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Co-expression of MyHC-15 with other known isoforms in rat muscle spindles

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ABSTRACT

Muscle spindles are skeletal muscle sensory receptors composed of intrafusal fibres, partially encapsulated by connective tissue capsule. This capsule encloses the central A and B regions while leaving the distal C region extracapsular. Several past studies in rat have shown that muscle spindles typically contain a single bag1 fibre, a single bag2 fibre, and two smaller chain fibres. Intrafusal fibres co-express multiple myosin heavy chain (MyHC) isoforms: -slow or -1, -slow-tonic, -α, -2a, -2b, -embryonic, and -neonatal. While MyHC-2x was previously thought absent, the recently discovered MyHC-15 isoform has been identified in the C region of rat bag fibres. Using antibodies specific for nine MyHC isoforms and analyzing four different rat skeletal muscles – soleus, extensor digitorum longus, and the lateral and medial heads of gastrocnemius – we aimed to further characterize the co-expression pattern of MyHC-15 with other known isoforms and to determine whether MyHC-2x is expressed in rat intrafusal fibres. While rodents are widely used as animal models in skeletal muscle research, notable species-specific differences in MyHC isoform expression exist. Our findings revealed that MyHC-15 expression in rat intrafusal fibres is less abundant than in human fibres. MyHC-15 was primarily observed in bag fibres but was not detected in the C region, contrary to previous reports in both rat and human. We confirmed the absence of MyHC-2x in rat intrafusal fibres. Similarly, MyHC-embryonic and -neonatal were not detected in the analyzed spindles, suggesting that previously used antibodies may have cross-reacted with MyHC-2a and -2b. While our results partially corroborate previous extensive studies, discrepancies suggest that MyHC expression in intrafusal fibres varies not only along the fibre length but also across muscles.

Key words: myosin heavy chain isoforms (MyHC); skeletal muscle; muscle spindle; intrafusal fibre; immunohistochemistry.

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Introduction

Muscle spindles are encapsulated mechanosensory receptors located within skeletal muscles, positioned in parallel with extrafusal muscle fibres and concentrated near nerve entry points and intramuscular blood vessels, particularly in deep and middle muscle regions.[1] These spindles contain small intrafusal fibres that detect changes in extrafusal fibre length and tension, conveying proprioceptive information to the central nervous system via afferent innervation. γ-motoneurons innervating intrafusal fibres regulate their sensitivity during extrafusal muscle contraction, enabling effective locomotion and postural control. Muscle spindles are particularly abundant in muscles requiring precise proprioception[2,3] and have recently been implicated in chronic musculoskeletal pain perception.[4,5]

Muscle spindles are divided into three regions: A, B and C.[6] The proximal A and B regions are encapsulated. Region A, containing intrafusal fibre nuclei and sensory nerve endings, is largely non-contractile. Region B contains γ-motoneuron endings and the contractile portions of intrafusal fibres. In the extracapsular C region intrafusal fibres fuse with extrafusal tissue.

Intrafusal fibres are classified into three types. The larger bag fibres have nuclei clustered in a «nuclear bag» and extend beyond the capsule into the C region. The smaller chain fibres, with nuclei arranged in a single row, are located exclusively within the encapsulated A and B regions. Bag fibres are further differentiated into bag1 and bag2 fibres based on size and staining characteristics following histochemical reactions for myofibrillar ATPase.[7] Rat muscle spindles typically contain four intrafusal fibres: one bag1, one bag2, and two chain fibres.[8-10]

The primary determinants of fibre classification and their contractile properties are myosin heavy chain (MyHC) isoforms, in vertebrates encoded by 11 sarcomeric myosin heavy chain (MYH) genes.[11] With the development of isoform-specific antibodies, several studies have investigated MyHC expression in intrafusal fibres.[10,12-17] In addition to MyHC isoforms expressed in extrafusal fibres (MyHC-1/β-cardiac, -2a, and -2b), intrafusal fibres express α-cardiac (MyHC-α) and slow-tonic (MyHC-st) isoforms, the latter more recently discovered to be coded by gene, initially termed MYH14, a later reassigned as MYH7b.[18] The reports about MyHC-embryonic (MyHC-emb) and -neonatal (MyHC-neo) expression remains controversial. While some studies have confirmed their presence,[19,20] others have reported absent or negligible expression levels.[10,15,17] Similarly, the expression of MyHC-2x in rat intrafusal fibres remains unconfirmed, having been previously presumed absent due to the lack of a specific antibody.[10] Despite this, up to seven MyHC isoforms have been identified within intrafusal fibres, demonstrating expression patterns that vary along the fibre length, across different muscle types, and with age, which may contribute to differences between studies.

Generally, bag1 fibres abundantly express MyHC-st throughout their length, in the B and C regions they may also express MyHC-1, -α, -2a and -emb. Bag2 fibres in soleus (SOL) express MyHC-1, -α, and -st throughout their length, with additional MyHC-2a and -2b expression in the A region and MyHC-2b in the B region. In fast extensor digitorum longus (EDL), bag2 fibres primarily express MyHC-st in the A region, while MyHC-emb and -neo are reportedly expressed throughout most of their length. Chain fibres are predominantly reactive to antibodies against MyHC-2a and -2b, with potential MyHC-α expression.[10,21-23] Some studies report weak chain fibre positivity for MyHC-emb and/or MyHC-neo.[2,10,15,17,19,20] However, there are differences in the pattern of MyHC expression not only along the intrafusal fibre length but are age-related as well.[14,15,17,24,25]

The more recently discovered MYH15,[18] which is an orthologue of a gene expressed in the adult chicken ventricle,[11] was reported to be expressed not only in the orbital layer of rat extraocular muscles, but in the extracapsular region of a subset of rat bag fibres,[26] in human extraocular muscles[27] and, most recently, in human intrafusal fibres.[28] Its corresponding protein, MyHC-15, shares similarities with cardiac MyHC isoforms and is hypothesised to possess slow contractile properties.[18] All these add an additional layer of complexity to the MyHC isoform profile of intrafusal fibres.[11] Therefore, in this study we aimed to further characterise the co-expression pattern of MyHC-15 with other known MyHC isoforms in rat intrafusal fibres and to determine whether MyHC-2x is expressed in rat intrafusal fibres using two distinct, isoform-specific antibodies.

