The MuSK activator agrin has a separate role essential for postnatal maintenance of neuromuscular synapses

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The motoneural control of skeletal muscle contraction requires the neuromuscular junction (NMJ), a midmuscle synapse between the motor nerve and myotube. The formation and maintenance of NMJs are orchestrated by the muscle-specific receptor tyrosine kinase (MuSK). Motor neuron-derived agrin activates MuSK via binding to MuSK's coreceptor Lrp4, and genetic defects in agrin underlie a congenital myasthenic syndrome (an NMJ disorder). However, MuSK-dependent postsynaptic differentiation of NMJs occurs in the absence of a motor neuron, indicating a need for nerve/agrin-independent MuSK activation. We previously identified the muscle protein Dok-7 as an essential activator of MuSK. Although NMJ formation requires agrin under physiological conditions, it is dispensable for NMJ formation experimentally in the absence of the neurotransmitter acetylcholine, which inhibits postsynaptic specialization. Thus, it was hypothesized that MuSK needs agrin together with Lrp4 and Dok-7 to achieve sufficient activation to surmount inhibition by acetylcholine. Here, we show that forced expression of Dok-7 in muscle enhanced MuSK activation in mice lacking agrin or Lrp4 and restored midmuscle NMJ formation in agrin-deficient mice, but not in Lrp4-deficient mice, probably due to the loss of Lrp4-dependent presynaptic differentiation. However, these NMJs in agrin-deficient mice rapidly disappeared after birth, and postsynaptic specializations emerged ectopically throughout myotubes whereas exogenous Dok-7–mediated MuSK activation was maintained. These findings demonstrate that the MuSK activator agrin plays another role essential for the postnatal maintenance, but not for embryonic formation, of NMJs and also for the postnatal, but not prenatal, midmuscle localization of postsynaptic specializations, providing physiological and pathophysiological insight into NMJ homeostasis.

neuromuscular junction | postsynaptic specialization | Dok-7 | Lrp4

A hallmark of the chemical synapse is the precise apposition of its pre- and postsynaptic specializations, placing the neurotransmitter release sites in nerve terminals opposite the clusters of neurotransmitter receptors in the postsynaptic membrane. The neuromuscular junction (NMJ) is a cholinergic synapse in mammals that controls skeletal muscle contraction where the motor nerve terminal is apposed to the endplate (the region of postsynaptic specialization on the myotube) (1, 2). To achieve efficient neuromuscular transmission, acetylcholine receptors (AChRs) must be densely clustered on the postsynaptic muscle membrane of the NMJ. Impaired formation and/or maintenance of NMJs leads to neuromuscular-transmission pathologies characterized by fatigable muscle weakness, such as congenital myasthenic syndromes (CMSs) and myasthenia gravis, which are inherited and autoimmune NMJ disorders, respectively (3, 4).

The muscle-specific receptor tyrosine kinase MuSK and the motor neuron-derived MuSK activator agrin play essential roles in the formation and maintenance of NMJs in the central region of each myotube, and their mutation has been reported in some types of CMSs (5–13). Before motor innervation during normal development, or in genetically engineered mouse embryos that lack motor neurons, AChR transcripts and proteins are expressed and clustered only in the central region of the skeletal muscle in a manner dependent on MuSK (1, 14). This MuSK-dependent, but nerve-independent, postsynaptic specialization, also known as muscle prepatterning, is further modified and fine-tuned by agrin, which counteracts the acetylcholine (ACh)-mediated dispersal of AChR clusters (15, 16). Consistent with this, agrin-deficient mice show muscle prepatterning, but fail to form NMJs due to rapid dispersal of AChR clusters upon motor innervation (14). In addition, because postnatal loss of MuSK or agrin causes disassembly of NMJs (6, 11), agrin-dependent MuSK activation is believed to be essential for the maintenance of NMJs. However, molecular mechanisms underlying this agrin-dependent NMJ maintenance remain to be studied.

Activation of MuSK also involves the cytoplasmic protein Downstream of kinases-7 (Dok-7) and the receptor protein Low-density lipoprotein receptor-related protein 4 (Lrp4) (17–22). We previously demonstrated that Dok-7 is an essential muscle-intrinsic activator of MuSK and that its mutations underlie *DOK7* myasthenia, a limb-girdle type of CMS with NMJ synaptopathy (17–19, 23, 24). Dok-7 directly interacts with the cytoplasmic portion of MuSK and activates the receptor kinase, and overexpression of Dok-7 in the muscle enhances MuSK activation and promotes NMJ formation in vivo (19). On the other hand, agrin, an extracellular activator of MuSK, binds to MuSK's coreceptor Lrp4 and indirectly induces MuSK activation (21, 22). However, no sign of MuSK activation, including muscle prepatterning, is observed in mice lacking Dok-7, and recombinant agrin cannot activate MuSK in Dok-7–deficient myotubes (17, 19), indicating a requirement for Dok-7 in the agrin-mediated activation of MuSK.

