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# Requirement for the ryanodine receptor type 3 for efficient contraction in neonatal skeletal muscles

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**The skeletal isoform of $Ca^{2+}$ release channel, RyR1, plays a central role in activation of skeletal muscle contraction. Another isoform, RyR3, has been observed recently in some mammalian skeletal muscles, but whether it participates in regulating skeletal muscle contraction is not known. The expression of RyR3 in skeletal muscles was studied in mice from late fetal stages to adult life. RyR3 was found to be expressed widely in murine skeletal muscles during the post-natal phase of muscle development, but was not detectable in muscles of adult mice, with the exception of the diaphragm and soleus muscles. RyR3 knockout mice were generated, and it was shown that skeletal muscle contraction in these mice was impaired during the first weeks after birth. In skeletal muscles isolated from newborn RyR3$^{-/-}$ mice, but not in those from adult mice, the twitch elicited by electrical stimulation and the contracture induced by caffeine were strongly depressed. These results provide the first evidence that RyR3 has a physiological role in excitation–contraction coupling of neonatal skeletal muscles. The disproportion between the low amount of RyR3 and the large impact of the RyR3 knockout suggests that this isoform contributes to the amplification of $Ca^{2+}$ released by the existing population of ryanodine receptors (RyR1).**

*Keywords*: $Ca^{2+}$ release channels/muscle contraction/ muscle development/ryanodine receptors

## Introduction

Ryanodine receptors (RyRs) are intracellular homotetrameric $Ca^{2+}$ release channels whose subunits are encoded by three different genes indicated as RyR1, RyR2 and RyR3 (Meissner, 1994; Sorrentino, 1995; Sutko and Airey, 1996). RyR1 channels are located in the terminal cisternae of the sarcoplasmic reticulum of skeletal muscle fibres, while RyR2 channels are found in the corresponding structures of cardiac myocytes (Meissner, 1994; Sutko and Airey, 1996). Both RyR1 and RyR2 are also found, although at much lower levels of expression than in striated muscles, in several other tissues and type of cells (Giannini *et al.*, 1995; Sorrentino, 1995). The third isoform, RyR3, is characterized by a wide pattern of expression but lack of a preferential association with one tissue (Giannini *et al.*, 1992, 1995). At variance with RyR1 and RyR2, for which a major functional role is to release $Ca^{2+}$ in order to regulate skeletal and cardiac contraction, there is no evidence of a physiological role for RyR3. Based on its wide expression, it has been proposed that RyR3 may participate in intracellular $Ca^{2+}$ release in conjunction with other $Ca^{2+}$ release channels, including the inositol trisphosphate ($InsP_3$) receptors and other RyR isoforms, via a mechanism of activation mediated by $Ca^{2+}$ itself, named $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) (Giannini *et al.*, 1992, 1995; Berridge, 1993).

Among other tissues, RyR3 was also detected in some mammalian skeletal muscle preparations (Giannini *et al.*, 1992; Conti *et al.*, 1996) and in cultured myocytes (Giannini *et al.*, 1995; Takeshima *et al.*, 1995; Tarrone *et al.*, 1997), although its expression in skeletal muscles is still debated (Buck *et al.*, 1997). In vertebrate skeletal muscles, RyR1 is essential for releasing $Ca^{2+}$ from the sarcoplasmic reticulum following electrical depolarization of the plasma membrane, a process referred to as excitation–contraction coupling (Fleischer and Inui, 1989; Schneider, 1994; Takeshima *et al.*, 1994; Nakai *et al.*, 1996; Sutko and Airey, 1996). The finding that RyR3 is present, even though at lower levels than RyR1, in mammalian skeletal muscles echoes the observation made in non-mammalian vertebrate skeletal muscles where equal amounts of two isoforms, α and β, are expressed (Sutko and Airey, 1996). These two isoforms have been shown, at least in frogs and chicken, to correspond to mammalian RyR1 and RyR3, respectively (Oyamada *et al.*, 1994; Ottini *et al.*, 1996). Although both α- and β-RyR isoforms are expressed in most muscles of birds, frogs and fishes, differential expression of RyR isoforms in specialized muscles of non-mammalian vertebrates correlates with particular contractile properties of those muscles (Sutko and Airey, 1996). Expression of the α-RyR isoform alone has been observed in the extraocular muscles of chicks and fishes, and in the swimbladder muscle of toadfish (O'Brien *et al.*, 1993). Since these muscles are among the fastest contracting muscles in vertebrates, it has been suggested that the β-RyR isoform is not compatible with fast contracting muscles (O'Brien *et al.*, 1995).

In addition to the biochemical data on the heterogeneity of RyR isoforms in vertebrate skeletal muscles, other lines of evidence suggest that two functionally distinct $Ca^{2+}$ release channels may be involved in the regulation of skeletal muscle contraction. Morphological analysis of fractured faces of sarcoplasmic and of T-tubule membranes have revealed that both RyRs and the voltage sensor, the di-hydropyridine receptors (DHPRs), are organized in regular arrays of particles (Block *et al.*, 1988). Accurate measurements of the distance between DHPRs on the

plasma membrane and between RyRs on the sarcoplasmic reticulum have shown that the distance between DHPRs is such that only one out of two RyRs may be in close contact with the DHPRs (Block *et al.*, 1988; Franzini-Armstrong and Kish, 1995; Flucher and Franzini-Armstrong, 1996). Since in skeletal muscle excitation–contraction coupling a direct contact between RyRs and DHPRs is important for activation of $Ca^{2+}$ release from the sarcoplasmic reticulum, it would appear that one out of two RyRs is not activated directly by this mechanism.

A second observation in favour of a two-release channel system comes from physiological studies of $Ca^{2+}$ release from the sarcoplasmic reticulum, where the existence of two components, a fast activating and inactivating component of large amplitude and a smaller more steady component, has been reported (Fleischer and Inui, 1989; Rios *et al.*, 1992; Meissner, 1994; Schneider, 1994). It has been proposed that the former represents a CICR component that is activated by the initial $Ca^{2+}$ released by the latter smaller component that is regulated directly by voltage (Rios *et al.*, 1992). The relationship between coupled and uncoupled RyRs and the proposed two components of $Ca^{2+}$ release in skeletal muscles has not yet been resolved. It should be noted that the structural organization with coupled and uncoupled RyRs has also been observed in muscles of non-mammalian and mammalian vertebrates that contain only one isoform of RyR (Block *et al.*, 1988; O'Brien *et al.*, 1993; Franzini-Armstrong and Kish, 1995). Thus, that particular structural organization seems to be a general motif in vertebrate skeletal muscles, but the presence of two RyR isoforms is not always required.