Materials and Methods

Muscle samples

Muscle spindles from the right hind limb of ten adult (33-week-old) male Wistar rats (HsdRccHan™: WIST) were analysed. The muscles sampled included three SOL, four EDL, two lateral gastrocnemius (GL), and three medial gastrocnemius (GM). Following euthanasia, muscles were excised and frozen in liquid nitrogen for storage at -80°C until cryosectioning. Serial muscle cryosections (5 μm) were prepared from each sample. For the SOL and EDL muscles from one animal, we performed serial cryosectioning to obtain multiple series of sections. In each series, 20 consecutive sections were cut. Then, from each series, one section was mounted per slide. This procedure was repeated 25 times, resulting in 25 series of sections. In other words, we ended up with 25 sets (or series) of 20 consecutive sections, each set representing different regions of the muscle spindles. For the remaining muscles (GL and GM), we prepared only one series of consecutive sections due to tissue availability. All animal procedures were conducted in accordance with Slovenian legislation and the European Convention ETS 123, Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes.[29] Ethical approval was granted by the National Medical Ethics Committee of the Republic of Slovenia (Licence No. 34401-61/2007/7). Animals were obtained from the Medical Experimental Centre (Ljubljana, Slovenia).

Immunohistochemistry

MyHC isoform expression in intrafusal fibres was assessed using a panel of commercially available and previously validated isoform-specific antibodies, most of which were previously described for human muscle spindles.[28] Table 1 provides details of the antibodies used, including their specificity, dilution, and source. MyHC-st and MyHC-15 were detected using two rabbit polyclonal antibodies, anti-MYH14 and anti-MYH15 respectively, kindly provided by Schiaffino and colleagues.[32] MyHC-1 was detected using BA-D5,[32] while MyHC-α was detected using BA-G5, either kindly provided by Schiaffino or purchased from Abcam (Cambridge, UK). MyHC-2a was detected using SC-71.[32] Two antibodies, 6H1 and 6F12H3, reported to be specific for MyHC-2x, were used to assess its expression, while MyHC-2b was detected using BF-F3 and 10F5.[32-34] Supernatants for antibodies BA-D5, SC-71, and BF-F3 were produced in-house at the Blood Transfusion Centre of Slovenia from cell lines obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Supernatants for 6H1 and 10F5 were purchased from the Developmental Studies Hybridoma Bank (DSHB, University of Iowa, USA). Antibody 6F12H3 was purchased from Merck (Merck KGaA, Darmstadt, Germany). In two muscle samples (SOL and EDL), sectioned into five consecutive series, MyHC-emb and MyHC-neo expression was assessed using antibodies MHC-d and MHC-n, respectively, both purchased from Leica Biosystems Newcastle Ltd. (Newcastle Upon Tyne, UK). Sections intended for incubation with anti-MYH14 and anti-MYH15 antibodies were pre-incubated in phosphate-buffered saline containing 0.5% bovine serum albumin (PBS/BSA) and 2% goat serum for 30 min. Binding of these antibodies was detected using a horseradish peroxidase-conjugated secondary antibody (PO448, Dako, Glastrup, Denmark). Sections were incubated overnight at 4°C in a humidified chamber with appropriate dilutions (Table 1) of primary antibodies BA-D5, BA-G5, MYH6, SC-71, BF-F3, 6H1, MHC-d, and MHC-n in PBS/BSA containing 2.5% rabbit serum. Binding of these primary antibodies was detected using a horseradish peroxidase-conjugated secondary antibody (PO260, Dako) diluted 1:100 in PBS/BSA containing 2.5% rabbit serum, with the exception of 6H1. Due to the weak staining observed with 6H1, its binding was also assessed using the NovoLink Polymer Detection System according to the manufacturer's instructions (Leica Biosystems, Newcastle, UK).

Sections intended for incubation with antibody 6F12H3 were treated according to a slightly modified protocol adapted from Sawano and co-workers.[34] Briefly, sections were fixed by microwave treatment for 5 min while immersed in PBS, followed by a 30-min cooling period and a brief wash in PBS containing 1% Triton X-100. After blocking for 1 h in PBS/BSA containing goat serum (1:40 dilution), sections were incubated overnight at 4°C with the primary antibody (2 µg/mL) diluted in PBS/BSA. Subsequently, sections were incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated goat-anti-rat IgG (H&L) secondary antibody diluted 1:250 in PBS containing goat serum (1:40 dilution).

Binding of the secondary antibody was visualised by incubation in 0.05% diaminobenzidine tetrahydrochloride hydrate (DAB) and 0.01% H_2O_2 in 0.2 M acetate buffer (pH 5.2) for approximately 7 min in the dark.[35,36] For each set of analysed samples, a negative control slide with serial sections was included. These control sections were incubated in PBS/BSA without primary antibody, followed by incubation with the appropriate secondary antibody or the NovoLink Polymer Detection System.

Muscle spindles analysis

Muscle spindles were identified in consecutive serial muscle sections stained with different antibodies. Images were captured using a digital camera (Kern, Germany) mounted on an Eclipse E80i microscope (Nikon, Japan). A total of 20 muscle spindles were analysed: four in SOL, eight in EDL, three in GL, and five in GM. Intrafusal fibres were classified as bag1, bag2, or chain fibres based on their MyHC expression patterns, as described in previous studies.[10,15,17] MyHC isoform expression was analysed in the A, B, and C regions of the spindles, as visualized in the muscle cross-sections. The B region provided the optimal visualisation for analysis. Staining intensity was visually estimated and classified into four categories: intensively stained (++), moderately stained (+), weakly stained (±), and unstained (-) (Table 2). Given the constraints of a limited sample size, our study was primarily descriptive in its aim, precluding the application of statistical analyses.

Results

Consistent with previous reports, most analysed muscle spindles contained two larger bag fibres and two smaller chain fibres within the encapsulated regions. Bag fibres were further classified as bag1 or bag2 based on established MyHC expression patterns.[10,15,17] Specifically, the generally smaller bag fibre, exhibiting strong MyHC-st positivity in the A and B regions, was classified as bag1, while the larger bag fibre with strong MyHC-1 positivity was classified as bag2. Table 2 summarises the staining characteristics of rat intrafusal fibres using antibodies specific to various MyHC isoforms.

MyHC expression pattern in the SOL muscle

In the slow SOL muscle, four muscle spindles were analysed: three within the B region only, and one in the B (Figures 1 and 2) and C regions (Figure 3 a-e). In addition to the predominant expression of MyHC-st, bag1 fibres exhibited weak to moderate MyHC-2a expression. Some bag1 fibres were also labelled by antibodies specific to MyHC-1, and in one sample, they were positive for MyHC-α in the B region. The anti-MYH15 antibody labelled bag1 fibres in the B region of two spindles with moderate to weak intensity, but no staining was observed in the C region. Bag2 fibres strongly expressed MyHC-1 and -α, and some also expressed MyHC-st and -2b. Notably, MyHC-2b was not detected in extrafusal fibres of the SOL, as expected. Chain fibres predominantly expressed MyHC-2a and -2b, with the latter detected by BF-F3 (Figure 2). Some chain fibres also expressed MyHC-α and -1. All intrafusal fibres were negative for MyHC-2x. In the C region, both bag fibres expressed only MyHC-1, mirroring the expression pattern observed in most extrafusal fibres. In the sample where antibodies specific for MyHC-emb and -neo were applied, no positive staining was observed in intrafusal fibres.