Significance

The neuromuscular junction (NMJ) is a synapse between the motor nerve and myotube essential for controlling skeletal muscle contraction. Motor nerve-derived glycoprotein agrin is indispensable for the formation and maintenance of NMJs, and genetic defects in agrin underlie a congenital myasthenic syndrome (CMS). Agrin's role has been thought to be activation of the muscle-specific receptor kinase MuSK. Here, we demonstrate that forced activation of MuSK in agrin-deficient mice restored embryonic formation, but not postnatal maintenance, of NMJs, demonstrating that agrin plays an essential role distinct from MuSK activation in the postnatal maintenance of NMJs. Given that CMSs frequently show postnatal onset, this finding provides key insights not only into NMJ homeostasis but also into CMS pathology with unknown etiology.

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Interestingly, as with mice lacking Dok-7, Lrp4-deficient mice do not show MuSK-dependent muscle prepatterning or NMJ formation (20), suggesting that both Dok-7 and Lrp4 are required for MuSK activation under physiological conditions, in contrast to our observation that Dok-7 can activate MuSK in the absence of Lrp4 or its ligand agrin, at least in vitro (19). Thus, we examined if Dok-7 can activate MuSK in the absence of Lrp4 or agrin in vivo and compensate for their deficiency in the formation and/or maintenance of NMJs.

Results

Forced Expression of Dok-7 Activates MuSK in Agrin-Deficient Mice. Although agrin is expressed in both motor neurons and myotubes, only the nerves produce an alternatively spliced isoform called "z-agrin," which is solely responsible for agrin-mediated activation of MuSK (9, 21, 22). MuSK activity has been widely monitored both in vivo and in vitro through tyrosine phosphorylation of MuSK itself or "autophosphorylation," including studies on z-agrin– and Dok-7–mediated activation of MuSK. Although not all phosphorylation sites necessarily correlate with kinase activity, this potentially imperfect surrogate provides a high degree of sensitivity. To address whether Dok-7 can activate MuSK in the absence of z-agrin (hereafter "agrin") in vivo, we crossed agrin-deficient mice with Dok-7 transgenic (Tg) mice, in which MuSK is strongly activated due to forced skeletal muscle-specific expression of Dok-7 tagged with enhanced green fluorescent protein (EGFP) (9, 19). We previously demonstrated that Dok-7 activates MuSK in cultured myotubes in the absence of agrin (17, 18) and thus anticipated similar strong activation of MuSK in Dok-7 Tg mice that lack agrin. Indeed, the level of MuSK phosphorylation was comparable in the skeletal muscle of Dok-7 Tg embryos with or without agrin at embryonic day 18.5 (E18.5) (Fig. 1 *A* and *B*), when agrin is indispensable for NMJ formation in wild-type (WT) embryos (9). By contrast, MuSK phosphorylation was undetectable in WT or agrin-deficient embryos, as also reported by others (25), suggesting that the endogenous, physiological level of MuSK phosphorylation is not detectable with the methods used in these studies. However, phosphorylation of AChR, which is triggered on activation of MuSK (26), was decreased compared with WT, but nevertheless detectable in agrin-deficient embryos, reflecting Dok-7–mediated activation of MuSK (19) (Fig. 1 *C* and *D*). In addition, this phosphorylation was greatly elevated in Dok-7 Tg embryos irrespective of agrin. These results indicate that forced expression of Dok-7 in vivo induces robust activation of MuSK in the absence of agrin.

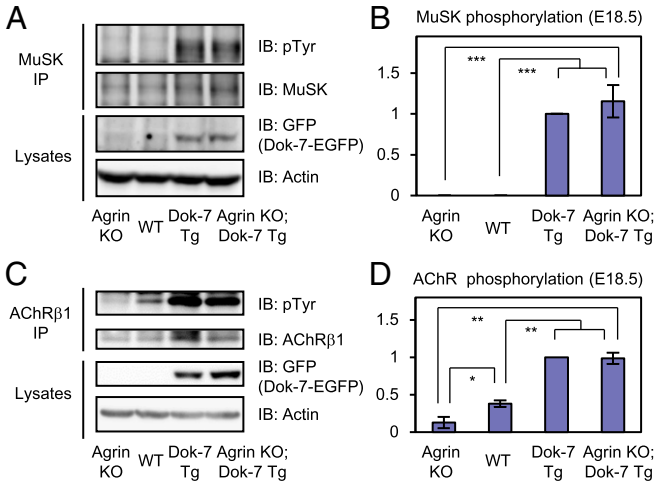

Fig. 1. Forced expression of Dok-7 activates MuSK in agrin-deficient mice. (*A* and *C*) The limb-muscle lysates and anti-MuSK or anti-AChR β1-subunit (AChRβ1) immunoprecipitates (IPs) from the lysates of wild-type (WT), Agrin KO, Dok-7 Tg, or Agrin KO;Dok-7 Tg embryos at E18.5 were subjected to immunoblotting (IB) with the indicated antibodies. pTyr, phosphotyrosine. (*B* and *D*) Ratio of tyrosine phosphorylation of MuSK or AChR to total amount of each protein was quantified. The relative intensity of MuSK or AChR phosphorylation in Dok-7 Tg was arbitrarily defined as 1. Error bars indicate mean ± SD ($n = 3$). Asterisks denote significant statistical difference: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