The reason for the seemingly redundant expression of RyR1 and RyR3 in skeletal muscle is therefore not clear. The central role of RyR1 in skeletal muscle excitation–contraction coupling has been confirmed by studies of RyR1 knockout mice (Takeshima *et al.*, 1994, 1995; Nakai *et al.*, 1996), in which skeletal muscle excitation–contraction coupling is abolished. RyR1 is also crucial for skeletal muscle excitation–contraction coupling in non-mammalian vertebrates, as exemplified by the cn/cn mutant, a naturally occurring chicken mutant that does not express RyR1 and in which skeletal excitation–contraction coupling is abolished although RyR3 expression levels remain high (Ivanenko *et al.*, 1995; Sutko and Airey, 1996). Thus the RyR3 isoform cannot substitute for RyR1 function, and whether RyR3 participates at all in the regulation of skeletal muscle contraction remains to be clarified (Sutko and Airey, 1996; Takeshima *et al.*, 1996).

In this study, we provide the first evidence that (i) RyR3 is widely expressed in murine skeletal muscles selectively during the neonatal phase of muscle development, while in adult muscles its expression is restricted mainly to the diaphragm and soleus muscles; (ii) skeletal muscle contraction in RyR3 knockout is impaired during the first weeks after birth, indicating that the RyR3 isoform has a functional role in optimizing muscle contractility during the neonatal phase of development of skeletal muscles; and (iii) the contribution of RyR3 to excitation–contraction coupling is apparently devoted to the $Ca^{2+}$ release amplification.

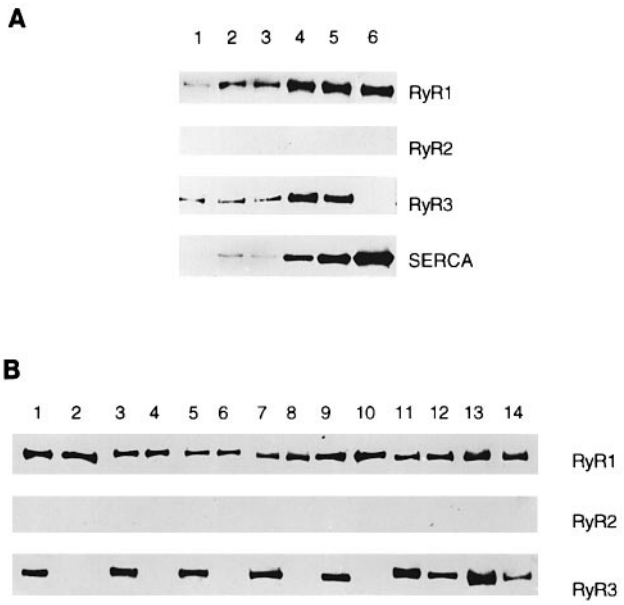


**Fig. 1.** Developmental expression of the RyR3 isoform in total hindlimb muscles. (**A**) Western blot analysis of RyR isoforms in microsomal proteins from total hindlimb muscles of day 18 embryos (lane 1); 2-day-old (lane 2); 5-day-old (lane 3); 15-day-old (lane 4); 25-day-old (lane 5); and adult mice (lane 6). Three, 50 and 20 μg of microsomal vesicles were loaded for RyR1, RyR2 and RyR3 immunodetection, respectively. (**B**) Expression of RyR1 and RyR3 in mouse skeletal muscles. Microsomes were prepared from tibialis anterior (lanes 1 and 2), biceps femoris (lanes 3 and 4), quadriceps femoris (lanes 5 and 6), extensor digitorum longus (lanes 7 and 8), abdominal (lanes 9 and 10), soleus (lanes 11 and 12) and diaphragm (lanes 13 and 14) muscles from 15-day-old (lanes 1, 3, 5, 7, 9, 11 and 13) and adult mice (lanes 2, 4, 6, 8, 10, 12 and 14). Three, 50 and 20 μg of microsomal vesicles were loaded for RyR1, RyR2 and RyR3 immunodetection, respectively.

## Results

### *RyR3 is widely expressed in neonatal but not in adult skeletal muscles*

The process of skeletal muscle development requires fine regulation of the expression of the contractile proteins and of proteins that accumulate and release $Ca^{2+}$ in the sarcoplasmic reticulum. Most muscle-specific proteins start to be expressed during embryonic development, yet a significant increase in the synthesis of these proteins occurs around birth and in the neonatal period of muscle development. Several muscle proteins undergo isoform transition during this period (Schiaffino and Reggiani, 1996). The developmental expression pattern of the RyR3 isoform of $Ca^{2+}$ release channel was investigated by Western blot analysis of microsomal fractions prepared from total hindlimb muscles isolated from mice at day 18 of fetal development, at day 2, 5, 15 and 25 after birth and from adult (i.e. 60-day-old) mice. Expression of RyR1 and RyR2 isoforms and of the sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) was analysed in parallel. An increase in RyR1 and $Ca^{2+}$-ATPase was observed in the early days after birth, with high levels maintained in adult mice. The RyR3 isoform was also detected in the hindlimb muscles of mice, where its expression attained the highest levels 15 days after birth (Figure 1A). Later, however, the RyR3 content was reduced in hindlimb muscles and became undetectable in adult mice.

The RyR3 content in post-natal skeletal muscles was

evaluated further in individual skeletal muscles including biceps and quadriceps femoris, extensor digitorum longus, soleus, tibialis anterior, diaphragm and abdominal muscles dissected from 15-day-old and from adult mice. As shown in Figure 1B, the RyR3 protein was expressed in all skeletal muscles of 15-day-old mice. It was, however, not detectable in the same muscles of adult mice, with the exception of the diaphragm and soleus muscles, where it remained present (Conti *et al.*, 1996) at lower levels than in younger mice. A similar pattern of expression for RyR3 was also found in skeletal muscles of rats (not shown). In both mouse and rat skeletal muscles, the cardiac isoform (RyR2) was never detected. Altogether, these results reveal a unique expression pattern for RyR3 in neonatal skeletal muscles. The wider pattern of expression of RyR3 in neonatal skeletal muscles compared with the restricted pattern observed in adult animals suggests that RyR3 could participate specifically in either $Ca^{2+}$ release in relation to metabolic processes or in excitation–contraction coupling, during that period of development.