Table 1. Antibodies used to detect myosin heavy chain (MyHC) isoforms in rat intrafusal fibres.

Antibody	Specificity	Dilution	Reference
MHC-d	MyHC-embryonic	1:20	Ecob-Prince *et al.*[31]
MHC-n	MyHC-neonatal	1:10	Ecob-Prince *et al.*[31]
BA-D5	MyHC-1 (β-cardiac)	1:200	Schiaffino *et al.*[32]
BA-G5	MyHC-α (α-cardiac)	1:100	Rudnicki *et al.* [30]
anti-MYH-14	MyHC-slow-tonic	1:100	Rossi *et al.*[26]
anti-MYH-15	MyHC-15	1:100-400	Rossi *et al.*[26]
SC-71	MyHC-2a	1:100	Schiaffino *et al.*[32]
6H1	MyHC-2x	1:50	Lucas *et al.*[33]
6F12H3	MyHC-2x	2 µg/mL	Sawano *et al.*[34]
10F5	MyHC-2b	1:20	Lucas *et al.*[33]
BF-F3	MyHC-2b	1:20	Schiaffino *et al.*[32]

MyHC expression pattern in the EDL muscle

In the fast EDL muscle, eight muscle spindles were analysed: one in all three regions (A, B, and C), one in the A and B regions, three in the B region (Figures 4 and 5), and three in the C region only (Figure 3 f-j). Bag1 fibres expressed MyHC-st throughout all three regions. In the A region, they also expressed MyHC-2b, and some were positive for MyHC-15. In the B region, most bag1 fibres expressed MyHC-1 and -α, with some also expressing MyHC-2a and -2b. In the C region, bag1 fibres expressed only MyHC-st, -1, and -α (the latter not shown in Figure 3). Bag2 fibres consistently expressed MyHC-st, -1, and -2b in the A region, with some also expressing MyHC-α, -15, and -2a. In the B region, MyHC expression was similar, except for weaker or absent MyHC-st expression. In the C region, bag2 fibres expressed only MyHC-1 and -α. Chain fibres moderately expressed MyHC-2a and -2b in the A region, with more pronounced MyHC-2b expression in the B region. Notably, the two antibodies used to detect MyHC-2b, BF-F3 and 10F5, exhibited differential labelling of intrafusal fibres, while staining extrafusal fibres equally (Figure 4). Specifically, 10F5 showed minimal labelling of intrafusal fibres compared to the distinct labelling observed with BF-F3. Some chain fibres also expressed MyHC-15 and -α. Despite the presence of numerous MyHC-2x positive extrafusal fibres in EDL, confirmed by both 6H1 and 6F12H3 antibodies, all intrafusal fibres were negative for MyHC-2x. Importantly, no bag fibres exhibited anti-MYH15 labelling in the C region (Figure 3h). Furthermore, no intrafusal fibres in any of the three regions of EDL were labelled by antibodies specific for developmental MyHC isoforms.

MyHC expression pattern in the lateral head of GL muscle

In the two GL muscles, three muscle spindles were analysed: one in the A region and two in the B region. Bag1 fibres expressed MyHC-st in both regions. In the A region, MyHC-st was co-expressed with MyHC-1 and -15, while in the B region, it was co-expressed with MyHC-α, and in some fibres, additionally with MyHC-1, -2a, and -2b. Bag2 fibres expressed MyHC-1 and -α in both regions. In the A region, these isoforms were co-expressed

Table 2. Myosin heavy chain (MyHC) isoform expression in intrafusal fibres of rat soleus, extensor digitorum longus, and gastrocnemius muscles.

A region			**B region**			**C region**	
m. soleus							
				(n=4)		**(n=1)**	
			bag1	**bag2**	**chain**	**bag1**	**bag2**
			st++	st+/ ±	-	-	-
			1-/++	1++	1-/±	1++	1++
			-	α++	α+/-	-	-
			15+/-	-	-	-	-
			2a+/±	-	2a++/±	-	-
			-	2b+/-	2b++/-	-	-
m. extensor digitorum longus							
	(n=2)			**(n=5)**		**(n=4)**	
bag1	**bag2**	**chain**	**bag1**	**bag2**	**chain**	**bag1**	**bag2**
st++	st++	-	st++	st-/±	-	st++/±	-
-	1++	-	1++/-	1++	-	1++	1++
-	α-/++	-	α++/-	α++	α+/±/-	α++/+	α++
15+/-	15+/-	-	15-/±/+	15-/±/+	15-/±	-	-
-	2a+/-	2a+/±	2a-/±	2a-/±	2a-/±	-	-
2b+	2b+	2b+	2b-/+	2b++/+/±	2b++/+/±	-	-
m. gastrocnemius, lateral head							
	(n=1)			**(n=2)**			
bag1	**bag2**	**chain**	**bag1**	**bag2**	**chain**		
st++	st++	-	st++	st+/-	-		
1++	1++	-	1+/-	1++	-		
-	α++	-	α++	α++/+	α++/+		
15+	15+	-	15+/-	-	-		
-	-	2a++	2a+/-	-	-		
-	2b+	2b+	2b-/+	2b± /-	2b++/+		
m. gastrocnemius, medial head							
	(n=1)			**(n=4)**			
bag1	**bag2**	**chain**	**bag1**	**bag2**	**chain**		
st++	st++	-	st++	st-/+	-		
-	1++	-	1++/-	1-/+/++	1+/-		
-	α++	α+/±/-	α+/++/-	α++	α+/++/-		
15±	15±	15±	15±	-	-		
2a-/±	2a-/±	2a-/±	2a±/-	2a±/-	2a-/±		
2b-/+	2b±/+/-	2b++/+	-	2b++/+/±	2b++/+/±		

Staining intensity of bag1, bag2, and chain fibres with antibodies against nine MyHC isoforms [MyHC-slow-tonic (-st), -1, -α, -2a, -2x, -2b, -15, -embryonic (-emb), and -neonatal (-neo)] is presented for different regions (A, B, C) of muscle spindles. Intrafusal fibres were classified into four categories based on staining intensity: intensively stained (++), moderately stained (+), weakly stained (±), and unstained (-).

with MyHC-st, -15, and -2b, while in the B region, some fibres also co-expressed MyHC-st and -2b. Chain fibres co-expressed MyHC-2a and -2b in the A region and MyHC-α and -2b in the B region. No intrafusal fibres were positive for MyHC-2x, although this isoform was expressed in some extrafusal fast fibres.

