The inbred mice (C57BL/6J strains) were injected daily (intraperitoneally - IP) with 45mg human IgG from two patients (anti-MuSK-2; patient 2, and anti-MuSK-3; patient 3, in Figure 5A and B) for more than 5 days with a single IP injection of cyclophosphamide monohydrate 24 hours after the first IgG injection to suppress immune reactions against the human protein. After 14 days of injections, mice developed signs of weakness such as chin down, flaccid tail, and limb weakness with a prominent cervicothoracic hump (Figure 5D), which may reflect weak cervical extensor muscles. These features were also observed in mice with EAMG induced by MuSK immunization (11, 12). Mice injected with control IgG showed neither weight loss nor muscle weakness compared with uninjected mice (Figure 5C). Decremental electromyographies typical of MG patients were recorded in the mice injected with human anti-MuSK antibodies (Figure 5E). The evidence of EAMG from the passive transfers clearly demonstrated that anti-MuSK antibodies in MG patients cause their disease rather than being just bystander antibodies.

2. Structures of NMJ

Before we discuss the pathogenic roles of MuSK antibodies in EAMG and patients, we should look at the structure of NMJs and MuSK functions. NMJs are the sites of synapses between motor nerves and muscle fibers (Figure 6). The NMJ comprises portions of three cells such as motor neurons, muscle fibers and Schwann cells (28, 29). The motor nerve terminal is specialized for neurotransmitter (acetylcholine:ACh) release. Synaptic vesicles storing ACh adjacent to specialized structures of the presynaptic membrane are called active zones. The active zones are

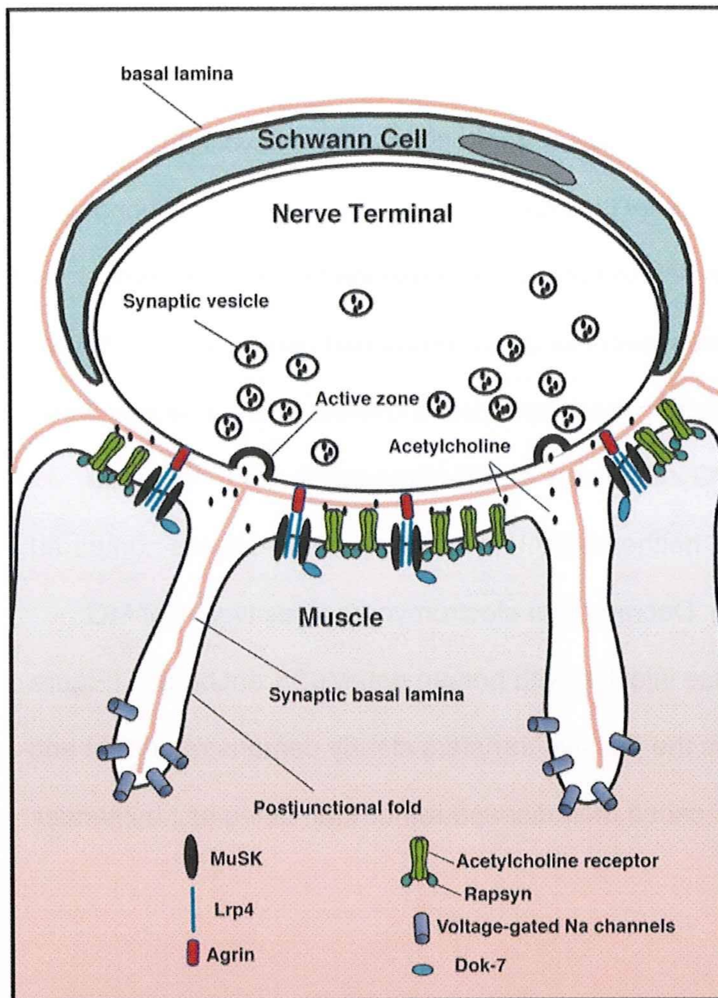

Figure 6. Structure and molecular architecture of NMJ. Details are discussed in the text.

precisely opposite mouths of the postjunctional folds (30, 31). AChRs are highly concentrated, with a density of about 12,000 receptors per μm^2(32), at the post-junctional membrane nearest the fold's peak (Figure 6) (33). Rapsyn is co-localized with AChRs as a scaffold molecule and required for the clustering of AChRs (34). When the nerve action potential reaches the terminal, depolarization opens voltage-gated Ca^+ channels on the presynaptic membrane (35, 36). This allows a Ca^+ influx that triggers the fusion of synaptic vesicles with the presynaptic membrane and the release of ACh

(37, 38). The postsynaptic membrane responds rapidly and dependably to ACh released from the overlying active zones in the nerve terminal. AChRs, by binding ACh, become transiently permeable to both Na^+ and K^+, then opening the associated voltage-gated ion channels in the depths of folds, which contribute to the action potential and muscle contraction (28). The synaptic cleft between nerve terminals and the postsynaptic membrane is about 50 nm wide (39, 40). A layer of connective tissue called basal lamina (basement membrane) sheaths each muscle fiber, passes through the synaptic clef and extends into the junctional folds (29). Both the presynaptic terminal and the muscle fiber secrete molecules including collagen IV, laminin, ectactin and heparan sulfate proteoglycans (agrin, perlcan, etc.) to the basal lamina. However, synaptic portions of the basal lamina contain their distinctive isoform composition separate from that of the extrasynaptic portions. Synaptic basal lamina contains the enzyme acetylcholinesterase, which quickly inactivates the ACh released from the presynaptic terminal by hydrolyzing it to acetate and choline. Acetylcholinesterase is clustered with AChRs by association with collagen Q and MuSK at the crest of the junctional folds (41). Concentrations of released ACh in the synaptic cleft decrease rapidly by diffusion and interaction with acetylcholinesterase, upon which the neuromuscular transmission terminates.

