AChR clustering in the absence of motor innervation. However, establishing a scenario for MuSK's participation in the process is somewhat complicated. For example, an element other than agrin may activate MuSK and trigger the postsynaptic specialization at NMJs. Simultaneously or alternatively, MuSK could act as a primary scaffold molecule without activation. The listed pleiotropic roles of MuSK in AChR clustering at developmental NMJs could also be required for the maintenance of mature NMJs (22, 23). Studies performed *in vivo* have shown that synaptic AChRs intermingle among themselves completely over a period of ~four days and that many extra-synaptic AChRs are incorporated into the synapse at the mature NMJ, although the synaptic membrane in adult muscle appears macroscopically to be stable (24). Therefore, the mechanisms at play during AChR clustering in developing NMJs are also required in mature NMJs where postsynaptic complexes including those with AChR and MuSK are dynamically turning over for the maintenance of muscle function.

Do MuSK antibodies cause MG?

In contrast to the well-accepted mechanisms by which AChR antibodies function in MG, the pathogenic role of MuSK antibodies has been unclear (25). First, no significant loss of AChR at NMJs was observed in biopsies from biceps brachii muscles of MuSK-positive patients with MG (26). Second, MuSK antibodies are mainly in the IgG4 subclass, which does not activate complement (14), and complement-mediated damage to postsynaptic membranes is considered a major source of pathogenicity in MG patients with AChR antibodies. Third, no research results have shown that passive transfer of MuSK serum from MG patients generates the equivalent disease in mice. Fourth, no experimental animal model of myasthenia gravis (EAMG) induced by immunization of MuSK protein has been developed. However MuSK antibodies from MG patients can inhibit MuSK functions *in vitro* (4).

The pathogenicity of AChR antibodies was simulated experimentally by the induction of muscle weakness and development of paralysis in rabbits immunized with AChR protein purified from the electric eel (3). This AChR protein induced the production of antibodies that cross-reacted with rabbit AChR at the NMJ. The flaccid paralysis that followed and electrophysiological studies of these animals provided a model that resembled the MG of humans. Therefore, the demonstration of experimental autoimmune MG in animals induced by MuSK antibodies was essential for proving

their pathogenicity and investigating their mechanisms of eliciting MG.

In 2006, we have found that immunization of rabbits with MuSK ectodomain caused myasthenic weakness and produced electromyographic findings that were compatible with a diagnosis of MG (23), as shown earlier by Patrick and Lindstrom (3). The extracellular segment of MuSK comprised five distinct domains, i.e., four immunoglobulin-like domains and one cysteine-rich region (Figure 2). The fusion protein expression constructs, which consisted of mouse MuSK ectodomain with the Fc region of human IgG1 or His-tag, were generated and transfected into COS-7 cells. The secreted recombinant MuSK-Fc and MuSK-His proteins were purified by using protein-A Sepharose and histidine affinity columns, respectively. New Zealand White rabbits were then immunized with 100 to 400 μg of purified MuSK recombinant protein. After three to four injections of MuSK protein, all of six rabbits manifested flaccid paralysis (Figure 3A). Sera from the paretic rabbits contained a high titer of MuSK antibodies that reacted specifically with MuSK molecules on the surfaces of C2C12 myotubes as observed in sera from MG patients who were positive for MuSK antibodies (23). Histological studies of the muscle tissues from the paretic rabbits, which had manifested severe exhaustion, revealed alterations in muscle fibers ranging from subtle to angular atrophy intermingled with normal muscle tissue (Figure 3B). The histological changes typical of atrophied muscle fibers can result from MG, reduced mechanical ability or cachexia. In repetitive electromyograms from one of these paretic rabbits, the retroauricular branch of facial nerve was stimulated at 20 Hz, and recordings were taken from adjacent retroauricular muscle (Figure 3C). The compound muscle action potential (CMAP) showed a decremental pattern, consistent with MG. However, injections of acetylcholine esterase inhibitor did not significantly reverse either the CMAP defect or the paralytic symptoms. Importantly, induction of myasthenia by MuSK antibodies is not confined to the rabbit, since we and others also produced myasthenia in mice by injection of MuSK protein (Figure 4) (27, 28).

How do antibodies to MuSK cause MG?