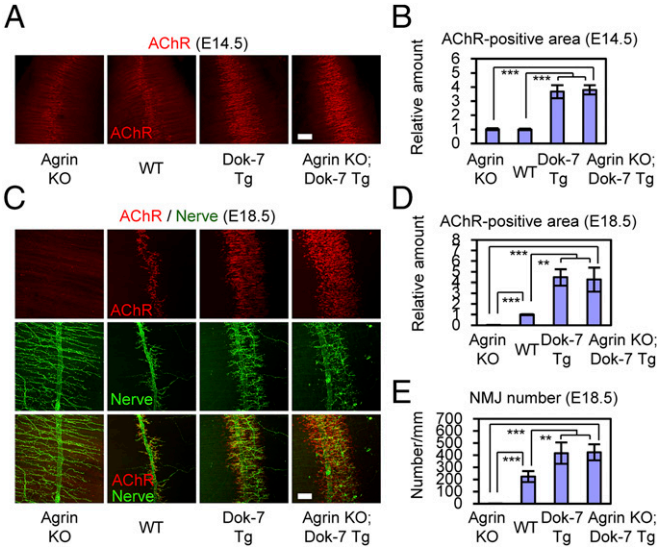

Fig. 2. Forced expression of Dok-7 restores NMJ formation in agrin-deficient mice. (*A* and *C*) Diaphragm muscles of WT, Agrin KO, Dok-7 Tg, or Agrin KO; Dok-7 Tg embryos at E14.5 (*A*) or E18.5 (*C*) were stained with Btx (AChR) and/or antibodies to neurofilament and synaptophysin ("Nerve"). (*B*, *D*, and *E*) The AChR-positive area at E14.5 (*B*) or E18.5 (*D*) and the number of NMJs at E18.5 (*E*) were quantified. The mean value of the AChR-positive area in WT was arbitrarily defined as 1. Error bars indicate mean ± SD. Data were collected from three or more diaphragm muscles for each genotype. Asterisks denote significant statistical difference: $**P < 0.01$ and $***P < 0.001$. (Scale bars, 100 μm.)

Forced Expression of Dok-7 Restores NMJ Formation in Agrin-Deficient Mice. As mentioned earlier, agrin is required for NMJ formation, but not for muscle prepatterning of postsynaptic specializations. Consistent with this, the lack of agrin did not affect prominent AChR clustering in the correct central region of myotubes at E14.5 before neuromuscular synaptogenesis, and forced expression of Dok-7 enhanced the prepatterning as we previously demonstrated (19) (Fig. 2 *A* and *B*). In accordance with an essential role for agrin in the preservation of postsynaptic specialization upon motor innervation at E18.5 when NMJs are normally formed in the central region of myotubes in WT embryos, only a few small AChR clusters were present and distributed throughout myotubes in agrin-deficient embryos as previously reported (8, 9) (Fig. 2 *C* and *D*). However, forced expression of Dok-7 in agrin-deficient embryos facilitated AChR clustering in the central region of muscle. Furthermore, visualization of motor axons and presynaptic nerve terminals revealed that Dok-7 Tg mice developed a significantly greater number of NMJs than the WT controls not only in the presence of agrin, as previously described (19), but also in the absence of it (Fig. 2 *C* and *E* and Fig. S1). Consistent with this, forced expression of Dok-7 in the skeletal muscle rescued all agrin-deficient mice from neonatal lethality. Together, these findings indicate that the exogenous Dok-7–mediated activation of MuSK compensates for agrin deficiency in prenatal NMJ formation.