3. MuSK functions in NMJ

MuSK, which is a receptor-tyrosine kinase, is a component of the agrin receptor with the low-density-lipoprotein receptor-related protein 4 (LRP4) at the postsynaptic

membrane (Figure 6) (8, 42-46). MuSK knock-out mice display devastating defects in both pre- and post-synaptic differentiation and die at birth because they cannot breathe, thus MuSK requires the formation of NMJs during neuromuscular development (8). In the knock-out mice, motor axons grow excessively and fail to form terminal arbors, and no AChR-rich clusters are present on myotubes opposing ingrowing motoneuron terminals as shown in Figure 7. Since MuSK is expressed in skeletal muscle but not in motor neurons (42), MuSK requires regulation of the retrograde signals for differentiation of pre-synaptic structures in NMJs (8).

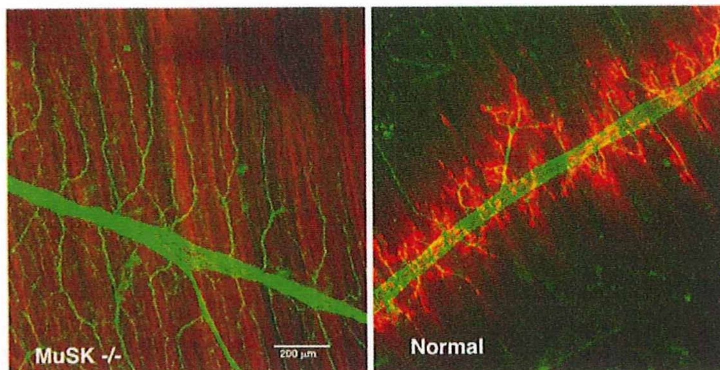

Figure 7. Aberrant structures of pre- and postsynaptic differentiation in diaphragm muscle from MuSK-/- mutant mouse. Motor axon (green), AChR(red).

MuSK also plays multiple roles in AChR clustering during development of the postsynaptic membranes of NMJs (47, 48). Contact of the motor-nerve growth cone with the muscle induces a narrow, distinct endplate zone in the mid-muscle that is marked by a high density of AChR clustering. In this step, innervation disperses aneural AChR clusters in nonsynaptic areas via muscle activity elicited by ACh (49, 50), whereas in synaptic regions, this negative activity is overpowered by agrin delivered by motor nerve terminals to induce AChR clusters (44, 48). However, since agrin does not bind MuSK, additional components are required to activate MuSK (44, 47). Recent

studies demonstrated that LRP4, a member of the LDLR family, is a long-sought receptor of agrin, forms a complex with MuSK, and mediates MuSK activation by agrin (45, 46). MuSK is also required for organizing a primary synaptic scaffold to create the post-synaptic membrane (48). Prior to muscle innervation, AChR clusters form at the central regions of muscle fibers, creating an endplate zone that is somewhat broader than that in innervated muscle (51, 52). Thus, MuSK is required for pre-patterning of AChR clustering in the absence of motor innervation. An intriguing finding is that MuSK has a cysteine-rich domain that shows homology to the Wnt receptor Frizzled (53, 54). Wnt is a family of secreted proteins that are implicated in neural development, neural outgrowth, navigation, and synaptogenesis (55-60). Wnt ligans bind to the receptor complex of Frizzled and LRP5/6 (61). Subsequently, signal is transmitted to the adaptor protein Dishevelled (Dvl), which interacts with Frizzled, to initiate intracellular canonical and noncanonical pathways. Like Frizzled, MuSK might interact on the membrane with LRP4 as a Wnt receptor and promote postsynaptic specialization at mammalian NMJ as in Zebrafish (62, 63). In fact, pre-patterning of AChR clustering disappears in LRP4 mutant mice as MuSK-deficient mice (46). Intriguingly, a number of Wnt signaling molecules including APC, β-catenin, Dvl have been implicated in MuSK signaling (63). Simultaneously or alternatively, MuSK could, thereby, form a primary scaffold molecule without activation by agrin.

The listed pleiotropic roles of MuSK in AChR clustering at developmental NMJs could also be required for the maintenance of mature NMJs throughout life. 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 NMJs, although the synaptic membrane in adult muscle appears macroscopically to be stable (64-66). Therefore, the mechanisms at play during AChR clustering in developing NMJs are also required in mature NMJ where postsynaptic complexes including those with AChR and MuSK dynamically turn over for the maintenance of muscle function. Furthermore, MuSK requires the development and maintenance of synaptic structures at NMJs after birth. The disassembly of NMJ structures *in vivo* can be induced by a reduction of MuSK expression using the RNA interference technology in single muscle fibers of adult rats (67). Postnatal inactivation of MuSK using the loxP/Cre system in mice also causes loss of AChRs and disassembly of the postsynaptic organization (68). The conditional inactivation of MuSK in mice during postnatal development leads to defects in NMJ maintenance and premature death. Studies of these dynamics indicated that MuSK is required for retrograde signals, so far unidentified, to maintain the pre-synaptic structure in mature NMJs (69). Moreover, histopathological studies disclosed the same changes of NMJs in mature animals with MuSK EAMG.

4. Pathogenic roles of anti-MuSK antibodies in EAMG

The establishment of MuSK EAMG, which reproduces the clinical and electrophysiological features of MG, enabled assessment of the pathogenic roles of the relevant autoantibodies in experimental animals (10). Histopathologic examination of