We have provided the first piece of evidence that active immunization with MuSK protein reproduces the MG-like disease in animals (23, 28). Next, we focused on how MuSK antibodies cause MG. The pathogenic role of MuSK antibodies in MG has been questioned, since the number of AChRs is not reduced and complement is not deposited

at the NMJs of biceps brachii muscles from MuSK-positive patients with MG (26). The mechanisms used by AChR antibodies to cause MG are well delineated (1, 2), but those mechanisms simply do not apply to MG associated with MuSK antibodies. MuSK antibodies have been identified as predominantly IgG4, which does not activate complement. However, antibodies binding to MuSK could accelerate the degradation of MuSK molecules (antigenic modulation) and/or inhibit MuSK functions directly. MuSK is essential for AChR clustering at the developing NMJs, and its deficiency may lead to the complete loss of junctional ultrastructure (22, 29). Further, MuSK may also play important roles in the maintenance of AChR clustering and the structure of mature NMJs. To reveal precisely how MuSK antibodies participate in MG, unraveling the way in which MuSK acts at mature NMJ is necessary.

To elucidate the mechanisms of AChR clustering at NMJs, numerous studies were performed using cultured C2C12 myotubes (Figure 5). Agrin induces clustering of AChR in C2C12 myotubes following autophosphorylation by MuSK. *In vivo*, this event represents a major cascade of AChR clustering at the NMJ after innervation by motoneurons. Laminin-1 and the *N*-acetylgalactosamine (GalNAc)-specific lectin *Vicia villosa* agglutinin (VVA-B4) also induce AChR clustering on C2C12 myotubes, without activation of MuSK. Neither the receptor nor the activation mechanisms of AChR clustering induced by agrin-independent inducers has been identified with certainty. However, these mechanisms may also be important for the formation and maintenance of NMJs, the latter via agrin-independent pathways as shown by genetic studies (23).

In a previous study, Hoch et al. observed that the MuSK antibodies of MG patients inhibited agrin-induced AChR clustering in C2C12 myotubes (4). We also found that agrin-induced clustering of AChR was strongly blocked in the presence of MuSK antibodies, whereas absorption of the antibodies with purified MuSK products prevented this blocking effect as illustrated in Figure 5 (23). These results showed that MuSK antibodies effectively inhibited the formation of agrin-induced AChR clustering. Intriguingly, the monovalent Fab fragments of MuSK antibodies from rabbits with experimental autoimmune MG also inhibited AChR clustering by agrin on C2C12 cells, indicating that complement-mediated mechanisms are not necessarily required for such inhibition (unpublished data). We also noted that MuSK-specific antibodies strongly inhibited AChR clustering induced by all known agrin-independent pathways as well as by agrin itself (Figure 5) (23).

We then examined the reduced expression of AChR at NMJs in soleus muscles of paretic and normal rabbits by using fluorescence microscopy after applying a rhodamine-conjugated AChR agonist, α-BTX (Figure 6). The use of a digital camera and staining with rhodamine-conjugated α-BTX enabled us to record the size and optical densities of AChR clusters. The resulting images were measured by using NIH image analysis software (23). The areas and intensity of AChR fluorescence in muscles of these paretic rabbits were significantly reduced compared with those in normal rabbits. In addition, the structure of NMJs in our paretic rabbits as well as the size and branching of the motor terminals were significantly reduced. Electron microscopic observations of NMJs in rabbits with EAMG induced by injection of MuSK protein demonstrated a significant loss of complexity in the convoluted synaptic folds but no destruction. A particularly important observation was that the EAMG model cited here resembles the phenotype of humans with MG and MuSK antibodies (Figure 7). In the intricate and convoluted synaptic folds, the high density of voltage gated sodium channels in the membranes' depths amplify the end-plate current, thus enhancing neuromuscular transmission and muscle contraction (30). A reduction in the size and branching of the motor terminals contributes to the reduced ACh output, and reduced post-synaptic folding increases the threshold for generation of muscle fiber action potential. These structural abnormalities in NMJ, including both pre- and post-synaptic structures, thus impair neuromuscular transmission in rabbits with EAMG (28).

Intriguingly, similar abnormalities of NMJ structure were also observed in rats with reduced expression of MuSK as noted by RNA interference (22), in a patient with congenital myasthenic syndromes (CMS) caused by MuSK mutations and also in mice expressing the MuSK missense mutation seen by electroporation experiments (31). MuSK knock-out mice also displayed presynaptic defects in addition to postsynaptic ones, indicating that MuSK is required for retrograde signals, so far unidentified, to maintain the pre-synaptic structure in mature NMJ.

Dok-7 is required for the maintenance of NMJs

In 2006, a MuSK-interacting protein called Dok-7 was discovered (32) and identified as a member of the Dok family of cytoplasmic proteins. Dok-7 is postulated to have three main functional domains: a pleckstrin homology (PH) domain, essential for membrane association, a phosphotyrosine-binding (PTB) domain involved in the Dok-7

induced activation of MuSK and a large C-terminal domain containing multiple tyrosine residues. Dok-7 knockout mice showed a marked disruption of neuromuscular synaptogenesis that was indistinguishable from the features found in MuSK-deficient mice. Thus, Dok-7 is essential for neuromuscular synaptogenesis through its interaction with MuSK.