### *Generation of RyR3 knockout mice*

To test whether RyR3 has a functional role in neonatal skeletal muscles, we took advantage of the homologous recombination technique. To disrupt the RyR3 gene, a replacement vector was constructed were the neomycin resistance gene was inserted into the sixth exon (counting from the last one). This eliminates the carboxy-terminus of the protein which includes the transmembrane segments involved in formation of the pore region of the $Ca^{2+}$ channel, thus generating a non-functional RyR3 allele (see Figure 2A). E14 embryonic stem (ES) cells were electroporated and two of the selected clones containing the mutated gene were used to generate chimeric mice. Homozygous mice were obtained by intercrossing heterozygous $RyR3^{-/+}$ mice, and the progeny were genotyped by Southern blot (Figure 2B). RyR3 knockout mice ($RyR3^{-/-}$) appeared normal and were detected at a normal frequency in the progeny, as also observed in an independent RyR3 knockout mouse line (Takeshima *et al.*, 1996). Western blot analysis confirmed the absence of the RyR3 protein in the microsomal fraction from diaphragm muscle (Figure 2C) and other organs (not shown). Expression levels of the RyR1 isoform in skeletal muscles were similar in $RyR3^{-/-}$ and $RyR3^{+/+}$ mice (Figure 2D). $RyR3^{-/-}$ mice were indistinguishable in appearance from wild-type controls. Histological examination of skeletal muscles of RyR3 knockout mice at the level of both the light and the electron microscope failed to reveal gross abnormalities in sarcomere and triad organization (not shown).

### *Impaired response to electrical stimulation in neonatal skeletal muscles of RyR3 knockout mice*

The contractile properties of skeletal muscles of RyR3 knockout mice were studied on muscle strips dissected from the diaphragm of neonatal (15 days) and adult (60–90 days) $RyR3^{-/-}$ and $RyR3^{+/+}$ mice. Although the preparations from neonatal animals developed less tension during fused tetani (50 Hz) than the preparations from adult mice, there were no significant differences between $RyR3^{-/-}$ and $RyR3^{+/+}$ mice (see Figure 3A). However, in 15-day-old mice, tension developed in a twitch at low stimulation frequency (0.1 Hz) was significantly lower in

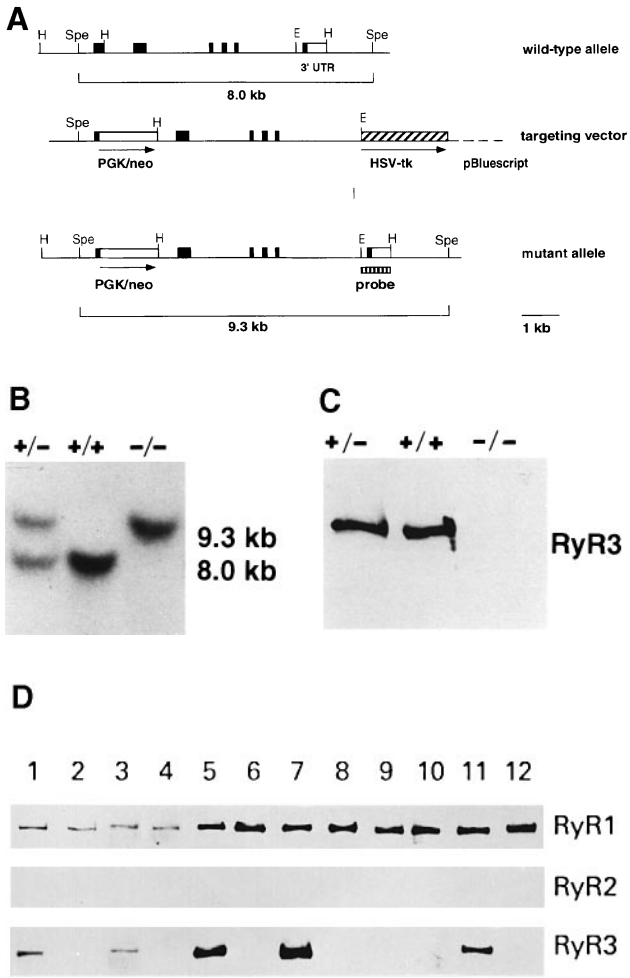


**Fig. 2.** Gene targeting of the RyR3 locus. (**A**) Map of restriction enzyme digestion sites within the RyR3 gene (top), scheme of the RyR3 targeting vector (middle) and the homologous recombination product (bottom). (**B**) Southern blot analysis. *Spe*I-digested DNAs were hybridized with the indicated 0.9 kb *Eco*RI–*Hin*dIII probe. The endogenous and targeted alleles are 8.0 and 9.3 kb, respectively. A Southern blot of tail DNA from three representative F2 mice is shown (lanes 1–3). (**C**) Immunoblot analysis of $RyR3^{-/-}$ mice. Twenty μg of microsomal proteins from diaphragm muscles of adult mice were fractionated on SDS–PAGE and analysed by Western blot with isoform-specific antibodies able to recognize selectively the RyR3 isoform. (**D**) Immunoblot analysis of RyR1, RyR2 and RyR3 isoforms in total hindlimb (lanes 1, 2 5, 6, 9 and 10) and diaphragm (3, 4, 7, 8, 11 and 12) muscles from 2-day-old (lanes 1–4), 15-day-old (lanes 5–8) and adult (lanes 9–12) $RyR3^{+/+}$ (lanes 1, 3, 5, 7, 9 and 11) and $RyR3^{-/-}$ (lanes 2, 4, 6, 8, 10 and 12) mice. Three, 50 and 20 μg of microsomal vesicles were loaded for RyR1, RyR2 and RyR3 immunodetection, respectively.

$RyR3^{-/-}$ than in $RyR3^{+/+}$ mice: expressed as a fraction of tetanic tension (twitch/tetanus ratio) it represented 0.23 ± 0.03 in $RyR3^{+/+}$ ($n$ = 10) and 0.15 ± 0.01 in $RyR3^{-/-}$ ($n$ = 10, $P<0.05$) (Figure 3B). The same was true for tension developed in unfused tetani (5 and 10 Hz). At higher stimulation rates, the difference was cancelled by fusion and summation of the responses. As can be seen in Figure 3C, the force–frequency curve appeared shifted downward and rightward in $RyR3^{-/-}$ compared with $RyR3^{+/+}$ mice. In contrast, in adult mice preparations, the differences in twitch/tetanus ratio and in the force–frequency curve disappeared (Figure 3D). The time course of the twitch showed a significant age-dependent change.