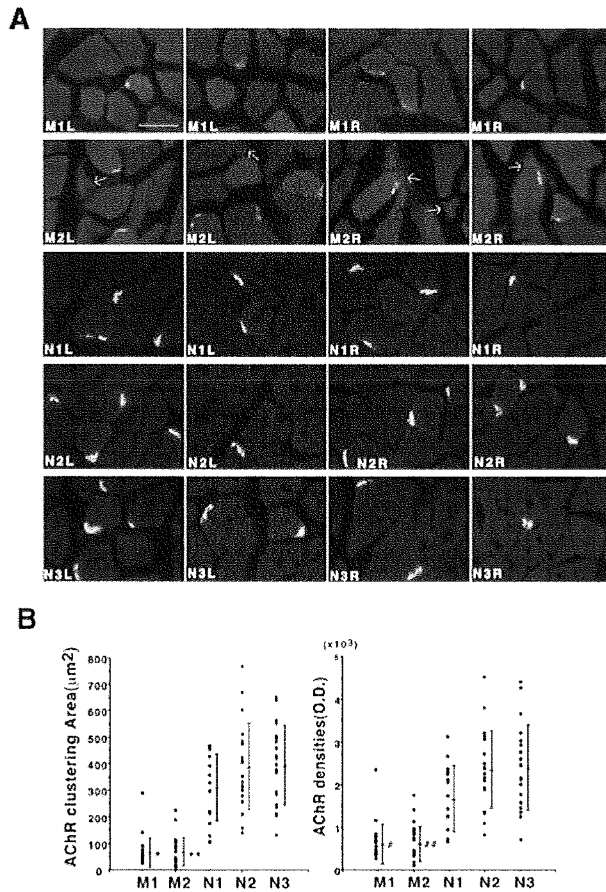

Figure 8. Reduction of the size and density of AChR clusters at the NMJs in paretic rabbits with MuSK EAMG. (A) Cross sections from the soleus muscles of 2 paretic (M1 and M2) and 3 normal rabbits (N1, N2, and N3). (B) Quantification of the area and intensity of AChR clustering. (Reprinted with permission from Shigemoto et al., *J Clin Invest* 116:1016-1024. 2006)

NMJs in rabbits with MuSK EAMG revealed that anti-MuSK antibodies interfere with MuSK functions at play in the maintenance of mature NMJs described in the previous section. Reduced expression of AChRs at the NMJs was observed by using fluorescence microscopy after applying a rhodamine-conjugated AChR agonist, α-BTX (Figure 8A and B) (10). The areas and intensity of AChR fluorescence at NMJs in muscles of paretic rabbits were significantly reduced compared with those in normal rabbits. Anti-MuSK antibodies in rabbits with EAMG blocked agrin-induced clustering of

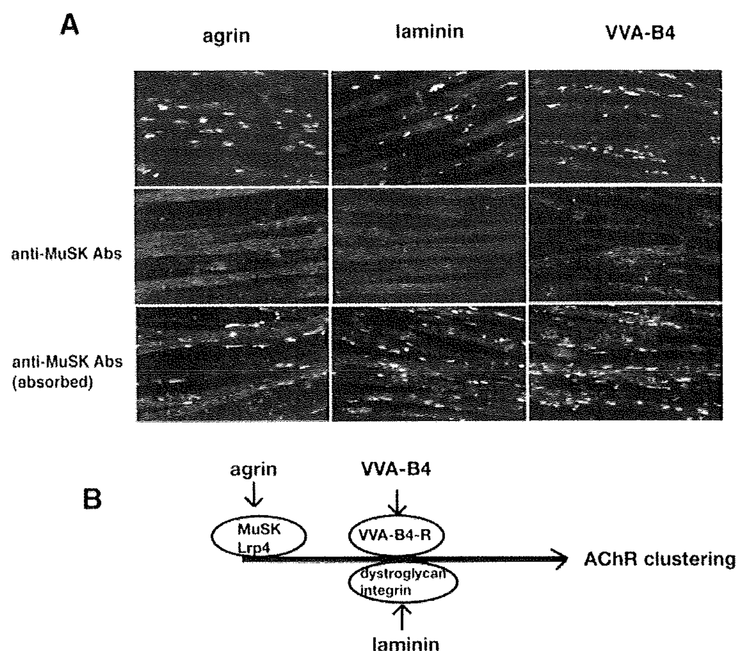

Figure 9. (A) Inhibition of agrin-induced and agrin-independent AChR clustering by MuSK antibodies. This inhibition was blocked by absorption of the MuSK antibodies with MuSK protein before treatment of the cells. (Reprinted with permission from Shigemoto et al., *J Clin Invest* 116:1016-1024. 2006) (B) VVA-B4 binds the unidentified receptors and induces AChR clustering. Laminin induces AChR clustering by binding with dystroglycan and integrin.

AChRs in C2C12 myotubes as well as the human antibodies, whereas absorption of the antibodies with purified MuSK products prevented this blocking effect as illustrated in Figure 9A.

To elucidate the mechanisms of AChR clustering at NMJs, numerous studies have been performed using cultured C2C12 myotubes (44). Agrin induces the clustering of AChRs in C2C12 myotubes following autophosphorylation by MuSK. *In vivo*, this event represents a major cascade of AChR clustering at the NMJs after innervation by motoneurons (42). These results showed that MuSK antibodies effectively inhibited the formation of agrin-induced AChR clustering. The monovalent Fab fragments of MuSK antibodies, from rabbits with MuSK EAMG also inhibited AChR clustering by agrin on C2C12 cells indicating that complement-mediated mechanisms are not necessarily required for such inhibition (unpublished data). In addition, the anti-MuSK antibodies in

rabbits strongly inhibited AChR clustering induced by all known agrin-independent pathways as well as by agrin itself (Figure 9A) (10). Soluble laminin and the *N*-acetylgalactosamine (GalNAc)-specific lectin *Vicia villosa* agglutinin (VVA-B4) also induce AChR clustering on C2C12 myotubes (Figure 9A and B), without activation of MuSK (10, 70-73). Laminin can induce clustering of AChRs via a pathway that involves tyrosine kinases downstream of MuSK by association with dystroglycan and b1integrin (Figure 9B) (74, 75). VVA-B4 selectively stains NMJs in skeletal muscles and AChR-rich portions of the C2C12 myotube surface (76, 77); however, the receptor remains to be identified (78). Laminin and VVA-B4 receptors lie downstream from and are common to agrin-mediated AChR clustering pathways (78). These receptors may be associated at postsynaptic membranes and cooperate in the maintenance of AChRs in NMJs. Without question, autoantibodies to MuSK will serve as useful tools for comprehending how MuSK functions at mature NMJs and the pathogenic mechanisms of MuSK MG.