Mutations in the Dok-7 protein cause the genetic form of limb-girdle myasthenia called CMS (33). Some clinical features of these patients resemble those in the severe type of MG accompanied by MuSK antibodies (34). Proximal muscles are usually more affected than those in distal regions, as evident in MuSK MG patients, and ptosis is often present. However, limb-muscle weakness is comparatively less severe. Previous studies showed no reduction of AChR clustering with significant changes in NMJ of MuSK MG patients (26), but further structural analysis of NMJ is required in muscles where severe weakness occurs commonly. The weakness and atrophy are not observed uniformly in muscles of these patients, although both MuSK and Dok-7 are essential for the formation of NMJs during the embryonic stage (32). Notably, one of the major distinctions between acquired MuSK MG and CMS with the Dok-7 mutation is the timing when weakness begins. The CMS patients typically have difficulty in walking after reaching that normal motor milestone during early childhood, whereas the onset of weakness of MG patients, in most instances, occurs in adulthood. Interestingly, AChR clustering and post-synaptic folds are reduced and have small motor terminals as observed at NMJs in patients with CMS and Dok-7 mutations (35). The effect of Dok-7 mutations on post-synaptic structures may also be an alteration of retrograde signaling to the pre-synaptic nerve terminals resulting in a reduced NMJ size in these patients (Figure 7). Dok-7, along with MuSK, is also required for the maintenance of NMJ, not only for synaptogenesis.

MuSK plays important roles in the maintenance of NMJs

We have shown that MuSK is required for the maintenance as well as the generation of NMJs (23, 28). Disruption of those mechanisms by MuSK antibodies causes MG in humans. Use of an experimental model for MG revealed that MuSK antibodies mediate the pathogenesis of this syndrome in rabbits and mice (23, 27, 28). In most cases, the symptoms take more than three months to manifest themselves in rabbits and more than four weeks in mice. Moreover, the symptoms were also induced experimentally by

passive transfer of MuSK antibodies from MG patients into animal hosts. However, the injection of a large amount of human MuSK antibodies into mice can barely induce EAMG (36). The mechanisms employed by these antibodies include multiple events during which MuSK functions stall in their process of regulating synapse formation and maintenance (37). MuSK antibodies against compound antigenic determinants in the extracellular domain may engage in their pathogenic activities through antigenic modulation and/or restraint of MuSK functions, and the consequences of these effects range from a partial to entire loss of MuSK function without the involvement of complement-mediated damage. The point that MuSK antibodies in MG patients are mainly of the IgG4 subclass, which does not activate complement, may be relevant here. These diverse possibilities reflect the complexity of clinical features seen in such patients ranging from typical MG throughout its many variants.

Aging and NMJs

How can we extend the studies of MG to understand *sarcopenia*? The structural changes of NMJs in aged rats have suggested that active remodeling mechanisms at the synapse between nerve and muscle may play crucial roles in the progression of *sarcopenia* (Figure 8) (38). Our studies of MG with MuSK antibodies demonstrated that the structure of NMJs is not statically maintained; rather, the nerve-to muscle and muscle-to nerve signals stimulate dynamic assembly and disassembly of NMJs' molecular complexes. A steady flow of molecular complexes at NMJs sustains both the structures and functions of the motor system including motoneurons and muscles. However, we do not completely understand the molecular mechanisms, although our animal models of MG demonstrated clearly that muscle-to-nerve signal transduction requires the maintenance of NMJs (37). We think that studying MG caused by the failure of NMJ maintenance will facilitate further progress in resolving the molecular basis of muscle atrophy. Additional areas of relevance are the many physical conditions, including aging, injury, cancer or AIDS, in which muscles shrink or atrophy. Understanding the molecular basis of NMJ maintenance promises to provide new targets for innovative therapeutics to create healthy, enduring muscles.

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Figure Legends

Figure 1. Structure and molecular architecture of the NMJ.
Drawings show progressive enlargement of segments of a NMJ. The presynaptic terminals consist of multiple swellings called synaptic terminals covered by a thin layer of Schwann cells. The nerve terminal occupies a shallow gutter in the muscle fiber and is capped by processes of Schwann cells. ACh from 150 to 200 vesicles is released from the active zones in the nerve terminal, which directly oppose junctional folds in the postsynaptic membrane. The terminals are separated from the postsynaptic cell by the synaptic cleft, which is about 50 nm wide. AChRs, MuSK and rapsyn concentrated at the peaks of postsynaptic folds are shown, with their subcellular localizations indicated by bars. Voltage gated sodium channels are localized in the depths of postsynaptic folds.