MyHC expression pattern in the medial head of GM muscle

In the three GM muscles, five muscle spindles were analysed: one in the A region and four in the B region (Figure 6). In the A region, bag1 fibres strongly expressed MyHC-st, with some exhibiting weaker expression of MyHC-2a and -2b. In the B region, most bag1 fibres expressed MyHC-1 and -α, in addition to MyHC-st and -2a. Bag2 fibres in the A region strongly expressed MyHC-1, -st, and -α, with less abundant expression of MyHC-15, -2a, and -2b. In the B region, bag2 fibre expression was more variable, with consistent and strong expression of MyHC-α, more pronounced MyHC-2b expression compared to the A region, and an absence of MyHC-15. In both regions, MyHC-2b was the most prominent isoform expressed in most chain fibres. Chain fibres in the A region exhibited variable positivity for MyHC-15, -α, and -2a. MyHC expression in the B region was similar, except for the absence of MyHC-15 and the presence of MyHC-1 in some chain fibres. As observed in other muscles, no intrafusal fibres were labelled by either of the two MyHC-2x specific antibodies.

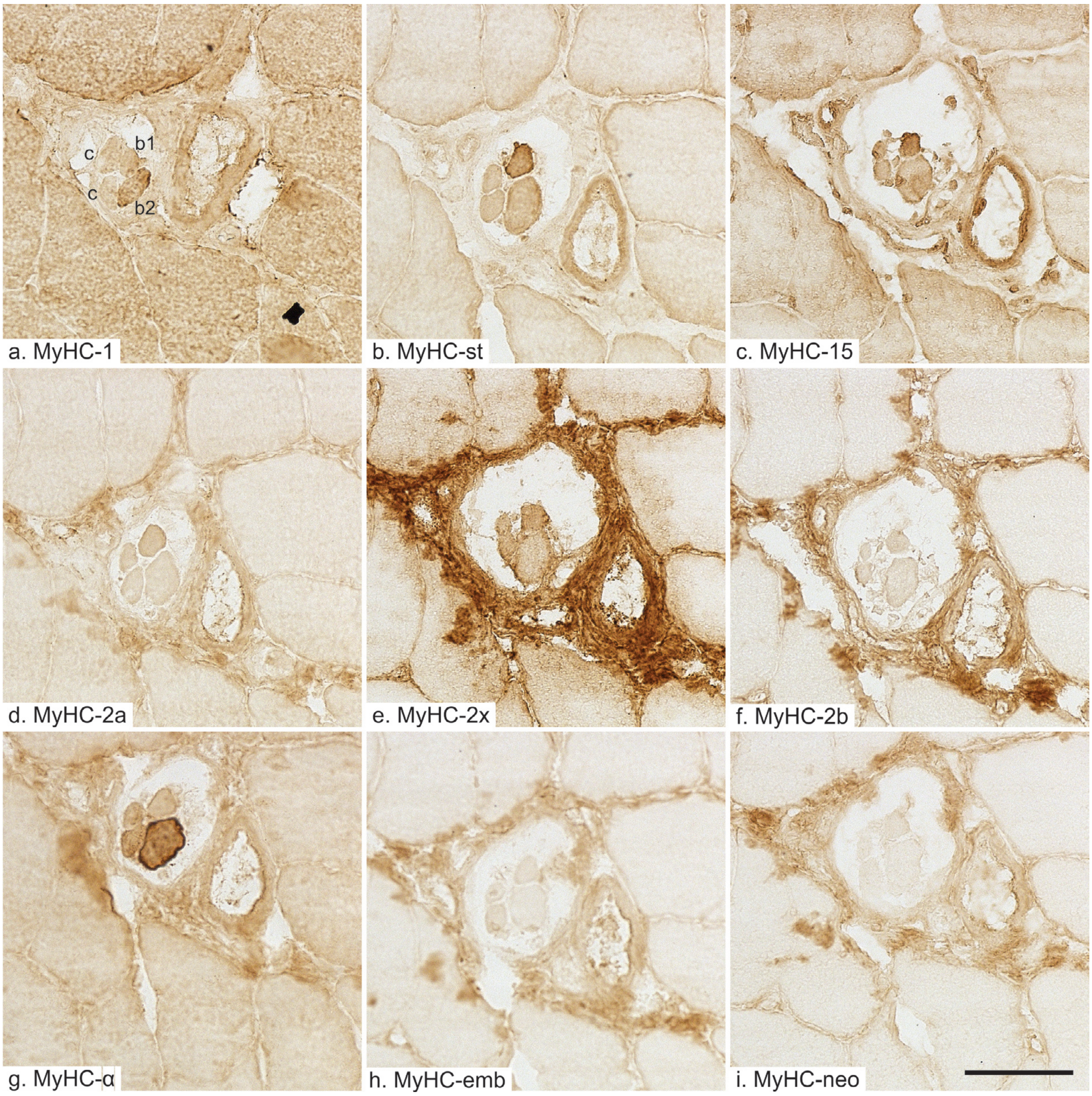

Figure 1. MyHC isoform expression in the B region of a muscle spindle in rat soleus muscle (magnification 40x). Serial muscle sections were stained with antibodies specific for MyHC-1 (**a**); MyHC-st (**b**); MyHC-15 (**c**); MyHC-2a (**d**); MyHC-2x (6H1 antibody) (**e**); MyHC-2b (BF-F3 antibody) (**f**); MyHC-α (**g**); MyHC-emb (**h**); MyHC-neo (**i**) Panel (**a**) indicates the location of bag1 (b1), bag2 (b2), and chain (c) fibres. Scale bars: 50 µm.