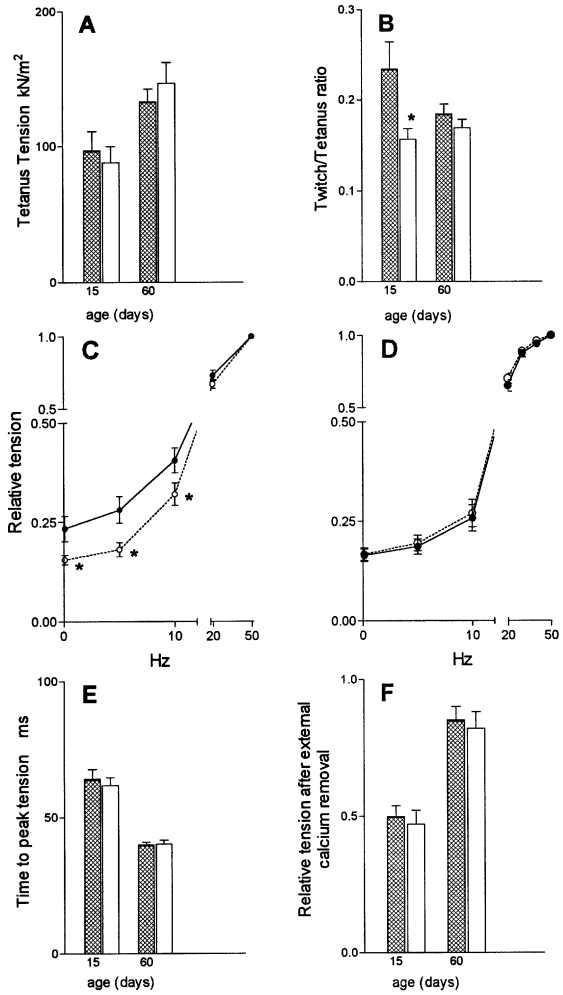


**Fig. 3.** Contractile properties of diaphragm strips from RyR3$^{-/-}$ (empty symbols and columns) and RyR3$^{+/+}$ (filled symbols and columns) mice at 15 (neonatal) and 60 days (adult). (**A**) Tension developed during tetanic stimulations at 50 Hz. (**B**) Twitch/tetanus ratio at the stimulation frequency of 0.1 Hz. (**C**) Force–frequency relationships in diaphragm strips from 15-day-old mice. (**D**) Force–frequency relationships in diaphragm strips from 60-day-old mice. (**E**) Time to peak tension in twitch responses. (**F**) Effect of $Ca^{2+}$ removal indicated by twitch tension 10 min after external $Ca^{2+}$ removal expressed relative to twitch tension in basal conditions. Means and standard errors of 10 neonatal and 20–28 adult RyR3$^{+/+}$ preparations and 10 neonatal and 16–22 adult RyR3$^{-/-}$ preparations. *$P$<0.05.

Time to peak tension (Figure 3E) and time to half-relaxation (not shown) became shorter in adult muscles, but no difference was detectable between RyR3$^{-/-}$ and RyR3$^{+/+}$ mice. Other contractile properties, including response to the fatiguing stimulation protocol (not shown) and depression of the contractile response produced by removing extracellular $Ca^{2+}$ (Figure 3F), were not different in RyR3$^{-/-}$ versus RyR3$^{+/+}$ mice, although, as expected (Dangain and Neering, 1991), the effect of lowering $Ca^{2+}$ concentration in the perfusing medium was greater in neonatal than in adult muscles.

### *The large contracture induced by caffeine in neonatal mice is strongly reduced in RyR3 knockout mice*

The most striking difference between muscles of RyR3$^{-/-}$ and RyR3$^{+/+}$ mice was found when tension development

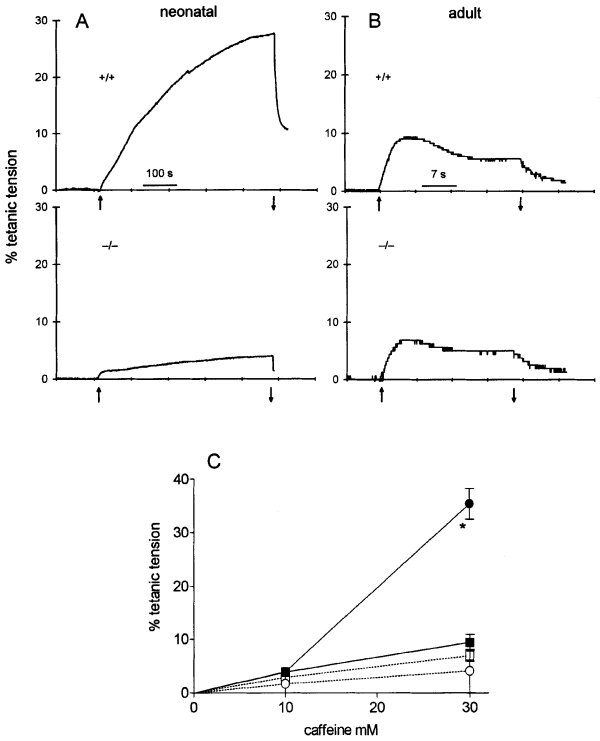


**Fig. 4.** Response to caffeine of diaphragm strips of neonatal (15-day-old) and adult (60-day-old) mice. (A and B) Experimental traces showing typical examples of tension development in neonatal (**A**) and adult (**B**) muscle strips, exposed to 30 mM caffeine. Tension is expressed as the percentage of tetanic tension. Note the large difference in the time scale. Arrows indicate addition and washing out of caffeine. (**C**) Tension developed in contractures elicited by exposing muscle strips to 10 and 30 mM caffeine. Means and standard errors of 10 neonatal and 35 adult RyR3$^{+/+}$ preparations and of 10 neonatal and 30 adult RyR$^{-/-}$ preparations. Symbols: circles refer to neonatal mice, squares to adult mice, filled symbols to RyR3$^{+/+}$ and empty symbols to RyR3$^{-/-}$. *$P$<0.05.

was induced by exposure to millimolar caffeine concentrations. Although mammalian muscles are less responsive to caffeine than amphibian muscles (Luttgau and Oetliker, 1968), release of calcium from sarcoplasmic reticulum and tension development without electrical stimulation can be induced by caffeine at concentrations >5 mM (Fryer and Neering, 1989). In RyR3$^{+/+}$ mice, neonatal diaphragm strips developed more tension in response to caffeine than adult diaphragm strips. At caffeine concentrations >10 mM, the difference increased abruptly: 30 mM caffeine induced a contracture four times greater in neonatal than in adult diaphragm strips (see Figure 4). The kinetics of tension development were markedly different, as tension peaked in ~10 s in adult and in 400–600 s in neonatal strips.