Figure 2. Schematic representation of the MuSK domain structure and expression of secretory MuSK proteins in COS-7 cells. The domain structures of recombinant secretory MuSK protein (MuSK-His and MuSK-Fc) and receptor-type MuSK are shown. The whole coding region of the MuSK extracellular domain was fused with the His-tag or Fc region of human IgG1 as shown.

Figure 3. Rabbits manifest myasthenia gravis (MG)-like paresis after immunization with MuSK protein.
(A) Two rabbits representative of four animals with positive outcomes developed myasthenic weakness after immunization with the recombinant MuSK protein. After three injections of MuSK protein, M1 and M2 rabbits manifested flaccid weakness within three and nine weeks, respectively. M2 rabbit developed severe exhaustion with muscle weakness. (B) Cross-sections from the soleus muscles of two paretic (M1 and M2) and a normal rabbits (Normal) were stained with H&E. Muscle fibers in M1 paretic rabbit showed only subtle changes in shape and size, whereas atrophy of muscles fibers in M2 paretic rabbit was observed as small angular fibers (indicated by arrows). Scale bar, 50mm. (C) Electromyograms recorded from M1 paretic rabbit. The retro-auricular branch of the facial nerve was continuously stimulated with constant square-wave pulses of 0.1 msec at 20 Hz delivered by a current stimulator, and the compound muscle action potential (CMAP, second peak observed on the oscilloscope

screen recorded at the indicated time-points during stimulation) showed a decremental pattern, consistent with MG.

Figure 4. Manifestations of MG after injection of purified MuSK proteins in a mouse.

Figure 5. Inhibition of agrin-induced and agrin-independent AChR clustering by MuSK antibodies. (A) C2C12 cells were treated with agrin, laminin-1, or VVA-B4. AChR clusters were stained with rhodamine-conjugated BTX. AChR clustering induced by agrin, laminin-1, and VVA-B4 was inhibited in the presence of MuSK antibodies. This inhibition was blocked by absorption of the MuSK antibodies with MuSK-AP before treatment of the cells. Scale bar: 20 mm. (B) Quantification of the inhibitory activity of the MuSK antibodies confirmed that they significantly inhibited agrin-, laminin-1–, and VVA-B4-induced AChR clustering. Preabsorption of the MuSK antibodies with MuSK-AP significantly blocked inhibition. Values represent means ± SEM of 10-15 fields for each of the 2 experiments per treatment. *$P < 0.01$ versus similar treatment without MuSK antibodies; #$P < 0.01$ versus similar treatment without preabsorption; ANOVA. J. Clin. Invest. 2006; 116:1016-1024. Copyright 2009 The American Society for Clinical Investigation.

Figure 6. Reduction of the size and density of AChR clusters at the NMJs in paretic rabbits. (A) Cross sections from the soleus muscles of 2 paretic (M1 and M2) and 3 normal rabbits (N1, N2, and N3) were stained with 10 nM rhodamine-conjugated BTX. Bright crescents of bound BTX, indicative of endplate AChR, were smaller and less intense in the paretic rabbits' muscle fibers than in those of the normal rabbits. Arrows indicate the small angular fibers in M2 soleus muscles. L, left; R, right. Scale bar: 50 mm. (B) Images of 10 AChR clusters at NMJs in the right and 10 in the left soleus muscles of the paretic and normal rabbits were randomly recorded by a digital imaging camera. Quantification of the area and intensity of AChR clustering in the unprocessed images were measured using NIH Image software. Bars indicate means ± SD. *$P < 0.01$ versus normal rabbits. J. Clin. Invest. 2006; 116:1016-24. Copyright 2009 The American Society for Clinical Investigation.

Figure 7. Schematic appearance of NMJs observed in normal humans and MG patients.

(A) Normal NMJ. AChRs are concentrated at the peaks of abundant and well-preserved, highly complex convoluted junctional folds. (B) and (C) NMJs observed in experimental animals that model MG induced by MuSK antibodies and in patients with the congenital myasthenic syndrome from MuSK or Dok-7 mutations. Small NMJs in both pre- and post-synaptic structures. (B) Attenuation of AChR and reduced complexity of synaptic folds at post-synaptic membranes without widened synaptic spaces. (C) Disappearance of post-synaptic folds with preserved synaptic space. (D) NMJs in MG patients with AChR antibodies. The myasthenic junction has a reduced number of AChRs, simplified synaptic folds and a widened synaptic space with a normal nerve terminal.