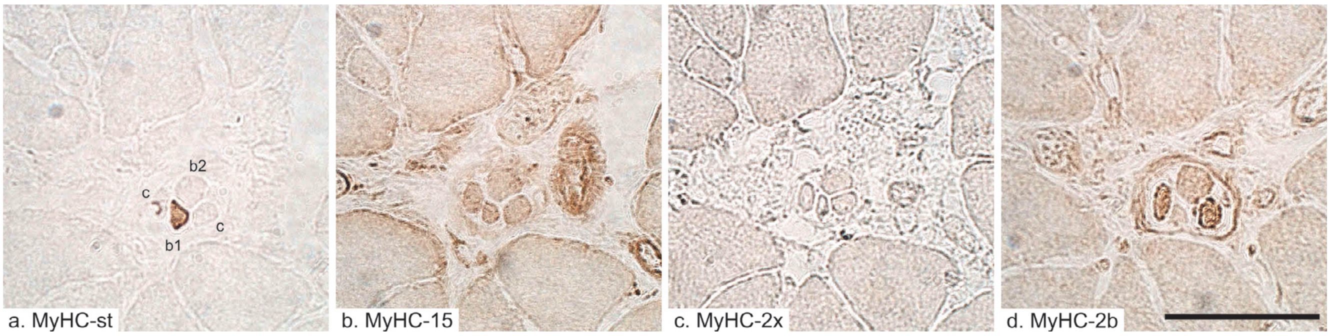

Figure 2. MyHC isoform expression in the B region of a muscle spindle in rat soleus muscle (magnification 40x). Serial muscle sections were stained with antibodies specific for MyHC-st (**a**); MyHC-15 (**b**); MyHC-2x (6F12H3 antibody) (**c**); MyHC-2b (BF-F3 antibody) (**d**). Panel (**a**) indicates the location of bag1 (b1), bag2 (b2), and chain (c) fibres. Note the expression of MyHC-2b in chain fibres, despite its absence in extrafusal fibres of this muscle. Scale bars: 50 µm.

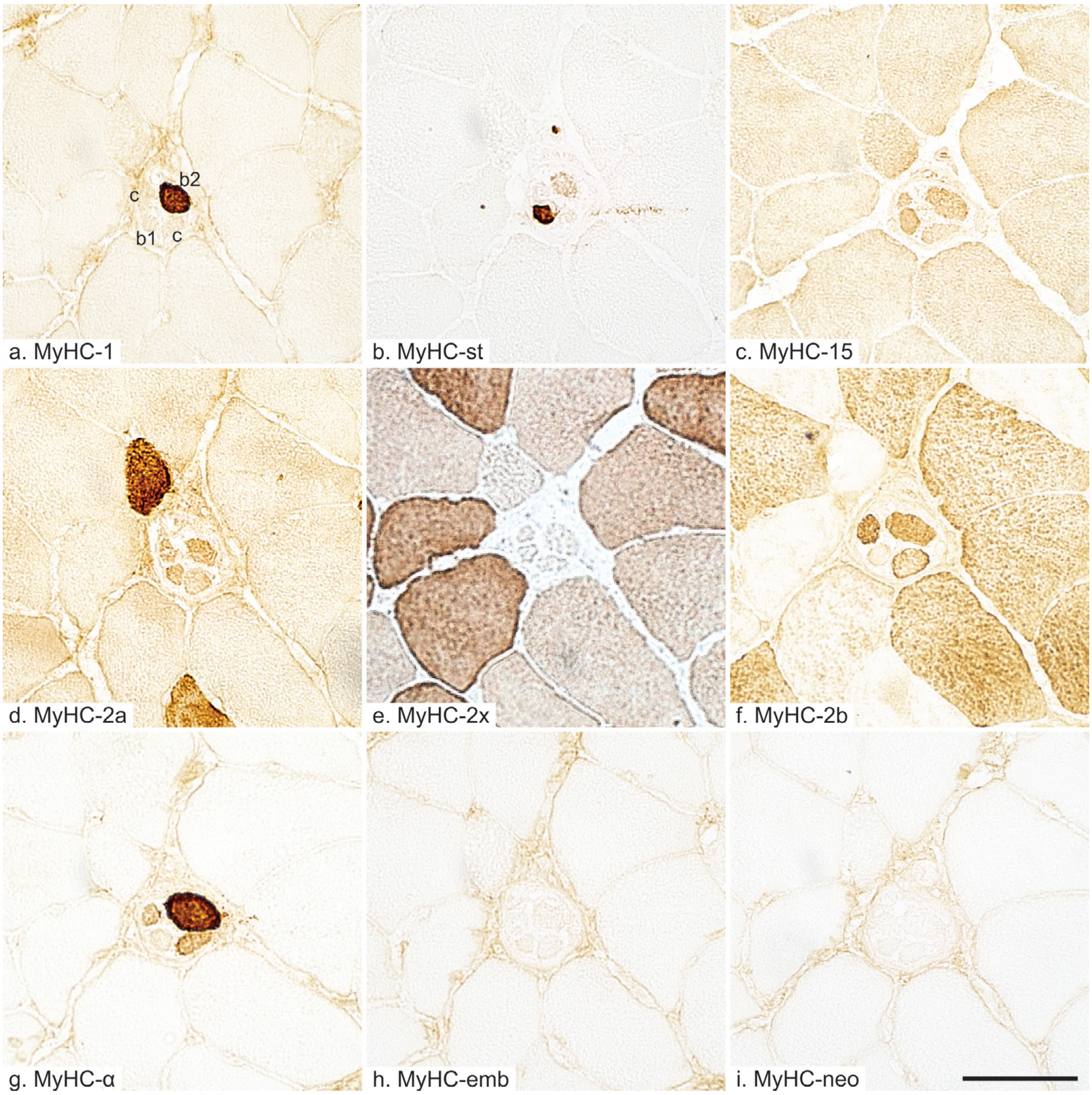

Figure 3. MyHC isoform expression in the B region of a muscle spindle in rat extensor digitorum longus muscle (magnification 40x). Serial muscle sections were stained with antibodies specific for MyHC-1 (**a**); MyHC-st (**b**); MyHC-15 (**c**); MyHC-2a (**d**); MyHC-2x (6F12H3 antibody) (**e**); MyHC-2b (BF-F3 antibody (**f**); MyHC-α (**g**); MyHC-emb (**h**); MyHC-neo (**i**). Panel (**a**) indicates the location of bag1 (b1), bag2 (b2), and chain (c) fibres. Scale bars: 50 µm.