Remarkably, the large response to caffeine observed in RyR3$^{+/+}$ neonatal diaphragm strips was abolished in knockout RyR3$^{-/-}$ preparations. The kinetics of tension development were, however, not altered, as tension development remained slower in neonatal than in adult strips (see Figure 4). The response to caffeine was also tested in extensor digitorum longus and soleus muscles with comparable results. The large contracture evoked by 30 mM caffeine in extensor digitorum longus (51% of tetanic tension $n$ = 3) and soleus (38% of tetanic tension $n$ = 3) of 15-day-old RyR3$^{+/+}$ mice was markedly reduced

in extensor digitorum longus and soleus muscles of $RyR3^{-/-}$ mice of the same age (7.4 and 8.6% of tetanic tension respectively, $n = 3$). In adult mice, the caffeine contracture was barely detectable in extensor digitorum longus, and ranged between 5 and 10% of tetanic tension in soleus without significant differences between $RyR3^{+/+}$ and $RyR3^{-/-}$ preparations (not shown).

## Discussion

We have found that, in mammalian skeletal muscles, RyR3 is already expressed in the late stages of fetal development and that it attains its higher levels of expression between 2 and 3 weeks after birth. The RyR3 levels then decrease and this isoform is no longer detected in adult muscles, with the notable exception of the diaphragm and the soleus muscles. While the RyR3 levels start to decrease at 3 weeks after birth, the levels of other sarcoplasmic proteins, including the predominant RyR1 isoform of $Ca^{2+}$ release channel and the $Ca^{2+}$-ATPase, increase steadily. The contrast between the wide pattern of expression of RyR3 in skeletal muscles of neonatal mice and the restricted expression observed in adult skeletal muscles suggests a specific requirement for RyR3, in addition to RyR1, at this stage of muscle development. Why neonatal skeletal muscles require the expression of RyR3 is not clear, but is worth remembering that usage of isoforms different from those present in adult fibres is observed commonly in neonatal skeletal muscles for some proteins of the contractile apparatus and sarcoplasmic reticulum (Schiaffino and Reggiani, 1996). Expression of RyR3 in the first 2–3 weeks after birth corresponds to a period where skeletal muscles undergo a series of major morphological and biochemical changes which precede the formation of adult skeletal muscles (Flucher and Franzini-Armstrong, 1996; Schiaffino and Reggiani, 1996).

The functional relevance of the RyR3 isoform during this period of muscle development is supported by results obtained with RyR3 knockout mice. These results indicate that, in agreement with the preferential expression of the RyR3 isoform in the post-natal period of mammalian skeletal muscle development, neonatal muscles lacking RyR3 are ~35% less efficient in translating electrical stimulation into force generation than muscles from control mice. Impairment of force generation in $RyR3^{-/-}$ mice following electrical stimulation represents the first physiological evidence that the RyR3 isoform participates in excitation–contraction coupling in skeletal muscles. Although the force deficit observed in skeletal muscles of $RyR3^{-/-}$ mice can be compensated easily by increasing the rate of motorneuron discharge, in agreement with preservation of mice motility in the absence of RyR3 (Takeshima *et al.*, 1996), an impairment of muscle performance in $RyR3^{-/-}$ mice under extreme conditions of muscle activity cannot be ruled out definitively.

The relevance of RyR3 in the response of skeletal muscles to contraction-inducing stimuli was even more evident when muscles from neonatal RyR3 knockout mice were exposed to high concentrations of caffeine, a known activator of $Ca^{2+}$ release through RyRs (Fryer and Neering, 1989; Meissner, 1994). In neonatal $RyR3^{-/-}$ mice, caffeine-induced contracture was in fact reduced to ~20% of that of control mice. Although several factors, including a disproportion between ability to release and to take up $Ca^{2+}$ into the sarcoplasmic reticulum (Luttgau and Oetliker, 1968; Makabe *et al.*, 1995), might also participate in setting the stage for the large caffeine contracture observed in neonatal mice, the depression of the caffeine response observed in $RyR3^{-/-}$ mice demonstrates that the RyR3 isoform is necessary for this large response to caffeine. This observation is in agreement with results obtained in muscles from RyR1 knockout mice which, although lacking excitation–contraction coupling, retained an unexpected high responsiveness to caffeine that tentatively was attributed to RyR3 (Takeshima *et al.*, 1994; Takeshima *et al.*, 1995; Nakai *et al.*, 1996). The functional consequences of the RyR3 knockout in neonatal skeletal muscle contractility reported here are even more surprising when we consider that available data indicate that RyR3 represents only 1/20–1/100 of the total RyRs in mammalian skeletal muscles (Conti *et al.*, 1996; Murayama and Ogawa, 1997; A.Conti and V.Sorrentino, unpublished data). Although we must await more work to establish the relative abundance of RyR3 and the structural and molecular organization of excitation–contraction coupling in developing muscles, these results strongly suggest that RyR3 could contribute to a secondary component of excitation–contraction coupling which creates an amplification mechanism for regulation of skeletal muscle contraction through a CICR mechanism (Fleischer and Inui, 1989; Rios *et al.*, 1992; Schneider, 1994; Takeshima *et al.*, 1995; Sutko and Airey, 1996).