NEUROSCIENCE

Lrp4 Is Required for Efficient Activation of MuSK by Dok-7 in Vivo. We previously purified bacterially expressed Dok-7 and the cytoplasmic region of MuSK (MuSK-cyt) including the kinase domain and showed that Dok-7 can directly activate the kinase in vitro (19). We also demonstrated that forced expression of either MuSK or MuSK-cyt alone did not result in activation of the expressed kinase, but did with forced and simultaneous expression of Dok-7 in HEK293T cells (17–19). However, muscle prepatterning is dependent not only on Dok-7 and MuSK, but also on Lrp4 (20), raising the possibility that in skeletal muscle Dok-7 might not be able to activate MuSK without Lrp4 also present. Thus, we crossed Dok-7 Tg mice with Lrp4-deficient mice (20) and found that forced expression of Dok-7 in the muscle induces MuSK activation in mice lacking Lrp4 as judged by MuSK and AChR phosphorylation (Fig. 3 and Fig. S2). However, the phosphorylation level of MuSK in Dok-7 Tg embryos lacking Lrp4 was significantly lower than that in Dok-7 Tg embryos with intact Lrp4 at both E14.5 and E18.5 (Fig. 3 *A* and *B* and Fig. S2 *A* and *B*). Together, these data indicate that Lrp4 is required for efficient activation of MuSK by Dok-7 in the muscle. Although AChR phosphorylation was detectable at E14.5 only in Dok-7 Tg embryos with intact Lrp4 (Fig. S2 *C* and *D*), its phosphorylation in Lrp4-deficient Dok-7 Tg embryos at E18.5 was detectable and comparable to that in WT embryos (Fig. 3 *C* and *D*), in which MuSK phosphorylation was undetectable and thus weaker than that in Lrp4-deficient Dok-7 Tg embryos (Fig. 3 *A* and *B*). This implies that Lrp4 may also play an important role in MuSK-dependent phosphorylation of AChR.

Forced Expression of Dok-7 Restores Muscle Prepatterning but Not NMJ Formation in Lrp4-Deficient Mice. Because forced expression of Dok-7 activates MuSK in the absence of Lrp4, we examined if it restores muscle prepatterning in Lrp4-deficient embryos. Indeed, forced expression of Dok-7 in Lrp4-deficient embryos promoted substantial AChR clustering in the central region of the muscle at E14.5 (Fig. 4 *A* and *B*). However, probably due to the decreased level of MuSK activation mentioned above, AChR clustering was less pronounced in Dok-7 Tg mice lacking Lrp4 than in those with intact Lrp4. Furthermore, these AChR clusters were not maintained, and NMJ formation occurred, but was

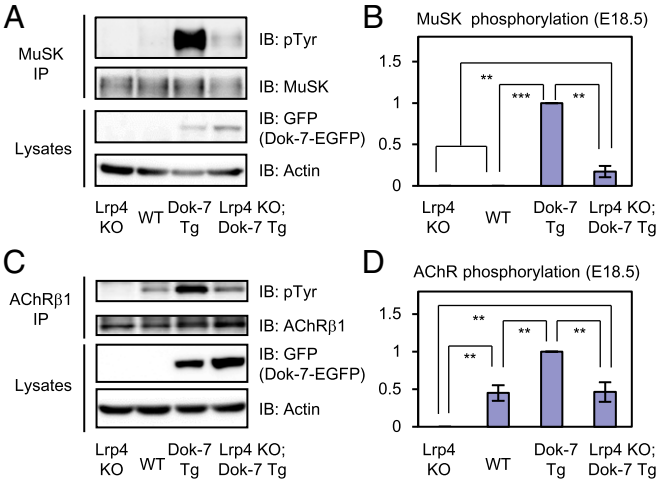

Fig. 3. Lrp4 is required for efficient activation of MuSK by Dok-7 in vivo. (*A* and *C*) The limb-muscle lysates and anti-MuSK or anti-AChRβ1 IP from the lysates of WT, Lrp4 KO, Dok-7 Tg, or Lrp4 KO;Dok-7 Tg embryos at E18.5 were subjected to IB with the indicated antibodies. (*B* and *D*) Ratio of tyrosine phosphorylation of MuSK or AChR to total amount of each protein was quantified. The relative intensity of MuSK or AChR phosphorylation in Dok-7 Tg was arbitrarily defined as 1. Error bars indicate mean ± SD ($n = 3$). Asterisks denote significant statistical difference: $^{**}P < 0.01$ and $^{***}P < 0.001$.

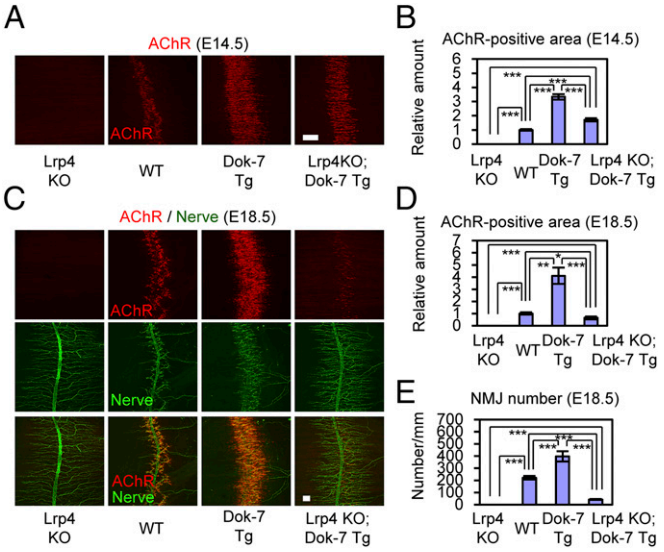

Fig. 4. Forced expression of Dok-7 restores muscle prepatterning but not NMJ formation in Lrp4-deficient embryos. (*A* and *C*) Diaphragm muscles of WT, Lrp4 KO, Dok-7 Tg, or Lrp4 KO;Dok-7 Tg embryos at E14.5 (*A*) or E18.5 (*C*) were stained with Btx (AChR) and/or antibodies to neurofilament and synaptophysin ("Nerve"). (*B*, *D*, and *E*) The AChR-positive area at E14.5 (*B*) or E18.5 (*D*) and the number of NMJs at E18.5 (*E*) were quantified. The mean value of the AChR-positive area in WT was arbitrarily defined as 1. Error bars indicate mean ± SD. Data were collected from three or more diaphragm muscles for each genotype. Asterisks denote significant statistical difference: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (Scale bars, 100 μm.)