Discussion

This study confirmed previous findings that MyHC-15, while not abundantly expressed, is present in rat intrafusal fibres. Its expression was almost exclusively localised to bag fibres within the encapsulated regions of the muscle spindle, with no detectable expression in the extracapsular region. Our findings also confirm previous suggestions that MyHC-2x is not expressed in rat intrafusal fibres. Furthermore, the absence of MyHC-emb and -neo expression in this study suggests that antibodies used in previous studies may have cross-reacted with the fast MyHC isoforms, -2a and -2b, both of which were clearly identified in our analysis. We observed some inter-muscle variations in MyHC expression patterns. While our results generally align with previous reports, some of discrepancies may be attributable to differences in animal age and antibodies used across studies. While MyHC-15 has been previously reported in the C region of some rat bag fibres,[26] our study did not detect its expression in this region. Conversely, we observed moderate to weak staining of most bag fibres in the A region with the anti-MYH15 antibody. In one GM spindle, weak labelling of chain fibres was also noted (*data not shown*). In the B region, MyHC-15 was predominantly localised to bag1 fibres in all muscles except EDL, where moderate to weak expression was observed in some bag2 and chain fibres. Similarly, our previous investigation of human muscle spindles revealed relatively low MyHC-15 expression, also primarily in bag fibres, but with pronounced expression in the C region.[28] MyHC-15 is presumed to be a slow isoform based on its high amino acid sequence homology with both cardiac isoforms.[11,18] Orthologues of MyHC-15 have been identified in the skeletal muscles of various species, including snakes and mammals. Beyond its role in skeletal muscle, MyHC-15 appears to have functional significance in other tissues, as evi-

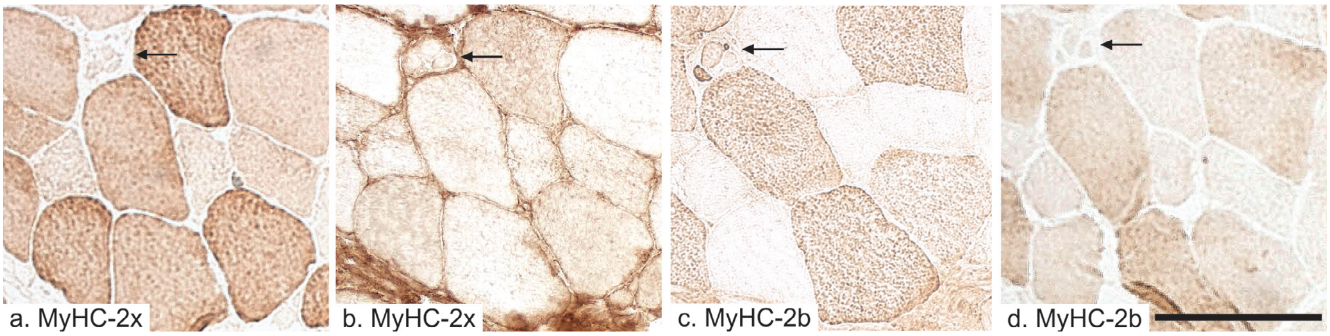

Figure 4. Differential staining of intrafusal (arrow) and extrafusal fibres in rat extensor digitorum longus muscle with antibodies specific for MyHC-2x and MyHC-2b (magnification 40x). Serial muscle sections were stained with 6F12H3 (MyHC-2x) (**a**); 6H1 (MyHC-2x) (**b**); BF-F3 (MyHC-2b) (**c**); 10F5 (MyHC-2b) (**d**). Note the absence of staining in all intrafusal fibres with both MyHC-2x antibodies and the 10F5 antibody (MyHC-2b), while BF-F3 labels both chain fibres (**c**). Also note the difference in staining intensity of extrafusal fibres with the two MyHC-2x antibodies. Scale bars: 50 µm.

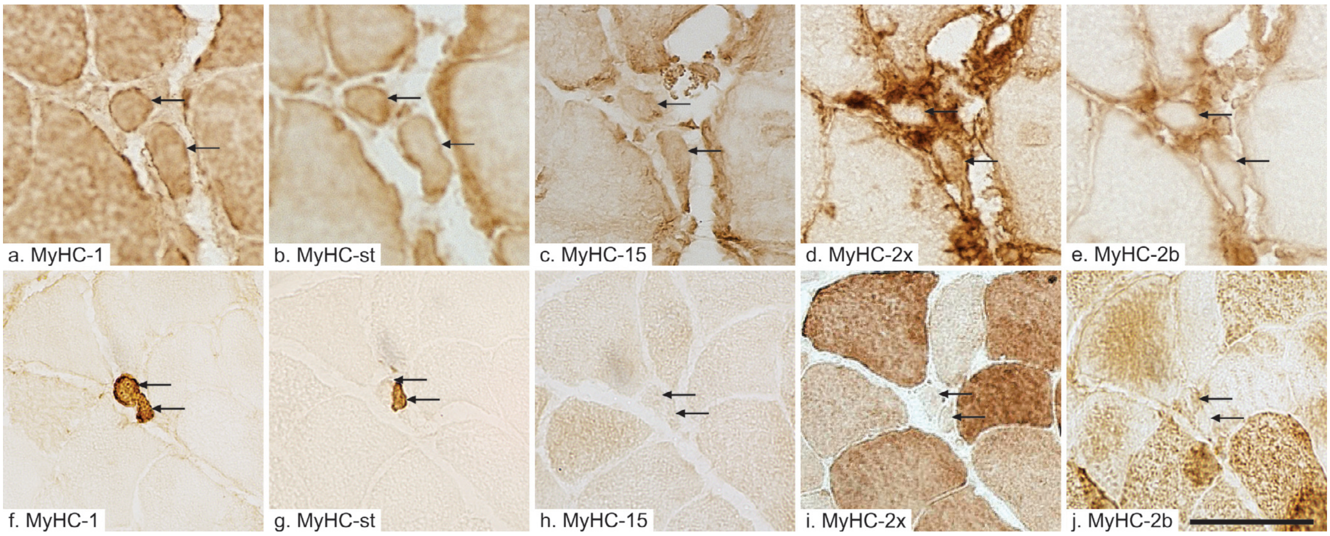

Figure 5. MyHC isoform expression in intrafusal bag fibres (arrows) within the C region of muscle spindles in rat soleus (**a-e**) and extensor digitorum longus (**f-j**) muscles (magnification 40x). Serial muscle sections were stained with antibodies specific for MyHC-1 (**a,f**); MyHC-st (**b,g**); MyHC-15 (**c,h**); MyHC-2x (6H1 antibody) (**d**); MyHC-2x (6F12H3 antibody) (**i**); MyHC-2b (BF-F3 antibody) (**e,j**). Note the absence of MyHC-2x and MyHC-15 staining in both bag fibres. Scale bars: 50 µm.

denced by its detection in pulmonary vascular endothelium and alveolar macrophages.[11,27,37]

Using two specific antibodies, we confirmed the absence of MyHC-2x expression in intrafusal fibres of the analysed rat muscle spindles, corroborating previous observations.[10] Initially, we employed the 6H1 antibody[33] as in our previous studies.[29,36] However, its weak reactivity, particularly in rat tissue, necessitated the use of the NovoLink detection system to enhance visualisation. This resulted in increased background staining (Figures 1e, 4b, 6g), hindering reliable assessment of MyHC-2x expression in intrafusal fibres. Fortunately, the 6F12H3 antibody,[34] exhibited more distinct and reliable staining. The clear demarcation between stained fast extrafusal fibres and unstained intrafusal fibres observed with 6F12H3 provides strong evidence for the absence of MyHC-2x in rat muscle spindles, as previously suggested.[10]