It is of interest to discuss our results within the framework of the excitation–contraction coupling model for skeletal muscles (see Figure 5), where, at triads, only one of every two $Ca^{2+}$ release channels on the sarcoplasmic reticulum faces the voltage sensor on the plasma membrane (Block *et al.*, 1988; Flucher and Franzini-Armstrong, 1996). It is accepted that the RyR1 isoform transduces, probably by mechanical coupling with the DHPR

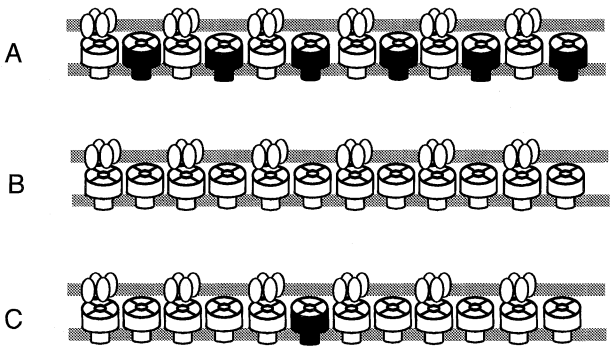


**Fig. 5.** Schematic representation of the relative position of RyR isoforms and DHPRs at triads of skeletal muscles. The T-tubule plasma membrane is depicted by the upper lines, where four DHPR molecules are grouped together to form the tetrads (Flucher and Franzini-Armstrong, 1996). The ryanodine receptors are aligned on the lower lines that represent the sarcoplasmic reticulum. Note that not all RyRs are in direct opposition to a DHPR-containing tetrad, according to the structural model proposed initially by Block *et al.* (1988). The white and black receptors on the sarcoplasmic reticulum are the RyR1 and RyR3 isoforms, respectively. (**A**) Triadic model for skeletal muscles which contain equimolar amounts of RyR1 and RyR3. (**B**) Triadic model for muscles that contain only the RyR1 isoform. (**C**) Triadic model for muscles that contain predominantly the RyR1 isoform, but also contain, although in lower amounts, the RyR3 isoform.

(Fleischer and Inui, 1989; Rios *et al.*, 1992; Schneider, 1994; Sutko and Airey, 1996), the signal between the plasma membrane and the sarcoplasmic reticulum (Takeshima *et al.*, 1994, 1995; Fleig *et al.*, 1996; Nakai *et al.*, 1996). This specialized function cannot be performed by the RyR3 isoform in either chicken or mice, as shown by lack of excitation–contraction coupling in natural chicken mutant and experimental mice lacking RyR1 (Takeshima *et al.*, 1994; Ivanenko *et al.*, 1995; Nakai *et al.*, 1996). On these bases, the RyR1 isoform is a logical candidate to build the coupled channels that face the voltage sensor. In most non-mammalian skeletal muscles, the RyR1 and RyR3 isoforms, given their equal ratio, could be organized in the working model shown in Figure 5A, with an alternating pattern where only RyR1 channels are opposed to DHPRs. In mammalian muscles that contain only the RyR1 isoform, as well as in those muscles of chicks and fishes that express only the α-RyR1 isoform, it has to be accepted that RyR1, in addition to contributing to the coupled channels, must also contribute to the channels that are not coupled with the voltage sensor, as shown in Figure 5B. In agreement with the expectation that contraction following electrical stimulation is controlled essentially by activation of $Ca^{2+}$ release channels containing the RyR1 isoform, RyR3 knockout mice possess a functional excitation–contraction coupling. However, in neonatal skeletal muscles of $RyR3^{-/-}$ mice, a strong reduction in the response to both electrical and caffeine stimulation was observed. We propose, therefore, that the RyR3 isoform, when expressed as in the neonatal stage of muscle development, forms uncoupled receptors which provide the CICR-based amplificatory component of excitation–contraction coupling (Fleischer and Inui, 1989; Rios *et al.*, 1992; Schneider, 1994; Sutko and Airey, 1996). Given the evidence that in mammalian skeletal muscles RyR3 is not in an equimolar ratio with RyR1, RyR3 could contribute to only a fraction of the uncoupled channels, as shown in Figure 5C. However, we should mention that, because of the current agreement that RyRs are homotetramers, the possibility of heterotetrameric channels has not been considered in Figure 5, although it cannot be excluded.

An obvious question arises from the results reported here. How can RyR3 channels, which represent only a small fraction of the total $Ca^{2+}$ release channels present in skeletal muscles, be responsible for the effects that we observe to be present in neonatal RyR3 knockout mice? Several models have been proposed to explain the functional significance of the relationship between DHPRs and RyRs in skeletal muscles, and probably the data reported here need to be incorporated into such models (Sutko and Airey, 1996). The use of the RyR3 isoform for the uncoupled release channels is likely to reflect specific regulatory properties of RyR3 (Percival *et al.*, 1994; O'Brien *et al.*, 1995; Takeshima *et al.*, 1995) that might make it more effective than RyR1 in a CICR mechanism. Specific activation/inactivation kinetics or differential sensitivity to $Ca^{2+}$ or other agonists and antagonists of channel activity might be the determinants of these properties (Percival *et al.*, 1994; O'Brien *et al.*, 1995; Sitsapesan *et al.*, 1995; Takeshima *et al.*, 1995; Rakovic *et al.*, 1996; Sutko and Airey, 1996). Thus, qualitative diversity between isoforms might account for the apparent discrepancy (see above) between the small amount of RyR3 detected in mammalian muscles and the large functional impact of its deletion described in this study. We can speculate that the presence of a more sensitive system for a CICR-mediated amplification might be of particular relevance in neonatal muscles in view of the as yet incomplete development of the triad structure (Luff and Atwood, 1971; Flucher and Franzini-Armstrong, 1996).

As a concluding remark, it should be considered that co-expression of various isoforms of $Ca^{2+}$ release channels, originating from both alternative splicing and expression of different genes from both the InsP3-R and the RyR families (Berridge, 1993; Furuichi *et al.*, 1994; Marziali, 1996; Striggow and Ehrlich, 1996; Sutko and Airey, 1996), has been observed with increasing frequency in numerous cell types. The involvement of multiple channels with different regulatory properties has been proposed to be a means of providing a more refined regulation of specific cellular functions which are dependent on $Ca^{2+}$ release (Berridge, 1993; Furuichi *et al.*, 1994; Kasai and Petersen, 1994; Clapham, 1995; Striggow and Ehrlich, 1996). Our finding that, in neonatal muscles, activation of the RyR1 isoform to achieve an optimal force development requires the action of the RyR3 isoform provides the first experimental evidence of such a case.

## Materials and methods

### *Microsomal vesicle preparations*

Skeletal muscles isolated from mice at the indicated ages were used to prepare the microsomal fractions. Muscle tissue was frozen quickly in liquid nitrogen, and microsomes were prepared as previously described (Conti *et al.*, 1996). Briefly, tissue samples were homogenized in ice-cold buffer A [320 mM sucrose, 5 mM Na-HEPES pH 7.4 and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] using a Dounce homogenizer. Homogenates were centrifuged at 7000 *g* for 5 min at 4°C. The supernatant obtained was centrifuged at 100 000 *g* for 1 h at 4°C. The microsomes were resuspended in buffer A and stored at –80°C. The protein concentration of the microsomal fraction was quantified using the Bradford protein assay kit (BioRad).