severely impaired at E18.5 in Lrp4-deficient embryos despite the presence of the Dok-7 transgene (Fig. 4 *C*–*E* and Fig. S3). Unlike Lrp4-deficient mice, which cannot move due to the lack of NMJs, those with the Dok-7 transgene were able to move for at least 10 min after birth, but died within 1 h, indicating the presence of severely impaired, but marginally functional NMJs at birth. Given that MuSK phosphorylation in Lrp4-deficient Dok-7 Tg embryos was greater than that in the WT controls at E18.5 (Fig. 3 *A* and *B*), these data suggest that Lrp4 plays a role distinct from MuSK activation in the maintenance of postsynaptic specialization in embryonic skeletal muscle. Consistent with this idea, it was recently demonstrated that Lrp4 plays an essential role as a retrograde signal in presynaptic differentiation of motor neurons (27, 28), raising the possibility that as-yet-unidentified signaling from presynaptic nerve terminals, which are lost in Lrp4-deficient embryos, is required for the maintenance of AChR clusters after motor innervation during normal development.

Agrin Is Required for the Postnatal Maintenance of NMJs Irrespective of MuSK Activation. Although forced expression of Dok-7 via a transgene restored embryonic NMJ formation and neonatal survival in all agrin-deficient mice, we found that these mice survived no longer than 8 wk after birth (Fig. S4*A*). However, Dok-7 Tg mice with intact agrin survived more than 1 y after birth (Fig. S4*B*), indicating forced expression of Dok-7 has no lethal pathogenic effects. By 5 wk of age, Dok-7 Tg mice lacking agrin, but not those with intact agrin, exhibited severe motor defects; they required at least 30 s to right themselves after being placed on their side and showed greatly reduced motor performance in rotarod tests (Fig. S5). They also showed abnormal curvature of the spine between the thoracic and lumbar vertebrae (thoracolumbar kyphosis), indicating a myasthenic phenotype (Fig. S6). Indeed, histological examination of the diaphragm muscle revealed that NMJs in Dok-7 Tg mice were much less numerous in the absence than in the presence of agrin at 5 wk of

age, whereas the number of NMJs was comparable in Dok-7 Tg mice at birth [postnatal day 0 (P0)] and 1 wk of age irrespective of agrin (Fig. 5 *A*–*D*). These results raise the possibility that MuSK activation might be impaired due to the lack of agrin in the mutant mice. However, even at 5 wk of age, the phosphorylation levels of MuSK and AChR were each comparable in Dok-7 Tg mice irrespective of the presence or absence of agrin and was significantly higher than that in WT mice, confirming that the exogenous Dok-7–mediated activation of MuSK was maintained until at least 5 wk of age (Fig. 5 *E* and *F* and Fig. S7). Thus, we further investigate the size of AChR clusters together with the cover ratio of presynaptic nerve terminals at NMJs to delineate defects in agrin-deficient Dok-7 Tg mice compared with NMJs in Dok-7 Tg mice with intact agrin. We found that both parameters were comparable irrespective of agrin at P0, but were lower in the absence than in the presence of agrin at 1 wk of age (Fig. S8). Interestingly, the coverage of presynaptic nerve terminals over AChR clusters, but not the size of the clusters, further decreased at 5 wk of age only in the absence of agrin, indicating an apparent defect in motor innervation (Fig. 5*G* and Fig. S8). Also, we found that the cover ratio at NMJs was lower in Dok-7 Tg mice than in WT mice even at P0 (Fig. S8*B*). Because the size of postsynaptic AChR clusters had already begun to enlarge at P0 in Dok-7 Tg mice compared with WT mice (Fig. S8*A*), this is probably due to lagging enlargement of presynaptic nerve terminals in response to the enlarged AChR clusters in the muscle.