In addition to the loss of AChR during EAMG, we have also noted the disassembly of NMJ structures and a reduction in the size and branching of the motor terminals in the paretic rabbits (Figure 10A). Electron microscopic observations of NMJs in rabbits with MuSK EAMG demonstrated a significant loss of complexity in the convoluted synaptic folds but not their destruction (unpublished data). Similarly, the NMJs of mice with MuSK EAMG incited by active immunization with MuSK (Figure 10B) and passive transfer of IgG from MuSK MG patients presented with the same histopathological picture (11-13). A reduction in the size and branching of the motor terminals contributes to a decreased ACh output, and a loss of AChRs and post-synaptic folding at NMJs increases the threshold for generation of muscle fiber action

potential. These structural abnormalities in NMJs, including both pre- and post-synaptic structures, thus impair neuromuscular transmission in animals with MuSK EAMG (10-13, 69).

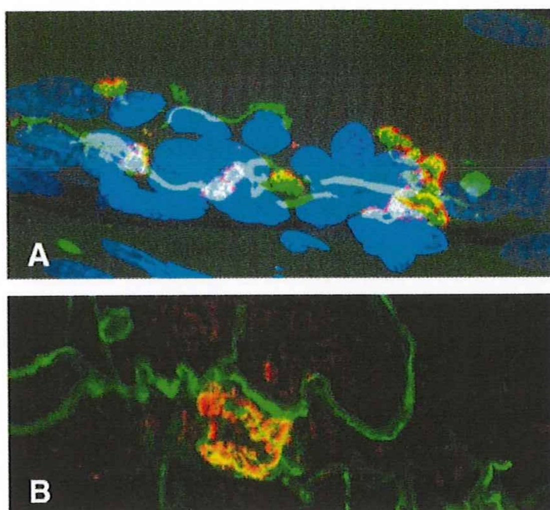

Figure 10. Disassembly of NMJs induced by anti-MuSK antibodies. Motor axon (green), AChR(red), subsynaptic nucleus (blue). (A) NMJs in a rabbit with MuSK EAMG. (B) NMJs in a mouse with MuSK EAMG.

The susceptibility to muscle weakness caused by anti-MuSK antibodies may vary among animal species, since the structures of NMJs differ greatly among them (79). Human NMJs are among the smallest found in the well-studied vertebrates. Therefore, the structural factors affecting the impairment of neuromuscular transmission by anti-MuSK antibodies may also vary among animals (79). However, anti-MuSK antibodies in rabbits and mice with MuSK EAMG inhibit agrin-induced AChR clustering on C2C12 myotubes much like that in MG patients (7), indicating the commonality of anti-MuSK antibodies as a cause of MG beyond the difference of species (10, 11).

The mechanisms used by anti-AChR antibodies to cause MG are well delineated (3, 4, 27), but those mechanisms simply do not apply to MG associated with anti-MuSK antibodies. One route of pathogenicity by anti-AChR antibodies is to damage the

postsynaptic membrane in NMJs, whereas antibodies to MuSK may interfere directly with the functions of MuSK required for maintaining NMJ structures (10, 12). Anti-MuSK antibodies in MG patients have been identified as predominantly IgG4, which does not activate complement (15, 16). Although the IgG1 of anti-MuSK antibodies is present in MG patients, only the titers of IgG4 were significantly associated with disease severity (17). Therefore, IgG4-antibodies binding to MuSK could accelerate the degradation of MuSK molecules (antigenic modulation) and/or inhibit MuSK functions directly (10, 69). In either case, anti-MuSK antibodies inhibit the functions of MuSK in NMJs.

We effectively generated EAMG in complement-deficient mice with severe symptoms by inoculating them with MuSK (unpublished data), whereas the deficient mice were highly resistant to EAMG from AChR immunization (80). Therefore tissue degradation by complement activation is not necessarily required for the manifestation of myasthenia by MuSK antibodies. Some MG patients with anti-MuSK antibodies also have low-affinity antibodies to AChRs found by testing with a highly sensitive immunofluorescence method (21, 81). Currently, the significance of anti-AChR antibodies in patients is not clear, but the clinical features of MuSK-MG are distinct from those for AChR-MG, thus anti-AChR antibodies may not produce visible symptoms in MuSK-MG patients.

Although significant AChR loss and structural changes in NMJs of animals with EAMG are commonly observed, those changes were not reported in the studies of biceps brachii muscles of anti MuSK-positive patients in a previous study (14). However, further histopathological analysis of NMJs in patients with severely weakened muscles would be beneficial for comparison with the animal under study for a similar condition. In

such patients, the weakness and atrophy are not observed uniformly; some anti MuSK-positive patients have more focal weakness in the neck, shoulder and respiratory muscles with prominent cranial and bulbar involvement than patients with anti-AChR antibodies. The limb muscles of individuals with MuSK-MG were less severely affected and inconsistently damaged, whereas patients with generalized AChR-MG patients often suffered from clearcut limb deterioration (82, 83). The mechanisms that favor particular sites of muscle weakness are unknown, and the nature of NMJs among various muscles may not be uniform.

5. The use of animals with EAMG to improve medication

MuSK MG is frequently a severe disease requiring emergent and aggressive therapies. The response to therapy for MuSK MG differs from that for AChR MG (84). Experimentation with animal models of EAMG will contribute to the development of more effective medication as the underlying pharmacological mechanisms become known. Acetylcolinesterase inhibitors are often used as symptomatic treatment and are effective for AChR MG. However, patients with MuSK MG are frequently unresponsive or develop cholinergic crises characterized by increasing muscle weakness that sometimes results in dysphagia and respiratory insufficiency (19, 20, 84, 85). Thus, differentiating a cholinergic crisis from a myasthenic crisis is critical for the decision on medical care of each patient (84, 86). Abnormal sensitivity to ACh after MuSK-MG patients receive acetylcolinesterase inhibitors can sometimes be recorded by electromyography (EMG) as extra discharges occurring after the CMAP with low-

frequency motor nerve stimulation, as shown in Figure 11 (86, 87). Such hypersensitivity to ACh may be caused by the interference of anchoring acetylcolinesterase to the synaptic cleft of postsynaptic membranes by MuSK (41). The same patterns of EMG showing hypersensitivity to ACh can be reproduced in mice with MuSK EAMG (unpublished data). The mechanisms of this hypersensitivity may be elucidated by studies of such animals with MuSK EAMG.