### *Western blot analysis*

Microsomal proteins were separated by SDS–PAGE, as described (Giannini *et al.*, 1995; Conti *et al.*, 1996). Proteins were then transferred to a nitrocellulose membrane (Schleicher & Schüll) by blotting gels for 5 h at 350 mA at 4°C in a transfer buffer containing 192 mM glycine, 25 mM Tris, 0.01% SDS and 10% methanol. Membranes were incubated for 3 h in a blocking buffer containing 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 0.2% Tween-20, 5% non-fat milk. Primary antibodies (diluted 1:3000) were incubated with membranes overnight at room temperature. Polyclonal rabbit antisera able to distinguish between the three RyRs were developed against purified GST fusion proteins corresponding to the region of low homology situated between the transmembrane domains 4 and 5 (divergent region 1, or D1) of the RyR1, RyR2 and RyR3 proteins, as previously described (Giannini *et al.*, 1995), and have been shown not to cross-react with each other. The Y1F4 monoclonal antibody against the $Ca^{2+}$-ATPase SERCA1 isoform (SERCA) was kindly provided by Dr J.M.East. Antigen detection was performed using the alkaline phosphatase detection method.

### *RyR3 gene targeting*

A library of 129/Sv strain mouse genomic DNA was screened with a murine RyR3 probe corresponding to the sequence encoding the pore region of the channel. Isolated clones were characterized by sequence analysis, and the exon–intron structure of the region encoding the last seven exons of the murine RyR3 gene was characterized (G.Giannini, A.Nori and V.Sorrentino, unpublished). An insertion targeting vector containing 1.0 and 6.0 kb fragments surrounding the neomycin resistance gene, and the herpes virus thymidine kinase gene, was constructed. The linearized vector was electroporated into the E14 ES cell line and

colonies were isolated following selection in G418 and gancyclovir, by standard methods. Double resistant cell lines were grown and characterized for homologous recombination by Southern blot after *Spe*I digestion and hybridization with a 0.9 kb *Eco*RI–*Hin*dIII probe. Four clones containing the targeted allele of 9.3 kb were isolated and injected into C57Bl/6 blastocysts. Transmission of the mutated allele to the progeny was obtained in four out of five chimeric mice obtained with two different ES clones. These mice were mated to C57Bl/6 mice to generate heterozygous mutant mice that were then cross-bred to generate homozygous RyR3-deficient ($RyR3^{-/-}$) mice.

### *Analysis of muscle contractile properties*

Diaphragm strips including the insertions on the rib and on the central tendon were dissected and mounted between a force transducer (AE 801Sensonor Horten, Norway) and a movable hook in a perfusion bath (volume 2 ml) containing bicarbonate Krebs solution. The solution was bubbled with $O_2$–$CO_2$ mixture and the temperature was kept constant at 22°C. The perfusion solution could be exchanged quickly (5–10 s) by flushing ~20 ml of the new solution through the chamber. The preparations were stretched just above slack length (resting tension ~5% of twitch tension) and supramaximally activated with field stimulation. The output of the tension transducer was stored in a PC after A/D conversion and recalled for analysis. CEA 1401 A/D converter and CEA Spike2 (CEA Cambridge UK) software were used.

After stabilization at 0.1 Hz, twitch and tetani (0.5 s duration) at frequencies of 5, 10, 20 and 50 Hz were recorded. Fatigue was induced with a protocol based on a 1:4 duty ratio with tetani of 0.5 s duration and 50 Hz stimulation frequency.

The depressing effect of external $Ca^{2+}$ removal was studied on twitches produced at the rate of 0.1 Hz. Contractures with increasing doses of caffeine (range 10–30 mM) in the absence of electrical stimulation were produced sequentially. Each contracture was followed by washing with Krebs solution without caffeine to produce a complete relaxation of the preparation.

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## References

Berridge,M.J. (1993) Inositol trisphosphate and calcium signalling. *Nature*, **361**, 315–325.

Block,B.A., Imagawa,T., Campbell,K.P. and Franzini Armstrong,C. (1988) Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. *J. Cell Biol.*, **107**, 2587–2600.

Buck,E.D., Nguyen,H.T., Pessah,I.N. and Allen,P.D. (1997) Dyspedic mouse skeletal muscle expresses major elements of the triadic junction but lacks detectable ryanodine receptor protein and function. *J. Biol. Chem.*, **272**, 7360–7367.

Clapham,D.E. (1995) Calcium signaling. *Cell*, **80**, 259–268.

Conti,A., Gorza,L. and Sorrentino,V. (1996) Differential distribution of ryanodine receptor type 3 (RyR3) gene product in mammalian skeletal muscles. *Biochem. J.*, **316**, 19–23.

Dangain,J. and Neering,I. (1991) Effect of low extracellular calcium and ryanodine on muscle contraction of the mouse during postnatal development. *Can. J. Physiol. Pharmacol.*, **69**, 1294–1300.

Fleig,A., Takeshima,H. and Penner,R. (1996) Absence of $Ca^{2+}$ current facilitation in skeletal muscle of transgenic mice lacking the type 1 ryanodine receptor. *J. Physiol.*, **496**, 339–345.

Fleischer,S. and Inui,M. (1989) Biochemistry and biophysics of excitation–contraction coupling. *Annu. Rev. Biophys. Biophys. Chem.*, **18**, 333–364.

Flucher,B.E. and Franzini-Armstrong,C. (1996) Formation of junctions involved in excitation–contraction coupling in skeletal and cardiac muscle. *Proc. Natl Acad. Sci. USA*, **93**, 8101–8106.

Franzini-Armstrong,C. and Kish,J.M. (1995) Alternate disposition of tetrads in peripheral couplings of skeletal muscle. *J. Muscle Res. Cell Motil.*, **16**, 319–324.

Fryer,M.W. and Neering,I.R. (1989) Actions of caffeine on fast- and slow-twitch muscles of the rat. *J. Physiol.*, **416**, 435–454.

Furuichi,T., Kohda,K., Miyawaki,A. and Mikoshiba,K. (1994) Intracellular channels. *Curr. Opin. Neurobiol.*, **4**, 294–303.