Figure 8. Scanning electron micrographs of NMJs in extensor digitorium longus muscles of young and aged subjects. (A) In a 4-month-old rat. Convoluted and winding synaptic gutters with numerous slit-like junctional folds. (B) In a 22-month-old rat. A number of cup-like depressions with slit-like junctional folds link together. A nerve ending, which faces toward the muscle apparatus, consists of numerous small protrusions of the terminal axons that may represent individual depressions. X3000 (scale bar 5 mm). Reprinted from Desaki et al. Virchows Arch. 2000;437:388-95.

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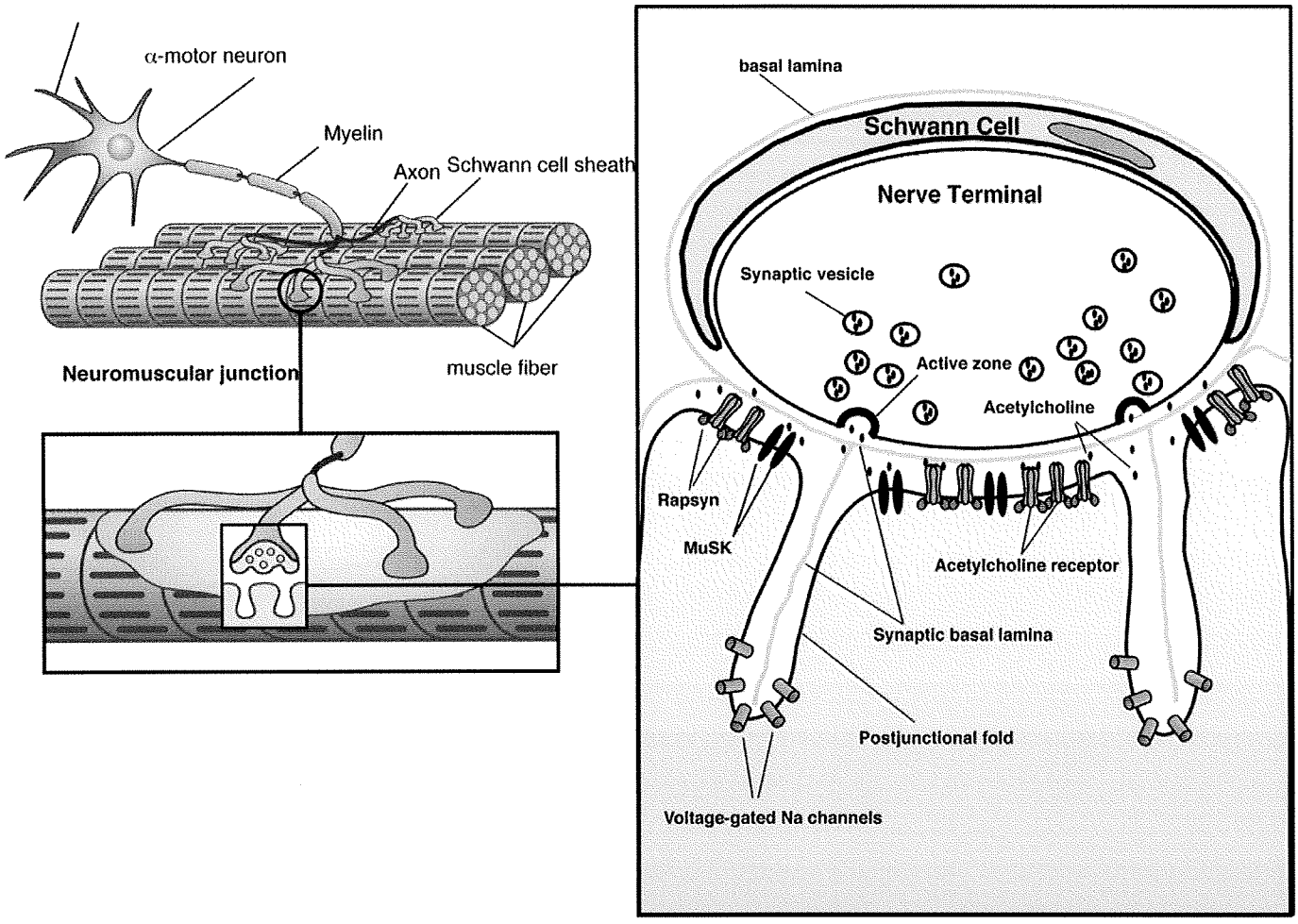
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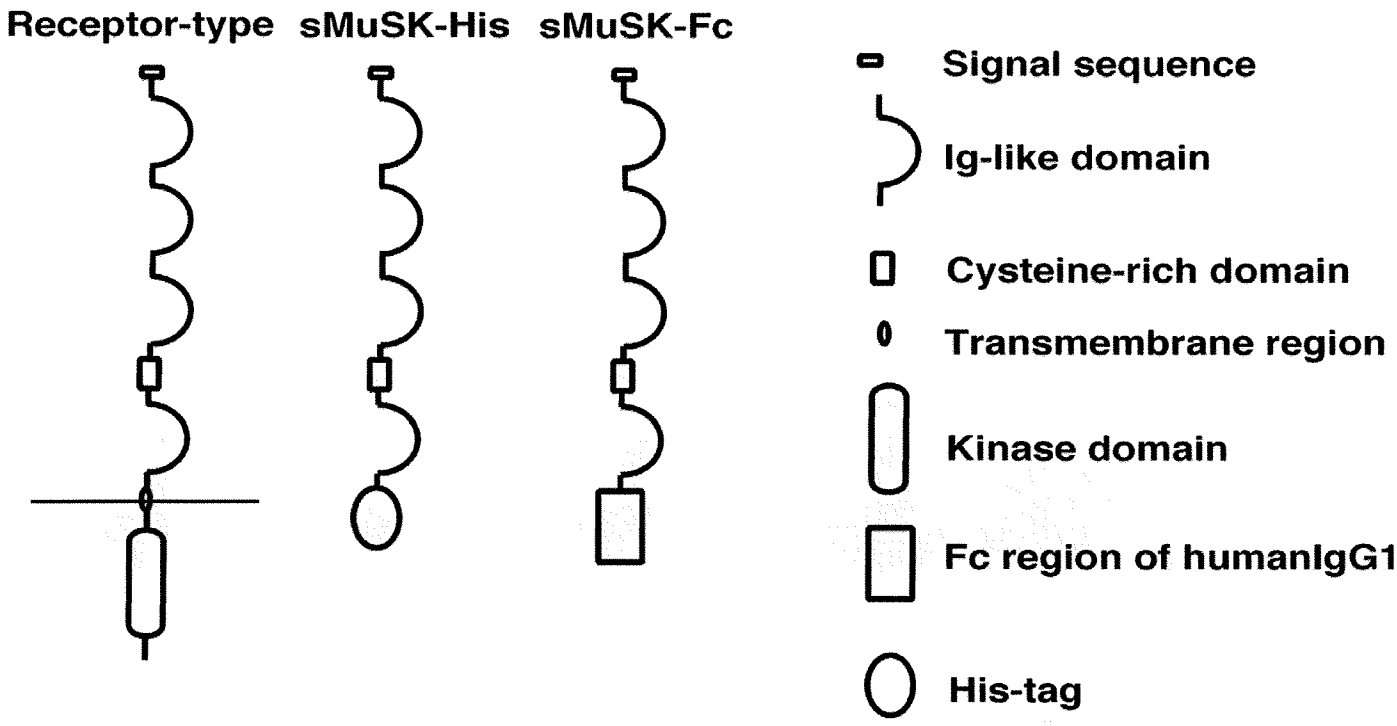