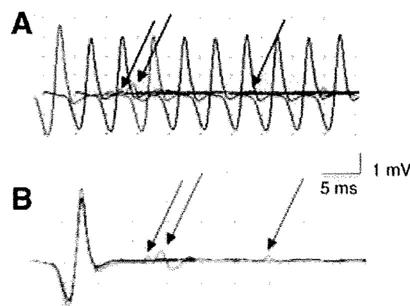

Figure 10. Extra repetitive discharges after the CMAP at low-frequency stimulation were recorded in a MuSK-MG patient with acetylcolinesterase inhibitor treatments. (Reprinted with permission from Punga et al., *Muscle Nerve* 34:111-115.)

Other symptomatic therapy such as 3,4-diaminopyridine, which increases ACh release from the nerve terminal, may be worth trying in MuSK-MG patients. Congenital myasthenic syndrome due to MuSK or Dok-7 mutations has been reported to respond favorably to 3,4-diaminopyridine (88-90). Dok-7 is essential for the formation of NMJs through its interaction with MuSK (91). Patients with MuSK MG apparently do not respond to immunosuppressive therapy as well as those with AChR MG; in fact, some of the former patients who did not respond to the therapy rapidly progressed to life-threatening muscle atrophy (20, 84). However, long-term treatment with higher dose steroids to unrefractory patients may accelerate the distortion of NMJ morphology by anti-MuSK antibodies (92). In view of the clinical variables involved, studying animals

with MuSK EAMG will provide important clues for developing and assessing the appropriate medication for all patients afflicted with MuSK MG.

ACKNOWLEDGMENTS

We thank Ms. P. Minick for excellent editorial assistance. Our studies reported in this review were supported by grants from the Health Science Research Grants for Research on Psychiatric and Neurological Diseases and Mental Health from the Ministry of Health, Labor, and Welfare, Japan, Scientific Research from the Ministry of Education, Science, and Culture, Japan, and the Kato Memorial Trust for Nambyo Research.

References

1. Patrick, J., and Lindstrom, J. 1973. Autoimmune response to acetylcholine receptor. *Science* 180:871-872.
2. Lindstrom, J.M., Seybold, M.E., Lennon, V.A., Whittingham, S., and Duane, D.D. 1976. Antibody to acetylcholine receptor in myasthenia gravis. Prevalence, clinical correlates, and diagnostic value. *Neurology* 26:1054-1059.
3. Conti-Fine, B.M., Milani, M., and Kaminski, H.J. 2006. Myasthenia gravis: past, present, and future. *J Clin Invest* 116:2843-2854.
4. Vincent, A., Lang, B., and Kleopa, K.A. 2006. Autoimmune channelopathies and related neurological disorders. *Neuron* 52:123-138.
5. Mossman, S., Vincent, A., and Newsom-Davis, J. 1986. Myasthenia gravis without acetylcholine-receptor antibody: a distinct disease entity. *Lancet* 1:116-119.
6. Burges, J., Vincent, A., Molenaar, P.C., Newsom-Davis, J., Peers, C., and Wray, D. 1994. Passive transfer of seronegative myasthenia gravis to mice. *Muscle Nerve* 17:1393-1400.
7. Hoch, W., McConville, J., Helms, S., Newsom-Davis, J., Melms, A., and Vincent, A. 2001. Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. *Nat Med* 7:365-368.
8. DeChiara, T.M., Bowen, D.C., Valenzuela, D.M., Simmons, M.V., Poueymirou, W.T.,

Thomas, S., Kinetz, E., Compton, D.L., Rojas, E., Park, J.S., et al. 1996. The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell* 85:501-512.

9. Lindstrom, J. 2004. Is "seronegative" MG explained by autoantibodies to MuSK? *Neurology* 62:1920-1921.
10. Shigemoto, K., Kubo, S., Maruyama, N., Hato, N., Yamada, H., Jie, C., Kobayashi, N., Mominoki, K., Abe, Y., Ueda, N., et al. 2006. Induction of myasthenia by immunization against muscle-specific kinase. *J Clin Invest* 116:1016-1024.
11. Jha, S., Xu, K., Maruta, T., Oshima, M., Mosier, D.R., Atassi, M.Z., and Hoch, W. 2006. Myasthenia gravis induced in mice by immunization with the recombinant extracellular domain of rat muscle-specific kinase (MuSK). *J Neuroimmunol* 175:107-117.
12. Shigemoto, K., Sachiho, K., Chen, J., Naohito, H., Yasuhito, A., Norifumi, U., Naoto, K., Kenji, K., Katsumi, M., Atsuo, M., et al. 2008. Experimentally induced myasthenia gravis with muscle-specific kinase
. *Ann N Y Acad Sci* 1132:93-98.
13. Cole, R.N., Reddel, S.W., Gervasio, O.L., and Phillips, W.D. 2008. Anti-MuSK patient antibodies disrupt the mouse neuromuscular junction. *Ann Neurol* 63:782-789.
14. Shiraishi, H., Motomura, M., Yoshimura, T., Fukudome, T., Fukuda, T., Nakao, Y., Tsujihata, M., Vincent, A., and Eguchi, K. 2005. Acetylcholine receptors loss and postsynaptic damage in MuSK antibody-positive myasthenia gravis. *Ann Neurol* 57:289-293.
15. McConville, J., Farrugia, M.E., Beeson, D., Kishore, U., Metcalfe, R., Newsom-Davis, J., and Vincent, A. 2004. Detection and characterization of MuSK antibodies in seronegative myasthenia gravis. *Ann Neurol* 55:580-584.
16. Ohta, K., Shigemoto, K., Fujinami, A., Maruyama, N., Konishi, T., and Ohta, M. 2007. Clinical and experimental features of MuSK antibody positive MG in Japan. *Eur J Neurol* 14:1029-1034.
17. Niks, E.H., van Leeuwen, Y., Leite, M.I., Dekker, F.W., Wintzen, A.R., Wirtz, P.W., Vincent, A., van Tol, M.J., Jol-van der Zijde, C.M., and Verschuuren, J.J. 2008. Clinical fluctuations in MuSK myasthenia gravis are related to antigen-specific IgG4 instead of IgG1. *J Neuroimmunol* 195:151-156.
18. Vincent, A., Bowen, J., Newsom-Davis, J., and McConville, J. 2003. Seronegative generalised myasthenia gravis: clinical features, antibodies, and their targets. *Lancet Neurol* 2:99-106.
19. Sanders, D.B., El-Salem, K., Massey, J.M., McConville, J., and Vincent, A. 2003. Clinical aspects of MuSK antibody positive seronegative MG. *Neurology* 60:1978-1980.
20. Evoli, A., Tonali, P.A., Padua, L., Monaco, M.L., Scuderi, F., Batocchi, A.P., Marino, M., and Bartoccioni, E. 2003. Clinical correlates with anti-MuSK antibodies in generalized seronegative myasthenia gravis. *Brain* 126:2304-2311.
21. Leite, M.I., Jacob, S., Viegas, S., Cossins, J., Clover, L., Morgan, B.P., Beeson, D., Willcox, N., and Vincent, A. 2008. IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis. *Brain* 131:1940-1952.
22. Bittner, R.E., Anderson, L.V., Burkhardt, E., Bashir, R., Vafiadaki, E., Ivanova, S., Raffelsberger, T., Maerk, I., Hoger, H., Jung, M., et al. 1999. Dysferlin deletion in SJL mice