The absence of MyHC-2x expression in intrafusal fibres is intriguing, especially as MyHC-2b, the fastest rat MyHC isoform, exhibited variable expression in intrafusal fibres of both fast and mixed muscles, including chain fibres of the slow SOL. Thus, the reason why MyHC-2x does not appear to meet the contractile requirements of rat intrafusal fibres remains unclear. It is noteworthy that MyHC-2x, the fastest MyHC isoform expressed in human extrafusal fibres[39] has been detected also in intrafusal fibres, particularly in chain fibres, albeit at much lower levels than MyHC-2b, which is not expressed in human extrafusal fibres.[40,41]

The MyHC expression patterns observed in rat intrafusal fibres in this study are only partially consistent with previous findings.[10,14-17,22-24] The differences may be partly attributed to the older age of the animals (7.6 months) used in our study compared to those in previous studies. It is well documented that the expression of MyHC isoforms in intrafusal fibres is subject to age-related modifications.[14,15,17,24,25] Furthermore, despite previous reports indicating no differences in MyHC isoform expression within intrafusal fibres between the slow soleus and fast tibialis anterior muscles,[10] our analysis revealed inter-muscle variations.

The expression pattern of MyHC-st in the A and B regions of bag fibres was generally consistent with previous reports.[2,10] Analysis of the C region, conducted exclusively in the SOL and EDL muscles, revealed a difference between these two muscles, with MyHC-st expressed in the fast EDL but not in the slow SOL.

The MyHC-1 expression was predominant in bag2 fibres within the A region of EDL and GM muscles, while both bag fibre types exhibited comparable expression levels in GL. In the B region, MyHC-1 expression remained generally more pronounced in bag2 fibres, with variable expression in bag1 fibres. Weak MyHC-1 labelling was also observed in some chain fibres of the slow SOL and mixed GM muscles. In the C region, both bag fibres expressed MyHC-1, consistent with previous findings.[10]

The expression of MyHC-1 in bag fibres is thought to be linked to their developmental origins. Bag2 fibres, likely originating from primary myotubes that express this slow isoform, would thus be expected to express MyHC-1.[24,42,43] Conversely, in bag1 fibres, which develop from secondary myotubes, the initial expression of MyHC-1 is presumably suppressed by afferent neuron innervation. Chain fibres, also developing from secondary myotubes expressing fast isoforms, were not expected to express MyHC-1.[10] However, our results suggest that some chain fibres in SOL and GM muscles may undergo a transition towards MyHC-1 expression, indicating inter-muscle differences in MyHC isoform expression profiles.

MyHC-α expression served as an additional marker for identifying muscle spindles in cross-sections. We employed the BA-G5

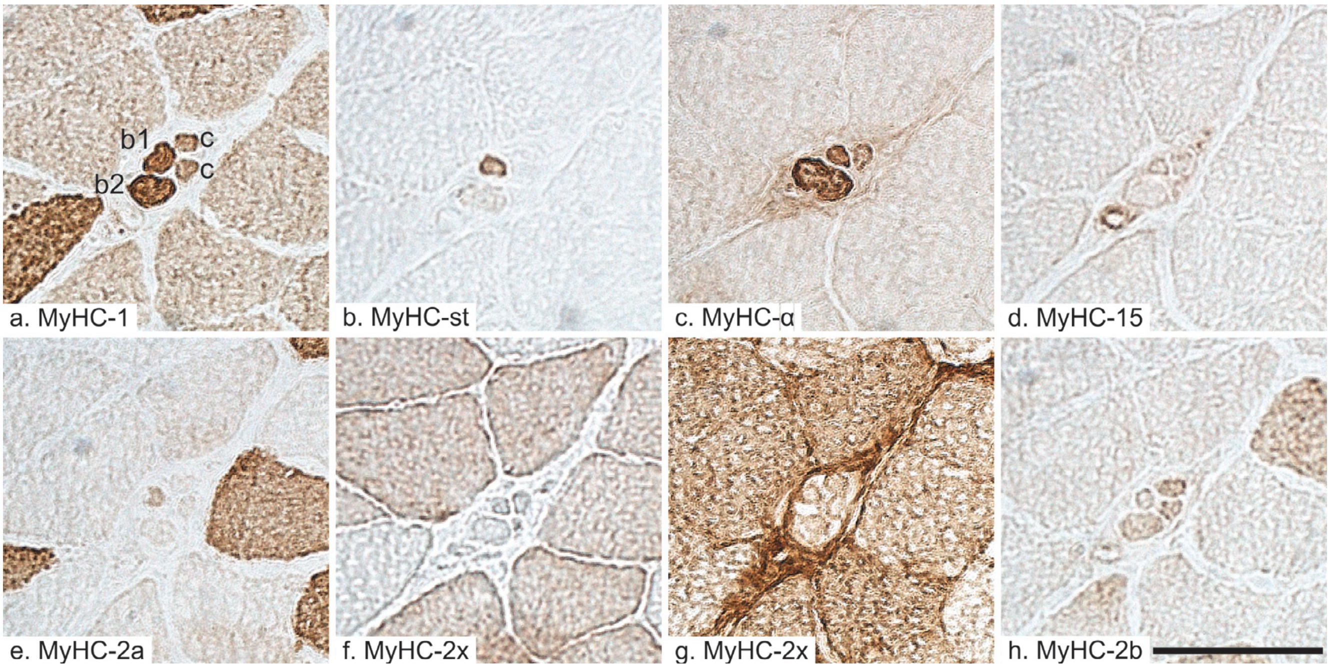

Figure 6. MyHC isoform expression in intrafusal fibres within the B region of a muscle spindle in the medial head of the rat gastrocnemius muscle (magnification 40x). Serial muscle sections were stained with antibodies specific for MyHC-1 (**a**); MyHC-st (**b**); MyHC-α (**c**); MyHC-15 (**d**); MyHC-2a (**e**); MyHC-2x (6F12H3 antibody) (**f**); MyHC-2x (6H1 antibody) (**g**); MyHC-2b (BF-F3 antibody) (**h**). Panel (**a**) indicates the location of bag1 (b1), bag2 (b2), and chain (c) fibres. Scale bars: 50 µm.