In addition to the NMJ defects, we also found that small AChR clusters formed uniformly throughout myotubes in agrin-deficient Dok-7 Tg mice (Fig. S9), suggesting noncentral, ectopic expression of MuSK because AChRs cluster where MuSK is expressed in the muscle (29). Indeed, the midmuscle-restricted expression of *MuSK* transcripts was lost, and instead their uniform expression was observed in Dok-7 Tg mice in the absence but not in the presence of agrin (Fig. 5*H*). Also, the loss of midmuscle-restricted expression of *AChRα1* transcripts, which is known to be dependent on MuSK (5), was confirmed in agrin-deficient Dok-7 Tg mice (Fig. S10*A*). By contrast, these transcripts were correctly restricted to the central region of muscle at E18.5 in agrin-deficient Dok-7 Tg embryos (Fig. S10*B*). Furthermore, because (*i*) Dok-7 Tg mice showed enhanced NMJ formation, compared with WT mice, in the correct central region of myotubes before and after birth and (*ii*) agrin-deficient Dok-7 Tg embryos also showed enhanced midmuscle NMJ formation (Figs. 2, 4, and 5 and Figs. S1, S3, S9, and S10), it is unlikely that forced expression of Dok-7 per se causes disassembly of NMJs and formation of small AChR clusters universally throughout myotubes in agrin-deficient Dok-7 Tg mice after birth, although nonphysiological effects of the transgene are not completely excluded. Together, these findings demonstrate that agrin plays an essential role distinct from MuSK activation in the postnatal maintenance of NMJs as well as in the postnatal, but not prenatal, midmuscle expression of MuSK.

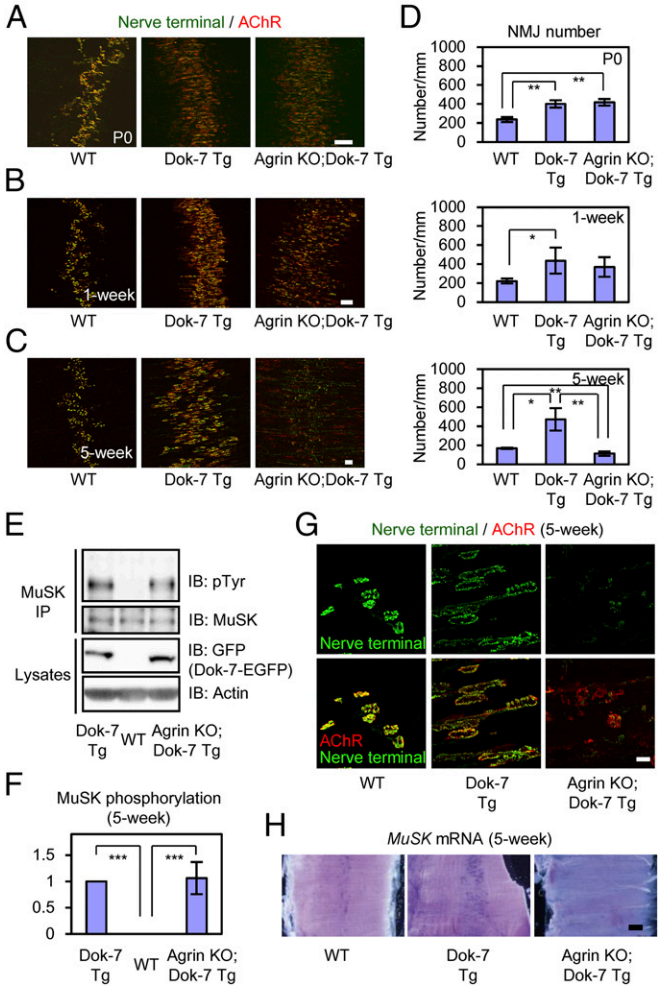

Fig. 5. Forced expression of Dok-7 maintains MuSK activation but not NMJs in agrin-deficient mice after birth. (*A*–*C*) Diaphragm muscles of WT, Dok-7 Tg, or Agrin KO;Dok-7 Tg mice at 0 (P0) (*A*), 1 (*B*), or 5 (*C*) weeks of age were stained with Btx (AChR) and antibodies to synapsin1 ("Nerve terminal"). (Scale bars, 100 μm.) (*D*) The number of NMJs in diaphragm muscles was quantified. Error bars indicate mean ± SD. Data were collected from three or more diaphragm muscles for each genotype. Asterisks denote significant statistical difference: $*P < 0.05$ and $**P < 0.01$. (*E*) The limb-muscle lysates and anti-MuSK IP from the lysates of 5-wk-old mice were subjected to IB with the indicated antibodies. (*F*) Ratio of MuSK tyrosine phosphorylation to total MuSK was quantified. The relative intensity of MuSK phosphorylation in Dok-7 Tg was arbitrarily defined as 1. Error bars indicate mean ± SD ($n = 3$). Asterisks denote significant statistical difference: $***P < 0.001$. (*G*) Magnified views of NMJs in diaphragm muscles of 5-wk-old mice are shown. (Scale bars, 20 μm.) (*H*) Diaphragm muscles of 5-wk-old mice were subjected to in situ hybridization with an antisense probe for *MuSK*. (Scale bars, 500 μm.)