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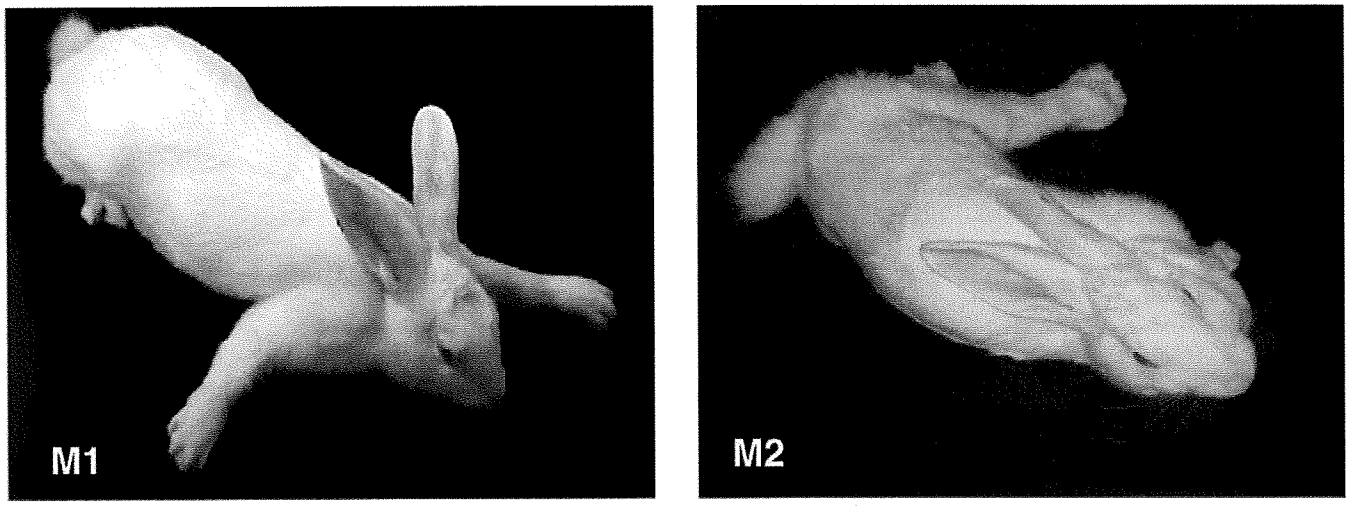
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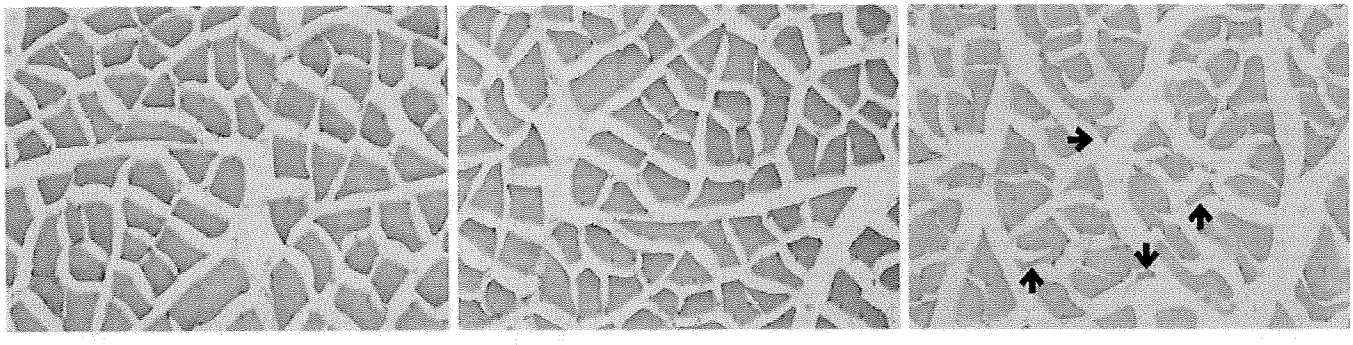
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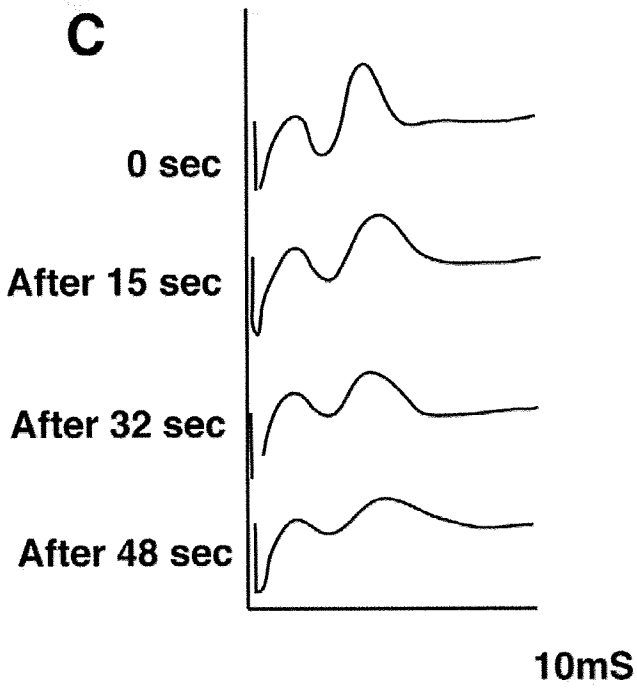
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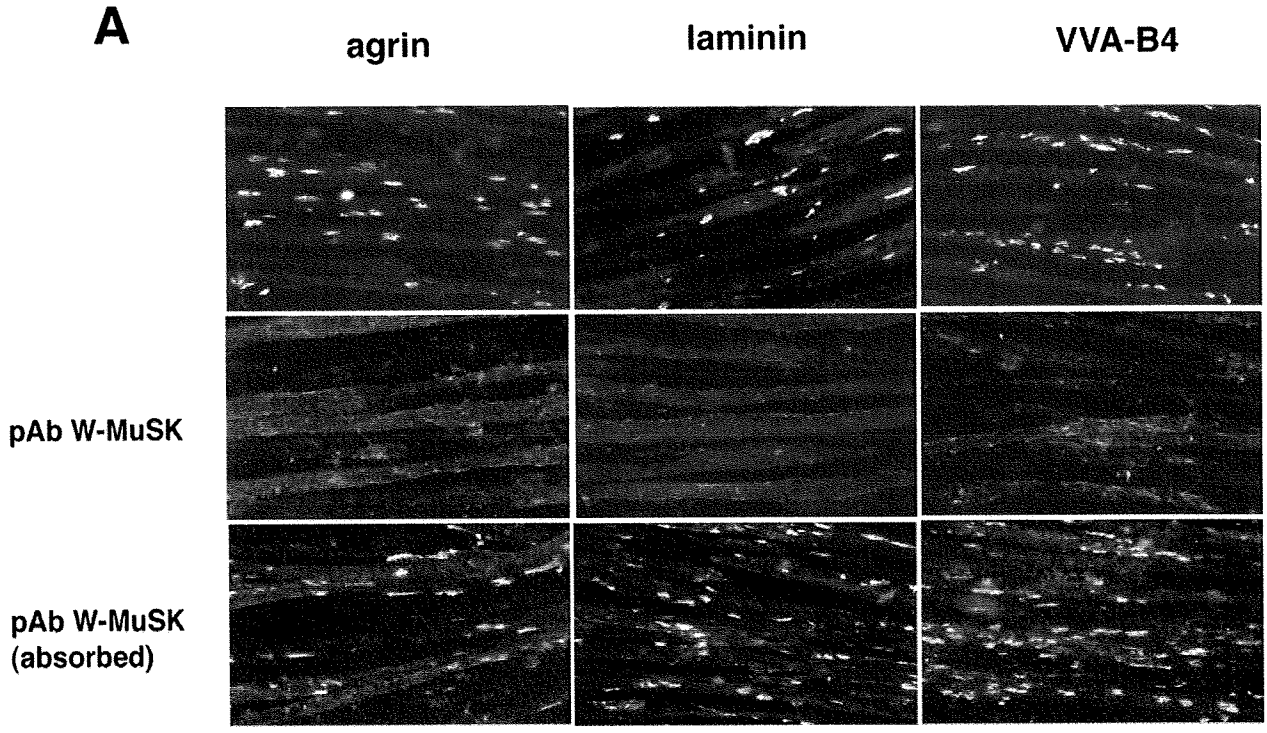