Giannini,G., Clementi,E., Ceci,R., Marziali,G. and Sorrentino,V. (1992) Expression of a ryanodine receptor-$Ca^{2+}$ channel that is regulated by TGF-β. *Science*, **257**, 91–94.

Giannini,G., Conti,A., Mammarella,S., Scrobogna,M. and Sorrentino,V. (1995) The ryanodine receptor/calcium channel genes are widely and differentially expressed in murine brain and peripheral tissues. *J. Cell Biol.*, **128**, 893–904.

Ivanenko,A., McKemy,D.D., Kenyon,J.L., Airey,J.A. and Sutko,J.L. (1995) Embryonic chicken skeletal muscle cells fail to develop normal excitation–contraction coupling in the absence of the alpha ryanodine receptor. *J. Biol. Chem.*, **270**, 4220–4223.

Kasai,H. and Petersen,O.H. (1994) Spatial dynamics of second messengers: $IP_3$ and cAMP as long-range and associative messengers. *Trends Neurosci.*, **17**, 95–101.

Luff,A.R. and Atwood,H.L. (1971) Changes in the sarcoplasmic reticulum and transverse tubular system of fast and slow skeletal muscles of the mouse during postnatal development. *J. Cell Biol.*, **51**, 369–383.

Luttgau,H.C. and Oetliker,H. (1968) The action of caffeine on the activation of the contractile mechanism in striated muscle fibres. *J. Physiol.*, **194**, 51–74.

Makabe,M., Werner,O. and Fink,R.H.A. (1995) The contribution of the sarcoplamic reticulum $Ca^{2+}$ transport ATPase to caffeine induced $Ca^{2+}$ transients of murine skinned skeletal muscle fibres. *Pflugers Arch.*, **432**, 717–726.

Marziali,G., Rossi,D., Giannini,G., Charlesworth,A. and Sorrentino,V. (1996) cDNA reveals a tissue specific expression of alternatively spliced transcripts of the ryanodine receptor type 3 (RyR3) calcium release channel. *FEBS Lett.*, **394**, 76–82.

Meissner,G. (1994) Ryanodine receptor/$Ca^{2+}$ release channels and their regulation by endogenous effectors. *Annu. Rev. Physiol.*, **56**, 485–508.

Murayama,T. and Ogawa,Y. (1997) Properties of homotetrameric RyR3 ryanodine receptor in mammalian diaphragm muscle. *Biophys. J.*, **A169**, 72.

Nakai,J., Dirksen,R.T., Nguyen,H.T., Pessah,I.N., Beam,K.G. and Allen,P.D. (1996) Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. *Nature*, **380**, 72–75.

O'Brien,J., Meissner,G. and Block,B. (1993) The fastest contracting muscles of nonmammalian vertebrates express only one isoform if the ryanodine receptor. *Biophys. J.*, **65**, 2418–2427.

O'Brien,J., Valdivia,H.H. and Block,B.A. (1995) Physiological differences between the α and β ryanodine receptors of fish skeletal muscle. *Biophys. J.*, **68**, 471–482.

Ottini,L., Marziali,G., Conti,A., Charlesworth,A. and Sorrentino,V. (1996) Alpha and beta isoforms of ryanodine receptor from chicken skeletal muscle are the homologues of mammalian RyR1 and RyR3. *Biochem. J.*, **315**, 207–216..

Oyamada,H., Murayama,Y., Takagi,T., Iino,M., Iwabe,N., Miyata,T., Ogawa,Y. and Endo,M. (1994) Primary structure and distribution of ryanodine-binding protein isoforms of the bullfrog skeletal muscle. *J. Biol. Chem.*, **269**, 17206–17214.

Percival,A., Williams,A., Kenyon,J., Grinsell,M., Airey,J.A. and Sutko,J.L. (1994) Chicken skeletal muscle ryanodine receptor isoforms: ion channel properties. *Biophys. J.*, **67**, 1834–1850.

Rakovic,S., Galione,A., Ashamu,G.A., Potter,B.V.L. and Terrar,D.A. (1996) A specific cyclic ADP-ribose antagonist inhibits cardiac excitation–contraction coupling. *Curr. Biol.*, **6**, 989–996.

Rios,E., Pizarro,G. and Stefani,E. (1992) Charge movement and the nature of signal transduction in skeletal muscle excitation–contraction coupling. *Annu. Rev. Physiol.*, **54**, 109–133.

Schiaffino,S.and Reggiani,C. (1996) Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol. Rev.*, **76**, 371–423.

Schneider,M.F. (1994) Control of calcium release in functioning skeletal muscle fibers. *Annu. Rev. Physiol.*, **56**, 463–484.

Sitsapesan,R., McGarry,S.J. and Williams,A.J. (1995) Cyclic ADP-ribose, the ryanodine receptor and $Ca^{2+}$ release. *Trends Pharmacol.*, **16**, 386–391.

Sorrentino,V. (1995) *Ryanodine Receptors*. CRC Press, Boca Raton, FL.

Striggow,F. and Ehrlich,B.E. (1996) Ligand-gated calcium channels inside and out. *Curr. Opin. Cell Biol.*, **8**, 490–495.

Sutko,J.L. and Airey,J.A. (1996) Ryanodine receptor $Ca^{2+}$ release channels: does diversity in form equal diversity in function? *Physiol. Rev.*, **76**, 1027–1071.

Takeshima,H., Iino,M., Takekura,H., Nishi,M., Kuno,J., Minowa,O., Takano,H. and Noda,T. (1994) Excitation–contraction uncoupling and muscular degeneration in mice lacking functional skeletal muscle ryanodine-receptor gene. *Nature*, **369**, 556–559.

Takeshima,H.,Yamazawa,T., Ikemoto,T., Takekura,H., Nishi,M., Noda,T. and Iino M. (1995) Calcium-induced calcium release in myocytes from dyspedic mice lacking the type-1 ryanodine receptor. *EMBO J.*, **14**, 2999–3006.

Takeshima,H., Ikemoto,T., Nishi,M., Shimuta,M., Sugitani,Y., Kuno,J., Saito,I., Saito,H., Endo,M., Iino,M and Noda,T. (1996) Generation and characterization of mutant mice lacking ryanodine receptor type 3. *J. Biol. Chem.*, **271**, 19649–19652.

Tarroni,P., Rossi,D., Conti,A. and Sorrentino,V. (1997) Expression of the ryanodine receptor type 3 (RyR3) calcium release channel during development and differentiation of mammalian skeletal muscle cells. *J. Biol. Chem.*, **272**, 19808–19813.

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