Discussion

In the current study, we demonstrated that forced expression of Dok-7 in Lrp4-deficient embryos induced MuSK activation and restored muscle prepatterning, showing that Lrp4 is not required for MuSK activation by Dok-7 at least in these experimental settings (Figs. 3 and 4 and Fig. S2). However, MuSK was activated to a greater extent in Dok-7 Tg embryos than in those lacking Lrp4. These findings suggest that Lrp4 facilitates Dok-7–mediated activation of MuSK, and thus at its normal expression levels Dok-7 needs Lrp4 to adequately activate MuSK for induction of muscle prepatterning. Therefore, interaction between Lrp4 and MuSK might induce conformational change in MuSK to promote its susceptibility to Dok-7–mediated activation in vivo. Indeed, overexpression of Lrp4 and MuSK in BaF3 or HEK293 cells induced partial activation of MuSK, and further stimulation with agrin led to higher MuSK activation (21, 22). Alternatively, but not mutually exclusively, the Lrp4-mediated partial activation of MuSK might induce a low level of phosphorylation of Tyr-553 that would stabilize binding of MuSK to Dok-7 via its phosphotyrosine-binding (PTB) domain and thus induce dimerization of the complex to facilitate further MuSK activation (30). However, we previously demonstrated that, in HEK293T cells, Dok-7 activates MuSK even in the presence of various types of mutations that disrupt their stable binding via the PTB domain and its target (17, 18). Therefore, further studies are required to determine whether the partial activation of MuSK by Lrp4 observed in heterologous cells is relevant to the apparent cooperation between Lrp4 and Dok-7 in MuSK activation in vivo.

Because agrin is secreted into the synaptic basal lamina and binds not only Lrp4, but also a variety of other NMJ proteins including laminins and dystroglycans (31), agrin could have roles other than activation of MuSK through such interactions. However, it was reported that modestly increased expression of MuSK in the skeletal muscle induces NMJ formation in agrin-deficient embryos, although highly increased expression induces postnatal lethality with abnormally scattered NMJ formation even in mice with intact agrin (29). These findings imply that, in the presence of Dok-7 and Lrp4, MuSK is activated as a result of increased expression to induce NMJ formation in agrin-deficient embryos, although MuSK activation per se was not examined in this study. In the current study, we demonstrate that forced expression of Dok-7 induced robust activation of MuSK and restored NMJ formation in agrin-deficient embryos (Figs. 1 and 2 and Fig. S1). These data together establish that MuSK activation can compensate for agrin deficiency in embryonic NMJ formation. In other words, agrin is required because Dok-7–mediated MuSK activation does not suffice for embryonic NMJ formation in the physiological setting. However, NMJs formed in agrin-deficient Dok-7 Tg embryos rapidly disappeared after birth (Fig. 5 *A*–*D*), and motor innervation and postsynaptic AChR clustering was impaired even in the remaining NMJs (Fig. 5*G* and Fig. S8). Given that the exogenous Dok-7–mediated MuSK activation was maintained at a high level (Fig. 5 *E* and *F* and Fig. S7), these findings indicate that agrin has a separate indispensable role as a synaptic organizer aside from MuSK activation for postnatal maintenance, but not embryonic formation, of NMJs. Uncovering the mechanisms by which agrin does this should illuminate why postnatal, but not prenatal, NMJs are vulnerable. This may also provide insight into CMS pathology because most CMSs show postnatal onset of muscle weakness as discussed further later.

Only a few elements that play indispensable roles in the postnatal maintenance, but not prenatal formation, of NMJs have been suggested (32). The laminin β2-subunit is one such element; mice lacking it show no defects in NMJ formation during embryogenesis and the first few postnatal days, but develop presynaptic defects by 1 wk after birth, leading to lethality during the third postnatal week (33–35). Interestingly, agrin-deficient mice are rescued by muscle-specific expression of "mini-agrin," which retains only agrin's laminin-binding and MuSK-activating (Lrp4-binding) regions (36). Therefore, agrin may evoke signals other than MuSK activation by binding to laminins and/or Lrp4. Because the laminin β2-subunit acts as an essential presynaptic organizer after, but not before, birth by binding to the presynaptic voltage-gated calcium channels at NMJs (33–35), agrin might promote the presynaptic specialization essential for NMJ maintenance via interaction with the laminins containing the β2-subunit. Notably, this hypothetical role for agrin could explain our finding that agrin deficiency impaired motor innervation as judged by the coverage of presynaptic nerve terminals over postsynaptic AChR clusters (Fig. 5*G* and Fig. S8*B*), which implies a presynaptic defect. However, it remains unclear if the presynaptic defect is solely responsible for the postnatal loss of NMJs in Dok-7 Tg mice that lack agrin.