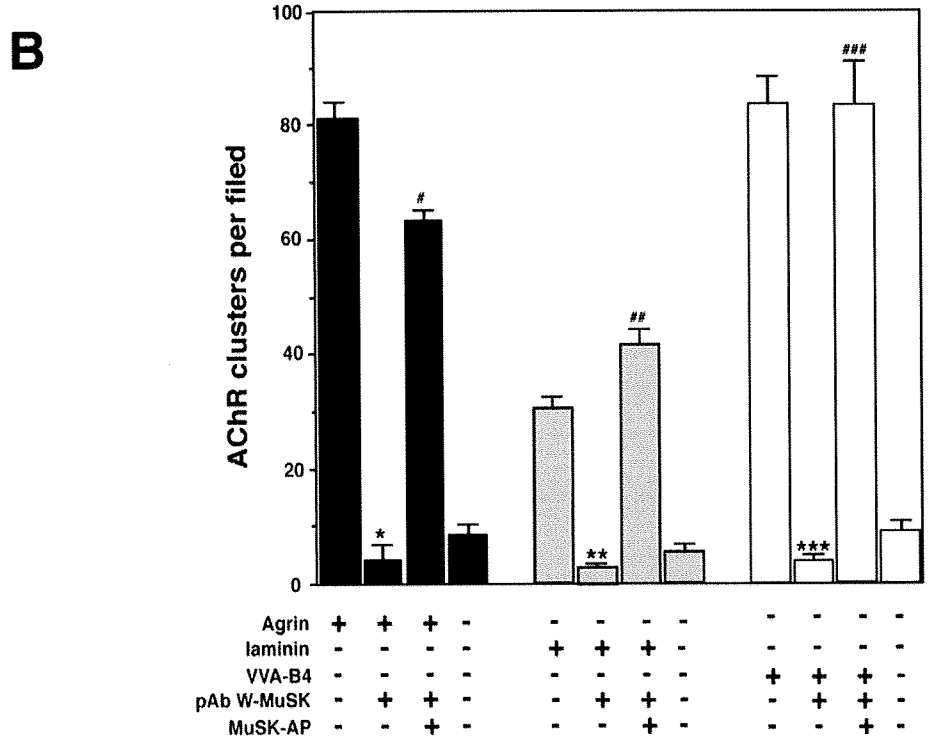
10mS


A
agrin
laminin
VVA-B4
pAb W-MuSK
pAb W-MuSK
(absorbed)


B
AChR clusters per filed
100
80
60
40
20
0
#
##
###
*
**
***
Agrin + + + - - - - - - - - - -
laminin - - - - + + + - - - - -
VVA-B4 - - - - - - - - + + + -
pAb W-MuSK - + + - - + + - - + + -
MuSK-AP - - + - - - + - - - + -