(SJL-Dysf) defines a natural model for limb girdle muscular dystrophy 2B. *Nat Genet* 23:141-142.
23. Niks, E.H., Kuks, J.B., Roep, B.O., Haasnoot, G.W., Verduijn, W., Ballieux, B.E., De Baets, M.H., Vincent, A., and Verschuuren, J.J. 2006. Strong association of MuSK antibody-positive myasthenia gravis and HLA-DR14-DQ5. *Neurology* 66:1772-1774.
24. Christadoss, P., Krco, C.J., Lennon, V.A., and David, C.S. 1981. Genetic control of experimental autoimmune myasthenia gravis in mice. II. Lymphocyte proliferative response to acetylcholine receptor is dependent on Lyt-1+23- cells. *J Immunol* 126:1646-1647.
25. Christadoss, P., Lennon, V.A., Krco, C.J., and David, C.S. 1982. Genetic control of experimental autoimmune myasthenia gravis in mice. III. Ia molecules mediate cellular immune responsiveness to acetylcholine receptors. *J Immunol* 128:1141-1144.
26. Bell, J., Rassenti, L., Smoot, S., Smith, K., Newby, C., Hohlfeld, R., Toyka, K., McDevitt, H., and Steinman, L. 1986. HLA-DQ beta-chain polymorphism linked to myasthenia gravis. *Lancet* 1:1058-1060.
27. Newsom-Davis, J. 2007. The emerging diversity of neuromuscular junction disorders. *Acta Myol* 26:5-10.
28. Ruff, R.L. 2003. *Neuromuscular Junction Physiology and Pathology*. Totowa, New Jersey: Humana Press.
29. Engel, A.G. 2004. *The Neuromuscular junction*. New York: McGraw-Hill, Medical Pub. Divisio.
30. Heuser, J.E., and Reese, T.S. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J Cell Biol* 57:315-344.
31. Robitaille, R., Adler, E.M., and Charlton, M.P. 1990. Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses. *Neuron* 5:773-779.
32. Salpeter, M.M., and Loring, R.H. 1985. Nicotinic acetylcholine receptors in vertebrate muscle: properties, distribution and neural control. *Prog Neurobiol* 25:297-325.
33. Salpeter, M. 1987. *The Vertebrate Neuromuscular Junction.* New York: Alan R. Liss.
34. Burden, S.J., DePalma, R.L., and Gottesman, G.S. 1983. Crosslinking of proteins in acetylcholine receptor-rich membranes: association between the beta-subunit and the 43 kd subsynaptic protein. *Cell* 35:687-692.
35. Engel, A.G. 1991. Review of evidence for loss of motor nerve terminal calcium channels in Lambert-Eaton myasthenic syndrome. *Ann N Y Acad Sci* 635:246-258.
36. Pumplin, D.W., Reese, T.S., and Llinas, R. 1981. Are the presynaptic membrane particles the calcium channels? *Proc Natl Acad Sci U S A* 78:7210-7213.
37. Smith, S.J., and Augustine, G.J. 1988. Calcium ions, active zones and synaptic transmitter release. *Trends Neurosci* 11:458-464.
38. Augustine, G.J., Adler, E.M., and Charlton, M.P. 1991. The calcium signal for transmitter secretion from presynaptic nerve terminals. *Ann N Y Acad Sci* 635:365-381.
39. Engel, A.G., and Santa, T. 1971. Histometric analysis of the ultrastructure of the neuromuscular junction in myasthenia gravis and in the myasthenic syndrome. *Ann N Y Acad Sci* 183:46-63.
40. Engel, A.G., Tsujihata, M., Lindstrom, J.M., and Lennon, V.A. 1976. The motor end plate in myasthenia gravis and in experimental autoimmune myasthenia gravis. A quantitative