antibody for its detection, despite reports of its cross-reactivity with MyHC-1 and -2b in rat tissue.[44] However, in our study, BA-G5 exclusively labelled intrafusal fibres, with a staining pattern distinct from that observed with BA-D5 (MyHC-1) and BF-F3 (MyHC-2b). The observed MyHC-α expression pattern aligns with previous findings.[10,14,17,21-23] In the A region, strong MyHC-α expression was exclusively localised to bag2 fibres, which continued to express this isoform in the B region. Bag1 and some chain fibres also exhibited variable MyHC-α positivity in the B region. In the C region, which was analysed only in EDL and SOL muscles, both bag fibres were strongly positive for MyHC-α in EDL, but negative in SOL (*data not shown*). Notably, MyHC-α expression was less abundant in human intrafusal fibres compared to rats in our analysis.[28]

The expression of fast MyHC-2a and -2b isoforms was previously found to be limited within short segments of bag1 and chain fibres.[2,10] In our study, MyHC-2a exhibited weak to moderate expression in the A region of chain fibres and some bag2 fibres, with minimal expression in bag1 fibres. Its expression in the B region varied across all three intrafusal fibre types, while no expression was observed in the C region. The most prominent MyHC-2a expression was detected in chain fibres of the SOL muscle within the B region. These findings differ somewhat from previous studies employing the same antibody (SC-71), which reported MyHC-2a expression in bag1 fibres across all three regions and in bag2 and chain fibres only within the A region.[10,22,23]

MyHC-2b, the fastest rat MyHC isoform, exhibited its strongest expression in chain fibres. In the fast EDL muscle, weak MyHC-2b expression was observed in all three intrafusal fibre types within the A region. In the B region, bag2 fibres generally displayed stronger expression than bag1 fibres. This contrasts with a previous study employing the same antibody (BF-F3), which reported MyHC-2b expression exclusively in the B region of chain fibres.[10] As mentioned previously, MyHC-2b was also detected in chain fibres of the slow SOL muscle, despite its absence in extrafusal fibres (Figure 2). Similarly, the 10F5 antibody, specific for MyHC-2b, labelled numerous human intrafusal fibres, particularly bag fibres,[28] even though MyHC-2b is not expressed in human skeletal muscle.[40,41]

Contrary to the conflicting reports on the expression of developmental MyHC isoforms, MyHC-emb and MyHC-neo, in rat intrafusal fibres using various antibodies,[2,15,26,46,47] we did not detect MyHC-emb or MyHC-neo in the SOL and EDL muscles with isoform-specific antibodies. Notably, these antibodies successfully identified both developmental isoforms in human muscle spindles.[28] Furthermore, we tested the F1.6521 antibody, previously considered specific for MyHC-emb,[10] and the N3.36 antibody, reported as specific for MyHC-neo.[48,49] However, neither antibody stained intrafusal fibres. Therefore, we suggest that antibodies used in previous studies may have exhibited insufficient specificity, leading to cross-reactivity with the fast MyHC isoforms, MyHC-2a and MyHC-2b, which were clearly identified in our analysis.

While it has been suggested that antibodies raised against extrafusal MyHC isoforms can reliably detect the expression of these isoforms in intrafusal fibres,[10] our findings highlight potential discrepancies. Specifically, we observed notable differences in the reactivity of two MyHC-2b specific antibodies, BF-F3 and 10F5. BF-F3 strongly labelled both extrafusal and intrafusal fibres, whereas 10F5 exhibited weak labelling of extrafusal fibres only (Figure 4 c,d). These observations underscore the potential for antibodies to bind multiple epitopes, a recognized limitation of immunohistochemistry that can lead to misinterpretation of results.[34]

It is well established that nerve stimulation patterns regulate MyHC expression. Therefore, the distinct MyHC isoform expression profiles observed in intrafusal fibres, which receive different innervation compared to their extrafusal counterparts, are not unexpected, even though both fibre types are thought to originate from common myoblasts.[2,42] Evidence suggests that primary afferent (Ia) innervation plays a crucial role in regulating MyHC expression in intrafusal fibres. For instance, in transgenic mice lacking the neurotropin 3 gene, which is essential for afferent neuron development, MyHC-st expression was absent, and muscle spindles failed to develop.[49]Furthermore, deafferentation at birth in rats resulted in downregulation of MyHC-st and upregulation of extrafusal-like MyHC isoforms (MyHC-1, -2a, and -2b).[21] Afferent innervation has been proposed to contribute to the retention of MyHC-emb and -neo in the intracapsular regions of intrafusal fibres. Conversely, in the extracapsular region, efferent neuron innervation is thought to attenuate the expression of developmental isoforms, leading to a more extrafusal-like MyHC expression pattern.[2,10,15,17] Efferent innervation of intrafusal fibres by γ motor neurons during development is crucial for inducing MyHC-α expression, whereas loss of this innervation (deefferentiation) inhibits its expression.[14,21]

Despite the low abundance of the presumably slow MyHC-15, its co-expression with the predominant slow MyHC isoforms, MyHC-st, MyHC-α, and MyHC-1, in intrafusal fibres aligns with observations that intrafusal fibres contraction speed is lower than that of extrafusal fibres.[2,50] In frog, it has been shown that the maximum shortening speed of intrafusal fibres is approximately half that of extrafusal fibers.[51] Furthermore, the tetanic force generated by intrafusal fibres is one-third to one-half of the average force produced by extrafusal fibres, and is higher in the extracapsular region compared to the intracapsular region. It has been demonstrated that while the extracapsular region shortens, the intracapsular region primarily stretches, which is thought to enable gradual adjustment of stretch receptor sensitivity.[50] This aligns with the requirement for intrafusal fibres to maintain sensitivity across a wide range of operating conditions, potentially enhancing the fidelity of afferent feedback to the central nervous system. However, it remains unclear how the expression of the faster MyHC-2b, coupled with the absence of the slower MyHC-2x isoform, is compatible with the contractile demands of intrafusal fibres, which require sustained, low-frequency adjustments to maintain spindle sensitivity rather than rapid, high-frequency contractions.

In conclusion, this study demonstrates that MyHC-15 is expressed at low levels in rat intrafusal fibres, but, contrary to previous reports in both rat and human, its expression is restricted to the intracapsular regions and is absent from the extracapsular C region.[26,28] Our findings confirm that, of the fast MyHC isoforms, only MyHC-2a and -2b are expressed in rat intrafusal fibres, while MyHC-2x is not. Furthermore, we did not detect MyHC-emb or -neo expression, suggesting that antibodies used in previous studies may have cross-reacted with MyHC-2a and -2b. Overall, these findings contribute significantly to our understanding of muscle spindle diversity and function, providing a foundation for improved insights the complex interplay between MyHC isoform expression, muscle fibre contractile properties, and proprioceptive mechanisms.

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