during postnatal development by different target-derived neurotrophins they receive from contact with primary and secondary myotubes respectively. In other words, primary and secondary myotubes may specify the tonic and phasic impulse pattern appropriate for their diverse functional roles in the mature EOM by influencing the synaptic inputs of their innervating ocular motoneurons. I propose that the very impulse patterns driving the various ocular movements induce the expression of the appropriate MyHC compositions and thus the mechanical properties of different EOM motor units to accomplish their tasks.[121]

Molecular mechanisms of post-tetanic potentiation and post-tetanic depression

Since the documentation of the phenomena of PTP and PTD during my PhD, the molecular basis of PTP had been progressively unfolded.[125] It is due to the phosphorylation of $MLC2_F$ by Ca^{2+}-calmodulin-activated myosin light chain kinase activated by the elevated intracellular Ca^{2+} level during the tetanus. Phosphorylation of $MLC2_F$ causes cross-bridges that are bound to the thick filament in the OFF state to swing away from the thick filament. This transition has been shown by electron microscopy of isolated thick filaments with phosphorylated $MLC2_F$.[126] In collaboration with Dr Maki Yamaguchi and colleagues, we show, using X-ray diffraction analysis of skinned rabbit fast fibres, that phosphorylation of $MLC2_F$ causes OFF cross-bridges to swing out towards the thin filament into the ON state.[82] The enhanced number of ON cross-bridges

increases the Ca^{2+}-sensitivity of the fibre and accelerates the rate of cross-bridge attachment at submaximal activating Ca^{2+} level,[127] generating PTP.

There has been no research on the molecular mechanism for PTD. A recent report showed that in slow muscle fibres, phosphorylation of s-MyBP-C by cAMP-activated protein kinase enhanced the transient force overshoot at low levels of Ca^{2+} activation and increased the rate of force development at all Ca^{2+} activation levels during the release and re-stretch manoeuvres.[128] It was proposed that unphosphorylated N-terminals of s-MyBP-C bind to thin filaments, thereby forming links between filaments which retard filament sliding, and that phosphorylation of N-terminals of s-MyBP-C by cAMP-activated protein kinase detaches them from thin filaments to bind to thick filaments, transforming OFF cross-bridges into the ON state. I proposed that this phosphorylation of s-MyBP-C is the molecular mechanism for the enhancement of slow muscle force due to β-adrenergic stimulation,[129] and that dephosphorylation of s-MyBP-C could provide a mechanism for PTD.[130] The elevated intracellular Ca^{2+} following a tetanus in a slow muscle fibre is postulated to activate the Ca^{2+}-calmodulin-activated phosphatase, calcineurin, to dephosphorylate s-MyBP-C. The N-terminal of dephosphorylated s-MyBP-C would then detach from thick filaments and attach to thin filaments, shifting ON cross-bridges into the OFF state and forming links between the filaments. This would reduce post-tetanic twitch force and the rate of force development, leading to PTD.

Looking back

In this sojourn I am thankful to Sir John Eccles for directing me into muscle research even though I initially deeply regretted it. I was fortunate to have had the excellent guidance of Dr Russel Close, and to have a succession of talented Honours and PhD students and research assistants, in particular, Susan Hughes, Angela Hamilton, Robin Fitzsimons, Gary Yeoh, Han Qin, Christine Lucas, Lucia Kang and Wendy Zhong. I am also thankful to many national and international collaborators for their valuable contributions over the years. I am extremely fortunate and grateful for the long-term and fruitful collaboration of Dr Gunther Rossmanith, a mathematician and biophysicist, in analysing myosin cross-bridge kinetics of cardiac and skeletal muscle fibres. This enabled us to perform the important task of relating the various myosin isoforms to their contractile speeds. I am also very grateful for the collaboration with Dr Maki Yamaguchi and colleagues at the Jikei University School of Medicine in Tokyo. This enabled us to use the synchrotron X-ray diffraction facilities at Spring8 to analyse cross-bridge orientation in masticatory muscle fibres and in fast skeletal fibres following the phosphorylation of $MLC2_F$ in connection with the mechanism for PTP. Finally, I am grateful to my wife for her love, patience and support over the years, which have been invaluable to my work.

I have enjoyed attending many international conferences over the years. One of the most memorable was the 29th Congress of the International Union of Physiological Sciences (IUPS) held in Sydney in 1983. On that occasion I invited a few muscle scientists, including Sir Andrew Huxley and Dr John Solaro, to dinner at home, and had an enjoyable evening in their company. In 2001, the 34th Congress of the IUPS was held in New Zealand and I took the opportunity to contribute to the muscle research community by organizing a satellite meeting in Sydney on "Muscle fibre types: development, function and regulation". It was attended by 30 delegates from Europe, US, Japan, China and Australia (Figure 1). An unforgettable fact was that the meeting took place a few days before the devastation of New York on September 11. Another memorable meeting was in Umea organized by Prof. Lars-Eric Thornell in the summer of 2002 (Figure 2). Being in Umea was the closest to the artic circle I have ever been, where one could read a

Figure 1. *Group photograph of attendees at the IUPS Satellite Meeting 2001 in Sydney.*

Figure 2. *Group photograph of attendees at the Muscle Meeting 2002 in Umea.*

book at midnight without artificial lighting! In 2003, I was honoured to have been invited by muscle scientists at the University of Padua to deliver some lectures on my work.

Figure 3. *With hosts at the University of Padua.*

I was accompanied by my wife, and we thoroughly enjoyed the hospitality of our hosts in Padua (Figure 3), most memorable was a horse-meat dinner in the home of Prof. Francesco Mascarello! The trip gave us a wonderful opportunity to tour Venice, Florence, Assisi and Rome, which we thoroughly enjoyed. I retired in 2004 and spent much time writing up unpublished works and reviews but regret not being able to continue to probe issue raised by the work done and test hypotheses proposed.

List of abbreviations

BDM, 2,3-butanedione monoxime
CLFS, chronic low frequency stimulation
CT, cricothyroid
EOM, extraocular muscle
F_{min}, stiffness minimum frequency
IBMX, Isobutylmethylxanthine
Mab, monoclonal antibody
MIFs, multiply-innervated fibres
MLC, myosin light chain
m-Tm, masticatory tropomyosin

m-TnI, masticatory troponin I
MyHC, myosin heavy chain
MyBP-C, myosin binding protein-C
PTD, post-tetanic depression
PTP, post-tetanic potentiation
SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIFs, singly-innervated fibres
TA, thyroarytenoid

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Conflict of interest

There is no conflict of interest to declare.

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