ultrastructural study. *Ann N Y Acad Sci* 274:60-79.
41. Cartaud, A., Strochlic, L., Guerra, M., Blanchard, B., Lambergeon, M., Krejci, E., Cartaud, J., and Legay, C. 2004. MuSK is required for anchoring acetylcholinesterase at the neuromuscular junction. *J Cell Biol* 165:505-515.
42. Valenzuela, D.M., Stitt, T.N., DiStefano, P.S., Rojas, E., Mattsson, K., Compton, D.L., Nunez, L., Park, J.S., Stark, J.L., Gies, D.R., et al. 1995. Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron* 15:573-584.
43. Ganju, P., Walls, E., Brennan, J., and Reith, A.D. 1995. Cloning and developmental expression of Nsk2, a novel receptor tyrosine kinase implicated in skeletal myogenesis. *Oncogene* 11:281-290.
44. Glass, D.J., Bowen, D.C., Stitt, T.N., Radziejewski, C., Bruno, J., Ryan, T.E., Gies, D.R., Shah, S., Mattsson, K., Burden, S.J., et al. 1996. Agrin acts via a MuSK receptor complex. *Cell* 85:513-523.
45. Zhang, B., Luo, S., Wang, Q., Suzuki, T., Xiong, W.C., and Mei, L. 2008. LRP4 serves as a coreceptor of agrin. *Neuron* 60:285-297.
46. Kim, N., Stiegler, A.L., Cameron, T.O., Hallock, P.T., Gomez, A.M., Huang, J.H., Hubbard, S.R., Dustin, M.L., and Burden, S.J. 2008. Lrp4 is a receptor for Agrin and forms a complex with MuSK. *Cell* 135:334-342.
47. Strochlic, L., Cartaud, A., and Cartaud, J. 2005. The synaptic muscle-specific kinase (MuSK) complex: new partners, new functions. *Bioessays* 27:1129-1135.
48. Kummer, T.T., Misgeld, T., and Sanes, J.R. 2006. Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost. *Curr Opin Neurobiol* 16:74-82.
49. Lin, W., Dominguez, B., Yang, J., Aryal, P., Brandon, E.P., Gage, F.H., and Lee, K.F. 2005. Neurotransmitter acetylcholine negatively regulates neuromuscular synapse formation by a Cdk5-dependent mechanism. *Neuron* 46:569-579.
50. Misgeld, T., Kummer, T.T., Lichtman, J.W., and Sanes, J.R. 2005. Agrin promotes synaptic differentiation by counteracting an inhibitory effect of neurotransmitter. *Proc Natl Acad Sci U S A* 102:11088-11093.
51. Lin, W., Burgess, R.W., Dominguez, B., Pfaff, S.L., Sanes, J.R., and Lee, K.F. 2001. Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature* 410:1057-1064.
52. Yang, X., Arber, S., William, C., Li, L., Tanabe, Y., Jessell, T.M., Birchmeier, C., and Burden, S.J. 2001. Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron* 30:399-410.
53. Saldanha, J., Singh, J., and Mahadevan, D. 1998. Identification of a Frizzled-like cysteine rich domain in the extracellular region of developmental receptor tyrosine kinases. *Protein Sci* 7:1632-1635.
54. Masiakowski, P., and Yancopoulos, G.D. 1998. The Wnt receptor CRD domain is also found in MuSK and related orphan receptor tyrosine kinases. *Curr Biol* 8:R407.
55. Burden, S.J. 2000. Wnts as retrograde signals for axon and growth cone differentiation. *Cell* 100:495-497.
56. Zou, Y. 2004. Wnt signaling in axon guidance. *Trends Neurosci* 27:528-532.
57. Ciani, L., and Salinas, P.C. 2005. WNTs in the vertebrate nervous system: from

patterning to neuronal connectivity. *Nat Rev Neurosci* 6:351-362.
58. Salinas, P.C. 2003. Synaptogenesis: Wnt and TGF-beta take centre stage. *Curr Biol* 13:R60-62.
59. Yoshikawa, S., McKinnon, R.D., Kokel, M., and Thomas, J.B. 2003. Wnt-mediated axon guidance via the Drosophila Derailed receptor. *Nature* 422:583-588.
60. Lyuksyutova, A.I., Lu, C.C., Milanesio, N., King, L.A., Guo, N., Wang, Y., Nathans, J., Tessier-Lavigne, M., and Zou, Y. 2003. Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling. *Science* 302:1984-1988.
61. Kikuchi, A., Yamamoto, H., and Kishida, S. 2007. Multiplicity of the interactions of Wnt proteins and their receptors. *Cell Signal* 19:659-671.
62. Jing, L., Lefebvre, J.L., Gordon, L.R., and Granato, M. 2009. Wnt signals organize synaptic prepattern and axon guidance through the zebrafish unplugged/MuSK receptor. *Neuron* 61:721-733.
63. Zhang, B., Xiong, W.C., and Mei, L. 2009. Get ready to Wnt: prepatterning in neuromuscular junction formation. *Dev Cell* 16:325-327.
64. Akaaboune, M., Culican, S.M., Turney, S.G., and Lichtman, J.W. 1999. Rapid and reversible effects of activity on acetylcholine receptor density at the neuromuscular junction in vivo. *Science* 286:503-507.
65. Akaaboune, M., Grady, R.M., Turney, S., Sanes, J.R., and Lichtman, J.W. 2002. Neurotransmitter receptor dynamics studied in vivo by reversible photo-unbinding of fluorescent ligands. *Neuron* 34:865-876.
66. Kummer, T.T., Misgeld, T., Lichtman, J.W., and Sanes, J.R. 2004. Nerve-independent formation of a topologically complex postsynaptic apparatus. *J Cell Biol* 164:1077-1087.
67. Kong, X.C., Barzaghi, P., and Ruegg, M.A. 2004. Inhibition of synapse assembly in mammalian muscle in vivo by RNA interference. *EMBO Rep* 5:183-188.
68. Hesser, B.A., Henschel, O., and Witzemann, V. 2006. Synapse disassembly and formation of new synapses in postnatal muscle upon conditional inactivation of MuSK. *Mol Cell Neurosci* 31:470-480.
69. Shigemoto, K. 2007. Myasthenia gravis induced by autoantibodies against MuSK. *Acta Myol* 26:185-191.
70. Vogel, Z., Christian, C.N., Vigny, M., Bauer, H.C., Sonderegger, P., and Daniels, M.P. 1983. Laminin induces acetylcholine receptor aggregation on cultured myotubes and enhances the receptor aggregation activity of a neuronal factor. *J Neurosci* 3:1058-1068.
71. Sugiyama, J.E., Glass, D.J., Yancopoulos, G.D., and Hall, Z.W. 1997. Laminin-induced acetylcholine receptor clustering: an alternative pathway. *J Cell Biol* 139:181-191.
72. Montanaro, F., Gee, S.H., Jacobson, C., Lindenbaum, M.H., Froehner, S.C., and Carbonetto, S. 1998. Laminin and alpha-dystroglycan mediate acetylcholine receptor aggregation via a MuSK-independent pathway. *J Neurosci* 18:1250-1260.
73. Marangi, P.A., Wieland, S.T., and Fuhrer, C. 2002. Laminin-1 redistributes postsynaptic proteins and requires rapsyn, tyrosine phosphorylation, and Src and Fyn to stably cluster acetylcholine receptors. *J Cell Biol* 157:883-895.
74. Schwander, M., Shirasaki, R., Pfaff, S.L., and Muller, U. 2004. Beta1 integrins in muscle, but not in motor neurons, are required for skeletal muscle innervation. *J Neurosci* 24:8181-8191.