In agrin-deficient Dok-7 Tg mice, we also found that the loss of postnatal, but not prenatal, midmuscle restricted expression of the *MuSK* and *AChRα1* transcripts (Fig. 5*H* and Fig. S10), which is important for the correct central localization of NMJs in skeletal muscle (2, 29). Although this transcriptional regulation is dependent on MuSK activity, these findings indicate that agrin plays an additional essential role distinct from MuSK activation in transcriptional regulation after, but not before, birth. Given that NMJs also disappeared after birth in agrin-deficient Dok-7 Tg mice, NMJs per se might be required for this midmuscle-restricted transcriptional regulation. However, this type of regulation occurs at E14.5 before NMJ formation and also occurs in the absence of neuromuscular transmission resulting from a lack of ACh (37, 38). Therefore, agrin may play two distinct roles: one in the postnatal maintenance of NMJs and the other in postnatal midmuscle-restricted transcriptional regulation. Alternatively, NMJs could have an essential role aside from neuromuscular transmission in exerting such transcriptional regulation.

Interestingly, among agrin mutations found in patients with CMS, one mutation, V1727F, reduced agrin's ability to activate MuSK in cultured myotubes (12), but another mutation, G1709R, showed no such reduction (13). However, injection of G1709R mutant agrin, but not the WT protein, into the skeletal muscle of 4-wk-old rats perturbed the organization of NMJs, implying that the G1709R mutation may impair agrin-mediated signaling to maintain postnatal NMJs without affecting its ability to activate MuSK. Similar disturbances in this agrin-mediated signaling pathway could also be involved in the pathogenesis of other types of CMSs with unknown etiology because CMSs frequently present with postnatal onset (39). Therefore, uncovering the role that agrin plays in the postnatal maintenance of NMJs, apart from its role in MuSK activation, would not only deepen our understanding of NMJs but also pave the way toward finding new therapeutic targets for CMSs.

Materials and Methods

Mouse Strains. The transgenic mice expressing Dok-7 tagged with EGFP (Dok-7–EGFP) under the control of the skeletal actin promoter and z-agrin–deficient or Lrp4-deficient (Lrp4^{mitt}) mice were previously described (9, 19, 20). All experiments described here were approved by the Animal Care and Use Committee at the Institute of Medical Science, University of Tokyo.

Immunoprecipitation and Immunoblotting. Tissue lysates were prepared from limb muscle with alkaline-lysis buffer (50 mM Tris·HCl, pH 9.5, 1 mM Na_3VO_4, 50 mM NaF, 1% sodium deoxycholate with a mixture of protease inhibitors). For immunoprecipitation, lysates were incubated with antibodies to MuSK (N-19 and C-19) or AChRβ1 (H-101) (Santa Cruz Biotechnology), followed by incubation with protein G-Sepharose (GE Healthcare). The immune complexes were washed four times and collected as immunoprecipitates. For immunoblotting, immunoprecipitates or tissue lysates were separated by SDS/7.5% or 9% (wt/vol)-PAGE and transferred to a PVDF membrane (Millipore), which was then incubated with antibodies to phosphotyrosine (4G10) (Millipore), MuSK (AF562) (R&D Systems), AChRβ1 (H101), GFP (B-2), actin (I-19) (Santa Cruz Biotechnology), and Dok-7 (R&D Systems), washed, and incubated with horseradish peroxidase-labeled anti-mouse, anti-rabbit (GE Healthcare), anti-goat (Santa Cruz Biotechnology) IgG, or anti-rabbit light-chain antibodies (Jackson ImmunoResearch Laboratories). Band intensities were measured and analyzed using a LAS4000 imager with ImageQuant TL software (GE Healthcare). Data were assessed by paired or unpaired *t* test. The differences between samples with P values <0.05 were considered to be statistically significant.

Whole-Mount Tissue Staining and in Situ Hybridization. For whole-mount tissue staining, diaphragm muscles from mouse embryos at E14.5 or E18.5 and mice at 0 (P0), 1, or 5 wk of age were fixed in 1% paraformaldehyde in PBS for 2 h or overnight at 4 °C and then rinsed with PBS. After dissection of the connective tissue, the muscles were permeabilized with 1% Triton X-100 in PBS for 1 h and then incubated with rabbit antibodies against synaptophysin (Invitrogen) or synapsin1 (Cell Signaling) to label presynaptic nerve terminals and/or against neurofilament (Millipore) to label motor axons, followed by incubation with Alexa 647-conjugated anti-rabbit IgG and/or Alexa 594-conjugated α-bungarotoxin (Btx) (Invitrogen) to label postsynaptic AChR clusters. Confocal Z serial images were collected with a confocal laser-scanning microscope (FV1000, Olympus), collapsed into a single image, and analyzed with photoshop (Adobe) and cellSens (Olympus) software (19, 28, 29). Data were assessed by unpaired *t* test. The differences between samples with P values <0.05 were considered to be statistically significant. For in situ hybridization, diaphragm muscles dissected from mouse embryos at E18.5 or 5-wk-old mice were probed with a digoxigenin-labeled antisense riboprobe corresponding to mouse cDNA of *AChRα1* or *MuSK*, and images were obtained using the stereo microscope SZX16 fitted with a DP-70 camera (Olympus) as described previously (19).

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