75. Nishimune, H., Valdez, G., Jarad, G., Moulson, C.L., Muller, U., Miner, J.H., and Sanes, J.R. 2008. Laminins promote postsynaptic maturation by an autocrine mechanism at the neuromuscular junction. *J Cell Biol* 182:1201-1215.
76. Sanes, J.R., and Cheney, J.M. 1982. Lectin binding reveals a synapse-specific carbohydrate in skeletal muscle. *Nature* 300:646-647.
77. Martin, P.T., and Sanes, J.R. 1995. Role for a synapse-specific carbohydrate in agrin-induced clustering of acetylcholine receptors. *Neuron* 14:743-754.
78. McDearmon, E.L., Combs, A.C., and Ervasti, J.M. 2001. Differential Vicia villosa agglutinin reactivity identifies three distinct dystroglycan complexes in skeletal muscle. *J Biol Chem* 276:35078-35086.
79. Slater, C.R. 2008. Structural factors influencing the efficacy of neuromuscular transmission. *Ann N Y Acad Sci* 1132:1-12.
80. Christadoss, P. 1988. C5 gene influences the development of murine myasthenia gravis. *J Immunol* 140:2589-2592.
81. Vincent, A., Leite, M.I., Farrugia, M.E., Jacob, S., Viegas, S., Shiraishi, H., Benveniste, O., Morgan, B.P., Hilton-Jones, D., Newsom-Davis, J., et al. 2008. Myasthenia gravis seronegative for acetylcholine receptor antibodies. *Ann N Y Acad Sci* 1132:84-92.
82. Deymeer, F., Gungor-Tuncer, O., Yilmaz, V., Parman, Y., Serdaroglu, P., Ozdemir, C., Vincent, A., and Saruhan-Direskeneli, G. 2007. Clinical comparison of anti-MuSK- vs anti-AChR-positive and seronegative myasthenia gravis. *Neurology* 68:609-611.
83. Wolfe, G.I., and Oh, S.J. 2008. Clinical phenotype of muscle-specific tyrosine kinase-antibody-positive myasthenia gravis. *Ann N Y Acad Sci* 1132:71-75.
84. Evoli, A., Bianchi, M.R., Riso, R., Minicuci, G.M., Batocchi, A.P., Servidei, S., Scuderi, F., and Bartoccioni, E. 2008. Response to therapy in myasthenia gravis with anti-MuSK antibodies. *Ann N Y Acad Sci* 1132:76-83.
85. Hatanaka, Y., Hemmi, S., Morgan, M.B., Scheufele, M.L., Claussen, G.C., Wolfe, G.I., and Oh, S.J. 2005. Nonresponsiveness to anticholinesterase agents in patients with MuSK-antibody-positive MG. *Neurology* 65:1508-1509.
86. Punga, A.R., and Stalberg, E. 2009. Acetylcholinesterase inhibitors in MG: To be or not to be? *Muscle Nerve* 39:724-728.
87. Punga, A.R., Flink, R., Askmark, H., and Stalberg, E.V. 2006. Cholinergic neuromuscular hyperactivity in patients with myasthenia gravis seropositive for MuSK antibody. *Muscle Nerve* 34:111-115.
88. Chevessier, F., Faraut, B., Ravel-Chapuis, A., Richard, P., Gaudon, K., Bauche, S., Prioleau, C., Herbst, R., Goillot, E., Ioos, C., et al. 2004. MUSK, a new target for mutations causing congenital myasthenic syndrome. *Hum Mol Genet* 13:3229-3240.
89. Beeson, D., Higuchi, O., Palace, J., Cossins, J., Spearman, H., Maxwell, S., Newsom-Davis, J., Burke, G., Fawcett, P., Motomura, M., et al. 2006. Dok-7 mutations underlie a neuromuscular junction synaptopathy. *Science* 313:1975-1978.
90. Palace, J., Lashley, D., Newsom-Davis, J., Cossins, J., Maxwell, S., Kennett, R., Jayawant, S., Yamanashi, Y., and Beeson, D. 2007. Clinical features of the DOK7 neuromuscular junction synaptopathy. *Brain*.
91. Okada, K., Inoue, A., Okada, M., Murata, Y., Kakuta, S., Jigami, T., Kubo, S., Shiraishi, H., Eguchi, K., Motomura, M., et al. 2006. The muscle protein Dok-7 is essential for

neuromuscular synaptogenesis. *Science* 312:1802-1805.
92. Farrugia, M.E., Robson, M.D., Clover, L., Anslow, P., Newsom-Davis, J., Kennett, R., Hilton-Jones, D., Matthews, P.M., and Vincent, A. 2006. MRI and clinical studies of facial and bulbar muscle involvement in MuSK antibody-associated myasthenia gravis. *Brain* 129:1481-1492.